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INCREASED PLATELET AGGREGABILITY FOLLOWING AN ATHEROGENIC DIET IN RABBITS

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In atherosclerosis researches different animal models are used but the most common is the rabbit, because of the easy development of atherosclerotic lesions. Atherosclerosis is a multicellular process and platelets play an important role in atherogenesis. Excessive plasma lipids stimulate platelet aggregability and thus atherosclerosis development. The effects of an atherogenic diet on lipid status, abdominal aorta wall structure, and platelet aggregability were studied in rabbits. Adult male Chinchilla rabbits were fed an atherogenic diet (2% edible oil solution of crystaline cholesterol at 8 mg cholesterol per kg b.wt.daily) for 8 weeks. Plasma lipid levels (triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, and LDL/HDL ratio) and platelet aggregability were then measured and compared to the values obtained for control animals. Histological analysis showed atherogenic changes in the abdominal aorta wall of the experimental animals. Total plasma cholesterol level, plasma LDL-cholesterol, and LDL/HDL ratio were significantly increased compared to the controls ($p<0.01$), as well as triglyceride and HDL-cholesterol levels ($p<0.05$). Platelet aggregability was also significantly increased ($p<0.01$) after the atherogenic diet in comparison with controls when ADP, at concentrations both of 5 and 10 μmol/L, was used as the aggregant. Disaggregation was common in control animals but this phenomenon was not recorded in the experimental animals. It can be concluded that an 8-week atherogenic diet in rabbits induced marked changes of the lipid parameters, provoked atherogenic changes in the abdominal aorta wall and increased platelet aggregability.

Key words: hyperlipoproteinemia, platelet aggregation, atherosclerosis, rabbits

INTRODUCTION

Quite a long sequence of specific events in the blood vessel wall precede atherosclerosis. The crucial incident is endothelial damage, which is the *conditio sine qua non* for pathologic interactions of blood cells and the blood vessel wall. A pro-thrombotic and pro-inflammatory state of the endothelium folowing vasoconstriction, activation and adhesion of platelets and white blood cells (WBCs), formation of fibrin deposits in the vessel wall and additional inflammatory interactions as a consequence of the co-aggregation of platelets and WBCs. The platelet CD40-ligand binds to CD40-receptors on endothelial cells, monocytes and B-cell membranes (Becker et al., 2000). Platelet activation and aggregation release bio-reactive molecules, such as platelet derived growth factor (PDGF) that initiates further cellular events in the vessel wall (Ross *et al.,* 1974; Grotendorst et al., 1982; Heldin and Westermark, 1990). Thus, platelet aggregation is an inevitable event in atherosclerosis development. The most common risk factor for atherosclerosis development is type II hyperlipoproteinemia (Brown and Goldstein, 1986). Platelet aggregatability is increased in hyperlipoproteinemia, because the extra lipid peroxidation products that then appear additionally damage the endothelium (Halliwell, 1995; Halliwell and Chirico, 1993). Platelet aggregatability in hyperlipoproteinemia is increased also in other species than man e.g. in rabbits (Velkovski, 1995). Rabbits are commonly used in accurate animal models for atherosclerosis research. This model was used for the first time at the beginning of the last century (Ignatowsky, 1908). The objective of the present study was to demonstrate the platelet aggregability patterns in hyperlipoproteinemia, using this animal model.

MATERIAL AND METHODS

Adult male Chinchilla rabbits (2.8 - 3.6 kg), bred at the Institute of Medical Physiology, Belgrade, were used. They were kept under a 12/12 hr light-dark cycle, at 22±2 ^{o}C and fed commercial rabbit chow ("Veterinarski Zavod", Zemun, Yugoslavia). Food and water were available *ad libitum*. The control animals (n = 16) were treated continued under this regime. The experimental animals (n = 16) in the same manner but they were also given intragastrically an atherogenic solution (20 g/L crystaline cholesterol in sunflower oil) at 8 mg cholesterol / kg body weight per day. Atherogenic diet was given for 8 weeks before platelet aggregability was measured.

Lipid status

At the end of the 8th week of the treatment, 3 mL blood from the marginal ear vein was collected in heparinized tubes. Triglycerides (TG), total cholesterol (CH) and HDL-cholesterol were determined in plasma using commercial enzymatic colorimetric kits ("Zastava Yugomedica", Kragujevac - Yugoslavia and "Lightning Instrumentation", Lausanne - Switzerland). Plasma levels of LDL-cholesterol were calculated using Friedewald's equation:

LDL (mmol/L) = CH (mmol/L) - HDL (mmol/L) - TG (mmol/L)/2.2

The LDL/HDL plasma ratio was also calculated.

Platelet aggregability

After the 8-week treatment, the left jugular vein was prepared under total i.v. anesthesia (thiopentone-sodium, Thiopentone Injection BP, HEFA-Frenon

Arzneimittel, 30 mg/kg). A plastic 16 G cannula was placed centrally in the jugular vein and 6 mL blood was drawn with a plastic syringe containing 0.67 mL 3.8% sodium-citrate. The blood was carefully transferred into a plastic tube and left at room temperature for 2 hours. Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by spinning the blood sample at 800 rpm for 10 min and 3000 rpm for 10 min, respectively. Platelet aggregability was measured in PRP as the percentage of maximum platelet aggregation using an aggregometer (Chrono-Log). Adenosine diphosphate (ADP) solutions (5 μmol/L and 10 μmol/L) were used as the aggregants.

Light microscopy

The animals were killed with a lethal dose of thiopentone-sodium. Abdominal aortas were excised, fixed in Bouins solution, embedded in paraffin and cut serially into 5 μm thick sections, which were stained with hematoxylin-eosin, and examined under a light microscope (Opton).

Statistical analyses

Lipid levels and platelet aggregation data obtained from both control and experimental groups were averaged and the standard deviation (SD) and error (SE) were calculated. The Mann Whitney U - test was used for statistical comparisons between groups. Values of P less than 0.05 were considered to indicate statistical significance of differences.

RESULTS

During the study the values for triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, and LDL/HDL ratio in the control animals remained within the normal ranges. In the experimental animals total cholesterol and LDL-cholesterol levels, as well as the LDL/HDL ratio increased with high statistical significance ($p<0.01$) in comparison with the controls. Triglycerides and HDL-cholesterol levels also increased significantly ($p<0.05$). Namely, triglycerides and HDL-cholesterol rose moderately, while total cholesterol and LDL-cholesterol rose four-fold and seven-fold respectively, the LDL/HDL ratio rose four-fold (Table 1).

Table 1. Lipid parameters in control animals and in experimental animals after the atherogenic diet (Mean ± SD)

	TG (mmol/L)	CH (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	LDL/HDL
C	1.01 ± 0.12	1.39 ± 0.11	0.56 ± 0.06	0.50 ± 0.08	1.05 ± 0.57
E	* 1.66 ± 0.26	*** 5.23 ± 0.67	* 0.80 ± 0.08	*** 3.68 ± 0.68	*** 4.32 ± 2.41

*: P less than 0.05 and ***: P less than 0.01, compared to the controls
C -controles, E experimentals after the diet

Platelet aggregability was in accordance with the lipid status changes and with the pathohistological pattern: Using ADP at 5 μmol/L platelet aggregability in the experimental animals (80.0%±3.4%) was significantly higher ($p<0.01$) compared to platelet aggregability in the controls. (46.2%±3.0%). When ADP at 10 μmol/L was used as the aggregant, platelet aggregability in the experimental animals (85.34%±3.7%) was also significantly higher ($p<0.01$) in comparison with the controls (57.8%±3.2%) (Figure 1). Disaggregation incidence was as high as 14 out of 16 (87.5%) in the controls with ADP at 5 μmol/L and 12 out of 16 (75.0%) when ADP was used at 10 μmol/L. Disaggregation was not recorded in the experimental animals (Figure 2).

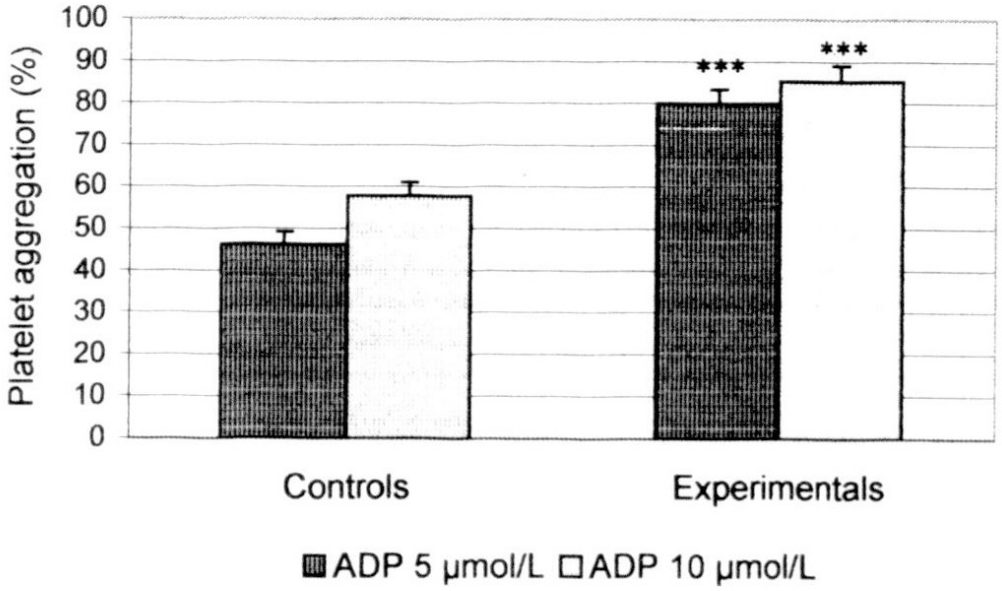

Figure 1. Platelet aggregation in control animals and in experimental animals after the atherogenic diet (Mean ± SE)

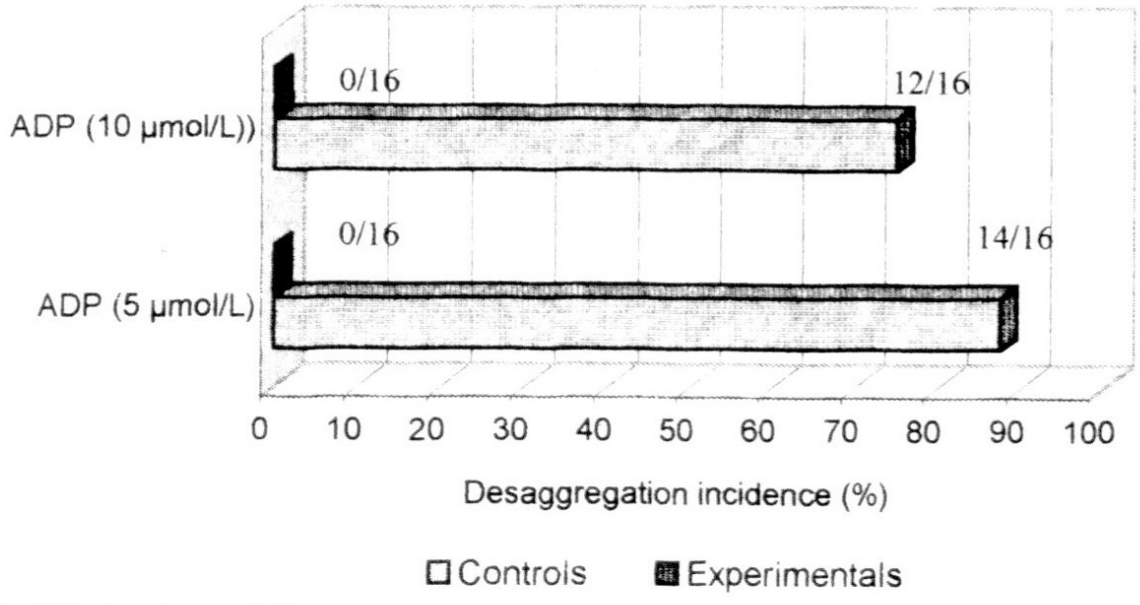

Figure 2. Incidence of the disaggregation phenomenon in control animals and in experimental animals after the atherogenic diet.

In the controls, the abdominal aorta wall showed a histological pattern typical for elastic arteries. Thus, the endothelium completely covered the intimal layer and there was no cell accumulation in the intima. The media consisted of several contractile phenotype SMC layers separated with distinctive elastic membranes. There were neither inflammatory nor any other pathohistologic changes in the adventitia (Figure 3). The abdominal aorta wall in the experimental animals was completely thickened and had a disturbed structure. The endothelium was partially damaged. The intima was thickened and macrophages and foam cell accumulations were present. The elastic membrane beneath the intima was wrinkled and fragmented. Medial layers were unclearly separated with elastic membranes that had a blurred structure. They also contained light areas from lipid accumulation and a remarkable number of secretory type SMCs (Figure 4).

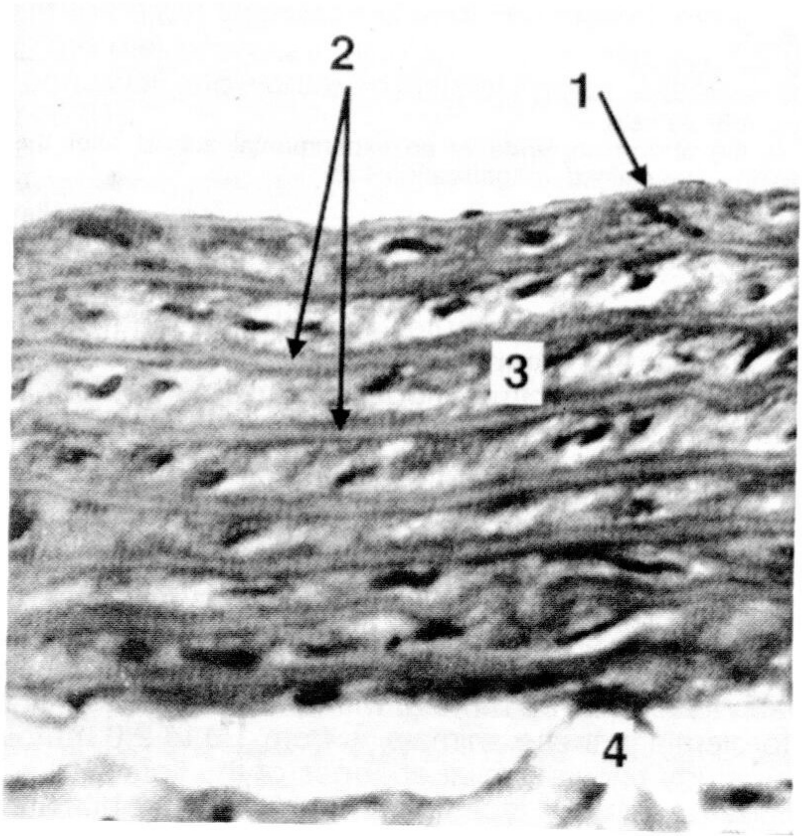

Figure 3. 1 - endothelium
2 - elastic membranes
3 - smooth muscle cells
Section of the abdominal aorta of a control animal (hematoxylin-eosin stained, magnification 128x)

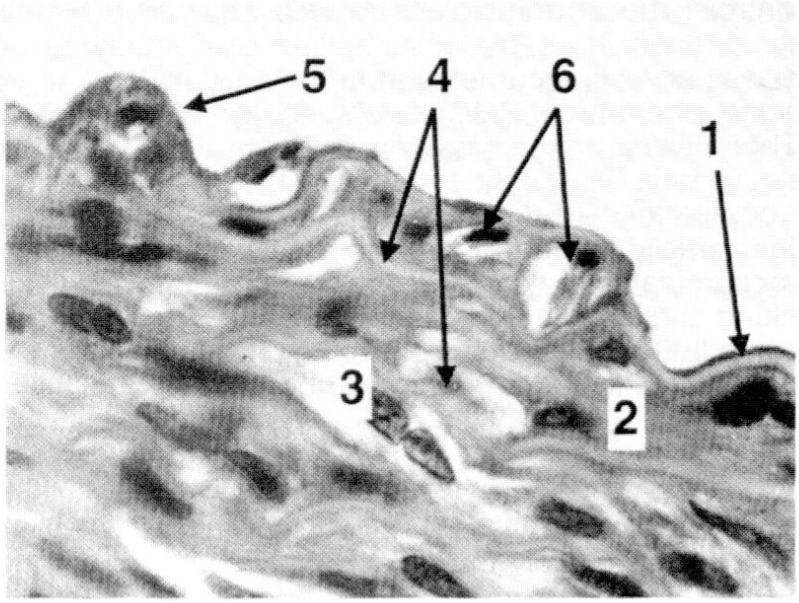

Figure 4. 1 - endothelium
2 - cell accumulations
3 - elastic membranes
4 - smooth muscle cells
Section of the abdominal aorta of an experimental animal after the atherogenic diet (hematoxylin-eosin stained, magnification 400x)

DISCUSSION

Atherosclerosis greatly contributes to morbidity and mortality in modern humans. Atherosclerosis is a complex multicellular event led by a very subtle and discrete mechanism. A good animal model is crucial for profound research of all the aspects of atherosclerosis including the molecular, early pathohistologic and discrete pathoanatomic changes, as well as possibilities for prevention and early treatment of atheromatic/atherosclerotic changes. Certain criteria were established for choosing the best animal model (Veselinovitch, 1988). Rabbits are most often used in experimental models of atherosclerosis because atherosclerotic changes may be easily provoked by a mild atherogenic diet. We demonstrated that only 2% cholesterol in oil could induce changes within 8 weeks. It has already been shown that after such a diet the plasma cholesterol level in the rabbit could reach as high as 38 mmol/L within 6 to 8 weeks, (Gross, 1992). The normal cholesterolemia in these animals, is from 1.3 to 2.0 mmol/L (Caisey and King, 1980). Our study shows similar changes of the lipid status. Besides these models, Watanabe Hereditary Hyperlipidemic (WHHL) rabbits are used enabling Brown and Goldstein to win the Nobel Prize for their discovery of LDL receptors (Brown and Goldstein, 1986). WHHL rabbits represent an excellent model for homozygous familial hypercholesterolemia in humans (Havel *et al.*, 1982; Buja *et al.*, 1983; Brown and Goldstein, 1986).

The most common places for early atheromatic changes in rabbits are the aortic arch and thoracic aorta. We have demonstrated that atheromatic changes

are evident after 8 weeks in the abdominal aorta wall, too. Spontaneous atherosclerotic changes in normal rabbits do not appear often, but when there are any, they resemble Monckeberg medial sclerosis in humans (Garbarsch *et al.*, 1970). Similar changes appear after an atherogenic diet and our results are in accordance with this. Regression of atherosclerotic lesions usually follows cessation of the atherogenic diet.

We have demonstrated that platelet aggregability rises significantly in cholesterol fed rabbits compared to the controls. In all experimental animals platelet aggregation was irreversible, while the incidence of disaggregation was high in the controls. These findings confirm the fact that ADP does not cause secondary, irreversible aggregation in healthy rabbits (Kinlough-Rathbone et al., 1983).

In hyperlipidemia, vascular production of different reactive species is increased due to lipid peroxidation (Kojda and Harrison, 1999). In recent years it has been shown that free radicals produced during lipid peroxidation directly stimulate platelet arachidonic metabolism in a similar manner to phospholipase A_2 (Pratico *et al.* 1992; Iuliano *et al.*, 1994). Beside lipid peroxides, native LDL may also directly stimulate atherogenetic events (Ozer *et al.*, 1995). This supports our expectation that platelet aggregatability should be increased in hyperlipidemia.

Finally, we can conclude that the atherogenic diet in rabbits led to statistically highly significant ($p<0.01$) increases of total cholesterol and LDL levels, and the LDL/HDL ratio. The diet provoked histologically proved atheromatic changes in the abdominal aorta wall and increased platelet aggregatability with high statistical significance ($p<0.01$).

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POVEĆANA AGREGABILNOST TROMBOCITA POSLE ATEROGENE DIJETE U KUNIĆA

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SADRŽAJ

U istrživanjima ateroskleroze korišćeni su razlčiti animalni modeli, ali najčešće korišćene životinje su kunići zbog toga što lako razvijaju aterosklerozne lezije. Ateroskleroza je multicelularni proces i trombociti igraju važnu ulogu u aterogenezi. Prekomerna koncentracija lipida u plazmi izaziva povećanu agregaciju trombocita i na taj način pospešuje razvoj ateroskleroze. Istraživali smo efekte aterogene dijete na lipidni status, promene na abdominalnoj aorti i agregabilnost trombocita u kunića. Odrasli mužjaci Činčila kunića bili su podvrgnuti aterogenoj dijeti koja se sastojala od 2% rastvora kristalnog holesterola u jestivom ulju u toku 8 nedelja. Dnevna doza holesterola bila je 8 mg/kg telesne mase. Po završetku dijete, određivane su koncentracije triglicerida, ukupnog holesterola, LDL holesterola i HDL holesterola, odnos LDL/HDL, kao i agregabilnost trombocita. Vrednosti su poređene sa kontrolnim životinjama. Histološka analiza je pokazala aterogene promene u zidu abdominalne aorte eksprimentalnih životinja. Koncentracije ukupnog i LDL holesterola u plazmi, kao i odnos LDL/HDL bili su visoko statistički značajno povećane ($p<0,01$). Koncentracije triglicerida i HDL-holesterola u plazmi takođe su bile statistički značajno povećane ($p<0,05$). Agregabilnost trombocita bila je statistički visoko značajno povećana ($p<0,01$) posle aterogene dijete, bez obzira na to da li je agregant ADP korišćen u koncentraciji 5 ili 10 μmol/L. Fenomen dezagregacije bio je uobičajen za kontrolne životinje, ali nije registrovan kod eksperimentalnih životinja. Može se zakljuciti da je osmonedeljna aterogena dijeta u kunića izazvala značajne promene lipidnih varijabli, izazvala nastanak aterogenih promena u zidu abdominalne aorte i povećala agregabilnost trombocita.