Journal of Animal and Feed Sciences, 5, 1996, 163 – 173

Effect of feeding cholesterol–enriched diet on plasma and tissue cholesterol and atherosclerosis in rabbits

Anna M. Konecka, T. Jezierski and Agata Wnuk

Institute of Genetics and Animal Breeding,
Polish Academy of Sciences
Jastrzębiec, 05-551 Mroków, Poland

(Received 3 November 1995; accepted 12 April 1996)

ABSTRACT

The aim of the experiment was to investigate the relationship between consumption of cholesterol-enriched diet and plasma and tissue cholesterol content as well as the degree of atherosclerosis in rabbits, to make further investigations on the role of emotional and behavioural factors in diet induced atherosclerosis more conclusive. The experimental rabbits (E) were offered 150 g standard pellet feed daily mixed with 1 egg yolk and 1.5 g cholesterol in substantia during 7 weeks, whereas the control rabbits (C) were fed the same amount of feed without egg yolk and supplementary cholesterol. Significant effect of diet type on total plasma cholesterol, HDL-cholesterol (high density lipoprotein cholesterol) and cholesterol content in liver, heart and muscle was found. However negative correlations between cholesterol food consumption and total plasma cholesterol ($r = -0.24$) and atherosclerosis ($r = -0.40$) were found in group E. The relative increase of total plasma cholesterol in E group was greater than that of plasma HDL-cholesterol. Females demonstrated significantly higher total plasma cholesterol and HDL-cholesterol than males. The degree of atherosclerosis was positively correlated with the total plasma cholesterol ($r = 0.73$, $P < 0.01$) and plasma HDL-cholesterol concentration ($r = 0.29$) but negatively correlated with ratio HDL/total cholesterol ($r = -0.66$, $P < 0.01$) as estimated after the end of the experiment.

KEY WORDS: feeding, plasma cholesterol, HDL-cholesterol, tissue cholesterol, atherosclerosis, rabbits

INTRODUCTION

Dietary cholesterol consumed in large amounts is believed to increase plasma cholesterol which in turn may raise risk of atherosclerosis. Moreover, the

absorption of high amounts of cholesterol from blood and its accumulation in liver probably may impair liver function (Beynen et al., 1986).

In the previous experiments we used rabbits as experimental models for investigation emotional and behavioural factors playing a role in diet induced atherosclerosis. These factors turned out to be biologically not neutral but are often unpredictable (Jezierski et al., 1993). To make these factors more discernible we tried to control individual differences in cholesterol feed consumption and to induce only a moderate atherosclerosis. We found no significant effect of feeding egg yolk on plasma cholesterol and development of atherosclerosis in rabbits (Jezierski and Konecka, 1994). In the present experiment apart from egg yolk supplementary cholesterol was added to rabbit standard diet in order to induce atherosclerosis. Relationship between cholesterol food consumption and primary plasma cholesterol content, the increase of plasma cholesterol and cholesterol content in liver, heart, muscle as well as the atherosclerosis were investigated. Particular attention was paid to differences between sexes. The results of the present experiment should help us to find the optimal experimental set-up for further experiments on the role of emotional and behavioural factors in diet induced atherosclerosis.

MATERIAL AND METHODS

Forty White New Zealand rabbits (23 males and 17 females) stemming from Institute's own outbred stock were used. The animals were three months old with an average body weights 3.65 kg for males and 3.77 kg for females at the beginning of the experiment. The rabbits were kept in individual wire cages 60 cm x 60 cm x 32 cm at the average room temperature of 14-18^{0}C. After weaning, till the begining of the experiment the rabbits were fed *ad libium* with standard pelleted rabbit feed containing 18.0% crude protein, 4.2% fat (bone-meat meal) and 13.1% crude fibre and 98 (SD = 7.2) mg cholesterol/100 g dry feed. Water was available *ad libitum*.

The experiment lasted seven weeks. During that time the experimental rabbits (E, n = 22) were offered 150 g standard feed daily mixed with 1 egg yolk and 1.5 g cholesterol in substantia, except for Saturdays and Sundays when they received standard feed only. The control rabbits (C, n = 18) received the same amount of feed but without egg yolk and without supplementary cholesterol. Since the rabbits sometimes did not consume their full daily dose, the refuse was weighted daily. All the rabbits were weighted weekly.

Before and after the experimental period blood samples were taken into heparinized tubes from an ear vein. Prior to blood sampling the rabbits were fasted for 24 h. After collection the blood samples were centrifuged at 3000 x g for

15 min at $+4^0$C. Plasma total cholesterol content in mg/100ml was determined using Biochemtest No. 1344-690-718061 (POCh, Gliwice, Poland). The estimation of total cholesterol for each sample was repeated 3 times the intra assay variability being 2%. Plasma HDL cholesterol was measured after precipitation of VLDL (very low density lipoprotein) and LDL (low density lipoprotein) with phosphotungstate/magnesium chloride at room temperature.

After killing each rabbit, the aorta ascendens, arcus aortae and aorta descendens up to the 6 th or 7 th arteria intercostalis were cut out and dissected. These arteries were stained with oil-red O (Romeis, 1968), pinned out and photographed in colour. Two colours tints were distinguised: (a) deep red indicating severe atheroma and (b) pink or pale indicating weak or no atheroma. Atheroma size was estimated by placing a transparent grid over the photograph, counting the deep red squares and presenting them as percentage of the total number of counted squares.

The liver, heart and muscle were removed and frozen at -20^0C, until used. The cholesterol was extracted from organs using the chloroform-metanol mixture (2:1, v/v) extraction procedure of Folch et al. (1957) and Rhee et al.(1982). Cholesterol was determined according to the colorimetric method of Searcy and Bergquist (1960). The results were expressed as mg/100g of tissue.

Statistical analysis

For the statistical analyses the following mixed model of two-way ANOVA was used:

$$Y(ijk) = u + D(i) + S(j) + DS(ij) + e(ijk)$$

where: u – mean value
D(i) – effect of diet (i = 1,2) – fixed effect
S(j) – effect of sex (j = 1,2) – random effect
DS(ij) – effects of diet x sex interaction
e(ijk) – error

Prior to the ANOVA (analysis of variance) the data on atherosclerosis degree and ratio HDL/Total plasma cholesterol were transformed using $\arcsin\sqrt{p}$.

The linear correlation coefficients between the traits investigated were calculated.

RESULTS

Rabbits in the control group as well as the females in both E and C groups tended to consume more feed. However these differences did not reach the significance level (Table 1).

TABLE 1

Feed consumption (g) in rabbits during the experimental period and degree of atherosclerosis, % of aorta surface

	Experimental group				Control group			
	n	mean	SEM	atheroscler	n	mean	SEM	atheroscler
males	12	6681	208.1	45.7	11	6872	158.4	0
females	10	6919	171.9	71.8	7	7236	44.1	0
total	22	6789	642.9	57.6	18	7014	448.9	0

Results of ANOVA

Source of variation		Significance of F	
		Feed consumption	Atherosclerosis (for E group only, data transformed)
	df		
diet	1	0.168	
sex	1	0.104	0.073
diet x sex	1	0.730	

Plasma total cholesterol concentration significantly increased in group E after the experiment ($P<0.000$), whereas a moderate decrease in the C group was observed (Table 2). Females had higher total plasma cholesterol than males especially after the experiment ($P=0.032$; Table 2). The same holds for the

TABLE 2

Total plasma cholesterol in mg/100 ml of blood plasma before and after the experimental period

	Before experiment			After experiment	
	n	mean	SEM	mean	SEM
Experimental group					
males	12	134.6	12.9	636.2	109.8
females	10	160.1	11.9	947.2	91.8
total group E	22	146.2	9.1	777.5	79.1
Control group					
males	11	140.7	9.5	60.7	3.6
females	7	151.9	10.9	118.8	14.8
total group C	18	145.1	7.1	83.3	9.1

Results of ANOVA

Source of variation		Significance of F	
	df		
diet	1	0.993	0.000
sex	1	0.135	0.032
diet x sex	1	0.557	0.130

TABLE 3

Plasma HDL-cholesterol in mg/100 ml of blood plasma and ratio HDL/total cholesterol before and after the experimental period

	Before experiment				After experiment		
	n	mean	SEM	HDL/t.chol.	mean	SEM	HDL/t.chol.
Experimental group							
males	12	63.0	5.0	.508	75.1	10.6	.214
females	10	74.8	5.4	.477	94.1	6.5	.113
total group E		68.4	3.8	.494	83.8	6.7	.168
Control group							
males	11	67.0	9.0	.479	45.7	6.0	.755
females	7	93.4	7.1	.626	70.2	5.9	.619
total group C		77.3	6.8	.536	55.2	5.1	.702

Results of ANOVA

Source of variation	df	Significance of F			
diet	1	0.115	0.390	0.003	0.000
sex	1	0.010	0.212	0.013	0.296
diet x sex	1	0.310	0.077	0.741	0.490

HDL-cholesterol concentration, however, the changes in HDL-cholesterol concentration after vs. before the experiment were relatively smaller (Table 3). The ratio HDL/Total plasma cholesterol decreased in group E after the experiment and increased in group C (in males only).

Intake of cholesterol-enriched diet resulted in almost three-fold increase of hepatic cholesterol and two-fold increase of heart and muscle cholesterol concentration in group E as compared to control animals ($P = 0.000$; Table 4).

The correlation coefficients between cholesterol feed consumed in group E and cholesterol content in plasma and tissues were negative and nonsignificant (Table 5).

Rabbits of the E group developed severe atherosclerosis after feeding with cholesterol-enriched diet, whereas no symptoms of atherosclerosis were ascertained in the group C. Typical aorta with and without atherosclerosis is shown on Figure 1. Females developed more severe atherosclerosis (on average 71.8 % aorta surface) than males (45.7 %) but this difference did not reach the significance level (Table 1). The correlation between cholesterol feed consumed in group E and the degree of atherosclerosis was negative ($r = -0.4$). The degree of atherosclerosis was positively correlated with total plasma cholesterol concentration ($r = 0.73$; $P < 0.01$) and negatively correlated with the ratio HDL/total plasma cholesterol after the experiment ($r = -0.66$; $P < 0.01$, Table 5).

TABLE 4

Cholesterol content (mg/100 g) in rabbit tissues

	Liver			Heart		Muscle	
	n	mean	SEM	mean	SEM	mean	SEM
Experimental group							
males	12	1073.0	112.6	295.3	34.5	117.3	13.9
females	10	1276.3	39.7	311.8	18.5	164.1	16.5
total group E		1165.4	66.9	302.8	20.6	138.6	11.6
Control group							
males	11	385.2	29.7	162.0	5.3	77.6	8.7
females	7	434.0	43.1	160.6	11.2	77.7	8.4
total group C		404.2	24.1	161.5	5.4	77.7	5.5

Results of ANOVA

Source of variation	df	Significance of F		
diet	1	0.000	0.000	0.000
sex	1	0.106	0.757	0.086
diet x sex	1	0.316	0.714	0.088

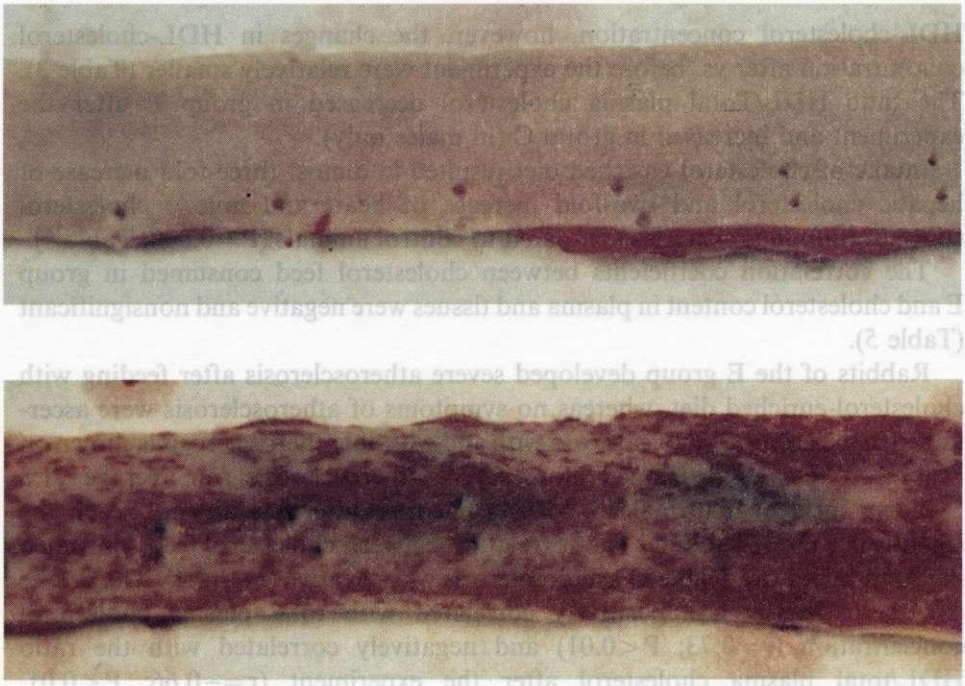

Figure 1. Typical aortas of rabbits without (a) and with (b) atherosclerosis

TABLE 5

Correlation coefficients between traits investigated (Experimental group n=22, Control group n=18)

	trait number									
	2	3	4	5	6	7	8	9	10	11
1. E	−.44	−.24	.20	.02	.45	.25	−.22	−.23	−.09	−.40
C	.02	.33	.39	.41	.44	.09	−.37	.11	.01	
2. E		.37	.39	.14	−.61*	−.36	.47	.26	.27	.43
C		.12	.48	.42	−.10	.19	.05	.09	−.11	
3. E			.20	.54*	−.14	−.79**	. 66**	.71**	.51*	.73**
C			.43	.60*	.44	−.37	.38	.16	.19	
4. E				.25	.43	.02	.31	.08	.30	.19
C				.52	.81**	.02	.25	.05	.35	
5. E					.04	−.36	.83**	.48	.23	.29
C					.31	.44	−.01	.23	.36	
6. E						.36	−.17	−.18	−.09	−.19
C						−.14	.26	.02	.44	
7. E							−.64**	−.46	−.48	−.66**
C							−.48	.02	.18	
8. E								.45	.36	.56*
C								.03	.04	
9. E									.47	.47
C									−.05	
10. E										.56*

Trait numbers

1. Feed consumed during the experiment
2. Total plasma cholesterol before the experiment
3. Total plasma cholesterol after the experiment
4. Plasma HDL-cholesterol before the experiment
5. Plasma HDL-cholesterol after the experiment
6. Ratio HDL/Total plasma cholesterol before the experiment
7. Ratio HDL/Total plasma cholesterol after the experiment
8. Liver cholesterol
9. Heart cholesterol
10. Muscle cholesterol
11. Atherosclerosis degree

DISCUSSION

As expected the rabbits fed with cholesterol-enriched diet, generally responded with significant elevation of cholesterol level in plasma and in all tissues investigated. This is consistent with results obtained by many authors on different species (Beynen et al., 1986; Grundy and Denecke, 1990; Jonnalagadda et al., 1993; Richard et al., 1990; Zhang et al., 1994). Rather unexpected however was the ascertained negative correlation between the amount of cholesterol feed consumed and cholesterol content in plasma, tissues and degree of atherosclerosis, as calculated on individual data in the group E. This would mean that consumption of greater amounts of cholesterol may not result in more atherosclerosis. The negative correlation may also be interpreted as a tendency in rabbits with a higher primary level of plasma total cholesterol to consume spontaneously less feed containing supplementary cholesterol. This may be regarded as a behavioural mechanism preventing diet induced atherosclerosis. The observed decrease of plasma total cholesterol and HDL-cholesterol in control group after the experimental period may be explained by possible seasonal variation in cholesterol level in animals, which was reported by some authors (e.g. Kristal-Boneh et al., 1993).

In our experiment the females demonstrated generally higher level of cholesterol especially in the group fed with cholesterol enriched diet and consequently more severe atherosclerosis. The gender differences in this respect seem to be very interesting and were confirmend by some authors. Van Ree et al. (1994) indicated that female mice had doubled serum cholesterol as compared to males and were more susceptible to develop atherosclerosis while on atherogenic diet. Similarily to our results Roberts et al. (1974) reported higher total cholesterol in female rabbits. Cobb et al. (1993) found higher HDL cholesterol in women. Apart of this the latter authors found a higher fall in HDL cholesterol in females, following the crossover from a low to high polyunsaturated to saturated fat diet. On the contrary, Lind et al. (1990) did not find any remarkable sex dependent differences with respect to HDL cholesterol in hyperlipidemic rabbits.

The highest increase of cholesterol content in the liver as compared to other tissues is not surprising as the accumulation of cholesterol from the diet takes places first of all in the liver and to a lesser degree in other tissues (Richard et al., 1990; Zhang et al., 1994). On the other hand in comparison of different organs and tissues in some animal species, liver did not contain the highest cholesterol level in all cases (Park et al., 1991). The liver cholesterol level in our C rabbits was comparable to that in hamsters with normal level of cholesterol in the plasma and in our E rabbits was comparable to that in hamsters with spontaneous high level of plasma cholesterol (Sicart et al., 1984).

Hypercholesterolemia is generally recognized as a risk factor for the atherosclerosis in several species (Tsuda et al., 1983; Remy,1993; van Ree et al., 1994). This was confirmed by positive and significant correlations between total plasma cholesterol and atherosclerosis degree in our experiment. Paigen et al. (1985) found much lower correlation ($r = 0.29$) between the total cholesterol level and susceptibility to lesions formation in the aortic wall in mice.

The results of the present experiment support the concept of the protective nature of the HDL-cholesterol as suggested by several authors for humans (Gordon et al., 1989) and other species (Liu et al., 1994). Of importance is the ratio HDL/total plasma cholesterol which was negatively correlated with the degree of atherosclerosis.

In conclusion, the diet induced atherosclerosis only roughly depends on cholesterol feed consumed as demonstrated by differences between the E and C groups and the negative correlation between amount of feed consumed and atherosclerosis degree in the E group. Taking into account the great individual variability in atherosclerosis degree and relationships between total plasma cholesterol, HDL cholesterol and atherosclerosis, for experiments on factors playing a role in diet induced atherosclerosis, we suggest to use animals with similar primary level of cholesterol.

ACKNOWLEDGEMENT

We are grateful to Mrs. Anna Stawicka for her care for the experimental animals.

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STRESZCZENIE

Wpływ diety wzbogaconej cholesterolem na poziom cholesterolu w osoczu krwi i tkankach oraz arteriosklerozę u młodych królików

Celem pracy było zbadanie zależności pomiędzy ilością spożytej paszy wzbogaconej cholesterolem a poziomem cholesterolu całkowitego i HDL-cholesterolu w osoczu krwi, tkankach (wątroba, serce, mięsień) oraz stopniem arteriosklerozy u królików. Ponadto celem doświadczenia było określenie optymalnych metod i uwarunkowań ilościowego wywoływania arteriosklerozy u królików dla badań nad rolą czynników emocjonalnych i behawioralnych w tym zakresie. Grupa

doświadczalna (E) była żywiona standardową paszą granulowaną (150 g/dzień), z dodatkiem 1 żółtka jaja kurzego oraz 1,5 g cholesterolu *in substantia* w ciągu 7 tygodni. Zwierzęta kontrolne (C) otrzymywały w tym samym okresie jedynie paszę standardową. Stopień arteriosklerozy określano w procentach powierzchni aorty ze zmianami sklerotycznymi. U królików z grupy (E) stwierdzono istotny wzrost poziomu cholesterolu całkowitego i HDL-cholesterolu w osoczu oraz cholesterolu w badanych tkankach, przy czym stosunkowo najwięcej wzrósł poziom cholesterolu całkowitego w osoczu, zaś zmniejszył się stosunek HDL:Cholesterol całkowity. Poziom cholesterolu całkowitego i HDL-cholesterolu był istotnie wyższy u samic w porównaniu z samcami. W grupie doświadczalnej poziom cholesterolu całkowitego w osoczu i stopień arteriosklerozy był ujemnie skorelowany z ilością spożytej paszy. Poziom cholesterolu całkowitego w osoczu krwi i tkankach po okresie żywienia paszą z cholesterolem był dodatnio skorelowany ze stopniem arteriosklerozy. Stopień arteriosklerozy był zależny od wzajemnych relacji HDL i cholesterolu całkowitego w osoczu krwi.