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INFLUENCE OF NON-OXIDISED AND OXIDISED RAPESEED OIL CONSUMPTION ON LIVER METABOLISM PATHWAYS AND NON-ALCOHOLIC STEATOHEPATITIS DEVELOPMENT IN RABBITS

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Abstract

For 24 weeks, rabbits were fed feed containing non-oxidised or oxidised rapeseed oil. At the beginning of the experiment and every six weeks the rabbits were weighed and blood was taken. After the experiment was completed, their liver was dissected for biochemical and histological examinations. The activity of alanine aminotransferase, aspartate aminotrasferase, glutamate dehydrogenase, sorbitol dehydrogenase, and aldolase in blood plasma and liver were determined. Enzymes of the protein and liver metabolic pathways were determined using kinetic and spectrophotometric methods. The content of fatty acids was determined by means of fatty acid methyl ester concentration measurement using gas chromatography. It was found that the applied diet with oxidised rapeseed oil caused the development of slight liver steatosis and disturbances in the activity of enzymes involved in the liver pathways, despite the fact that it was a balanced diet, and differed only in the ratio of saturated to unsaturated fatty acids. The obtained results indicate that more profound oil oxidation and its increased supply in diet may result in the development of liver steatosis.

Key words: rabbit, oxidised rapeseed oil, liver steatosis, enzymes.

Although non-alcoholic steatohepatitis (NASH) has become a common disease worldwide, pathogenesis of this disease remains unsolved. Establishment of a proper animal model is one of urgent issues to analyse the molecular mechanisms (11). Increasing evidence suggests that NASH is associated with progressive fibrosis, cirrhosis, and eventually hepatocellular cancer. The medical conditions that are most frequently associated with NASH are obesity, diabetes mellitus, dyslipidaemia and hypercholesterolaemic atherosclerosis. All these conditions can be induced by feeding animals diets that are high in fat or sucrose (7). Importantly, strain-related factors are significant in influencing vulnerability to the dietary effects.

In previous investigations we found that oxidised rapeseed oil, administered to animals, caused the oxidative stress and development of atherosclerosis changes, observed both in biochemical and histological analyses (19). It seems to be very interesting to find out if such a diet can exert an influence on liver metabolism pathways enzymes and cause the development of liver steatosis.

Among the most important enzymes related to hepatic metabolism are: alanine aminotransferase (ALT EC 2.6.1.2), asparate aminotransferase (AST EC 2.6.1.1), and glutamate dehydrogenase (GLDH EC 1.4.1.2), which participate in protein metabolism. Among enzymes that take part in carbohydrate metabolism are sorbitol dehydrogenase (SDH EC 1.1.1.21) and aldolase (ALD EC 4.1.2.13). All of these enzymes are useful in estimation of liver function and their increased activity in plasma could serve as an indicator of liver injury (2).

The aim of this study was to evaluate the effects of non-oxidised and oxidised rapeseed oil consumption on the liver metabolism pathways and non-alcoholic steatohepatitis development using the rabbit as an experimental model.

Material and Methods

Preparation of oil. The oil was oxidised for 7 d at 120^0C. The oil samples were prepared according to the procedure described in the previous publication (19).

Animals and experimental design. Studies were performed on 12 adult mixed European male rabbits (16) weighing 2,500 ±100 g and were conducted following the experimental protocol approved by the Committee for Research and Animal Ethic in the Silesian Medical University. The rabbits were housed in separate cages, at temperature controlled room (22-25^0 C) with 12:12 h light/dark cycle. After initial selection (lipid parameters), the rabbits were divided into two equal groups. The animals were kept on balance diet

(21% of protein, 34% of fats, 45% of carbohydrates) containing isocaloristic addition of non-oxidised (group C) or oxidised (group O) rapeseed oil (12 g/200 g of fodder) included in the total fat content. All animals had fodder (200 g/d) and water *ad libitum.* The fodder was prepared in the Animal Nutrition Unit of the Zootechnical Institute in Brzeziny, Poland. The experiment lasted 24 weeks. At the beginning and every six weeks (0, 6, 12, 18, and 24 weeks), after 12 h fasting with free access to water, the rabbits were weighed and 5 ml of blood samples were taken from the central auricular vein at 08:00 to 09:00 a.m. In order to obtain the blood plasma, ethylenediaminetetra acetate (EDTA) or heparin were used as an anticoagulant. After the experiment was completed, the animals were anaesthetised with pentobarbital (60 mg/kg) and their livers were dissected out for biochemical and histological examinations.

Determination of enzymes of the protein and liver metabolic pathways in plasma and liver. Protein concentration in the supernatants from tissue homogenates was measured by the Lowry method (9). The activity of ALT and AST were determined by kinetic methods using commercial kits (Alpha–Diagnostic, Germany). The activity of SDH, GLDH, and ALD were determined according to Krawczyński's method (8). Activity of SDH was measured as a decrease in the $NADH_2$ concentration after application of oxidised fructose as a substrate. Decrease in absorbance was measured at 340 nm. Activity of GLDH was determined as a decrease in the concentration of $NADH_2$ after reaction with α-ketoglutarate acid and ammonium. Decrease in absorbance was measured at 340 nm. Activity of aldolase diphosphofructose was based on degradation of phosphobisfructose to phosphoglycerol and phosphodihydroxyacetone, which reacted with 2,4-dinitrophenylhydrazine after hydrolysis, and the complex formation was measured at 366 nm. Enzyme activity was expressed in international units (IU) per litre in plasma and in IU/mg protein in liver homogenates.

Determination of free fatty acids (FFA) concentration in oil samples. The content of FFA, measured by means of fatty acid methyl esters (FAME), was determined by gas chromatography GC-FID according to the procedure described in the previous publication (18). Data are shown as FFA% in 100 g of oil.

Histological examination. Samples of liver tissue were fixed in 10% formalin. Paraffin sections were stained with haematoxylin and eosin. Neutral fats were visualised by Sudan III staining of frozen sections (20). Histological examination of the slides was performed using concentrated light microscope Docuval and photographic camera Carl Zeiss Jena.

Statistical analysis. In the comparison of changes between groups C and O, the U Mann-Whitney test was used. $P<0.05$ was considered as statistically significant.

Results

The results are shown in Table 1 and Figs 1–6. Results are presented as the mean ± SEM.

Oxidised rapeseed oil characterisation. As it was described in the previous publication, the rapeseed oil oxidised for 7 d at 120^0C showed an increased content of palmitic and oleic acids by 24% and 19%, respectively, and a decreased content of linolic and linolenic acids by 70% and 90%, respectively. At the same time, the peroxide value (PN) increased 39 times and iodine value decreased about 2% (18).

The activity of liver metabolic pathways enzymes in plasma and liver. No statistically significant differences in the activity of ALD in plasma and liver were found. A statistically significant increase in plasma activity of ALT by 6%, AST by 21%, GLDH by 28%, and SDH by 13% was observed between groups C and O (Table 1). Simultaneously, a decrease in the activity of the determined enzymes in the liver was observed. The activity of ALT, AST, SDH, and GLDH decreased by 16%, 38%, 48%, and 47%, respectively (Figs 1-4).

Table 1
Plasma activity (IU/L) of ALT, AST, GLDH, and SDH in various stages of the experiment

	0 week	6 week	12 week	18 week	24 week	P 24 week C *vs* O
ALT						
group C	29.2 ± 3.2	33.8 ± 4.1	36.2 ± 2.3	35.4 ± 2.3	34.7 ± 2.3	0.05
group O	29.6 ± 2.5	36.7 ± 2.5	36.9 ± 1.0	37.0 ± 2.2	37.0 ± 1.9	
AST						
group C	26.5 ± 2.7	28.0 ± 3.2	28.8 ± 3.2	27.3 ± 5.4	27.5 ± 4.2	0.05
group O	27.1 ± 4.2	25.5 ± 1.3	28.7 ± 1.5	30.9 ± 1.6	33.3 ± 2.3	
GLDH						
group C	14.1 ± 2.9	13.5 ± 1.6	16.3 ± 3.0	22.2 ± 3.0	22.1 ± 1.2	0.05
group O	13.9 ± 1.9	14.0 ± 1.1	20.6 ± 1.0	26.3 ± 2.0	28.4 ± 1.3	
SDH						
group C	32.1 ± 3.5	34.3 ± 5.6	32.7 ± 4.6	35.5 ± 4.5	26.4 ± 2.3	0.05
group O	32.6 ± 3.5	41.0 ± 6.6	32.2 ± 4.1	43.4 ± 1.2	29.8 ± 3.6	

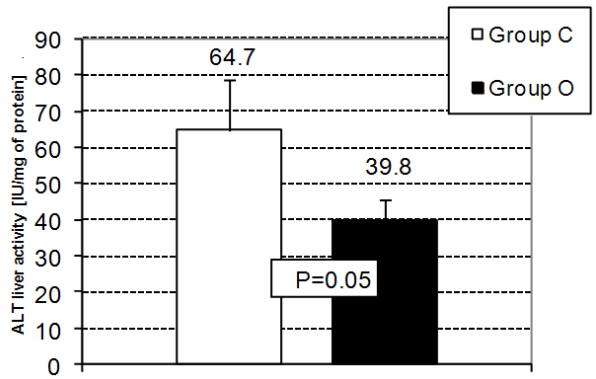


Fig. 1. The effect of non-oxidised (C) and oxidised (O) rapeseed oil on liver ALT activity after 24 weeks of experiment.

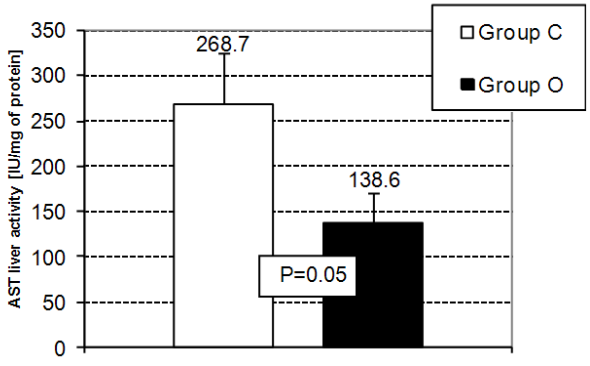


Fig. 2. The effect of non-oxidised (C) and oxidised (O) rapeseed oil on liver AST activity after 24 weeks of experiment.

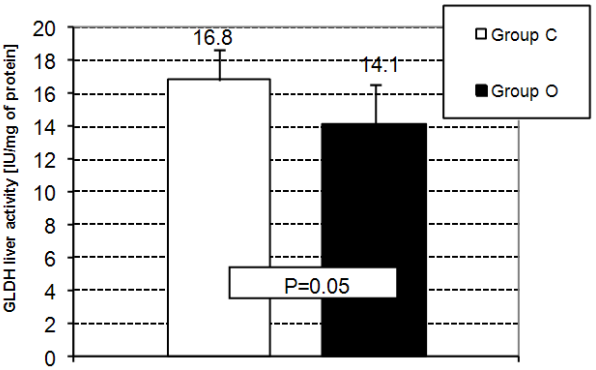


Fig. 3. The effect of non-oxidised (C) and oxidised (O) rapeseed oil on liver GLDH activity after 24 weeks of experiment.

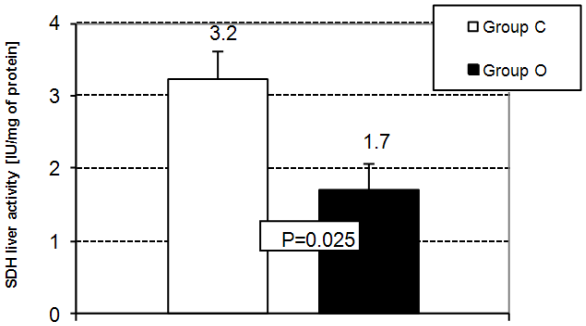


Fig. 4. The effect of non-oxidised (C) and oxidised (O) rapeseed oil on liver SDH activity after 24 weeks of experiment.

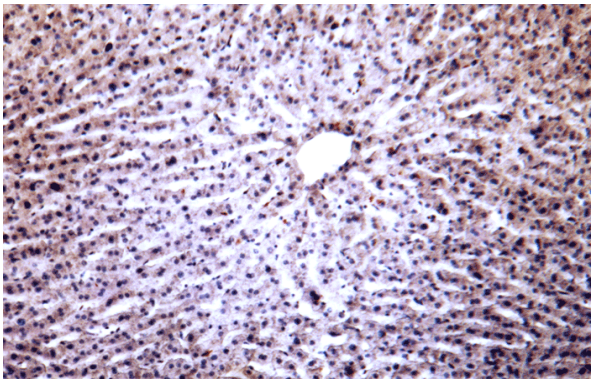


Fig. 5. Group C. Normal appearance of the liver of rabbits receiving non-oxidised rapeseed oil Sudan III staining. 150x.

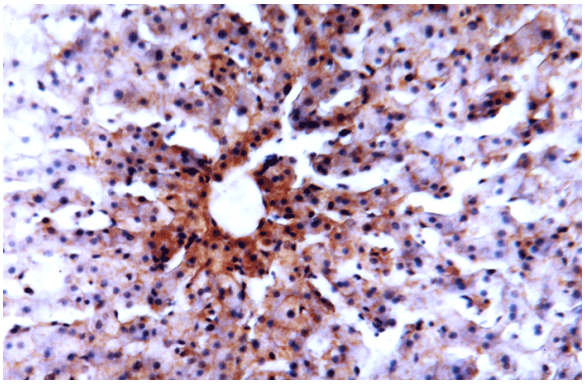


Fig. 6. Group O. The liver of rabbits receiving oxidised rapeseed oil. Slight steatosis of hepatocytes. Sudan III staining. 150x.

Histological examination of liver tissue. Microscopic image of the liver from the group C is shown in Fig. 5. No changes in the liver structure were observed. In group O (Fig. 6), slight steatosis of hepatocytes was noted. It had a character of regressive change, defined as parenchymatous degeneration. Sudan III staining confirmed the presence of fat in hepatocytes.

Discussion

Oxidised oil causes the oxidation, hydrolysis, and polymerisation of the oil. Oxidation produces hydroperoxides and low molecular volatile compounds (such as aldehydes or ketones), which play an important role during oxidative stress. Polymers in oxidised plant oils are rich in oxygen and the oxidised polymer compounds accelerate the oxidation and degradation of oil, increase oil viscosity, produce foam during oxidation, and develop undesirable colour. They are also highly conjugated dienes, and thus play a role during peroxidation and oxidative stress. Hydrolysis increases the amount of free fatty acids, mono- and diacylglycerols and glycerol as well (3).

Consumption of fresh plant oil has beneficial health effects (12). However, the fact that it contains monounsaturated- and polyunsaturated fatty acids makes it prone to oxidation upon heating, producing species that cause increased oxidative stress that has been implicated in the pathogenesis of several diseases (4). The liver is particularly exposed to cooking-oils products.

A major cause of steatosis is increased fatty acid flux to the liver caused by a high availability of plasma FFA in relation to peripheral oxidative requirements. Several conditions increase the fatty acid flux to the liver. One of them could be an increase in exogenous fat at high-fat feeding. This increase in hepatic triacylglycerols (TG) content can occur within 10 d after starting the high-fat diet in mice (5). Fatty acid delivery to the liver can also be increased because of disturbances in fatty acid/TG partitioning between organs. This was observed in several studies. Mice lacking CD36, a fatty acid transporter in muscle and adipose tissue, have increased plasma fatty acid levels and show liver steatosis (6). Several intra-hepatic mechanisms also induce steatosis. These changes involve alterations in hepatic glucose and fatty acids metabolism. Increased *de novo* hepatic synthesis of fatty acids and subsequent esterification into TG is an important cause of steatosis. Mc Devitt *et al.* (10) proved that high-surcose feeding induces liver steatosis by increased lipogenesis. Inhibition of glucose-6-phosphatase by S4048 results in hepatic entrapment of glucose and *de novo* lipogenesis, leading to massive steatosis within several hours (1). An inhibition of fatty acid oxidation in the liver is another intra-hepatic cause of the development of liver steatosis.

Deep-frying oil could also disturb liver functioning. In our previous experiments, we have observed an increase in TG concentration and free fatty acids content in serum (18, 19). It confirmed lipid disturbance caused by diet with an addition of oxidised rapeseed oil (but not high-fat diet). On the other hand, these studies revealed a temporary increase in homocystein and 7-ketocholesterol concentrations in the groups of animals receiving oxidised oil. We also observed that addition of oxidised oil resulted in increased production of oxygen radicals, evidenced by elevated plasma MDA production in rats (17). We concluded that such diet causes the development of atherosclerotic changes. However, it is still not known how such diet influences liver steatosis development.

Although AST and ALT are important factors for the diagnosis of liver and kidney disturbances, only a few animal studies on the toxicity of oxidised oil measured the AST/ALT ratio. GLDH, ALD, and SDH are also connected with certain processes taking place in the liver but we did not find any studies describing changes of these enzymes' activity during oxidised oil supplementation. During this experiment we have observed a statistically significant decrease in activities of ALT, AST, GLDH, and SDH in the liver with simultaneous increase in the activity of these enzymes in blood plasma. Sorbitol dehydrogenase catalyses the conversion of sorbitol to fructose. It is also an enzyme that can be used in assessment of liver functioning, being specific for that organ. Its activity increases already in the early stage of viral hepatitis and decreases as the clinical condition improves. A drop in its activity, as well as increase in plasma levels, indicates the disturbances in liver functioning. It seems to be a result of the disturbance of fructose metabolism during this experiment.

AST, ALT, and GLDH participate mainly in protein metabolism. Enzymes of protein metabolism are indicative enzymes, non-specific in organs. They are characterised by the fact that under physiological conditions they demonstrate a moderate activity in the plasma, which is a symptom of continuous necrosis of cells. Only under condition of organ injury, an increase in their activity in the plasma points at the degree of organ injury and is proportional to the damage. AST, ALT, and GLDH – when appearing in high quantities in the plasma – indicate the hepato-cellular damage. In the performed research, only a slight increase in their activity in the plasma has been found. It has been noted, however, that their activity appeared to drop in the liver at the threshold of statistical significance for the detected enzymes. That testifies that hepatocyte lesion has not been the case, but only disturbances in liver functioning.

Totani *et al.* (14) studied for several years the effect of thermally oxidised oil on rats. The animals were fed a diet containing 7% oil (soybean and rapeseed) heated in 180^0 C for 10 h. They found that levels of glucose, triacylglycerol, phospholipids, and cholesterol were significantly lower than those of control group receiving fresh oil. They found that ALT activity in both group was almost the same while AST activity was much higher than max (118 IU/L) in group receiving oxidised oil (in two of eight rats).

In the next study conducted by Totani and Ojiri (13), rats were fed the same diet but they compared fresh oil to recovered oil from food manufacturing. There were no statistically significant differences in the levels of serum glucose, TG, phospholipids, free fatty acid, cholesterol, and insulin between the groups, but the activity of AST and ALT increased statistically significantly in group receiving recovered/heated oil. Increased activity of these enzymes demonstrated the damage of the liver and kidneys after mild ingestion of the recovered oil. Another study on consumption of

frying soybean and rapeseed oil showed that consumption of plant oil heated at 180^0C for 20 h caused similar changes to those described above (15).

During histological examinations of the liver, no steatosis changes were observed in the control group C, while in the group O, slight steatosis of hepatocytes has been noted in 3/6 animals. Changes of regressive character were defined as parenchymatous degeneration. Sudan staining confirmed the presence of fat in hepatocytes.

Totani *et al.* (13-15), during examination of the liver, revealed dark-red patches due to dotted bleeding on the surface of the liver obtained from animals receiving frying oil recovered from Japanese food manufacturing companies (recovered oil), which indicated the organ degeneration. The number of dark-red patches corresponded well with the high AST activity. These reports suggest that cytotoxicity of thermally oxidised oil is attributable to low-molecular-weight of decomposition products.

Assessing liver functioning in the examined rabbits, it can be assumed that the applied diet caused slight disturbances, despite the fact that it was a balanced diet and differed only in the ratio of saturated to unsaturated fatty acids, and was not a high-fat diet. The obtained results indicate that more profound oil oxidation and its increased supply in diet may result in the development of liver steatosis.

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