

## EFFECTS OF DIFFERENT DIETARY FATTY ACID SUPPLEMENTS UPON LIPOPROTEIN METABOLISM AND LIPID PEROXIDES PRODUCTION IN HYPERLIPIDEMIC RATS

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**Abstract:** It has been well documented that hypercholesterolemia represents both a common and a dominant, although non-obligatory, risk factor in the progression of atherosclerosis. Research was conducted upon experimentally induced hyperlipidemic animals by means of a custom-tailored atherogenic diet. Cell susceptibility to nonenzyme-induced oxidative stress appears to be influenced by membrane fatty acid composition. This study was undertaken to determine whether differences in lipid peroxidation in steady-state and induced lipid peroxidation is a result of a different fatty acid supplementation.

Adult *Wistar* strain rats of male gender were exposed to an atherogenic diet for a period of 160 days, before randomization into 6 dietary groups with different intragastral oil supplementation. Lipid peroxidation products were measured in 2.5% (w/v) of fresh liver homogenates (Tris-HCl, pH 7.4), by the assay of a thiobarbituric acid reactive substances (TBARS) formation using the procedures described by Okhawa (1979), including modifications (1989) in three different experimental conditions: steady-state (which corresponds to concentration of lipid peroxides *in vivo*), spontaneous and metal-stimulated lipid peroxidation. Results were expressed as nmol TBARS per g of liver homogenate, calculated from the absorbency at 532 nm, using TEP as an external standard. This study shows that prolonged atherogenic dietary treatment causes moderate hypercholesterolemia and enhanced hypertriglyceridemia (+34.1% and +114.8,  $p < 0.001$ , respectively). Despite the lowering effects of the lipoprotein profiles, resulting from a fatty acid supplementation, at the end of each supplementation period,  $\omega$ -6 fatty acids (soybean and corn oil) revealed an enhancement in the production of lipid peroxi-

des (TBARS formation) measured in steady-state levels (+22.9%,  $p < 0.05$  and +22.6%,  $p < 0.05$ , respectively). When liver homogenates were exposed to  $\text{Fe}^{2+}$  and ascorbic acid-induced oxidative stress, lipid peroxidation (LPO) was enhanced in the group treated with soybean oil ( $\omega$ -6) and fish oil ( $\omega$ -3; +48.4 %,  $p < 0.001$  and +44.1%,  $p < 0.001$ , respectively), but not in the group receiving corn oil. The achieved results support the hypothesis that the process of lipid peroxidation is not always in correlation with the number of double bonds in fatty acids esterified in phospholipid molecules. Consequently, it can be concluded that supplementation of unsaturated fatty acids, in the therapy of cardiovascular diseases, should include the administration of antioxidants, in order to prevent fatty acid decomposition in the case of oxidative insult.

**Kew words:** rats, hypercholesterolemia, lipoproteins, lipid peroxidation (LPO), inductive fero-ascorbate system,  $\omega$ -6,  $\omega$ -3 fatty acids, liver.

### Introduction

Alteration in lipoprotein metabolism in circulating blood plays an important role in the pathogenesis of atherosclerosis. The etiology of this progressive and degenerative disease, despite the influence of existing determinants (congenital hyperlipoproteinemias, metabolic hyperlipidemia, gender, age, arterial hypertension and diabetes; Bergeron and Havel, 1997; Fielding, 1997; Mc Gill, 1998; Hanna, 1998), includes the impact of lipid peroxides as metabolites which enhance the risk of atherosclerosis (Rankin *et al.*, 1991; Reaven *et al.*, 1993; Reaven and Witztum, 1996; Brown and Goldstein, 1989, according to McKenney and Hawkins, 2001). The latter are a product of the peroxidation of unsaturated fatty acids and their esters, triggered by activated oxygen species, transition metal ions and the breakdown of primary lipid hydroperoxides. Thus formed lipid peroxides are cytotoxic products which cause damage to and destruction of the cell's membrane apparatus (Hatefi and Hanstein, 1970; Hasselbach and Migala, 1975; Haliwell and Gutteridge, 1985).

There has been a considerable increase in scientific research over the last two decades into the potential role of lipid peroxidation and peroxidation products in the process of degeneration, associated with aging acceleration, and in the pathogenesis of several clinically significant diseases such as: Alzheimer's and Parkinson's diseases (neurodegenerative diseases), Amyloidosis, Amyotrophic lateral sclerosis, Cataracts, Diabetes Mellitus, Bloom syndrome and Fanconi's anaemia, including the two most common diseases: Cancer and Atherosclerosis (Plaa and Witschi, 1976; Burk, 1979; Litov *et al.*, 1981; Gee and Tapel, 1981; Prilipko *et al.*, 1982, 1983; Poli *et al.*, 1985; Janero, 1990, Esterbauer and Chesman, 1990). Namely, the experiments of Cosgrove *et al.* (1987) demonstrate that the sensitivity of endothelial membrane phospholipids to lipid peroxidation correlates with the unsaturation index (a measure based upon the concentration

and number of double bonds). In support of the above is the study of Hidgon *et al.* (2000), which suggests that a diet relatively rich in unsaturated fatty acids might cause controversial effects, as it causes pronounced cellular prooxidative activity, despite its favorable effect on lipoprotein profiles.

Scientific controversy over the potential role of nutritional factors in the prevention of atherosclerosis was both a challenge and motive to the authors in their effort to highlight the consequences of peroxidative decomposition of unsaturated fatty acids which yield reactive lipid peroxides as key factors in the development of this degenerative disease.

This study examines the degree of lipid peroxidation evaluated in the liver homogenates of rats with experimentally induced moderate hyperlipidemia, and evaluates lipid peroxidation products incubated in the presence and absence of a peroxidation initiator.

### *Materials and methods*

Four-month-old white *Wistar* strain rats of male gender ( $n = 72$ ), weighing  $277 \pm 30$  g. each at the start of the experiment were used in this experimental research. The animals were obtained from the animal facility of the Department of Physiology and Biochemistry of the Faculty of Natural Sciences and Mathematics, Skopje. Prior to the start of the experiment all the animals were fed a commercial pellet diet (Zemun, Republic of Serbia and Montenegro). Except for the fatty acid supplementation, all the rats (exclusive of the control group) received a custom-tailored atherogenic diet (a modification of the original ICN Atherogenic diet for Mice diet; Research Diets Cat. No. 900865) supplied by Coopens, International, B.V., Helmond, Holland. The commercial animal feed for rats contained approximately 1300 Cal/kg of diet. Bearing in mind that the required dose of feed is 10g of pelleted diet, specifically 13 Cal/100g body weight/day (according to the American Institute of Nutrition AIN-76; <http://www.-icnpharm.com>), the amount of calories received by the groups with atherogenic treatment was thus three times higher and amounted to 42.6 Cal/100 g body weight/day. In the supplementary period the rats were fed intragastrally 2.0 ml of oil/day for a period of 20 days and had *ad libitum* access to commercial feed and water throughout the experiment. Different oils (with the exception of the commercial soybean oil) were obtained from Sigma Chemicals Co.

**Experimental design** The rats were randomized in 6 groups of 12 animals each: (1) intact rats receiving commercial laboratory feed throughout the experiment (**Group K** – control animals); (2) rats receiving an atherogenic diet for 160 days, with no oil supplement (**Group A**); (3) rats receiving the same diet as the afore-mentioned, followed by intragastral supplementation with olive oil, for a period of 20 days (**Group B**); (4) rats treated with an atherogenic diet for a

period of 160 days, followed by intragastral corn oil supplementation for a period of 20 days (**Group C**); (5) rats exposed to an atherogenic diet for the same period of time as the previous groups, followed by fish oil supplementation (**Group D**) and (6) rats treated with an atherogenic diet supplemented with soybean oil (**Group E**).

The animals were sacrificed by decapitation, without sedative (due to possible lipolytic effects). All tissues were harvested between 10–11 a.m., except for those animals which died during the experiment. Lipoprotein levels in circulating blood were determined by means of diagnostic kits (enzyme-colorimetric test) at 20-day intervals. Blood samples were collected in tubes after an overnight fast of 12 hours. Lipid peroxidation products were measured in 2.5% (w/v) of liver homogenates, measuring the concentration of thiobarbituric acid reactive substances according to a method described by Okhawa, including modifications by Stroev and Makarova (1989). Dehydrated paraffin-treated fresh liver slices fixed in 10% neutral formalin were used for histological section analysis. The 5µm paraffin sections were treated with classical staining procedure for light-microscopy (Haematoxylin & Eosin).

Statistical data processing for lipoprotein profiles was performed using multiple analyses of variance for repeated measurements (MANOVA-Duncan's multiple range tests). Differences between groups with different numbers of animals were tested using a t-test for independent samples ( $p < 0.05$  value was considered significant).

## Results

***Cholesterol and triglycerides fluxes during atherogenic diet*** Considering the fact that all the animals were treated equally until day 160, when they were divided into different groups according to supplementation (of the same age and with approximately equal body mass), the average values presented in figures 1 and 2 respectively apply to all of the groups. There follows our analysis of lipoprotein levels until the start of the supplementation period for a duration of 160 days.

The results, as shown in Figure 1, indicate similar fluctuations of cholesterol and triglyceride values, with significant enhancement of concentrations throughout the atherogenic treatment in comparison with the control group. The positive coefficient of correlation ( $r = 0.7318$ ) reveals a significant reliance between the two parameters. Cholesterol concentrations measured every 20 days did not correlate with the function of time, regardless of the significant enhancement according to the baseline ( $r = 0,0727$ ). The obtained results, as per Figure 1, show a higher level of cholesterol concentration on the 40th day of treatment, followed by significant enhancement of the triglyceride level (40:k +68.7% for cholesterol, 40:k' +65.81% for TG). However, the TG level reached its peak at the end of

the atherogenic treatment (day 160), increasing by +114.8% with respect to the control, or specifically by +17.2% according to the level attained on day 40. In general terms the data acquired during the atherogenic dietary treatment (for a period of 160 days) showed moderate hypercholesterolemia (+34.1%) and enhanced hypertriglyceridemia (+114.8%). The group exposed to an extended atherogenic diet (160+20) did not reveal significant alterations in the two measured parameters as compared to day 160 of the dietary treatment (a:160 n.s.; a':160 n.s.).

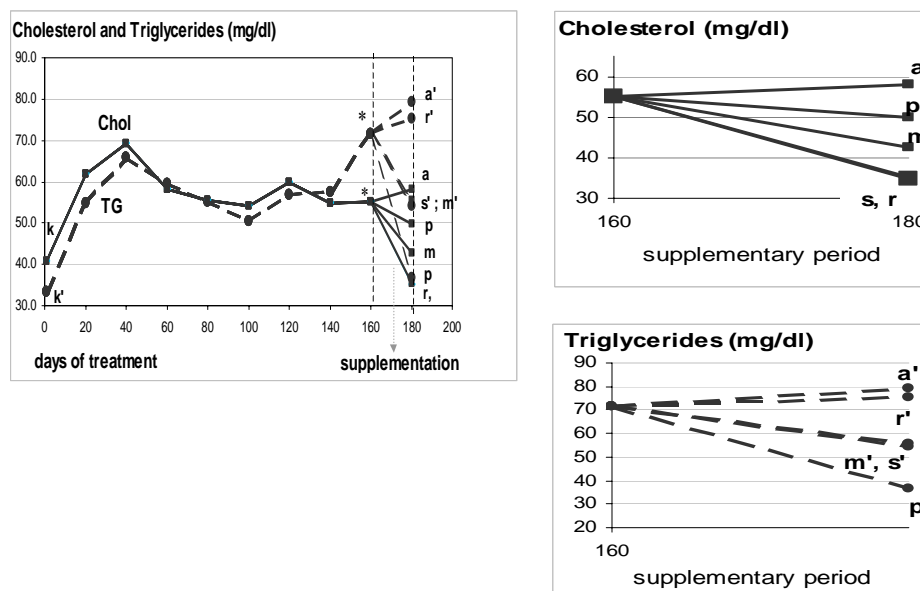


Figure. 1a

<i>cholesterol</i>		<i>%</i>		<i>triglycerides</i>		<i>%</i>	
20:k	p <sub>n</sub> 0.001 (+50.8)	100:k	p <sub>n</sub> 0.001 (+31.9)	20:k'	p <sub>n</sub> 0.001 (+64.9)	100:k'	p <sub>n</sub> 0.001 (+51.7)
40:k	p <sub>n</sub> 0.001 (+68.7)	120:k	p <sub>n</sub> 0.001 (+50.0)	40:k'	p <sub>n</sub> 0.001 (+98.0)	120:k'	p <sub>n</sub> 0.001 (+70.8)
60:k	p <sub>n</sub> 0.001 (+42.0)	140:k	p <sub>n</sub> 0.001 (+33.9)	60:k'	p <sub>n</sub> 0.001 (+78.8)	140:k'	p <sub>n</sub> 0.001 (+72.9)
80:k	p <sub>n</sub> 0.001 (+34.8)	160:k	p <sub>n</sub> 0.001 (+34.1)	80:k'	p <sub>n</sub> 0.001 (+66.0)	160:k'	p <sub>n</sub> 0.001 (+114.8)

<i>supplementary period with</i>		<i>cholesterol</i>		<i>supplementary period with</i>		<i>triglycerides</i>	
a:160	n.s.	r:160	p <sub>n</sub> 0.001 (-36.7)	a':160	n.s.	r':160	n.s.
a:k	p <sub>n</sub> 0.001 (+41.0)	r:k	n.s.	a':k'	p <sub>n</sub> 0.001 (+138.8)	r':k'	p <sub>n</sub> 0.001 (+125.1)
m:160	p <sub>n</sub> 0.005 (-22.8)	s:160	p <sub>n</sub> 0.001 (-36.1)	m':160	p <sub>n</sub> 0.025 (-23.9)	s':160	p <sub>n</sub> 0.0025 (-22.3)
m:k	n.s.	s:k	n.s.	m':k'	p <sub>n</sub> 0.001 (+65.0)	s':k'	p <sub>n</sub> 0.001 (+66.7)
p:160	n.s.			p':160	p <sub>n</sub> 0.001 (-48.3)		
p:k	p <sub>n</sub> 0.05 (+21.3)			p':k'	n.s.		

Figure and Table 1 – Time-course of cholesterol and triglycerides fluxes in male rats serum treated with atherogenic diet for a period of 160 days, followed by intragastral

supplementation with different types of oil; \* $p < 0.05$ . Fig. 1a. Concentrations of circulating cholesterol and triglycerides after supplementary period of 20 days.

Legend 1:  $k$  – control value (baseline) of cholesterol,  $k'$  – control value of triglycerides (TG);  $a$ ,  $a'$  – achieved values of cholesterol and triglycerides respectively, of animals treated with atherogenic diet for a period of 160 days (group A);  $m$ ,  $m'$  – achieved values of cholesterol and TG respectively, after the supplementary period with olive oil (group B);  $p$ ,  $p'$  – achieved values of cholesterol and TG respectively, after the supplementary period with corn oil (group C);  $r$ ,  $r'$  – achieved values of cholesterol and TG respectively, after the fish oil supplementation group D);  $s$ ,  $s'$  – achieved values of cholesterol and TG respectively, after the soybean oil supplementation (group E).

Граф. и Таб. 1 – Компаративен приказ на флукуациите на холестерол и триацилглицероли во серум кај машки стаорци третирани 160 дена со атерогенична диета по која следи 20. дневен интрагастрален третман со различни диетни масла; \* $p < 0.05$ . Граф. 1a. Концентрации на холестерол и TG по 20. дневна суплементација со различни масла.

Легенда 1:  $k$  – контролна вредност за холестерол,  $k'$  – контролна вредност за триацилглицероли;  $a$  – вредност за холестерол на 160. ден од третманот со атерогена диета;  $a'$  – вредност за TG на 160. ден од атерогената диета, група A),  $m$ ,  $m'$  – вредност за холестерол, односно TG по периодот на суплементација со маслиново масло (група B);  $p$ ,  $p'$  – вредност за холестерол (TG) по периодот на суплементација со пченкарно масло (група C);  $r$ ,  $r'$  – вредност за холестерол (TG) по периодот на суплементација со рибино масло (група D);  $s$ ,  $s'$  – вредност за холестерол (TG) по периодот на суплементација со соино масло (група E).

**Fatty acid supplementation** (Fig.1a) The dietary treatment with olive oil for a period of 20 days resulted in a successful decrease of total cholesterol concentration ( $m:160$ ,  $p < 0.005$ ,  $-22.8\%$ ) as well as a decrease in triglycerides ( $m':160$ ,  $p < 0.025$ ,  $-23.9\%$ ) by  $\frac{1}{4}$  of the value achieved before the supplementary period. A greater decrease of TG level was registered with the group treated with corn oil ( $\omega$ -6 fatty acids), achieving a similar value to the control group ( $p':k'$ , n.s.), but lower than the value achieved on day 160 by  $-48.3\%$  ( $p':160$ ,  $p < 0.001$ ). In contrast, circulating cholesterol values remained unchanged following supplementation. The intragastral supplementation of fish oil ( $\omega$ -3 fatty acids) provoked a decrease in cholesterol concentration ( $34.942 \text{ mg/dl} \pm 3.576$ ), achieving a value lower by  $36.7\%$  compared to day 160 ( $r:160$ ,  $p < 0.001$ ). Triglycerides levels remained unchanged ( $r':160$  n.s). With regard to the results measured in the group with soybean supplementation ( $\omega$ -6 fatty acids), the concentration of cholesterol was close to the level achieved by fish oil supplementation ( $35.261 \text{ mg/dl} \pm 8.004$ ), but lower than that achieved in groups receiving a corn and olive oil diet ( $-29\%$ ;  $-17.3\%$ , respectively). TG levels in the same experimental group decreased by  $\frac{1}{4}$  in comparison to day 160 ( $s':160$   $-22.3\%$ ,  $p < 0.0025$ ). Attained TG values amounted to  $55.424 \text{ mg/dl} \pm 15.568$ , and are approximately equal to that with olive oil supplementation ( $+2.2\%$ ), and

lower than that with fish oil (-26.3%), but increasingly higher than the concentrations with corn oil supplementation (+50%).

**Circulating lipoprotein fluxes during the atherogenic diet** Lipoprotein measurements (Fig. 2) indicate a considerable variance of the parameters measured with, primarily, a significant increase in the values attained in comparison with the baseline. The measured parameters relevant to the time of exposure show a low coefficient of correlation ( $r = -0.1112$  for *LDL* and  $r = -0.1511$  for *HDL*), with a moderate correlation between lipoproteins (*LDL:HDL*  $r = 0.5773$ ).

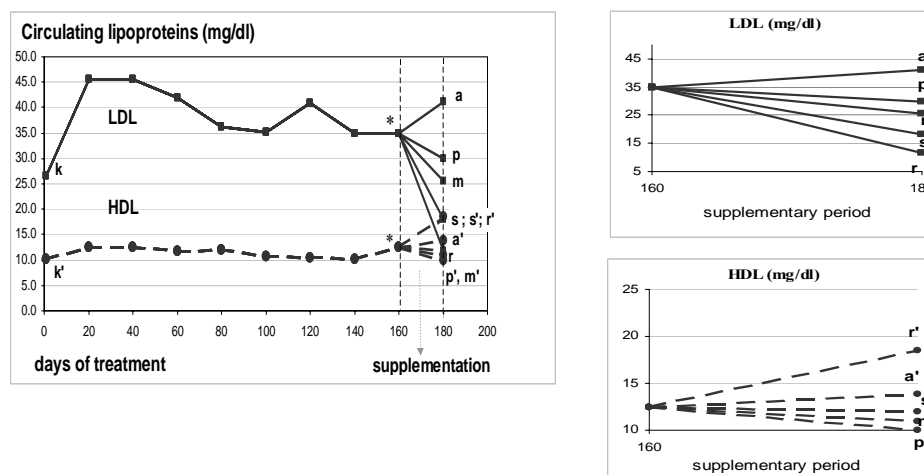


Figure 2a

<i>LDL</i> %		%		<i>HDL</i> %		%	
20:k	p<0.001 (+72.3)	100:k	p<0.001 (+32.3)	20:k'	p<0.001 (+21.0)	100:k'	n.s.
40:k	p<0.001 (+72.0)	120:k	p<0.001 (+54.11)	40:k'	p<0.001 (+21.7)	120:k'	n.s.
60:k	p<0.001 (+58.0)	140:k	p<0.001 (+32.0)	60:k'	p<0.001 (+13.7)	140:k'	n.s.
80:k	p<0.001 (+36.2)	160:k	p<0.0025 (+23.5)	80:k'	p<0.001 (+17.2)	160:k'	p<0.001 (+21.2)

<i>supplementary period with different fatty acids</i>				<i>supplementary period with different fatty acids</i>			
<i>LDL</i> %		%		<i>HDL</i> %		%	
a:160	n.s.	r:160	p<0.001 (-65.6)	a':160	n.s.	r':160	p<0.001 (+48.7)
a:k	p<0.001 (+58.3)	r:k	p<0.001 (-57.4)	a':k'	p<0.001 (+47.5)	r':k'	p<0.001 (+80.2)
m:160	p<0.001 (-42.8)	s:160	p<0.001 (-43.6)	m':160	n.s.	s':160	n.s.
m:k	p<0.01 (-29.4)	s:k	p<0.01 (-30.3)	m':k'	n.s.	s':k'	p<0.001 (+35.6)
p:160	n.s.			p':160	p<0.001 (-45.0)		
p:k	p<0.001 (+41.2)			p':k'	p<0.0025 (-33.4)		

Figure and Table 2 – Time-course of circulating lipoprotein fluxes in male rats treated with atherogenic diet for a period of 160 days, followed by intragastral supplementation with different types of oil; \* $p < 0.05$ . Fig. 2a. Lipoprotein concentration after supplementary period of 20 days.

Legend 2: k – control value (baseline) of LDL particles, k' – control value of *HDL* particles; a, a' – achieved values of LDL and HDL lipoproteins respectively, of animals treated with atherogenic diet for a period of 160 days (group A), m, m' – achieved values of LDL and HDL lipoproteins respectively, after the supplementary period with olive oil (group B); p, p' – achieved values of LDL and HDL lipoproteins respectively, after the supplementary period with corn oil (group C); r, r' – achieved values of LDL and HDL lipoproteins respectively, after the fish oil supplementation (group D); s, s' – achieved values of LDL and HDL lipoproteins respectively, after the soybean oil supplementation (group E).

Граф. и таб. 2 – Компаративен приказ на флукуациите на циркулирачките липопротеини во серум кај машки стаорци третирани 160 дена со атерогенична диета по која следи 20 дневен интрагастрален третман со различни диетни масла; \* $p < 0.05$ . Граф. 2а. Концентрации на липопротеините по 20 дневна суплементација со различни масла.

Легенда 2: k – контролна вредност за *LDL*, k' – контролна вредност за *HDL*; a, a' – вредности за *LDL* и *HDL* соодветно, на 160-тиот ден од третманот со атерогена диета (група А), m, m' – вредност за *LDL*, односно *HDL* по периодот на суплементација со маслиново масло (група В); p, p' – вредност за *LDL*, односно *HDL* по периодот на суплементација со пченкарно масло (група С); r, r' – вредност за *LDL*, односно *HDL* по периодот на суплементација со рибино масло (група D); s, s' – вредност за *LDL*, односно *HDL* по периодот на суплементација со соино масло (група E).

A prominent enhancement of *LDL* lipoprotein fraction was measured on the 20th day of the supplementation period (20:k +72.3%,  $p < 0.001$ ), maintaining a plateau for an additional 20 days, but with a tendency to decline, achieving a 24% higher value compared with the control (160:k +23.0%,  $p < 0.0025$ ). The latter is the lowest achieved value (34.978 mg/dl) following the atherogenic treatment (although the achieved concentration of TG was the highest measured since the baseline, +114.8%). With reference to *HDL* levels, the latter achieved a significant increase (from +17 to +21%), with a peak of +21.2% on day 160 (160 (a'): k',  $p < 0.001$ ). The *HDL* fraction as a percentage of the total cholesterol amounted to 25.06% at the start of the experiment, reaching 22.58% at the end of the supplementary period (%  $HDL_{160} = 22.58\%$ ).

In the group with extended atherogenic treatment (160+20), there was established a significant enhancement of *LDL* levels (a:160 +25.3%,  $p < 0.001$ ), achieving high value equal to that on days 60 and 120, respectively, but 50% higher than the control group (a:k +54.7%,  $p < 0.001$ ). The coefficient of correlation indicates a moderate correlation between the two measured parameters ( $r = 0.5466$ ).

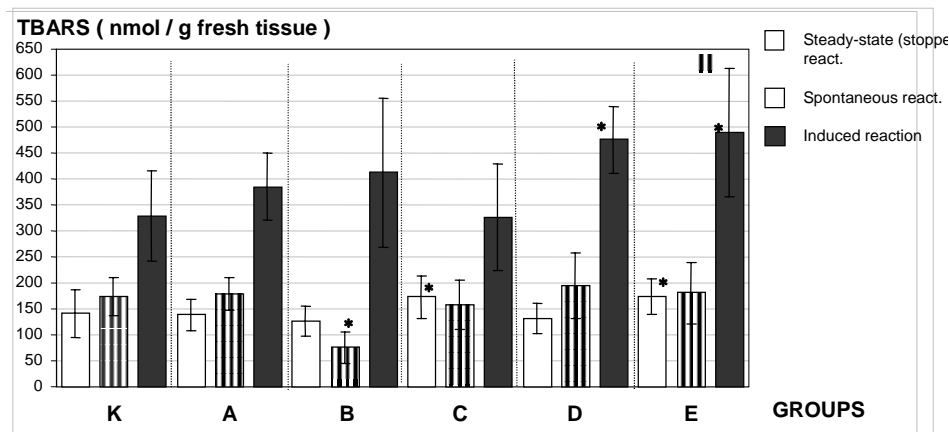
***Circulating lipoprotein fluxes during the supplementary period*** (Fig. 2a) In the group with intragastral supplementation with olive oil, there was established an approximately 50% decrease in the *LDL* fraction (m:160 -42.8 %,  $p < 0.001$ ), achieving a value of 17.8433 mg/dl. Despite *LDL* levels, *HDL* lipoproteins remained unchanged, with a similar value to the control group (m':160



n.s.). The *HDL* fraction, as a percentage of the total cholesterol, showed an insignificant alteration in comparison with the start of the experiment (25% of total cholesterol). A moderate positive correlation was established between the two parameters (*LDL* and *HDL*), expressed by the coefficient of correlation, which was statistically significant ( $r = 0.5635$ ). In the animals treated with corn oil, the *LDL* fraction remained unchanged relative to day 160 (p: 160 n.s.). The corn oil administration revealed a negative side-effect upon *HDL* levels, reducing it by 45%, which represents the lowest value as a percentage of total cholesterol (with a 13% share of total cholesterol concentration). The intragastral fish oil administration over a period of 20 days provoked a rapid decrease in *LDL* levels of ca. 2/3 (r:160, -65.6%), or more precisely a decrease of 57% according to the baseline (r:k,  $p < 0.001$ ). The *LDL* values of lipoprotein fraction achieved at the end of the supplementary period is even lower than the achieved *HDL* fraction, amounting to 11.635 mg/dl (*HDL* = 18.538 mg/dl). The superiority of the administered fish oil is attributed to the high yield of *HDL* lipoproteins, which increased to 48.7 % in comparison to day 160 (r':160,  $p < 0.001$ ), by 53.05% of total cholesterol (the highest measured value compared with the groups exposed to dietary treatment). A moderately negative correlation was established between the two measured parameters (*LDL:HDL*,  $r = -0.6267$ ). Consequently, the *LDL* fraction in the group receiving an additional soybean oil diet (for 20 days), achieved 18.47 mg/dl, which is 47% less than the value attained on day 160 (s:160,  $p < 0.001$ ). The latter value was insignificant as compared to the value achieved in the group treated with olive oil, which represents a higher yield in contrast to the group with fish oil supplementation (+64%), but lower than the value attained in the group with the corn oil diet (-51%). The *HDL* level in the same group of animals was insignificant (s':160 n.s.) both prior to and following the supplementary period (20 days), maintaining a plateau of 34.11% of total cholesterol.

***Lipid peroxide production in groups of animals treated with varied fatty acid supplementation*** Figure 3 presents the results of the lipid peroxide values in liver homogenates, measured as thiobarbituric reactive substances (TBARS or MDA – equivalent). Namely, the first histogram (i) represents a steady-state concentration which corresponds to lipid peroxide formation *in vivo*; histogram (ii) marks the reaction of spontaneous oxidation of liver homogenate (with an incubation time of 20 minutes), while the third bar (iii) represents the generated or induced reaction of lipid peroxidation triggered by the ferro-ascorbate system. Based on the obtained results of steady-state levels of lipid peroxides (i) it is possible to assess that groups with corn oil and soybean supplementation marked a 22% increase in comparison with the untreated group (C:K +22.6%, E:K, +22.9%,  $p < 0.05$ ). Lipid peroxide levels, measured in the same type of incubation, were insignificant in groups without supplementation and in groups receiving olive and fish oil. Spontaneous oxidation of liver homogenates of male rats (ii) showed a significant decrease only in the group treated with

olive oil (B:K -56.6%,  $p < 0.001$ ). Lipid peroxide formation in Group A, treated with saturated dietary fatty acids, remained unchanged throughout the different protocols of incubation. When homogenates were exposed to a peroxidation initiator ( $\text{Fe}^{2+}$ -ascorbic acid induced oxidative stress), lipid peroxidation products were enhanced by 44% in the group with fish oil supplementation (D:K,  $p < 0.001$ ) and by 48% in the group treated with soybean oil (E:K,  $p < 0.001$ ).



**TBARS (nmol/g fresh tissue) HEPAR / rats of male gender**

	stopped reaction	spontaneous reaction	induced reaction
	%	%	%
A:K	n.s.	A:K n.s.	A:K n.s.
B:K	n.s.	B:K $p < 0.001$ (-56.6)	B:K n.s.
C:K	$p < 0.05$ (+22.6)	C:K n.s.	C:K n.s.
D:K	n.s.	D:K n.s.	D:K $p < 0.001$ (+44.1)
E:K	$p < 0.05$ (+22.9)	E:K n.s.	E:K $p < 0.001$ (+48.4)

Figure and Table 3 – TBARS formation in liver homogenates of male rats treated with different dietary fatty acid supplement (i) histogram represents real concentration of lipid peroxides in tissue sample without incubation of probe (steady-state reaction); (ii) histogram represents concentration of lipid peroxides after incubation of probe: either without (spontaneous) or (iii) with  $4 \times 10^{-5}$  M  $\text{Fe}^{2+}$  -2.6 mM ascorbic acid (stimulated or generated lipid peroxidation). Incubation was performed for 20 minutes at  $37^\circ\text{C}$  under air. K – control value, \*significant changes in TBARS concentration compared with control group,  $p < 0.050$ ;

Legend 3: K – control group of rats (group 1); A – group of animals receiving atherogenic diet for 160 days (group 2); B – group of rats receiving atherogenic diet followed by intragastral supplementation with olive oil (group 3); C – group of rats receiving atherogenic diet followed by intragastral supplementation with corn oil (group 4); D – group of rats receiving atherogenic diet followed by intragastral supplementation with fish oil (group 5); E – group of rats receiving atherogenic diet followed by intragastral supplementation with soybean oil (group 6).

Граф. и Таб. 3 – TBARS продукција во хомогенат од хепар кај машки стаорци третирани со различен маслен суплемент. (i) хистограмски столб – количество на липидни пероксиди добиени без инкубација на системот (steady-state или стопирана реакција); (ii) хистограмски столб – количество на липидни пероксиди добиени по инкубација: 20 минутна аерација на системот (спонтанa реакција) како и (iii) хистограмски столб – количество на липидни пероксиди добиени при индуцирана реакција со фero-аскорбатен систем  $4 \cdot 10^{-5}$  M  $Fe^{2+}$  – 2.6 mM AA (стимулирана липидна пероксидација); k – контрола, \*сигнификантни промени во концентрацијата на TBARS во компарација со соодветната контрола,  $p < 0.05$ .

Легенда 3: K – контролни стаорци (група 1); A – група стаорци третирани само со атерогена диета во период од 160 дена; B – стаорци третирани 160 дена со атерогена диета + 20 дена интрагастрален третман со маслиново масло (група 3); C – стаорци третирани 160 дена со атерогена диета + 20 дена интрагастрален третман со пченкарно масло (група 4); D – стаорци третирани 160 дена со атерогена диета + 20 дена интрагастрален третман со рибино масло (група 5); E – стаорци третирани 160 дена со атерогена диета + 20 дена интрагастрален третман со соино масло (група 6).

***Histopathological analysis of liver tissue samples under chronic exposure to atherogenic diet supplemented by soybean oil*** Histologic analysis was conducted as a result of the established enhancement of steady-state levels of lipid peroxides in the group, responsible for the disruption and disintegration of the cell, which can be seen in the histological section.

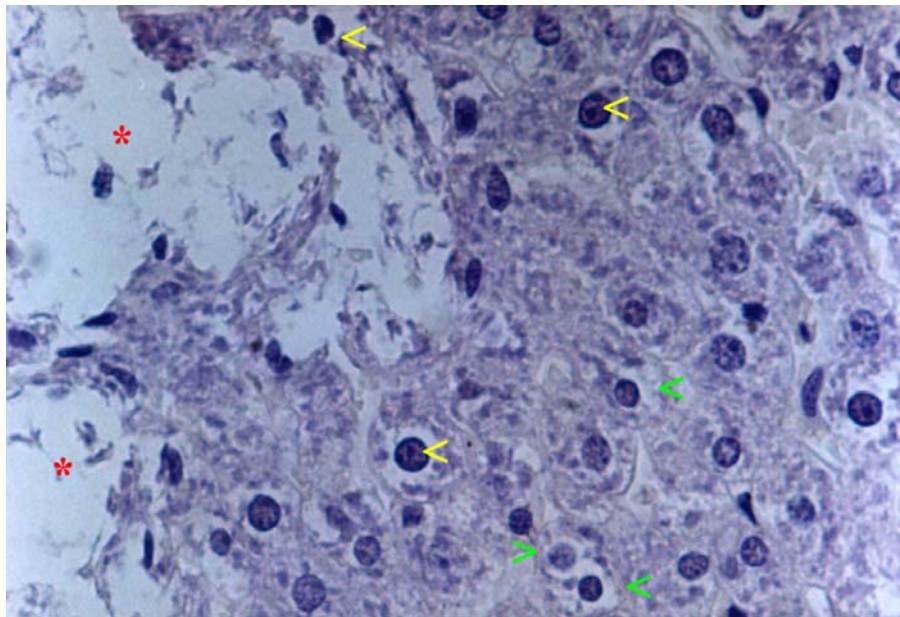


Figure 4 – Extensive necrosis (\*asterix) in liver parenchyma of hyperlipidemic rats of male gender (160 days of treatment with atherogenic diet) followed by 20 days soybean

oil therapy. Detritus containing necrotic areas are surrounded by hepatocytes with picnotic nuclei. Mild lymphocyte infiltration is evident; H&E  $\times$  400 (yellow arrow – picnotic nuclei; green arrow – lymphocytes).

Слика 4 – Масовна некроза на хепаталниот паренхим (\*свездички) кај хиперлипидемични машки стаорци (160 дена атероген третман) под 20-дневна орална суплементација со соино масло. Некротичниот регион е исполнет со детритус и опкружен со изумрени хеатоцити со пикнотични јадра. Присутна е слаба лимфоцитна инфилтрација; H&E  $\times$  400 (жолти стрелки – пикнотични јадра; зелени стрелки – лимфоцити).

At this stage of tissue damage, a total retreat of lipid steatosis was marked in hepatocytes, which was a typical reaction for the group undergoing atherogenic treatment (Dimitrova, 2002). However, the above analysis indicates clear morphological signs of focal and extensive hepatic necrosis, often present in rats of male gender (Fig. 4).

### Discussion

Summarizing the results of the measured lipid parameters of all the animal groups during the period of the atherogenic diet, it can be assumed that the applied diet caused moderate hypercholesterolemia (160:  $k+34.1\%$ ) and enhanced triglyceridemia (TG, 160:  $k'+114.8\%$ , Fig. 1). A prominent enhancement of the *LDL* lipoprotein fraction was measured on the 20th day of the supplementation period (20:  $k+72.3\%$ ,  $p < 0.001$ ), maintaining a plateau for an additional 20 days, but with a tendency of decline, achieving a 24% higher value compared with the control (160:  $k+23.0\%$ ,  $p < 0.0025$ , Fig. 2). The latter is the lowest achieved value (34.978 mg/dl, Fig. 2) following the atherogenic treatment (although the achieved concentration of TG was the highest measured since the baseline,  $+114.8\%$ ). The coefficient of correlation between total cholesterol flux and *LDL* lipoprotein fraction in the groups of rats under atherogenic treatment showed a high ratio of correlation ( $r = 0.9443$ ), which indicates that the change of flux in one of the parameters consequently implies similar changes in the other parameter. With reference to *HDL* levels, the latter achieved a significant increase (from  $+17$  to  $+21\%$ ), with a peak of  $+21.2\%$  on day 160 (160 (a'):  $k'$ ,  $p < 0.001$ ). The *HDL* fraction as a percentage of total cholesterol amounted to 25.06% at the start of the experiment, reaching 22.58% at the end of the supplementary period. ( $\% HDL_{160} = 22.58\%$ ). A moderate coefficient of correlation was registered between the analyzed parameters (*HDL:LDL*,  $r = 0.5773$ ).

The above results concerning the moderate cholesterol levels established (following atherogenic treatment) correlate with the research of Smith *et al.* (1998), which suggests that the relative stability of cholesterol levels in circulation (an increase of ca. 50% in comparison with the baseline) is a result of the

different mechanisms of metabolizing of chronic exogenous cholesterol influx vis-à-vis the metabolizing process in humans and rabbits. According to Schaefer (1997), the rats' response to dietary cholesterol provokes a suppression of its synthesis and enhances biliary acid production, which provides for the stability in its concentration. The relatively long time-course of provoking continuous hyperlipidemia in the rats supports the hypothesis that the liver capacity in the process of metabolism of cholesterol results in a lesser susceptibility to dietary cholesterol (Hassel, 1998). In conformity with the above is the frequency of lipoprotein concentrations achieved, which does not alter linearly with cholesterol flux in liver tissue (Fig. 1 and Fig. 2).

Following the intragastral fatty acid supplementation over a period of 20 days, fish and soybean oil demonstrated the best effects in reducing the levels of circulating cholesterol (r, s: 160 – 36.7%), while the olive oil supplementation achieved a lesser effect (m: 160 – 22.8%, Fig. 1a). The decrease of total cholesterol, as a consequence of dietary supplementation with different types of edible oil, resulted in lower *LDL* levels. The administration of fish oil resulted in *LDL* reduction superiority (r: 160 – 65.6%; Fig. 2a and Tab.2). In support of the presented results are the studies by Hanna, (1998), Nenster and Drevon (1996), Chan, S. *et al.* (1998) in which the latter decreases in *LDL* are explained by the fact that reduction of TG and cholesterol levels are the consequence of the suppressive effect of fish oil upon *VLDL* production and apoB protein synthesis in the liver. The effects of fish oil administration in lowering the *LDL* fraction were similar to the effect caused by gemfibrosyl.

Although the corn oil supplementation did not alter cholesterol concentrations, it showed, in contrast, the best effects in reducing TG levels, reducing the TG value by half (p': 160 – 48.5%, Fig. 1a). Equal effects were registered in the olive and soybean oil supplementation (m': 160 – 23.95; s': 160 – 22.3%, Fig. and Tab. 1a), versus the fish oil diet, which did not cause a significant alteration (r': 160 n.s.).

The results in the present study which refer to the influence of dietary supplementation with edible oils on TG levels, correspond to the similar studies by Chan *et al.* (1998). Hence, the above research suggests that hydrolysis of lipoprotein particles rich in TG is faster in those particles which contain short-chain unsaturated fatty acids, whereas triglycerides which contain  $\omega$ -3 polyunsaturated fatty acids (e.g. fish oil) hydrolyzed at a lower pace in contrast to the TG which contain  $\omega$ -6 polyunsaturated fatty acids (e.g. corn and soybean oils). It is assumed therefore that the greater solubility of  $\omega$ -6 fatty acids contained in the surface of emulsified particles makes them susceptible to easy breakdown and rapid elimination of *remnant particles* by the enzymatic activity of lipoprotein lipase. Differences in the registered values of TG levels between corn and soybean oil (despite the fact that both oils are of the same group according to their respective fatty acid composition), are probably a result of the different

percentages of their fatty acid composition. Namely, the presence of 7% of linoleic acid (LNA), as an  $\omega$ -6 fatty acid, implies a faster hydrolysis of those TG which contain this type of oil. In contrast, soybean oil contains an approximately equal percentage of ALA ( $\omega$ -3,  $\alpha$ -linolenic acid) and 9% of highly saturated palmitinic acid (according to Guy Inchbald, 29/09/00, <http://www.queehill.demon.co.uk/seedoils>). Bearing in mind the above findings concerning triglycerides and cholesterol, it may be assumed that the intensive intravascular catabolisation of *VLDL* in the group under corn oil administration (achieved low levels of TG in circulating blood) provoked a high *LDL* concentration.

The superiority of the administered fish oil is attributed to the high yield of *HDL* lipoproteins, which increased to 48.7 % in comparison to day 160 ( $r$ :160,  $p < 0.001$ , Fig. 2a, Tab. 2), by 53.05% of total cholesterol (the highest measured value among the groups exposed to dietary treatment). The *HDL* level in the group treated with soybean oil was insignificant ( $s$ :160 n.s.) both prior to and following the supplementary period (20 days), maintaining a plateau of 34.11% of total cholesterol, while corn oil supplementation led to a reduction by 45%. In support of the above are the results expressed in the research by Grundy *et al.*, 1986, 1990; Matsson *et al.*, 1985, (according to Reaven 1993), by which the replacement of dietary polyunsaturated with monounsaturated fatty acids is recommended, due to the fact that the latter type of oil, by reducing total cholesterol, does not affect the *HDL* levels.

Discrepancies in analyzed references of the effects of unsaturated dietary fatty acids upon lipid peroxides production (LPO), as well as the fact that the greater number of scientific analyses refer to the mutual effect of different types of fatty acids (except for lipid peroxide oxidation products), was the reason for focusing this research on the potential instability of fatty acids with different numbers of double bonds (Aust *et al.*, 1982; Bast *et al.*, 1986). The impact of transitional metals on lipid peroxide production (Magnusson *et al.*, 1994; Ehrenwald *et al.*, 1994; Ehrenwald i Fon, 1996; Sempos *et al.*, 1994, according to Stocker, 1994) was the reason why Fe-ion was used as the generator of the LPO process, thus monitoring the stability of the dietary fatty acids administered.

Figure 3 presents the results of the lipid peroxide values in liver homogenates, measured as thiobarbituric reactive substances (TBARS or MDA – equivalent) in different conditions *in vitro*. Hence the presented results (Fig. 3) of TBARS production in liver homogenate indicate alterations both in relation to the type of incubation, as much as the supplementation applied. Namely, the first histogram represents the steady-state concentration which corresponds to the physiological values of lipid peroxide formation (concentration *in vivo*). Based on the obtained results of steady-state levels of lipid peroxides, it is possible to assess that groups with corn oil and soybean supplementation showed a 22% increase in comparison with the untreated group (C:K+22.6%, E:K +22.9%,  $p < 0.05$ , Fig. 3). Lipid peroxides levels, measured in the same type of incubation,

were insignificant in groups without supplementation and in groups receiving olive and fish oil. In tribute to the above results is the supposition that a rapid infusion with  $\omega$ -6 fatty acids may lead to setting the fatty acid esterification in the process of sythesis of membrane phospholipids off balance which, in turn, is attributed to an elevated production of peroxidative catabolites (Chan and McCowen, 1998). Furthermore, high concentrations of  $\omega$ -6 fatty acids in diets inhibit neutrofile and macrophage function, suppressing the activity of the reticuloendothelium system in the case of rapid and/or masive administration (Chan *et al.*, 1998). The above is considered as the major cause of increased mortality provoked by infection of patients whose diet consists, predominantly, of  $\omega$ -6 fats.

In support of the latter asumptions are a number of studies which stress that a major degradation product of peroxidative decomposition of  $\omega$ -6 polyunsaturated fatty acids is 4-hydroxynonenal (4-HNE), since it has been shown to possess cytotoxic, hepatotoxic, mutagenic and genotoxic effects (Esterbauer, 1985; Esterbauer and Cheesman, 1990; Schaur *et al.*, 1990; Janero and Burghard, B, 1989).

Likewise, our histological slices reveal necrotic regions in the liver of male rats which received soybean oil (Fig. 4 in Results). Morphological analysis clearly indicated focal and hepatic necrosis, registered in both genders of the tested animals, with initial or rare necrotic regions in female rats, vis-à-vis progressive and frequent changes in male rats (Dimitrova, 2002).

When homogenates were exposed to a peroxidation initiator ( $\text{Fe}^{2+}$  – ascorbic acid induced oxidative stress), lipid peroxidation products were enhanced by 44% in the group with fish oil supplementation (D:K,  $p < 0.001$ ) and by 48% in the group treated with soybean oil (E:K,  $p < 0.001$ ). Consequently, it may be assumed that the different respone to dietary supplementation with soybean versus corn oil is a result of the different percentages of unstaured fatty acids in the two types of oil. The authors suggest that the presence of 7% of alpha-linolenic acid (ALA) in soybean oil is the probable cause of its instability, or, in contrast, the content of 11% of saturated fatty acids (18:0) in corn oil adds to its stability (composition of oils as per USDA Nutrient Database of Standard Reference, August 1997).

In conclusion, the results of this study suggest that the administration of different unstaured fatty acids in cardiovascular therapy requires, in parallel, an adequate administration of a quantity of antioxidants, in order to increase the stability of fatty acids in diets with oil supplementation.

### Conclusions

Hereby follow the conclusions as a result of this pilot study:

- ✓ Atherogenic supplementation causes moderate hypercholesterolemia (+34.1%) and enhanced hypertriglyceridemia (+114.8 %);

- ✓ The results indicate similar fluctuations of cholesterol and triglyceride values, with significant enhancement of concentrations throughout the atherogenic treatment in comparison with the control group;
- ✓ The positive coefficient of correlation ( $r = 0.9443$ ) reveals a significant reliance between fluxes of total cholesterol and the *LDL* lipoprotein fraction;
- ✓ The correlation coefficient between total cholesterol and *HDL* fraction is lower than that achieved between total cholesterol and the *LDL* fraction, amounting to  $r = 0.577$ ;
- ✓ The *HDL* fraction as a percentage of total cholesterol amounted to 25.06% at the start of the experiment, reaching 22.58% at the end of the supplementary period. (%  $HDL_{160} = 22.58\%$ ). The *LDL* fraction before the start of the experiment amounted to a 65% share of total cholesterol level, reaching an increase of 23.5% above the baseline;
- ✓ Following 20 days of oral supplementation with different types of edible oil, cholesterol concentration marked a significant decrease which correlates with a decrease in lipoprotein levels of the *LDL* fraction in the same order: fish oil = soybean oil > olive oil > corn oil. The group treated with corn oil is an exception, due to the fact that the *LDL* fraction remained unchanged relative to day 160;
- ✓ A greater decrease in TG levels was registered in the group treated with corn oil, with a simultaneous decrease in *HDL* levels, reducing them by 45%. In mathematical terms the effects of oil supplementation in the *HDL* fraction enhancement is as follows: fish oil > soybean oil > olive oil > corn oil;
- ✓ The superiority of the administered fish oil is attributed to the lower yield of *LDL* lipoproteins;
- ✓ Lipid peroxides levels (steady-state reaction) measured in liver tissue in groups with corn and soybean oil supplementation ( $\omega$ -6 fatty acids), expressed as TBARS values, were significantly increased. The histological findings of necrotic regions in liver slices correlate with the established enhancement of steady-state levels of lipid peroxides in the group treated with soybean oil;
- ✓ The fact that lipid peroxidation was enhanced more in groups treated with  $\omega$ -6 fatty acids (soybean and corn oil) than that established in the group treated with  $\omega$ -3 fatty acids (fish oil) supports the hypothesis that the process of lipid peroxidation is not always in correlation with the number of double bonds in fatty acids esterified in phospholipid molecules.



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#### Резиме

### ЕФЕКТОТ НА РАЗЛИЧНИ МАСЛЕНИ СУПЛЕМЕНТИ ВРЗ НИВОТО НА ЛИПОПРОТЕИНИТЕ И ПРОДУКЦИЈАТА НА ЛИПИДНИ ПЕРОКСИДИ КАЈ ХИПЕРЛИПИДЕМИЧНИ СТАОРЦИ

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Поаѓајќи од сознанието дека хиперхолестеролемијата е доминантен, иако необлигаторен фактор на ризик во развојот на атеросклерозата, извршивме испитувања врз експериментално индуцирани хиперлипидемични стаорци, со примена на сопствен модел на атерогена диета. Постои уверување дека чувствителноста на клетките на неензимски индуциран оксидативен стрес, зависи од степенот на незаситеност на масните киселини, естерски врзани во

фосфолипидите и липопротеините. Тоа беше предизвик да го испитаме ефектот на диетната суплементација со различни масни киселини врз процесот на липидна пероксидација во ткивен хомогенат, со и без присуство на прооксиданси.

Во експерименталниот процес се користени адултни *Wistar* стаорци од машки пол, поделени во шест групи и изложени на третман со атерогена диета во период од 160 дена, по што следеше 20-дневна интрагастрална суплементација со различни видови масла. Липидните пероксиди се определени во свеж 2,5% хепатичен хомогенат, преку мерење на супстанциите кои реагираат со тиобарбитурната киселина (TBARS), екстрахирани во органски растворувач (по метод на Okhawa, 1979, модифицирано според Stroeve и Makarova, 1989). Добиените резултати покажаа дека третманот со атерогена диета предизвика умерена хиперхолестеролемија и изразена хипертриглицеридемија (+34,1% и +114,8,  $p < 0.001$ , соодветно). Метаболичка супериорност во регулацијата на циркулирачките липопротеини покажа администрација со рибино масло.

Наспроти остварениот поволен ефект врз липопротеинските фракции, маслените суплементи се покажаа нестабилни во присуство на генератори на процесот на липидната пероксидација. Во тој контекст,  $\omega$ -6 (n-6) масните киселини манифестираа најголема нестабилност при различни услови на инкубација, што беше особено изразено кај стаорците под администрација со соино масло (+22,9%,  $p < 0,05$ ). Хистолошката слика со некротични фокуси во хепатичното ткиво корелира со измереното високо ниво на липидни пероксиди кај групата третирана со истото масло. Кога хепарни хомогенати беа изложени на фero-аскорбатен систем (индуциран оксидативен стрес), липидната пероксидација беше зголемена кај групите третирани со соино и рибино масло (+48,4% и +44,1%,  $p < 0,001$ , соодветно), но не и кај групата што беше на диетна суплементација со пченкарно масло. Добиените резултати ја потврдуваат хипотезата дека степенот на липидна пероксидација не е секогаш повисок кај масните киселини што содржат поголем број на двојни врски. Следствено, се наметнува и заклучокот дека примената на незаситени масни киселини во терапијата на кардиоваскуларни заболувања, претпоставува и администрација со соодветно количество на антиоксиданси, заради зголемување на стабилноста на маслените суплементи во услови на оксидативен инсулт.

**Клучни зборови:** стаорци, хиперлипидемија, липопротеини, липидна пероксидација (LPO), индуцирачки фero-аскорбатен систем,  $\omega$ -6,  $\omega$ -3 масни киселини, хепар.

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