Rocz Panstw Zakl Hig 2018;69(1):37-43

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ORIGINAL ARTICLE

EFFECT OF CALORIC RESTRICTION ON LIVER FUNCTION IN YOUNG AND OLD APOE/LDLR$^{-/-}$ MICE

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ABSTRACT

Background. Caloric restriction (CR) leads to decrease metabolic intensity, which results in a reduction of oxygen consumption and the amount of free radicals. This can affect the function of the liver. Studies show that caloric restriction does not alter or significantly increase the enzyme activity associated with gluconeogenesis, but the effect was different according to the age of the model animals.

Objective. The aim of the study was to determine the effect of caloric restriction on liver function in young and old ApoE/LDLr$^{-/-}$ mice.

Material and methods. Dietary experiments were performed on 2 and 5 month old male ApoE/LDLr$^{-/-}$ mice. Animals were divided into 3 experimental groups (n=6) and fed AIN'93G diet for 8 and 5 weeks, respectively. Control animals were fed *ad libitum* (AL) and housed in a colony cages. These animals were checked for dietary intake. The second group were also fed *ad libitum* but the animals were kept individually in cages (stress AL- sAL). Similarly to sAL group, the animals from the CR group were kept individually but received a 30% less diet compared to AL group. At the end of the experiment animals were euthanized and the blood, liver and adipose tissue have been collected. Alanine aminotransferase (ALT) as well as aspartate aminotransferase (AST) were measured in plasma. Fatty acid profile was evaluated (relative %) in adipose tissue (GC-MS). Liver's stetosis was assessed. Results were analyzed statistically (ANOVA, STATISTICA v.10.0).

Results. CR ApoE/LDLr$^{-/-}$ mice showed significantly lower body weight compared to animals, both AL and sAL. There were no significant differences between ALT and AST in both younger and older animals. However, negative tendencies were more pronounced in younger animals. In young animals CR significantly increased liver weight compared to AL (4.14 vs 3.73g/100g). In adipose tissue fatty acid profile differed in CR mice compared to control in young animals.

Conclusions. Caloric restriction did not affect liver enzymes in mice. Caloric restriction showed similar but not identical metabolic activity in young and old mice.

Key words: *caloric restriction, liver, ApoE/LDLr$^{-/-}$ mice*

STRESZCZENIE

Wprowadzenie. Restrykcje kaloryczne (CR) prowadzą do spadku intensywności metabolizmu, co wiąże się ze zmniejszeniem zużycia tlenu i ilości powstających wolnych rodników. Tym samym mogą mieć wpływ na funkcjonowanie wątroby. Badania wykazują, że ograniczenia kaloryczne nie zmieniają lub znacząco zwiększają aktywność enzymów związanych z glukoneogenezą, w tym kluczowego enzymu jakim jest aminotransferaza alaninowa (ALT). Obserwowany efekt był różny w zależności od wieku modelowych zwierząt.

Cel badań. Celem pracy było określenie wpływu restrykcji kalorycznej na czynność wątroby u młodych i starszych myszy ApoE/LDLr$^{-/-}$.

Materiał i metody. Doświadczenie żywieniowe przeprowadzono na 2. i 5. miesięcznych samcach myszy ApoE/LDLr$^{-/-}$. Zwierzęta podzielono na 3 grupy doświadczalne (n=6) i żywiono dietą AIN'93G przez okres 8 i 5. tygodni. Zwierzęta z grupy kontrolnej (AL) żywione były *ad libitum* i przetrzymywane zbiorowo w klatkach. Spożycie diety było sprawdzane. Grupa druga otrzymywała dietę *ad libitum* przy czym zwierzęta przetrzymywano w klatkach indywidualnie (sAL). Analogicznie do grupy drugiej zwierzęta z grupy restrykcji kalorycznych (CR) były trzymane indywidualnie, jednak otrzymywały 30% mniej diety w porównaniu do grupy kontrolnej AL. Po zakończeniu doświadczenia zwierzęta poddano eutanazji

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i pobrano wątrobę w celu oceny histologicznej (barwienie H&E i ORO) oraz krew, w której metodą spektrofotometryczną przeprowadzono oznaczenie enzymów aminotransferazy alaninowej (ALT), jak również aminotransferazy asparaginowej (AST). W tkance tłuszczowej oznaczono profil kwasów tłuszczowych (GC-MS). Wyniki poddano analizie statystycznej (ANOVA , STATISTICA v.10.0).
Wyniki. Zarówno młodsze, jak i starsze myszy ApoE/LDLr$^{-/-}$ poddane restrykcji kalorycznej wykazały istotnie niższą końcową masę ciała w porównaniu do zwierząt z grup kontrolnych, zarówno AL, jak i sAL. Nie stwierdzono istotnych statystycznie różnic pomiędzy grupami w poziomie ALT, jak również AST zarówno u zwierząt młodszych, jak i starszych, przy czym negatywne tendencje były wyraźniej widoczne u zwierząt młodszych poddanych restrykcji kalorycznej. Również u młodszych zwierząt CR miało istotny niekorzystny wpływ na masę wątroby w porównaniu do kontroli (4,14 vs 3,73 g/100g). Zaobserwowano istotny wpływ CR na profil kwasów tłuszczowych w tkance tłuszczowej u zwierząt młodszych.
Wnioski. Restrykcje kaloryczne nie miały wpływu na próby czynnościowe wątroby u myszy. Restrykcje kaloryczne wykazały podobną, ale nie identyczną aktywność metaboliczną u młodszych i starszych zwierząt.

Słowa kluczowe: *restrykcje kaloryczne, wątroba, myszy ApoE/LDLr$^{-/-}$*

INTRODUCTION

A balanced diet can provide proper proportions of nutrients to organisms. In 1935 McCay [13], for the first time documented the hypothesis that in rodents the increase in life expectancy is correlated with the reduction of the consumption of a diet called caloric restriction (CR) [7]. Caloric restriction (CR) is defined as restricting the diet of an organism to fewer calories (20–50%) than *ad libitum* feeding without altering the levels of vitamins, minerals and amino acids in order to include all essential nutrients. Initially it was speculated that caloric restriction prolongs life, because it causes a slowdown of growth and development, and thereby contributes to the inhibition of "gene age". This theory, however, was rejected. Recent studies suggest that caloric restriction causes the body's low stress levels and thus stimulates intracellular signaling pathways. This protects cells and tissues against the effects of aging. In addition, CR regulates the metabolism of glucose, fats and proteins in a way that increases the chances of survival under stress conditions [21]. However, the actual mechanism that CR can effectively lengthen lifespan remains controversial. Many of the early hypotheses to explain this effect were based on it being a passive alteration in metabolism, however recent data support the idea that CR is not simply a passive effect. It has therefore been hypothesized that the beneficial effects of the CR diet are due to increased activity or efficiency of cellular antioxidant defenses. Some studies have supported this hypothesis [14, 17], other studies have failed to demonstrate consistent enhancement of antioxidant defenses in association with the CR diet [4, 19, 22]. There is evidence that CR is an active, highly conserved stress response that evolved early in life's history to increase organism's chance of surviving adversity [21].

CR has also been proven to be an effective treatment for NAFLD [1, 23]. Nonalcoholic fatty liver disease (NAFLD) is a disease spectrum that includes hepatic steatosis, steatohepatitis, fibrosis and liver cirrhosis. The fatty liver has been shown to be insulin resistant and to overproduce glucose, VLDL, CRP, and coagulation factors leading to hyperglycemia and lipid disorders.

Tauriainen et al. [24] reported that CR almost completely prevented fatty liver formation in mice. The mechanisms underlying the beneficial effects of CR is not well understood.

Therefore, CR is the most potent and reproducible intervention demonstrated to not only extend lifespan but also delay the negative physiologic consequences of chronic diseases associated with aging. However, *Harisson* et al. [6] has shown, that 40% CR increases mortality in C57BL/6J mice when started just after weaning (i.e. 4 weeks of age), but increases lifespan when started in middle age. Unfortunately, little is know about effect CR on mice in different age.

The aim of the study was to determine the effect of caloric restriction on liver function in young and old ApoE/LDLr$^{-/-}$ mice.

MATERIAL AND METHODS

Animals and and feeding

All procedures involving animals were conducted according to the Guidelines for Animal Care and Treatment of the European Union and were approved by the Local Animal Ethics Commission. ApoE/LDLr$^{-/-}$ male mice were originally obtained from Jackson Lab (USA) and bred in house. Animals were housed in a temperature-controlled environment (22-25°C) with a 12 hour light/dark cycle.

Two and five month old male ApoE/LDLr$^{-/-}$ mice were divided into 3 experimental groups (n = 6) and fed AIN'93G diet (Table 1) for 8 and 5 weeks, respectively. Control animals were fed *ad libitum* (AL) and housed in a cages. Daily dietary intake were monitored. Stressed AL group (sAL) received *ad libitum* diet, but animals were kept individually in cages. Similarly to sAL group the animals from the CR group were kept

individually but received a 30% less diet compared to the AL group. Body weight of mice were monitored weekly. At the end of the experiment animals were euthanized. The mice were injected intraperitoneally with 1000 IU of heparin (Sanofi-Synthelabo; Paris, France) and after 10 min, anesthetized with ketamine/xylazine given intraperitoneally. Finally mice have been sacrificed by cervical dislocation.

Table1. Composition of experimental diet (%)

Corn starch	53.2486
Caseine	20
Sucrose	10
Soybean oil	7
Celulose powder	5
Mineral mixture *	3.5
Vitamin mixture *	1
Choline	0.25
Tert-butylhydrochinone	0.0014

* According to Reeves, 1993

Blood sampling and measurements of biochemical markers

Blood samples were taken from the vena cava and were collected into test tubes and centrifuged (13 000 x g, 4 min) to obtain plasma samples. The plasma were deep frozen (-80°C) and stored until further analysis using commercially available kits for alanine transaminase (ALT), aspartate aminotransferase (AST) (Pentra 400, Horiba).

Determination of fatty acids composition in adipose tissue

Adipose tissues were collected from each mice. The samples (10 mg) were placed in vials and treated with 2 ml solution of 0.5 M KOH in methanol. The samples were heated at 57°C for 15 min. Next, 2 ml of 14% BF_3 in methanol was added and heated at 57°C for 15 min. After cooling, 2 ml of hexane and 2 ml saturated sodium chloride was added. The mixture was vortexed. The upper n-hexane layer was transferred to eppendorf tubes and dried with anhydrous Na_2SO_4. The analysis of fatty acids methyl esters (FAME) was performed on a SHIMADZU GC-MS- QP 5050A equipped with a SP-2560 capillary column (100 m x 0.25 mm i.d. x 0.25 μm film thickness, Supelco). Helium was the carrier gas and operated at flow rate of 1.8 ml/min. Injector temperature was maintained at 245ºC, detector temperature was 200ºC. The total FAME profile in a 1 μl injection at a split mode was determined. The oven temperature was operated at 60°C for 5 min, then the temperature programmed at 5°C/min to 180°C, held for 16 min, programmed at 5°C/min to 220°C, held for 7 min. FAME identification was validated and based on electron impact ionization mode.

Histological analysis

The livers were excised and weight. Part of liver from each mice was formalin fixed for routine histopathological examination. Tissues were placed in sucrose for 24 h, dried and frozen in OCT. Slides (7 μm sections) were stained with hematoxylin and eosin (H&E) and oil red O (ORO).

Statistical analysis

Results are expressed as mean ± SEM. Where appropriate, the data were subjected to analysis of variance calculated in the STATISTICA 10 package (StatSoft Inc., USA), followed by *post-hoc Tukey* multiple range test. Differences were considered significant at $p<0.05$.

RESULTS

Effect of CR on body weight

Regardless of age, body weight in ApoE/LDLr$^{-/-}$ caloric restricted mice significantly decreased compared to the AL and sAL animals (Table 2). Young animals have reduced body weight by 6.52 g, while old animals by 5.97 g. Both in young as well as old animals the highest decrease in body weight were observed after first week of experiment (5.9 g in young mice and 7 g in old mice, data not shown).

Effect of CR on liver

In young animals CR significantly affected the liver. Liver weight (g/100g b.w.) significantly increased (by 11%) in mice fed CR diet. However, in old animals no significant differences in liver weight were observed (Table 2).

Table 2. Effect CR diet on ApoE/LDLr$^{-/-}$ mice in different age ($p < 0.05$. $^{a.b}$ young animals, $^{A.B}$ old animals)

	Experimental groups					
	AL_y	sAL_y	CR_y	AL_o	sAL_o	CR_o
	% fatty acids					
Body weight (g)	26.20 ± 1.24 ^{a}	26.26 ± 0.45 ^{a}	19.68 ± 0.85 ^{b}	27.96 ±0.37 ^{A}	27.59 ± 0.80 ^{A}	21.99 ± 0.92 ^{B}
Liver weight (g/100g)	3.73 ± 0.08 ^{a}	3.77 ±0.04 ^{a}	4.16 ± 0.16 ^{b}	4.11 ± 0.12	4.08 ± 0.28	3.62 ± 0.11
ALT(U/L)	47.15 ± 11.93	31.85± 11.50	90.45 ± 11.84	53.86 ± 8.60	53.75 ± 7.31	66.42 ± 8.18
AST(U/L)	147.59 ± 37.65	128.37± 46.16	322.94 ± 46.30	234.16 ± 52.76	145.86 ± 14.39	346.93 ± 75.76

* – różnica istotna statystycznie przy $p<0.05$

In old mice ALT as well as AST levels were higher compared to young animals (by 14% for ALT and by 59% for AST). After CR treatment no significant differences between ALT and AST both in young and old animals were observed, however negative tendencies (i.e. increase) were more pronounced in young animals compared to old (ALT: 91% vs 23% AST: 118% vs 48%).

No significant differences were observed in livers stained ORO. However, compared to AL slightly more fat in liver has been observed in young animals after CR treatment.

Table 3. Fatty acid profile in adipose tissue in ApoE/LDLr$^{-/-}$ mice ($p < 0.05$, $^{a.b}$ young animals, $^{A.B}$ old animals)

Fatty acids	Experimental groups					
	AL_y	sAL_y	CR_y	AL_o	sAL_o	CR_o
	Fatty acids (%)					
C14:0	0.52 ± 0.02 ^{a}	0.62± 0.03 ^{a}	0.91± 0.03 ^{b}	1.83 ± 0.09	1.77 ± 0.07	2.12 ± 0.14
C14:1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.09 ± 0.01	0.11 ± 0.01	0.15 ± 0.02
C15:0	0.06 ± 0.01	0.04 ± 0.02	0.01 ± 0.01	0.27 ± 0.01	0.25 ± 0.01	0.20 ± 0.05
C16:0	14.16 ± 0.21 ^{a}	15.04 ± 0.41 ^{a}	20.52 ± 0.51 ^{b}	17.16 ± 0.37 ^{AB}	15.94 ± 0.32 ^{A}	17.89 ± 0.85 ^{B}
C3-16:1	0.40 ± 0.04 ^{a}	0.34 ± 0.02 ^{ab}	0.18± 0.07 ^{b}	0.24 ± 0.09	0.32 ± 0.14	0.21 ± 0.07
C9-16:1	3.57 ± 0.31	3.09 ± 0.29	3.87 ± 0.38	6.76 ± 0.47	6.20 ± 0.17	8.27 ± 0.47
C17:0	0.13 ± 0.02 ^{a}	0.09 ± 0.01 ^{ab}	0.07 ± 0.01 ^{b}	0.38 ± 0.03	0.34 ± 0.01	0.28 ± 0.02
C8-17:1	0.20 ± 0.03 ^{a}	0.07 ± 0.01 ^{b}	0.05 ± 0.02 ^{b}	0.73 ± 0.05	0.71 ± 0.02	0.64 ± 0.07
C18:0	1.82 ± 0.07 ^{a}	1.83 ± 0.08 ^{a}	4.54 ± 0.49 ^{b}	3.99 ± 0.34	3.91 ± 0.17	4.28 ± 0.55
C9-18:1 n-9	33.84 ± 0.20	33.55 ± 0.25	34.63 ± 0.41	30.87 ± 0.33	31.32 ± 0.38	30.32 ± 0.43
C9.12-18:2 n-6	41.73 ± 0.22 ^{a}	41.72± 0.33 ^{a}	32.32 ± 1.18 ^{b}	30.89 ± 0.46 ^{A}	31.90 ± 0.44 ^{A}	28.93 ± 1.62 ^{B}
C20:0	0.14 ± 0.04	0.12 ± 0.04	0.25 ± 0.02	0.60 ± 0.04	0.68 ± 0.03	0.57 ± 0.11
9.12.15-18:3 n-3	3.40 ± 0.11 ^{a}	3.49 ± 0.12 ^{a}	2.63 ± 0.19 ^{b}	5.29 ± 0.28	5.77 ± 0.26	6.01 ± 0.35
C6.9-18:2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.08 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
C8.11-20:2 n-9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.11 ± 0.01	0.10 ± 0.01	0.08 ± 0.01
other (e.g.C20:3)	0.06± 0.01	0.03 ± 0.02	0.03 ± 0.01	0.58 ± 0.03	0.63 ± 0.05	0.94 ± 0.13

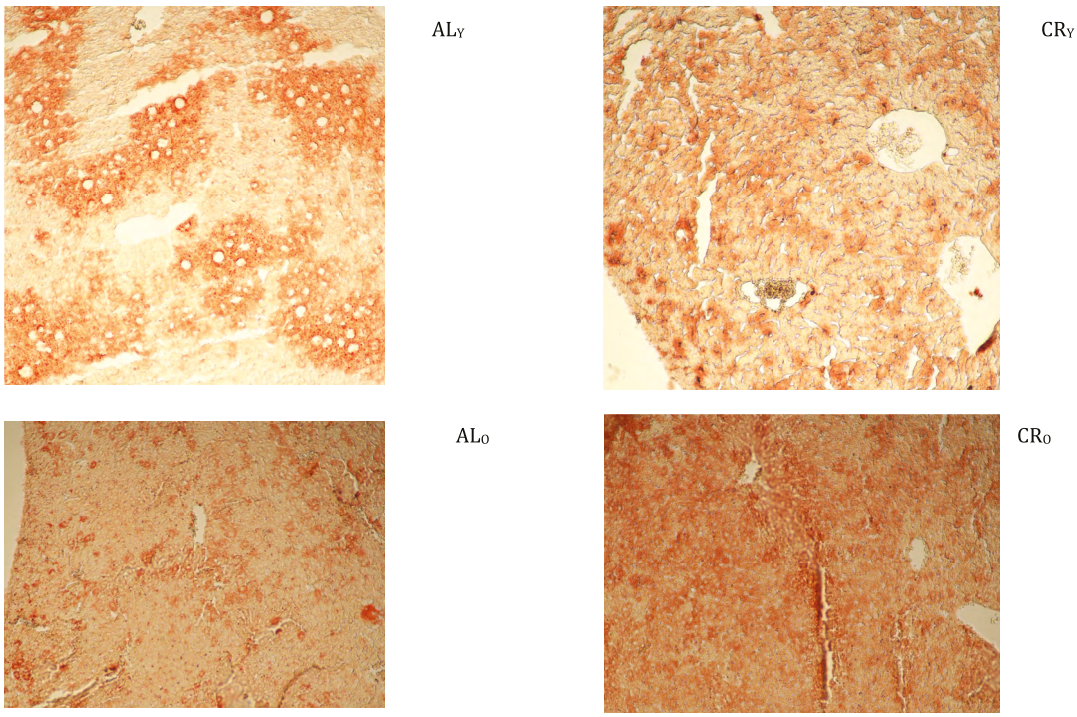

Photo 1. Livers of young (AL_y, CR_y) and old (AL_o, CR_o) ApoE/LDLr$^{-/-}$ mice stain ORO

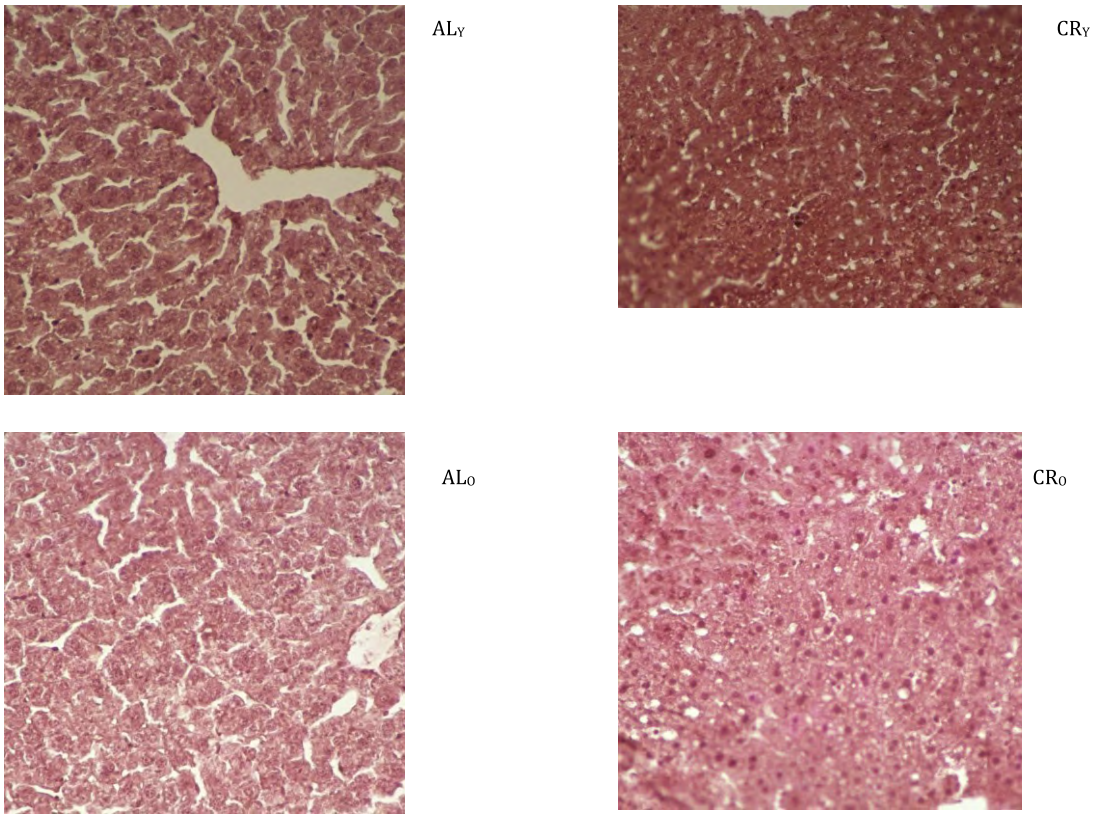

Photo 2. Liver of young (AL_y, CR_y) and old (AL_o, CR_o) ApoE/LDLr$^{-/-}$ mice stain H&E

Effect of CR on fatty acid profile in adipose tissue

Fatty acid profile in adipose tissue changed significantly after caloric restriction. In young animals negative changes in individual fatty acids levels were observed. Compared to control SFA level has significantly increased, whereas PUFA level was significantly decreased. In old animals significant difference was observed only in linoleic acid level (Table 4). No differences in SFA level in old animal have been observed.

Table 4. Saturated fatty acids, monounsaturated fatty acid and polyunsaturated fatty acids in ApoE/LDLr$^{-/-}$ mice adipose tissue ($p < 0.05$, $^{a.b}$ young animals, $^{A.B}$ old animals)

Fatty acids	Experimental groups					
	AL_y	sAL_y	CR_y	AL_o	sAL_o	CR_o
	Fatty acids (%)					
∑ SFA	16.83 ± 0.24 ^{a}	17.73 ± 0.49 ^{a}	26.30 ± 1.0 ^{b}	24.23 ± 0.79	22.90 ± 0.49	25.22 ± 1.35
∑ MUFA	38.00 ± 0.29 ^{b}	37.04 ± 0.35 ^{a}	38.72 ± 0.52 ^{b}	38.44 ± 0.81 ^{A}	38.34 ± 0.29 ^{AB}	39.38 ± 0.85 ^{B}
∑ PUFA	45.13 ± 0.19 ^{a}	45.21 ± 0.25 ^{a}	34.95 ± 1.32 ^{b}	36.26 ± 0.44^{AB}	37.78 ± 0.22^{A}	34.27 ± 1.99^{B}

DISCUSSION

While excessive calorie intake are associated with several health problem also NAFLD, caloric restriction (CR) with adequate nutrition ameliorates metabolic disturbances. A strong connection between caloric intake and aging has been developed. Caloric restriction significantly increases lifespan and decreases the rate of occurrence of most age-associated degenerative diseases in rodents. CR leads to a decrease in metabolic intensity. This is associated with a reduction in oxygen consumption and the amount of free radicals produced, which can affect the liver's function. *Tauriainen* et al. [24] has shown that CR almost completely prevented fatty liver formation in mice. However, the impact of caloric restriction on liver is not fully understood. Therefore, the aim of the study was to determine the effect of caloric restriction on liver function in young and old ApoE/LDLr$^{-/-}$ mice.

Presented study confirmed, that CR affect the body weight both in young and old animals. *Omodei* et al. [15] review that caloric restriction is one of the most effective nutritional interventions that protects against obesity. The use of caloric restriction causes

a reduction in body fat and thus losing weight, thereby lowering leptin synthesis and simultaneously an increase in adiponectin. The recent evidence suggests that even when implemented over a short period, caloric restriction is a safe and effective treatment.

In our study we observed that CR had no significant effect on liver's enzymes. However, ALT and AST levels tended to increase mostly in young animals after CR treatment. Additionally, CR significantly increased liver weight in young animals, whereas no differences were observed in old animals. ORO staining showed that hepatic steatosis was similar in old amimals regardless of the type of nutrition. In young animals liver histology was slighty impaired by CR.

Caloric restriction (CR) is commonly recommended for improvement of obesity-related diseases such as NAFLD [9]. *Kim* et al [9] found that CR reverted hepatic steatosis of db/db mice. H&E and ORO staining showed that hepatic steatosis in db/db mice was reduced by CR administration. Additionally, they found that hepatic enzymes (ALT and AST) were significantly decreased by CR treatment. On the contrary, *Hagopian* et al. [5] observed that short-term (1 month) and long-term (28 months) calorie restriction diet in mice significant increased liver transaminases including ALT and AST as well as multiple other gluconeogenic enzymes, e.g. pyruvate carboxylase, phosphoenopyruvate carboxykinase, fructose-1-6-biphosphatase, and glucose-6-phosphatase.

Many authors have proved, that the transaminases are important to effectively utilize amino acids for gluconeogenesis during caloric restriction. ALT plays a significant role in gluconeogenesis and amino acid metabolism. Therefore, it is not just a marker of cellular injury. Liver ALT will increase in response to dietary protein as well as fasting [20]. This suggests some ALT elevations may reflect a metabolic adaptive response and not necessarily indicate hepatocellular injury. Thus should not be considered adverse [18]. However, in our study in young animals liver's weight significantly increased after CR treatment. The same was true for liver histology which indicated slighty more fat (i.e. intensified steatosis).

The mechanisms underlying the accumulation of fat in the liver may include excess dietary fat, increased delivery of free fatty acids (FFA) to the liver, inadequate fatty acid oxidation and increased de novo lipogenesis [26]. Plasma FFA normally originate from adipose tissue lipolysis and are the major circulating lipid fuel.

Because the vast majority of the fatty acids stored in adipose tissue originate from dietary fat, studying this aspect of adipose tissue physiology is warranted [8].

Fatty acid profile in mice's adipose tissue fed caloric restricted diet were analysed. Linoleic acid (18:2 n-6) significantly decreased after CR treatment in both young and old animals. Therefore PUFA decreased after CR treatment. However, this effect was more visible in young animals.

This may reflect lower dietary intake, where soybean oil is a source of fat in AIN93G diet. Composition of soybean oil is: linoleic acid (51%), oleic acid (23%), palmitic acid (10%), linolenic acid (7%) and stearic acid (4%). When examining the effect of CR on fatty acid composition in young mice, it was observed that most of the fatty acid changes occurred. SFA level statistically increased in young animals after CR treatment (16,83 vs 26,30%). In old animals no effect on SFA has been observed.

Phinney et al. [16] found that there was a decrease of EPA and linolenic acid (18:3 n-3) in the adipose tissue of subjects consuming weight-reducing diets for 3-5 months. These observations suggest that essential fatty acid content in weight-reducing diets may be inadequate.

When investigating the effect of CR (CR-soy vs. control) on mitochondrial phospholipid fatty acid composition, increase of ($P<0.05$) total n-6 fatty acid content was observed [2]. CR also resulted in a decrease in monounsaturated fatty acid (MUFA) content due to decreases in the content of 16:1n-7 and 18:1n-7.

It has been reported that CR increases the content of 18:2n-6 and decrease long chain polyunsaturated fatty acids of membrane phospholipids in liver [10, 11], spleen [25] and heart [12]. However, we found no evidence that CR increases the level of 18:2n-6. The results of the present study are consistent with the idea that CR decreases long chain polyunsaturated fatty acids. However, it is possible that additional changes in fatty acid composition may occur with age and/or duration of CR. In support of this idea, it has been reported that both, level of CR and length of CR influence skeletal muscle phospholipid fatty acid composition [3, 6].

Thus, additional studies at multiple time points are likely needed to completely characterize the influence of CR on mice model.

CONCLUSIONS

1. Caloric restriction did not affect liver enzymes in mice.
2. Caloric restriction showed similar but not identical metabolic activity in young and old mice.

Conflict of interest

The authors declare no conflict of interest.

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Received: 21.08.2017
Accepted: 14.12.2017