EFFECT OF OXIDISED RAPESEED OIL WITH GARLIC ON THE CONCENTRATION OF 7-KETOCHOLESTEROL, MALONDIALDEHYDE, AND FREE FATTY ACIDS IN HYPERCHOLESTEROLAEMIC RABBITS

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Abstract

The influence of a balanced diet (21 g% protein, 34 g% fat, 45 g% carbohydrate) with an isocaloric addition of non-oxidised or oxidised rapeseed oil, with and without garlic, on the development of hypercholesterolaemia was examined in 18 adult male rabbits divided into 3 equal groups. The rabbits from group I were fed fresh rapeseed oil, group II – received oxidised rapeseed oil, and group III – was given oxidised rapeseed oil and garlic. The concentration of 7-ketocholesterol and malondialdehyde (MDA) in plasma and free fatty acids (FAME) in blood serum was determined. The experiment lasted 24 weeks. At the beginning, and every six weeks, the rabbits were weighed and blood was taken. After the experiment was completed, the aorta was dissected for histological examinations. It was found that oxidised rapeseed oil caused an increase in the concentration of 7-ketocholesterol and FAME at the end of the experiment. MDA concentration increased in the 6th week of the experiment but did not appreciably change at the end of it. The obtained results suggested that the diet caused the development of hypercholesterolaemic alterations in the aorta wall and increased temporarily the level of 7-ketocholesterol, FAME, and MDA. Diet rich in oxidised oil modified significantly homeostasis of lipids in plasma and blood serum. The administration of garlic in such a diet inhibited atherosclerotic changes in the aorta wall and this seemed to be related to the decreasing concentration of 7-ketocholesterol and MDA in plasma and FAME in blood serum.

Key words: rabbit, garlic, rapeseed oil, oxidation, 7-ketocholesterol, MDA, FAME.

Hypercholesterolaemia, and in particular high levels of low density lipoprotein (LDL), is a well established risk factor for the development of coronary heart disease, but the biological mechanism by which LDL promote the formation of atherosclerosis plagues is still the object of many investigations (21). During the last decade, several lines of evidence have suggested that the oxidative modification of LDL is a key process in this respect (25). The oxidative modification of LDL is a primary event before its uptake in macrophages and cellular accumulation of cholesterol, leading to the formation of foam cells (16). The oxidatively modified LDL contains substantially greater amounts of cholesterol oxidation products: cholest-5-ene-3β, 7α-diol, cholesta-3,5-diene-7-one, cholest-5-ene-3β,5,6-α-epoxy-5 α-cholestan-3β-ol, 3β-hydroxycholest-5-ene-7-one (7-ketocholesterol), and cholest-5-ene-3β,25-diol (7). Moreover, the cholesterol oxidation products were identified as major cytotoxic compounds of oxidised LDL, capable of exerting proatherosclerotic activity. They are known to exert cytotoxicity to rabbit aortic endothelial cells in cell culture (13), to induce compositional changes in aortic elastin, to increase platelet adhesion and decrease prostacyclin production, and to inhibit lysosomal sphingomyelin and non-esterified cholesterol in arterial wall macrophages (3, 11). Oxidised LDL lipoproteins were identified in vivo, and a continuous exposure to cholesterol oxidation products transported by those lipoproteins may represent a substantial oxidative and cytotoxic burden to vascular tissues (6).

A large number of studies, link fats with the regulation of plasma lipids levels as a risk factor of atherosclerosis development in animal models (20, 23). The vegetable oils containing unsaturated fatty acids have been used in diets as a factor preventing atherosclerosis through their hypolipaemic action. However, the eating of foods prepared by deep frying in oils or animal fat may constitute a health risk. The results of a number of studies suggest that garlic or garlic extracts may have favourable effects against the development of hypercholesterolaemia and atherosclerosis (4, 8).

In previous investigations it was found that oxidised rapeseed oil, administered to animals, caused the development of atherosclerotic alterations in the
aorta wall and increased the homocystein content in blood serum. The administration of garlic in the diet inhibited atherosclerotic changes in the aorta wall and this seemed to be related to the decreasing concentration of triacylglyceroles in blood serum (26).

The present study was aimed to investigate the effect of non-oxidised and oxidised rapeseed oil with garlic on the concentration of 7-ketocholesterol, MDA, and fatty acids during experimental hypercholesterolaemia in rabbits.

Material and Methods

**Preparation of oil.** The “Kujawski” rapeseed oil, (ZT Kruszwica S.A., Poland) was used in the study. The oil was oxidised for 7 d at 120°C. The oxidation process was controlled by determining the peroxide value in accordance with the Polish Standardising Committee - Animal and vegetable oils and fats - (PN – ISO 3960) (14), iodine value in accordance with the Polish Standardising Committee - Animal and vegetable oils and fats - (PN – ISO 3961) (15), and the content of fatty acids by means of gas-chromatography (12).

**Animals.** The study was performed on 18 adult male mixed breed European rabbits, 3 months of age and weighing 2.563±50 g. The experimental protocol was approved by the Committee for Research and Animal Ethic in the Silesian Medical University. The rabbits were housed in separate cages, in a temperature controlled room (22-25°C) with 12:12 h light/dark cycle. After initial selection (lipid parameters), the rabbits were divided into 3 equal groups. All the animals had fodder ad libitum.

**Experimental design.** The animals were kept on a balanced diet with isoaloric addition of fresh (non-oxidised) or oxidised rapeseed oil with and without garlic. The rabbits from group I were fed fresh rapeseed oil, group II – received oxidised rapeseed oil, and group III – was given oxidised rapeseed oil and garlic (Allium sativum L). Garlic was prepared according to the procedure described in the previous publication (26). The detailed experimental design and fodder content are presented in Table 1.

The experiment lasted 24 weeks. At the beginning and every six weeks (weeks 0, 6, 12, 18, and 24) after fasting for 12 h but with free access to water, the rabbits were weighed and blood was taken (in sterile conditions) from the central auricular vein at 08:00 to 09:00 a.m. in the volume of 5 ml each time. In order to obtain blood plasma, ethylene diamine tetracetic acid (EDTA) or heparin were used as an anticoagulant. After the experiment was completed, all the animals were anaesthetised with pentobarbital 60 mg/kg and aorta was dissected out for histological examination.

**Determination of malondialdehyde (MDA) concentration.** Plasma concentration of MDA was determined spectrofluorimetrically using its reactivity with thiobarbituric acid (2). The blood was transferred immediately after drawing into vials containing 0.01% of butylated hydroxytoluene (BHT) and 1.5 mg of EDTA/mL of blood, and plasma was obtained by centrifugation. The samples were stored under argon at −75°C. Oxysterol content is not affected for at least three months in such storage conditions (personal communication, A. Sevanian). Total 7-ketocholesterol measurement was preceded by cold alkaline hydrolysis. In brief, 400 µl of plasma was incubated with 2 ml of 20% potassium hydroxide in methanol. Then the lipids were extracted with 2.5 ml of diethyl ether for 3 h at room temperature under argon. Hydrolysis was stopped by addition of 20% acetic acid and hexane. The mixture was shaken for 30 s and then centrifuged. The hexane and ether phase layer was evaporated under vacuum and re-dissolved in 200 µl of heptane-isopropanol 95:5 (v/v) mixture for NP- HPLC analysis. Afterwards 100 µl of the sample was injected on Nucleosil-100 10 µm 250x2 mm ID column (KNAUER, Berlin, Germany). The flow rate of hexane-isopropanol-acetonitrile 95:4:9:0.1 (v/v/v) solvent was 0.22 ml/min, the wavelength was 234 nm.

**Determination of 7-ketocholesterol concentration.** Plasma concentration of 7-ketocholesterol was determined using high-pressure liquid chromatography - HPLC (KNAUER, Germany) (22).

The blood was transferred immediately after drawing into vials containing 0.01% of butylated hydroxytoluene (BHT) and 1.5 mg of EDTA/mL of blood, and plasma was obtained by centrifugation. The samples were stored under argon at −75°C. Oxysterol content is not affected for at least three months in such storage conditions (personal communication, A. Sevanian). Total 7-ketocholesterol measurement was preceded by cold alkaline hydrolysis. In brief, 400 µl of plasma was incubated with 2 ml of 20% potassium hydroxide in methanol. Then the lipids were extracted with 2.5 ml of diethyl ether for 3 h at room temperature under argon. Hydrolysis was stopped by addition of 20% acetic acid and hexane. The mixture was shaken for 30 s and then centrifuged. The hexane and ether phase layer was evaporated under vacuum and re-dissolved in 200 µl of heptane-isopropanol 95:5 (v/v) mixture for NP-HPLC analysis. Afterwards 100 µl of the sample was injected on Nucleosil-100 10 µm 250x2 mm ID column (KNAUER, Berlin, Germany). The flow rate of hexane-isopropanol-acetonitrile 95:4:9:0.1 (v/v/v) solvent was 0.22 ml/min, the wavelength was 234 nm.

**Determination of methyl esters of free fatty acids (FAME) concentration in blood serum and oil samples.** The content of FAME was determined by gas chromatography – GC-FID (Perkin Elmer) (12). 0.5 ml of blood serum was dissolved in acetone (0.5 ml) to remove protein. After centrifugation, 2 ml of 12% BF₃ in methanol was added. Boron trifluoride in methanol was used as a transesterification catalyst, and in particular as a rapid means of esterifying free fatty acids. The mixture was heated at 80°C for 1 h in a stopped tube. Then 40% NaCl solution was added and the required esters were extracted with hexane (2 x 2 ml), using Pasteur pipettes to separate the layers. The hexane layer was washed with acetonitril (4 ml) and dried over anhydrous sodium sulphate. The solution was evaporated under vacuum and re-dissolved in 1 µl of hexane for GC-FID analysis. Non-polar lipids, such as cholesterol esters or triacylglycerols, are not soluble in reagents composed predominantly of methanol, and will not react in a reasonable time. Data are shown as nmol/L of plasma.
Table 1
Experimental design and fodder composition

<table>
<thead>
<tr>
<th>Group I – control (C)</th>
<th>Group II – experimental (O)</th>
<th>Group III – experimental (OG)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fodder composition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Fats</td>
<td>Saccharide</td>
</tr>
<tr>
<td>21%</td>
<td>34%</td>
<td>45%</td>
</tr>
<tr>
<td>Fresh rapeseed oil (non-oxidised) (12g/200g of fodder, calculated as total fat content)</td>
<td>Oxidised rapeseed oil (12g/200g of fodder, calculated as total fat content)</td>
<td>Oxidised rapeseed oil (12g/200g of fodder, calculated as total fat content) and 1g of garlic</td>
</tr>
</tbody>
</table>

FAME in oil samples was preceded by the addition to 100 µl of the oil 3% anhydrous solution of thionyl chloride in methanol. Then, 2 ml of 60% KOH in methanol were added. The mixture was heated at 80ºC for 1 h in a stoppered tube. Then 6 ml of 40% NaCl solution was added, and the required esters were extracted with hexane (2 x 2 ml), using Pasteur pipettes to separate the layers. The hexane layer was washed with acetonitrile (4 ml) and dried over anhydrous sodium sulphate. The solution was evaporated under vacuum and re-dissolved in 1 µl of hexane for GC-FID analysis. Contents of fatty acids in rapeseed oil are shown in percentages.

**Chromatography conditions:** Column: DB-WAX 30 m x 0.32 mm x 25 µ; Gas: helium 40 cm³/min; Detector FID; Oven temp: 190-260ºC.

**Histological examination.** Samples of the aorta were fixed in 10% formalin and embedded in paraffin. Histopathological sections were stained with Sudan III (29) and examined under a Decuval light microscope.

**Statistical analysis.** The comparison of changes between specific groups of the animals was carried out using the Kruskal-Wallis test ANOVA. In order to compare a given parameter in the control group and the O and OG groups and between these groups, the U Mann-Whitney test was used. Changes with P<0.05 were considered statistically significant.

**Results**

The concentration of 7-ketocholesterol and MDA in plasma are presented in Figs 1 and 2, the content of FAME in blood serum in Fig. 3, and the results of histological examinations of the aorta in Figs 4-6. Tables 2 - 5 present the percentage content of FA in rapeseed oil before and after its oxidation (Table 2), peroxide value and iodine value (JV) in rapeseed oil before and after oxidation (Table 3), coefficient of fatty acid chemical change (Table 4), and weight of rabbits (Table 5). As can be seen from the Table 2, the rapeseed oil oxidised for 7 d at 120ºC showed an increased content of palmitic acid by 24% and oleic acid by 19%, and a decreased content of linolic acid by 70% and linolenic acid by 90%. At the same time, the peroxide value increased 39 times and iodine value decreased by 2% (Table 2). After oxidation, the content of unsaturated fatty acids (UFA) was reduced by 3%. The proportion of oleic acid to palmitic acid before and after oxidation was the same. A decrease in the proportion of linolic and linolenic acids to palmitic acid was observed (Table 4). There were no differences in weight vs. control group at the beginning and at the end of the experiment (Table 5).

The concentration of 7-ketocholesterol in plasma increased temporarily (the 18th week of the experiment) by 65% (P=0.04) in group receiving oxidised oil (O) and by 70% (P=0.016) in group receiving oxidised oil with garlic (OG) vs. control group receiving non-oxidised oil (C). In the 24th week of the experiment, the concentration of 7-ketocholesterol increased by 86% (P=0.037) in O group and by 45% (P=0.004) in OG vs. C group. The concentration of the compound decreased in OG vs. O group at the end of the experiment by 22% (P=0.006) (Fig.1).

In the 6th week of the experiment, a statistically significant (P=0.016) increase in MDA concentration (by 30%) was noted in the O group vs. C group, which was administered non-oxidised oil only. At the same time, the concentration of MDA decreased significantly (P=0.01) in OG group vs. O group (by 26%). At the end of the experiment there were no differences in the concentration of that parameter between all investigated groups. (Fig. 2).

At the end of the experiment the concentration of FAME increased in O and OG groups vs. C group by 162% (P=0.004) and 36% (P=0.025) and decreased in OG group vs. O group by 48% (P=0.004). (Fig 3).

Histological examinations of aorta specimens revealed that the presence of non-oxidised rapeseed oil in a diet caused no histopathological changes in the aorta membrane (Fig. 4), whereas the presence of oxidised rapeseed oil in diet caused focal thickening of the wall, with foam cells in the intima in all animals examined (Fig. 5). In rabbits receiving oil diet supplemented with garlic, focal thickening of the internal layer of the aorta was noted only in one animal. There was no atheromatous plaque formation observed (Fig. 6).
Table 2
Percentage content of fatty acids in rapeseed oil before and after its oxidation for 7 d at 120°C

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Content of fatty acids (%) in rapeseed oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before oxidation</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>16:0</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>18:1</td>
</tr>
<tr>
<td>Linolic acid</td>
<td>18:2</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>18:3</td>
</tr>
</tbody>
</table>

± SD; all results are averages of 4 analyses

Table 3
Peroxide value (PV) and iodine value (JV) in rapeseed oil before and after oxidation

<table>
<thead>
<tr>
<th></th>
<th>Before oxidation</th>
<th>After oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV</td>
<td>4.7±0.30</td>
<td>183.2±0.10</td>
</tr>
<tr>
<td>JV</td>
<td>104.0±0.25</td>
<td>102.0±0.20</td>
</tr>
</tbody>
</table>

± SD; all results are averages of 4 measurements

Table 4
The coefficient of fatty acid chemical change in rapeseed oil during oxidation

<table>
<thead>
<tr>
<th>Content of UFA/palmitic acid</th>
<th>Content of oleic, linolic and linolenic acids/palmitic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before oxidation 16</td>
<td>After oxidation 13</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>12</td>
</tr>
<tr>
<td>Linolic acid</td>
<td>2.72</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 5
Body weight of rabbits (g) at the beginning and end of the study

<table>
<thead>
<tr>
<th>Weight (g)</th>
<th>C</th>
<th>O</th>
<th>OG</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>2528±119</td>
<td>2583±93</td>
<td>2578±200</td>
<td>0.947</td>
</tr>
<tr>
<td>Week 24</td>
<td>4175±107</td>
<td>4090±152</td>
<td>4250±173</td>
<td>0.837</td>
</tr>
</tbody>
</table>

± standard deviation (SEM);

Fig.1. The concentration of 7-ketocholesterol (µg/mL) in rabbits’ plasma (average ± SEM).
The concentration of MDA (µmol/L) in rabbit’s plasma (average ± SEM).

Fig. 2.

The content of methyl esters of fatty acids (FAME) (nmol/L) in blood serum at the end of the experiment (average ± SEM).

Fig. 3.
Discussion

It was noted that the oxidation of rapeseed oil caused a significant increase in the peroxide and iodine values, as well as changes in the percentage content of fatty acids in the oil, which indicate the disadvantageous process taking place during the oxidation (Tables 2 and 3). We obtained similar results in our earlier in vitro (27, 28) and in vivo (26) studies.

It is known that there is a decrease in the biological and nutritional properties in edible oils due to the oxidative reactions. Factors precipitating the reaction are temperature, ultraviolet light, and some metals. The obtained results showed changes in the content of fatty acids in rapeseed oil oxidised at 120°C. Although oil saturation requires some specific catalysts, we supposed that oxidation process leads also to the saturation of polyunsaturated fatty acids. After determining the coefficient of fatty acid chemical change in rapeseed oil as content of UFA in proportion to palmitic acid we could see that the content of UFA was reduced by 3% (Table 4). Consequently, the amount of palmitic and oleic acids increased (Table 2). The proportion of oleic to palmitic acid before and after oxidation remained the same. However, the percentage of linolic and linolenic acids decreased (Table 4).

Analysing those results it can be noted that the increase in acid content, in particular of oleic acid, is probably a result of the chemical changes (reduction) affecting linolic and linolenic acids. At the same time, the increased content of the palmitic acid indicates a probable degradation, in particular of the linolenic acid, by elimination of 2 methine groups. Obviously, during oxidation there are formed some oxidation products, such as lipid hydroxides, aldehydes or ketones. The observed changes are caused by all those factors and compounds, so it needs further research, which is in progress.

Due to the fact that numerous studies indicate that diets rich in saturated fatty acids may induce atherosclerotic lesions, it could then be assumed that a diet enriched with rapeseed oil oxidised in the way presented above would cause the development of atherosclerotic lesions in the given experimental model (17, 19, 26).

The results of our previous research revealed that the supplementation of a diet with oxidised rapeseed oil and oxidised rapeseed oil with garlic did not cause changes in the level of total cholesterol or HDL cholesterol in comparison with control, but with the addition of garlic, reduced the level of TG compared to the group receiving non-oxidised oil (26). It could be assumed then that garlic acted effectively in the initial phase of lipid accumulation. On the other hand, the study revealed a temporary increase in homocystein concentration in the groups of animals receiving oxidised oil, which was; however, lower in the group receiving garlic. It seems interesting how such a diet influences oxidative stress by protection against oxidation in vivo.

The findings of this study show that three different markers for lipid oxidation were consistently associated with accelerated progression of atherosclerosis in rabbits. These findings are consistent with observations concerning the association between elevated of 7-ketocholesterol, total amount of fatty acids, and MDA.

7-oxygenated oxysterols are the major oxysterols formed in connection with lipid peroxidation in biological membranes and lipoproteins. The two hydroperoxides are the primary labile products that are
rapidly reduced to the corresponding hydroxy derivative or dehydrated to the o xo derivative. There is substantial formation of 7-oxygenated cholesterol products during oxidation of LDL particles, in particular 7-oxocholesterol and 7β-hydroxycholesterol (1, 19). Most of the 7β-hydroxycholesterol in the circulation is present in esterified form in the LDL fraction, and it is generally believed that most, if not all of this oxysterol is of a non-enzymatic origin. 7α-hydroxycholesterol in plasma may be derived both from non-enzymatic oxidation and from the hepatic cholesterol 7β-hydroxylase. In view of this, 7β-hydroxycholesterol (7-ketocholesterol) can be expected to be a better marker for lipid peroxidation than 7α-hydroxycholesterol (5, 9, 18).

In the present study, fodder enriched with oxidised oil had a beneficial effect upon the concentration of 7-ketocholesterol at the end of the experiment. Similar research was carried out by Mahfouz and Kummerow (10). They found that in rabbits, cholesterol feeding produced severe hypercholesterolaemia (43-fold increase) and increased plasma and liver lipid peroxidation. The total as well as the individual oxysterol contents of 7α-, 7β-hydroxycholesterol, α-epoxy, β-epoxycholesterol, cholestane triol, 7-keto, and 27-hydroxycholesterol, significantly increased in the plasma of hypercholesterolaemic (HC) rabbits.

In our study, the addition of garlic to fodder enriched with oxidised oil had a beneficial effect upon the concentration of 7-ketocholesterol at the end of the experiment. The study showed also that garlic is supportive in modulating lipid peroxidation and it is consistent with higher protection against oxidation in vivo. Durak et al. (4) received similar results. In their study dietary supplementation of garlic extract decreased peroxidation in aortic tissue. They also observed a reduction in atherosclerotic plaque area, which was mirrored in our experiment.

In our study the increased rate of 7-ketocholesterol was followed by the increased fatty acids content in serum. These data confirm lipid disturbance caused by a diet rich in saturated fatty acids.

The protective effect of garlic is also confirmed by the concentration of MDA, which did not change during the experiment in the group receiving oxidised oil with garlic vs. control group while transitory increase of MDA was observed in the group receiving only oxidised oil.

No changes were observed throughout the experiment in animal body weight in all experimental groups. Still, improved condition of the hair and claws was noted in the group receiving garlic, which may testify to its multi-directional effects.

We conclude that a diet with a prevalence of saturated fatty acids; though it does not increase body mass, significantly modifies lipid metabolism favouring the atherosclerosis. We also indicate a beneficial role of garlic during this experiment. Although further studies are required to elucidate the mechanism involved, the protective activity of garlic may be due to its antioxidant properties.

References


