The effect of cholesterol and orotic acid administration and methionin-cholin deficiency on liver DNA synthesis and lipid metabolism in rats

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Summary

Aim: *The aim of study was to assess the influence of cholesterol and orotic acid dietary administration and methionin-cholin deficient diet on liver steatosis development, liver DNA synthesis and on selected biochemical markers.*

Material and methods: *After institutional approval 32 male Wistar rats were divided into 4 groups and fed using different diets ad libitum for 29 days as follows: group 1 (control): standard laboratory diet (SLD), group 2: methionine choline deficient diet (MCDD), group 3: diet with addition of 4 % of cholesterol (CHOL), group 4: diet with 1 % of orotic acid (OA). The ^{3}H-thymidine was applied i.v. 1 hour before sacrifice by exsanguination from the abdominal aorta. Liver DNA synthesis was determined by methyl ^{3}H- thymidine on Beckman Coulter analyzer (Beckman Coulter, USA). The cholesterol, LDL-cholesterol and HDL-cholesterol (mmol/L, Modular Roche), transaminases (μkat/L), leptin and insulin (pg/mL, EIA, R&D Systems, USA) serum was estimated. Liver tissue for histopathological examination was obtained. Statistics: a t-test (mean and SEM) was performed using SigmaStat software (Jandel Scientific, USA).*

Results: *The body weight was higher in CHOL rats, but lower in OA rats. The CHOL and MCDD rats had higher liver DNA synthesis in liver. The OA rats had significantly smaller adrenal glands than other. The CHOL rats had higher triacylglycerols, LDL and glucose in serum and lower insulin and leptin. The MCDD rats had lower HDL and LDL, but higher leptin and insulin. The OA rats had higher transaminases, glucose and insulin in serum but lower LDL and triacylglycerols.*

Conclusion: *Feeding with the CHOL diet led to an increase of glycaemia, concentration of cholesterol, LDL, TAG in serum, but markers of hepatic damage have not been elevated. The MCDD diet affects fat metabolism, especially TAG. The choline and methionine deficiency in the diet negatively affected bile production. The OA diet also inhibits production of LDL, but fat deposits in the liver lead to their damage.*

Key words: *cholesterol, rat, liver, steatosis, orotic acid, methionine-choline deficient diet*

Souhrn

Vliv diety obohacené o cholesterol nebo kyselinu orotovou a methionin-cholin deficitní diety na syntézu DNA v játrech a metabolismus lipidů u potkanů

Cíl studie: *Cílem studie bylo zhodnotit vliv diety obohacené cholesterolem nebo kyselinou orotovou nebo naopak diety methionin-cholin deficitní na rozvoj jaterní steatózy, syntézu jaterních DNA a na vybrané biochemické ukazatele.*

Materiál a metodika: *Pokusy byly provedeny na dospělých potkanech – samcích kmene Wistar (n = 32) rozdělených do 4 skupin, živených dietami ad libitum po 29 dní: standardní laboratorní dietou (SLD), dietou methionin-cholin deficitní (MCDD), dietou se 4 % cholesterolu (CHOL), a dietou s 1 % kyseliny orotové (OA). Potkanům byl před usmrcením podán ^{3}H-thymidin i.v. a 1 hod poté byli usmrceni vykrvácením z břišní aorty. V jaterní tkáni byla vyhodnocena specifická aktivita DNA (Beckman Coulter, USA) a zhotoveny histologické preparáty. V séru byly stanoveny aminotransferázy (μkat/L), cholesterol celkový, HDL i LDL (mmol/l, Modular Roche), triacylglyceroly (TAG, mmol/l), leptin a inzulin (pg/ml, EIA, RD Systems, USA) a ukazatele jaterního poškození. Statistické zhodnocení bylo provedeno s využitím SigmaStat software (Jandel Scientific, USA).*

Výsledky: *Nárůst tělesné hmotnosti byl nejvyšší u skupiny CHOL, naopak nejnižší u skupiny OA. V játrech byla vyšší syntéza jaterních DNA u skupiny CHOL a MCDD. Potkani OA měli nižší hmotnost nadledvin než ostatní. Potkani CHOL měli vyšší sérové triacylglyceroly, LDL a glukózu a nižší inzulin i leptin. Potkani MCDD měli v séru nižší HDL i LDL, a zvýšený leptin i inzulin. Potkani OA měli vyšší sérové aminotransferázy, glukózu i inzulin, naopak nižší LDL, TAG.*

Závěr: *Podávání diety CHOL vedlo k vzestupu glykémie, koncentrací cholesterolu, LDL, TAG v séru, ale ukazatele jaterního poškození nebyly zvýšeny. Dieta MCDD ovlivnila metabolismus tuků, především TAG. Deficit cholinu a methioninu v dietě negativně ovlivnil tvorbu žluči. Dieta OA sice bránila tvorbě LDL, ale jeho depozita v játrech vedla k jejich poškozování.*

Klíčová slova: *cholesterol, potkan, jaterní steatóza, kyselina orotová, methionin-cholin deficitní dieta*

Liver steatosis may not be always caused by serious disease or chronic alcoholism. Even small changes in diet can participate in steatosis development; some excess or shortage of some special diet parts. We try to present how serious the results of the developed state are for the body and how different the final pathological status could be. It is not yet known how to treat liver steatosis and one of the reasons might be its etiological disarticulation.

Inadequate nutrition (quantity or quality) has recently been discussed. There is no problem with food shortage (developed countries), but its excess and in some cases its bad compound. Food with a higher fat content (triacylglycerols and cholesterol), animal proteins rich in methionine, is regularly eaten. A lot of studies have dealt with genetic influence on failure of the metabolism. However, it is obvious that most failure of the metabolism is related or worsened by unsuitable food content. This leads to obesity and in the end lifestyle diseases: diabetes mellitus, liver steatosis, atherosclerosis and cardiovascular disorder.

Non-alcoholic fatty liver disease (NAFLD) is a major cause of liver-related morbidity and is frequently associated with obesity and metabolic syndrome [19]. It is associated with increased mortality and morbidity after liver resection [20] and survival depends on the regeneration and functions of the remnant liver.

Intra-abdominal adiposity and insulin resistance are risk factors for diabetes mellitus, dyslipidaemia and arteriosclerosis. Hypercholesterolaemia is a risk factor for the development and progression of atherosclerosis and cardiovascular diseases. Leptin, a fat-derived protein encoded by the ob gene, is a sensor of energy storage in adipose tissue capable of mediating a feedback signal to sites involved in the regulation of energy homeostasis [10].

We asked in our experiment how various diet precautions would change liver histological pictures and biochemical findings. All our diets always had the same amount of fat - i.e. 5.5 % corn oil. We could thus compare the effects of other matters of diet.

The 1st experimental diet (MCDD) leading to liver steatosis demonstrates a special type of malnutrition (choline and methionine deficiency). Choline is a component of the liver cell membranes and organelles, therefore a choline-deficient diet results in fatty liver in mice and rats [24,29] and induces changes in membrane phospholipids of the endoplasmatic reticulum and of other organelles of liver cells [7]. In our experiment we replaced methionine with amino acid L-arginine. The arginine stands for 4 % in defined diets for rodents (SLD, CHOL, OA), but the relative amount of L-arginine was increased to 7 % in the MCDD diet, which proved to have essential influence on the development of liver steatosis.

The 2nd “high-cholesterol” diet (CHOL, 4%, 5.5% corn oil) shows liver changes during cholesterol excess. People aggravate cholesterol excess by their fat surplus.

The 3rd diet was created using orotic acid (OA, 1 %), formerly designate as B13 vitamin. This acid inhibits the export of newly synthesised hepatic lipoprotein from the liver [11]. It is very important, because the liver is the major source of cholesterol containing lipoproteins. Diet with orotic acid significantly increased rate-limiting enzyme of triglyceride synthesis [3], and prevented lipoprotein transport through the Golgi apparatus, causing liver infiltration with triglyceride and free fatty acids [27]. Other effects of orotic acid are elevation of superoxide anion concentration in the liver and a decrease in superoxide dismutase activity followed by liver damage [1].

The fat and cholesterol metabolism is significantly influenced by insulin and leptin too, hence we monitored these hormones. Qi et al [21] stated that although insulin resistance can promote fatty liver, excessive hepatic accumulation of fat can promote insulin resistance and contribute to the pathogenesis of the metabolic syndrome. Insulin resistance, through the inhibition of lipid oxidation and increased fatty acid and triglycerides synthesis, is believed to be a key factor in the development of non-alcoholic fatty liver [19].

In hepatocytes, leptin has complex effects on the insulin response. It stimulates glucose transport and turnover, however it up-regulates gluconeogenesis and contributes to hepatic insulin resistance [25]. On the contrary leptin production and secretion by adipocytes is regulated by insulin, glucocorticoids and cytokines [22]. The liver participates in leptin degradation, hence the leptin serum rises when liver function is impaired [13].

MATERIAL AND METHODS

Preparation of the laboratory diet

The diet was prepared according to available literature (www.testdiet.com, www.dyets.com) from casein (PML Inc., Nový Bydžov, Czech Republic), cornstarch (Škrobárny Pelhřimov, Czech Republic), cellulose (Phrikolat, Chemische Erzeugnisse GmbH, Germany), choline chloride, L-cysteine, L-arginine and sucrose (Fisher Scientific Ltd., Pardubice, Czech Republic), corn oil (CANO Ltd., Heřmanův Městec, Czech Republic), DL-methionine (Sigma-Aldrich Ltd., Praha, Czech Republic), vitamin and mineral mixture – AIN-93M Maintenance Purified Diet 5801-M (TestDiet, Bethlehem, USA). This primary substance of the dietary base was identical and served as a control diet - standard laboratory diet (SLD). The 1st diet was not enriched by methionine and choline (MCDD, methionine 1.7 g/kg of diet

vs. others 11.7 g/kg, L-arginine 7 % vs. others 4 %, without choline vs. others diets choline 9 g/kg). The 2nd diet was enriched with cholesterol (CHOL, 4 %). The 3rd diet was enriched with orotic acid (OA, 1 %). The diets were made into pellets and dried at 60 °C in a food dryer.

Animals

A special committee approved the experiment protocol. All operations were performed in total ether anaesthesia. Male Wistar rats (6 weeks old, Biotest Inc., Konarovice, Czech Republic) were placed in plastic cages according to standard conditions. They were housed at a standard room temperature of 22 ± 2 °C, 12 hours light/dark system, air humidity 30–70 %. The rats were randomly divided into 4 groups, 8 rats each with starting body weight 256 ± 15 g. They were fed with the diets and drank tap water ad libitum for 29 days. The 1st group (SLD), were fed with standard laboratory diet, the 2nd group (MCDD), were fed by methionine-choline deficient diet, the 3rd group (CHOL), were fed with cholesterol enriched diet, and the 4th group (OA), were fed with orotic the acid enriched diet.

The ^{14}C-cholesterol (37 kBq/100 g of body weight, AP Czech, Praha, Czech Republic) was applied p.o. at 24 hours and the ^{3}H-thymidin (740 kBq/100 g of body weight, Lacomed Ltd., Řež u Prahy, Czech Republic) was applied i.v. at 1 hour before sacrifice by exsanguination from the abdominal aorta.

Analyses

Serum glucose (mmol/l), cholesterol (mmol/l), HDL-cholesterol (HDL, mmol/l), LDL-cholesterol (LDL, mmol/l), triglyceride concentrations (TAG, mmol/l) and transaminase activities (ALT, AST, µkat/l) were measured by automated methods (Modular Roche, Mannheim, Germany). Leptin (pg/ml, leptin EIA-4015, DRG, Marburg/Lahn, Germany) and insulin (pg/ml, EIA, Biovendor, Brno, Czech Republic) were measured using commercially available immunoassay kits.

The liver DNA content was determined with diphenylamine reagent [4]. Liver DNA synthesis was determined with methyl ^{3}H-thymidine [26]. We determined the blood and tissue activities of ^{14}C-cholesterol (Bq/g or ml) after ethanol-acetone extraction [28]. The radioactivity of the samples was measured by means of liquid scintillation on Beckman Coulter LS6000LL.

Liver tissue for histopathological examination was obtained from one standard site (processus anterior dexter et processus caudatus lobi caudati) and fixed in 10% buffered formalin. The 3 µm paraffin sections were stained with haematoxylin-eosin or blue trichrome.

Statistics

Statistical analyses were performed using the SigmaStat 3.1 (Jandel Scientific software, San Rafael, USA). The identical symbols indicate compared couples. 1 symbol represents statistical a significance of $p<0.05$, 2 symbols are $p<0.01$ and 3 symbols represent $p<0.001$. Results are expressed as mean ± SEM, leptin as median ± 25th and 75th percentiles.

RESULTS

Our results are summarised in Figs 1-5 and Tabs 1-3. The body weight increased in CHOL rats yet on the contrary decreased in OA rats. The CHOL and MCDD rats had a higher DNA synthesis in intact liver. The total volume of liver DNA decreased in CHOL and OA rats, but increased in the MCDD group. The OA rats had a significantly lower weight of adrenal glands in comparison with others.

The CHOL rats have some statistical significant decreases in activities of ^{14}C-cholesterol in blood and in monitored tissues. On the other hand, the MCCD rats had insignificantly higher ^{14}C-cholesterol activities in tissues (blood, liver, lungs, heart, thymus, kidneys and muscle – the results are not presented).

The CHOL rats had higher concentrations of TAG, LDL and glucose in serum and lower insulin and leptin concentrations. The MCDD rats had lower HDL and even LDL concentrations in serum and on the contrary they had higher leptin and insulin concentrations. The OA rats had higher transaminase activities and higher glucose and even insulin concentration in serum but lower LDL and TAG concentrations.

DISCUSSION

Male liver steatosis is a very frequent disease connected with several serious diseases (diabetes mellitus, obesity) and chronic alcoholism. It also can be encountered as the result of relatively insignificant malnutrition. Mild liver steatosis is often underestimated, because there has been no known effective treatment and under certain circumstances (e.g. persistence of high cholesterol intaking [16]) this may switch to steatofibrosis and cirrhosis. Experimental liver steatosis in mice or rats is evoked by ethanol, glucose realimentation after short-term starvation, high cholesterol diet (with high lipid content) and lastly by deficiency of methionine-choline. There is very intensive study of steatosis therapy in the experiments, the inhibition of steatosis development by synthetic lipoprotein lipase activator [29], reactive oxygen species inhibitors (glutathione [15]), IL-6 administration [8], L-arginine administration [12] etc.

At present, the non-alcoholic steatohepatitis is followed up to a large extent besides NAFLD (non-alcoholic fatty acid liver). Our study pursues some pathogenetic moments which might help steatotic patients. Speaking about liver transplantation, the intention is there to choose which

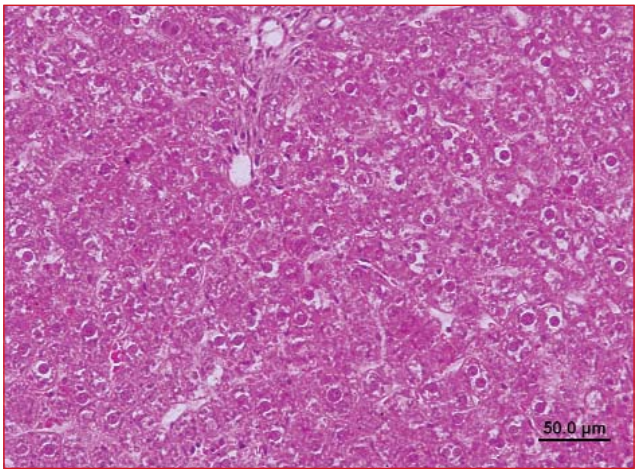

Fig. 1.
Histological examination of liver tissue – SLD. The figure shows normal liver histology, no evidence of steatotic changes is seen (bar 50 µm), haematoxylin-eosin, magnification 200×.

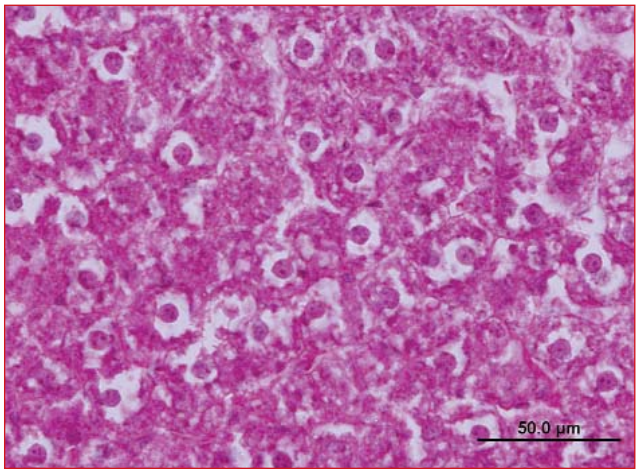

Fig. 2.
Histological examination of liver tissue – MCDD. Normal liver histology was found with no evidence of steatotic changes. Perinuclear spaces are autolytic in the course of liver dystrophia (bar 50 µm), haematoxylin-eosin, magnification 400×.

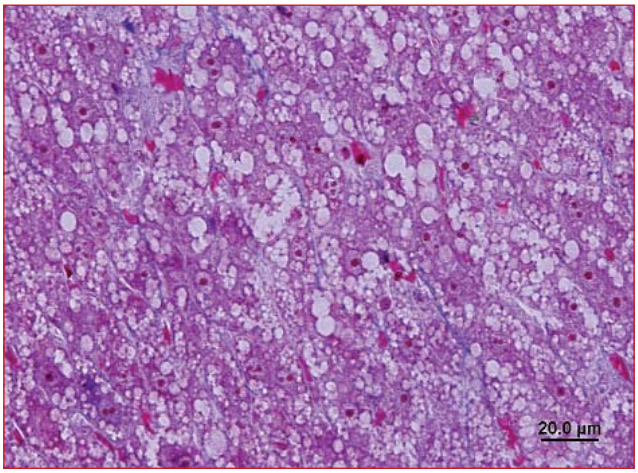

Fig. 3.
Histological examination of liver tissue – CHOL. The picture shows steatotic changes in all hepatocytes. Microvesicular and macrovesicular steatosis is present (bar 20 µm), blue trichrome staining, magnification 400×.

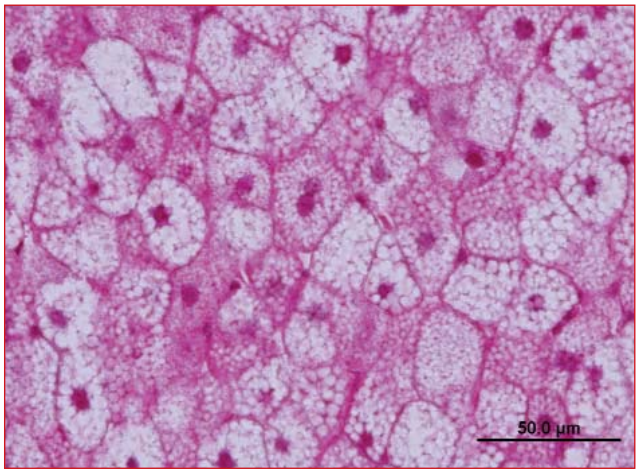

Fig. 4.
Histological examination of liver tissue – OA. This picture presents severe steatotic changes in all hepatocytes. Microvesicular and macrovesicular steatosis is present (bar 50 µm), haematoxylin-eosin, magnification 400×.

steatotic liver is able to be transplanted with hazards for recipient.

Our experiment showed the induction of the liver steatosis (histologically proved) in CHOL and OA rats but surprisingly not in the MCDD group (discrepancy with literature). We assume that it was caused by supplementation of a higher amount of L-arginine [12], which could replace methionine. Arginine participates in NO metabolism and is described as an inhibitor of steatosis formation [12].

Body weight and diet consumption

In our experiment we used rats, which were 6 weeks old, their growth had not finished and that was reflected in the weight curve. The highest weight occurred in CHOL rats and the lowest ones in OA rats. This could be caused by loss of appetite or induction of dyspepsia. There could also be some hormonal changes, particularly a shortage of anabolic steroids [27], because the OA rats had significantly lower weight of adrenal glands. This finding validated the fact that steroid production was blocked in livers by orotic acid, decreased expression of CYPs2C11 and 2E1 - biotransformation of steroid and fatty acid in the liver [27].

The CHOL rats had an increased weight of the spleen and heart that could be connected with their higher body weight. It is described in the pertinent literature, that an MCDD diet led to decrease in body weight (reducing of growth) [23], however we did not prove this fact.

Liver and DNA synthesis

The SLD control rats had lower liver DNA synthesis compared to CHOL and MCDD rats. This augmentation reflects liver tissue stimulation by components of diets. Damage to liver cells (hepatocyte, Kuppfer cell, interstitial cell) leads to an increasing count of mitosis, which is manifested by an increase in DNA synthesis. The damage of non-hepatocytes by steatosis is probably the cause of primary dysfunction after transplan-

tation and in patients after liver resection [20].

The total content of liver DNA was lowest in CHOL and OA rats. Both groups had liver steatosis (histologically proved), fat accumulation in cytoplasma to the detriment of the nucleus, whereas the SLD and MCDD group had lesser hepatocytes and liver steatosis was not proved.

Cholesterol metabolism

We applied ^{14}C-cholesterol to rats p.o. 24 hours before their sacrifice for observation of cholesterol absorption and metabolism. With regards to MCDD and OA groups, there were ^{14}C-cholesterol activities in tissues comparable with control SLD. The CHOL rats had significantly lower ^{14}C-cholesterol activities in all investigated body compartments. This was caused by feeding them a cholesterol diet where the mentioned compartments are completely saturated previously with higher conversion of cholesterol into bile acids [5]. Considering the fact that the rats in all groups had the same volume of fat in their diet, we may assume that steatosis development (CHOL group) had to be evoked by the cholesterol itself. This cholesterolaemic steatosis could have been caused by joining of steroids and the neutral fat metabolism [9].

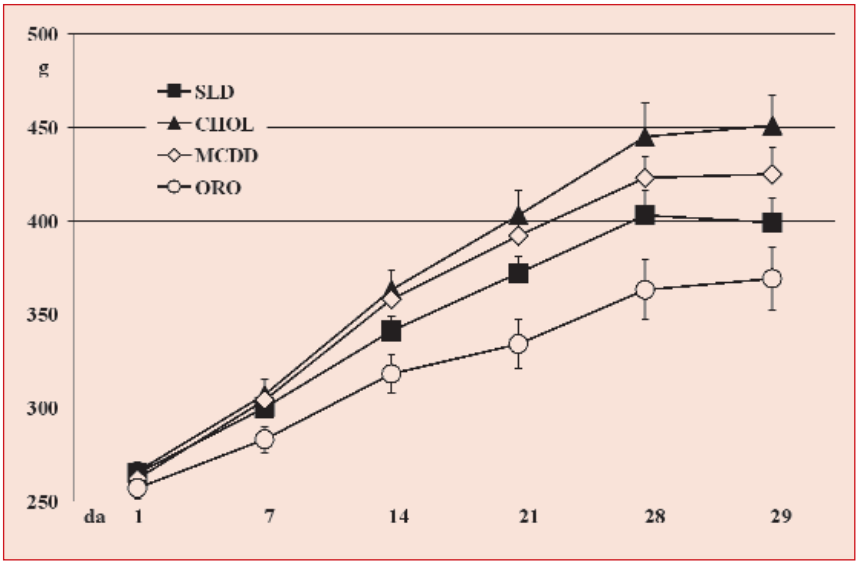

Fig. 5.
Body weight of the rats during experiment (g).

Serum insulin and leptin concentration

Our results prove that the rats with histologically proved steatosis (group CHOL and OA) had lower leptin concentrations than ones without it (SLD and MCDD) and miss the effect of this. The leptin is able to mobilise the fat [17] and avoid fat storage in liver [2], and stimulate beta-oxidation [17]. Leptin delivery leads to improvement of steatosis, because leptin even has a stimulatory effect on the proliferation and protein synthesis in liver cells [14]. There were not lower leptin concentrations supported by the reparation liver process in our experiment.

The rats without liver steatosis (SLD, MCDD) had higher leptin concentrations and lower glucose ones than the rats with steatosis (CHOL, OA). It is a known fact that leptin modulates insulin sensitivity of the liver [6]. No significant differences occurred in serum insulin concentration among the groups, hence we assume that insulin reactivity was not responsible for liver steatosis in rats.

CONCLUSIONS

Liver steatosis could be a result of relatively non significant dietary

Table 1. Content and synthesis of liver DNA and organs weight (g, mg). The statistical significance is present in cells and the all results are compared with SLD group.

	SLD	MCDD	CHOL	OA
s.a. DNA (Bq/ g DNA)	0.170 ± 0.031	0.363 ± 0.098 p = 0.097	0.325 ± 0.021 p = 0.003	0.255 ± 0.026 p = 0.069
c.o. DNA (mg/g)	1.68 ± 0.036	1.88 ± 0.071	1.48 ± 0.13	1.46 ± 0.14 p = 0.007
liver (g)	13.43 ± 0.81	14.20 ± 0.88	19.86 ± 1.40 p = 0.004	17.06 ± 1.52 p = 0.068
spleen (g)	0.837 ± 0.073	0.773 ± 0.058	1.097 ± 0.067 p = 0.03	0.786 ± 0.054
heart (g)	0.990 ± 0.034	1.080 ± 0.072	1.143 ± 0.040 p = 0.019	0.931 ± 0.028
adrenals (mg)	648 ± 39	716 ± 30	738 ± 39	440 ± 21 p = 0.002

Table 2. ^{14}C-cholesterol activities in rat tissue (kBq/g of tissue, or kBq/mL of blood).

	SLD	MCDD	CHOL	OA
intestine	1 952 ± 797 vs. CHOL	1 505 ± 218	335 ± 64	1157 ± 190
blood	616 ± 44 vs. CHOL	681 ± 85 vs. CHOL	228 ± 36	616 ± 77 vs. CHOL
liver	833 ± 123 vs. CHOL	1 237 ± 158 vs. CHOL, OA	312 ± 71	454 ± 45 vs. SLD, MCDD
hearth	152 ± 14 vs. MCDD	283 ± 39 vs. CHOL	103 ± 15	233 ± 26 vs. CHOL
spleen	1 276 ± 182 vs. CHOL	1 245 ± 206 vs. CHOL	635 ± 122 vs. CHOL	1 256 ± 112 vs. CHOL

The statistical significance is $p<0.05$ in all results. The compared groups are indicated in cell.

Table 3. Serum glucose, total cholesterol, HLD, LDL, TAG, AST, GMT, leptin and insulin concentration and transaminase activities. The identical symbols indicate compared couples. 1 symbol represents statistical significance $p<0.05$, 2 symbols are $p<0.01$.

	SLD	MCDD	CHOL	OA
glucose (mmol/l)	5.93 ± 0.21 ** ++	7.01 ± 0.70	8.15 ± 0.28 **	8.25 ± 0.47 ++
cholesterol (mmol/l)	2.28 ± 0.33	1.78 ± 0.08 **	3.05 ± 0.22 ** ++	1.95 ± 0.24 ++
HDL-cholesterol (mmol/l)	1.37 ± 0.13	0.93 ± 0.06 *	1.07 ± 0.06	1.51 ± 0.17 *
LDL-cholesterol (mmol/l)	0.21 ± 0.08	0.13 ± 0.02	0.54 ± 0.05 *	0.11 ± 0.02 *
TAG (mmol/l)	1.41 ± 0.21 ***	2.32 ± 0.19 •••	3.27 ± 0.43 *** +++	0.65 ± 0.17 +++ •••
ALT (µkat/l)	0.75 ± 0.09 **	0.55 ± 0.16 ••	0.71 ± 0.07 ++	1.74 ± 0.40 ** ++ ••
AST (µkat/l)	2.14 ± 0.21	2.00 ± 0.25	2.02 ± 0.32	3.22 ± 0.76
leptin (ng/ml)	8.59 ± 0.53 ** xxx	8.81 ± 1.14	5.78 ± 0.76 **	5.17 ± 0.44 xxx
insulin (ng/ml)	1.91 ± 0.17	2.01 ± 0.19	1.50 ± 0.19	2.41 ± 0.19

abnormalities. The liver DNA synthesis was lower in the control rats so we assumed that hepatocytes as well as non-hepatocytes were stimulated by some components of experimental diets. The rats without liver steatosis (SLD, MCDD) had higher leptin concentrations and lower glucose ones then the rats with steatosis (CHOL, OA). No significant differences occurred in serum insulin concentration among the groups, hence we assume that insulin reactivity was not responsible for liver steatosis development in rats.

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