The Effect of Combined Diet Containing n-3 Polyunsaturated Fatty Acids and Silymarin on Metabolic Syndrome in Rats

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Summary

The risk of development of metabolic syndrome can be increased by hypertriglyceridemia. A search for effective therapy is a subject of considerable attention. Therefore, our hypothesis is that the fish oil (containing polyunsaturated fatty acids; n-3 PUFA) in a combination with silymarin can more effectively protect against hypertriglyceridemia-induced metabolic disturbances. The study was conducted using a unique non-obese strain of rats with hereditary hypertriglyceridemia an accepted model of metabolic syndrome. Adult male rats were treated with n-3 PUFA (300 mg/kg/day) without or with 1 % micronized silymarin in a diet for 4 weeks. The treatment with the diet containing n-3 PUFA and silymarin significantly reduced concentrations of serum triglycerides (-45 %), total cholesterol (-18 %), non-esterified fatty acids (-33 %), and ectopic lipid accumulation in skeletal muscle (-35 %) compared to controls. In addition, an increase in Abcg5 and Abcg8 mRNA expression (as genes affecting lipid homeostasis) as well as in protein content of ABCG5 (+78 %) and ABCG8 (+232 %) transporters have been determined in the liver of treated rats. Our findings suggest that this combined diet could be used in the prevention of hypertriglyceridemia-induced metabolic disorders.

Key words

Polyunsaturated fatty acids • Silymarin • Metabolic syndrome • ABCG5/8

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Introduction

There is consistent evidence that hypertriglyceridemia is associated with an increased risk of coronary heart disease (Sarwar *et al.* 2007) and other risks of disorders development, such as liver disease, insulin resistance, and type 2 diabetes. Its prevalence is steadily rising and affects approximately 33 % of the US population or 28 % of the population as found in the Copenhagen General Population Study (Nordestgaard 2016). Therefore, the search for effective treatment of hypertriglyceridemia and its complications is a subject of considerable attention.

Polyunsaturated fatty acids, especially those of marine origin – docosahexaenoic acid (DHA, C22:6 n-3) and eicosapentaenoic acid (EPA, C20:5 n-3), were identified as potent triglycerides-lowering agents. In general, n-3 polyunsaturated fatty acids (n-3 PUFA) reliably reduce triglyceridemia by 20-50 % and affect both fasting and nonfasting levels (Catapano *et al.* 2016). Interestingly, Engler *et al*. (1994) already published that EPA and DHA have an antihypertensive effect in spontaneously hypertensive as well as in normotensive rats. They explained this effect on direct action on the vascular smooth muscle. Other studies support the role of n-3 PUFA in the prevention of cardiovascular diseases and amelioration of non-alcoholic fatty liver disease (Arca *et al.* 2018). In contrast, the long-term placebo-controlled clinical trials have produced inconclusive data

on effects of n-3 PUFA in the management of cardiovascular risk, liver fat and fibrosis, or anti-obesity effects (Dasarathy *et al.* 2015). Different findings may be affected by the dose, duration of administration, and the proportion of DHA and EPA in the fish oil concentrates used. Clinical trials have shown that both DHA and EPA reduced triglycerides. However, DHA was more effective than EPA in increasing HDL-cholesterol and modulating specific markers of inflammation but it also had a negative effect on LDL cholesterol (Wei and Jacobson 2011, Allaire *et al.* 2016).

One possibility to increase the efficacy of n-3 PUFA against metabolic disorders associated with hypertriglyceridemia could be a combination with silymarin (SM) which has proven hepatoprotective and antioxidative effects (Surai 2015). Silymarin is an extract from the seeds of milk thistle, *Silybum marianum*, from the family of *Asteraceae*. It is a mixture of polyphenolic compounds such as silybin and others. Silymarin or silybin are traditionally used for its hepatoprotective, anti-inflammatory, anti-oxidative, and anti-fibrotic properties for treatment of liver and other chronic diseases (Neha *et al.* 2016, Eraky *et al.* 2018). Additionally, hypolipidemic and glucose-lowering properties of SM have been observed as well (Skottova and Krecman 1998, Skottova *et al.* 2003). Recently, it was found that the micronized form of SM provides more pronounced effects than standardized extract, mainly due to its better solubility (Zhang *et al.* 2009, Poruba *et al.* 2015).

In the current study, we tested a hypothesis that fish oil standardized to higher DHA content (50 % of DHA and 10 % of EPA) in the combination with a micronized form of SM can protect against hypertriglyceridemia-induced metabolic disturbances. The therapeutic effects of the n-3 PUFA with SM have not yet been tested. Therefore, we used a unique non-obese strain of rats with genetic hypertriglyceridemia (HHTg) that originates from Wistar rats and exhibits dyslipidemia, liver steatosis, insulin resistance, hyperinsulinemia, oxidative stress, and inflammation (Vrana and Kazdova 1990, Klimes *et al.* 1995, Kazdova *et al.* 1997, Zicha *et al.* 2006). Previously, we have found that the administration of SM as 1 % dietary supplements reduced a high sucrose diet-induced oxidative stress and improved plasma lipoprotein cholesterol profile in HHTg rats (Skottova *et al.* 2004).

The aim was to find out whether n-3 PUFA and their combination with SM have an effect on dyslipidemia, ectopic triacylglycerol accumulation, and oxidative stress parameters which may play an important role in the heart failure, the pathogenesis of non-alcoholic fatty liver disease (NAFLD), skeletal muscle insulin resistance, and pancreatic β-cells dysfunction (Sattar and Gill 2014). Further, we focused on changes in mRNA and protein content of lipid transporters, and receptors important in lipid metabolism (Wang and Smith 2014). The mRNA expression of liver cytochromes P450 (CYP2E1 and CYP7A1) also deserves attention because they are involved among others in the reactive oxygen production (Wang *et al.* 2003).

Materials and Methods

Animals

Hereditary hypertriglyceridemic rats (HHTg) were obtained from the Institute for the Clinical and Experimental Medicine (Prague, Czech Republic). The animals were housed in a temperature-controlled room under a 12:12 h light-dark cycle and allowed free access to chow and water. All experiments were performed in accordance with the Animal Protection Law of the Czech Republic Act. No 359/2012 Coll. All procedures with animals were approved by the Ethics Committee, Ministry of Youth and Sports, Czech Republic.

Chemicals

All used chemicals in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). A mixture of n-3 polyunsaturated fatty acids (n-3 PUFA; with a content of 50 % DHA and 10 % EPA) was obtained from Smart City S.A. (Luxembourg), micronized silymarin (SM) was purchased from Favea (Koprivnice, Czech Republic).

Study design and sampling

HHTg rats (4-month-old) were fed a standard laboratory diet (SLD) as a control group (control; n=6), or SLD supplemented with n-3 polyunsaturated fatty acids (n-3 PUFA; 300 mg/kg/day; n=6), or SLD supplemented with n-3 PUFA and micronized silymarin (n-3 PUFA+ SM; 1 % of silymarin in SLD; n=6) for 4 weeks. The food intake was not different between groups as we measured it daily. At the end of the study, rats were decapitated in a postprandial state and their blood serum and tissues were collected for final biochemical analyses.

Lipid parameters assays

Determination of triglycerides in liver, heart, skeletal muscle and measuring of cholesterol in liver were performed as described previously (Qi *et al.* 2002). Briefly, tissues were powdered under liquid N_2 and extracted in the mixture of chloroform: methanol (2:1). Afterwards, the solution of 2 % potassium dihydrogenphosphate was added and an organic phase formed in the mixture was taken and evaporated under N_2. The residue was dissolved in isopropyl alcohol. Triglycerides (TAG) and cholesterol concentrations were determined by enzymatic assay (TG L 250S, Erba-Lachema, Brno, Czech Republic). Blood glucose concentrations were measured by the glucose oxidase assay (Erba-Lachema, Brno, Czech Republic) using tail vein blood drawn into 5 % trichloracetic acid and then centrifuged. Serum triglycerides and cholesterol concentrations were determined by standard enzymatic methods (Erba-Lachema, Brno, Czech Republic). Serum nonesterified fatty acids (NEFA) levels were analyzed using an acyl-CoA oxidase-based colorimetric kit (Roche diagnostics, Mannheim, Germany). Serum insulin concentrations were measured using a rat insulin ELISA kit (Mercodia, Uppsala, Sweden).

Oxidative stress parameters

The activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GR), and glutathione transferase (GST) were analyzed using Cayman Chemicals assay kits (Ann Arbor, MI, USA). Catalase (CAT) activity, concentration of lipoperoxidation products, such as thiobarbituric-reactive substances (TBARS) and conjugated dienes, were determined as described previously (Malinska *et al.* 2010). The reduced form of glutathione was determined with glutathione HPLC diagnostic kit (Chromsystems, Gräfelfing, Germany). Paraoxonase 1 (PON-1) activity was measured by the rate of hydrolysis of paraoxon to p-nitrophenol (Malinska *et al.* 2010). All parameters were adjusted to the tissue protein concentration or serum volume.

Gene expression assays

Total mRNA was isolated from liver tissue using RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA) and thereafter 1 µg of isolated RNA with a Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) was used for the synthesis of cDNA. For the analyses, cDNA of samples, PCR primers, TaqMan probes and other components important for real-time polymerase reaction were added into 1536-well plates (Roche Diagnostics, Mannheim, Germany) using Echo 555 acoustic liquid handler (Labcyte Europe, Dublin, Ireland). The analyses and quantification of CYPs mRNA expression were performed on LightCycler 1536 Instrument (Roche Diagnostics, Mannheim, Germany). Obtained data were normalized to the housekeeping genes (Actb1 and Hprt1) and calculated using the $^{\Delta\Delta}$Ct method.

TaqMan probes for Abca1, Abcg5, Abcg8, Hmgcr, Ldlr, Cyp2e1, Cyp7a1, Actb1, and Hprt1 were purchased from Life Technologies (Carlsbad, CA, USA).

Determination of an expression of selected proteins

The electrophoresis was used for the determination of ABCG5/8 protein expression in prepared samples (containing 20 µg of proteins). Proteins in samples were separated on the Mini-PROTEAN® TGX™ gel (Bio-Rad Laboratories, Hercules, CA, USA). After the electrophoresis, the proteins were transferred onto a PVDF membrane (Trans-Blot® Turbo™ Midi PVDF Transfer Packs, Bio-Rad Laboratories, Hercules, CA, USA) using Trans-Blot® Turbo™ Transfer System from the same company. The membranes were subsequently incubated with primary specific anti-rat ABCG5 and anti-rat ABCG8 antibodies (Santa Cruz, CA, USA) and then with secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA). For a loading control, the mouse monoclonal anti-rat GAPDH antibody (Sigma-Aldrich, St. Louis, MO, USA) was used. The detection of expression of ABCG5/8 proteins was performed with Luminol Reagent (WB, Luminol Reagent, Santa Cruz, CA, USA) and medical X-Ray films (Agfa, Belgium). CanoScan Toolbox software, ver. 5.0 (Canon Europa, Amstelveen, the Netherlands) and ElfoMan software, ver. 2.6 (Semecky Inc., Prague, Czech Republic) were used for the quantification of obtained results.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). All data obtained in this study were analyzed by Statistica software (ver. 12, Statsoft CZ, Prague, Czech Republic). All statistical analyses were performed by ANOVA because all data had a normal distribution. For detailed comparisons, *post hoc* Bonferroni tests were used to avoid false positive results. Statistical significance was defined as $p<0.05$.

Results

Body weight and food intake

As shown in Figure 1, the body weights of rats in all groups were equal at the beginning of the experiment. During the experiment, the body weight gain was slightly lower in the group treated with n-3 PUFA with or without added SM in comparison with control group. At the end of the experiment, the body weight of the rats supplemented with n-3 PUFA+SM diet was statistically significant lower by 5 % ($p<0.05$) compared to the control group. There were no significant differences in food intake among the groups. Similarly, the weight of epididymal fat as well as of perirenal fat was decreased in animal fed diet containing n-3 PUFA or n-3 PUFA and 1 % micronized SM compared to controls. Data are shown in Table 1.

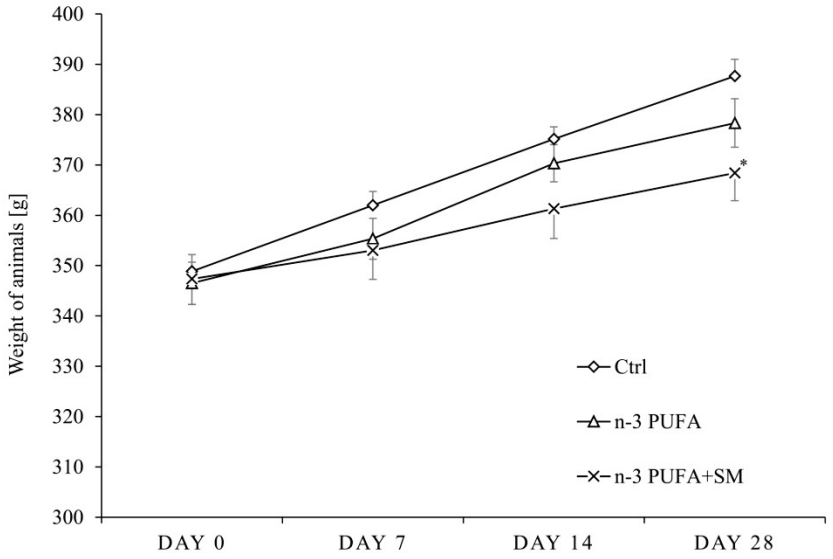

Fig. 1. Effect of n-3 PUFA and n-3 PUFA+SM on body weight of HHTg rats during the experiment lasting 4 weeks. Data are presented as mean ± SEM; n=6. *$p<0.05$ vs. control group. Ctrl. – control group, n-3 PUFA – experimental group of rats treated with n-3 polyunsaturated fatty acids, n-3 PUFA+SM – experimental group of rats treated with n-3 polyunsaturated fatty acids and micronized silymarin.

Table 1. Adipose tissue weights in control and experimental HHTg rats.

Adipose tissue	Control	n-3 PUFA	n-3 PUFA+SM
Epididymal [g]	6.70 ± 0.25	6.38 ± 0.14	5.53 ± 0.56*
Perirenal [g]	4.00 ± 0.19	3.38 ± 0.12	2.82 ± 0.25**

Data are presented as mean ± SEM; n=6. Data are statistical significant between groups (n-3 PUFA+SM vs. control): *$p<0.05$, **$p<0.01$. n-3 PUFA – experimental group of rats treated with n-3 polyunsaturated fatty acids, n-3 PUFA+SM – experimental group of rats treated with n-3 polyunsaturated fatty acids and micronized silymarin.

Serum level of lipids

Concentrations of triglycerides, total cholesterol, HDL-cholesterol, and NEFA were measured in serum of HHTg rats. The administration of n-3 PUFA significantly decreased the serum level of triglycerides by 34 % ($p<0.01$) and in the combination with SM by 45 % ($p<0.001$) compared to the controls (Table 2). Similarly, both diets containing n-3 PUFA with or without SM significantly decreased the total serum cholesterol level by 18 % ($p<0.01$) or 16 % ($p<0.01$), respectively, compared to the control group. The serum level of HDL-cholesterol was not affected by n-3 PUFA supplementation or in the combination with SM. As for NEFA (Table 2), the diet of n-3 PUFA+SM significantly decreased their serum level by 33 % ($p<0.05$) in comparison to the control rats.

Tissue lipid accumulation

Concentrations of triglycerides were also measured in the liver, skeletal muscle, and heart (Table 2). The triglycerides content in the liver was not changed by the treatment with n-3 PUFA or their combination with SM. On the other hand, the cholesterol content in the liver was significantly increased by 23 % ($p<0.05$) following n-3 PUFA treatment in comparison to the controls. Diet containing n-3 PUFA and SM caused a significant decrease of cholesterol concentration in rat

liver in comparison to the group treated with n-3 PUFA. While n-3 PUFA treatment did not affect the content of triglycerides in skeletal muscle, these triglycerides was reduced by 35 % in rats with a treatment of the combination of n-3 PUFA+SM in comparison to the control rats and n-3 PUFA treated rats. The concentration of triglycerides in the myocardium was not changed after n-3 PUFA diet neither with nor without SM.

Table 2. Serum levels lipids, glucose, insulin, and parameters of lipid metabolism measured in tissues of control and experimental HHTg rats.

	Parameters	Control	n-3 PUFA	n-3 PUFA+SM
SERUM	*TAG (mmol/l)*	4.73 ± 0.22	3.10 ± 0.28**[1]	2.61 ± 0.24***[2]
	Total CH (mmol/l)	1.52 ± 0.02	1.28 ± 0.02**[1]	1.25 ± 0.03**[2]
	HDL-CH (mmol/l)	0.71 ± 0.03	0.69 ± 0.04	0.67 ± 0.05
	NEFA (mmol/l)	0.43 ± 0.03	0.39 ± 0.02	0.29 ± 0.04*[2]
	Glucose (mmol/l)	8.33 ± 0.71	8.10 ± 0.59	7.48 ± 0.17
	Insulin (nmol/l)	0.25 ± 0.01	0.28 ± 0.02	0.23 ± 0.05
TISSUES	*Liver TAG (μmol/g)*	6.95 ± 0.83	6.92 ± 0.34	7.24 ± 0.70
	Liver CH (μmol/g)	8.41 ± 0.59	10.35 ± 0.36*[1]	8.43 ± 0.54*[3]
	M. gastrocnemius TAG (μmol/g)	4.89 ± 0.62	4.88 ± 0.56	3.20 ± 0.31*[2,3]
	Heart TAG (μmol/g)	2.39 ± 0.41	2.08 ± 0.41	2.09 ± 0.53

Data are expressed as mean ± SEM; n=6. Data are statistical significant between groups: *p<0.05, **p<0.01, ***p<0.001. Statistical significant difference between groups: [1]n-3 PUFA vs. control, [2]n-3 PUFA+SM vs. control, [3]n-3 PUFA+SM vs. n-3 PUFA. NEFA – nonesterified fatty acids, CH – cholesterol, TAG – triglycerides, n-3 PUFA – experimental group of rats treated with n-3 polyunsaturated fatty acids, n-3 PUFA+SM – experimental group of rats treated with n-3 polyunsaturated fatty acids and micronized silymarin.

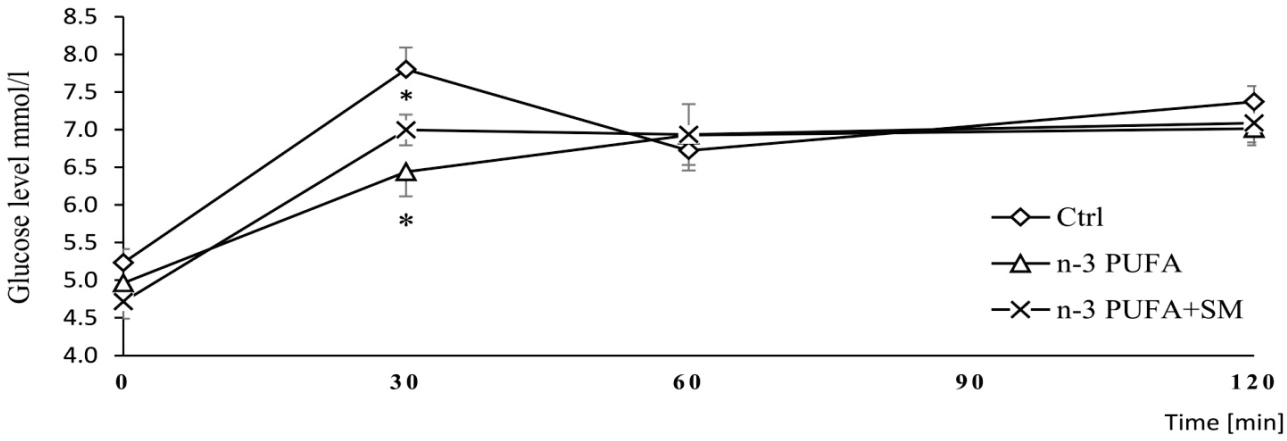

Fig. 2. Effect of n-3 PUFA and n-3 PUFA+SM on serum glucose concentration during oral glucose tolerance test in HHTg rats. Data are presented as mean ± SEM; n=6. *p<0.05 vs. control group. Ctrl. – control group, n-3 PUFA – experimental group of rats treated with n-3 polyunsaturated fatty acids, n-3 PUFA+SM – experimental group of rats treated with n-3 polyunsaturated fatty acids and micronized silymarin.

Glucose and insulin level

Glucose tolerance was measured by an oral glucose tolerance test (OGTT). The fasting blood glucose level did not differ between groups at the beginning of the test. Nevertheless, the change in blood glucose level was observed 30 min after glucose administration in rats treated with n-3 PUFA with or without SM compared to controls (Fig. 2). In postprandial state (Table 2), although there is an obvious decrease in serum glucose level following administration of n-3 PUFA+SM, the decrease did not reach the statistical significance in comparison to the control group or group treated with n-3 PUFA alone. The combined diet of n-3 PUFA+SM as well as n-3 PUFA diet had likewise no effect on the level of insulin (Table 2).

Changes in mRNA expression of selected genes in rat liver

Figure 3 shows mRNA levels of genes important for cholesterol or lipid homeostasis. The diet containing n-3 PUFA alone or in the combination with SM significantly increased the mRNA expression of both Abcg5 (Fig. 3A) and Abcg8 (Fig. 3B) genes. The administration of n-3 PUFA alone caused a 5-fold increase in mRNA expression of Abcg5 ($p<0.01$) and a 7-fold increase in mRNA expression of Abcg8 ($p<0.01$), whereas the application of n-3 PUFA+SM caused a 9-fold increase for Abcg5 ($p<0.001$) and a 13-fold increase for Abcg8 gene ($p<0.001$). The expression of other following studied genes, including Abca1, Hmgcr, Ldlr, Cyp7a1, and Cyp2e1, were not significantly affected by both experimental treatments (data not shown).

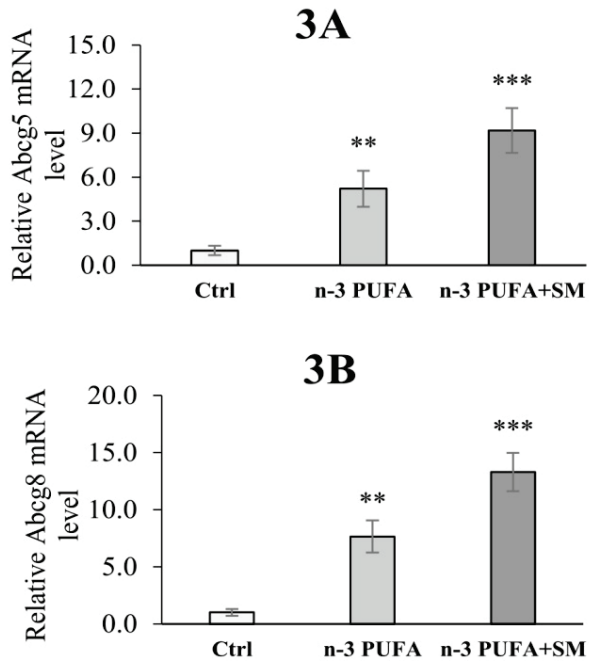

Fig. 3. Effect of n-3 PUFA and n-3 PUFA+SM on the mRNA expression of Abcg5 (**A**) and Abcg8 (**B**) in rat liver. Data are presented as mean ± SEM; n=6. *p<0.05, **p<0.01, ***p<0.001 vs. control group. Abcg5 and Abcg8 – genes for ATP-binding cassette (ABC) cholesterol transporters G5 and G8, Ctrl. – control group, n-3 PUFA – experimental group of rats treated with n-3 polyunsaturated fatty acids, n-3 PUFA+SM – experimental group of rats treated with n-3 polyunsaturated fatty acids and micronized silymarin.

Changes in protein content of ABCG5 and ABCG8 in rat liver

The protein content of ABCG5 transporter (Fig. 4A) was significantly increased by 78 % ($p<0.05$) in animals treated with n-3 PUFA+SM compared to the control group. In the case of the n-3 PUFA treatment, the increase in protein content of ABCG5 was observed but this change was not statistically significant. Similarly, the ABCG8 protein content (Fig. 4B) was significantly increased (by 151 %; $p<0.01$) in n-3 PUFA treated group and more pronounced in n-3 PUFA+SM supplemented group (by 232 %; $p<0.001$) in comparison to the controls.

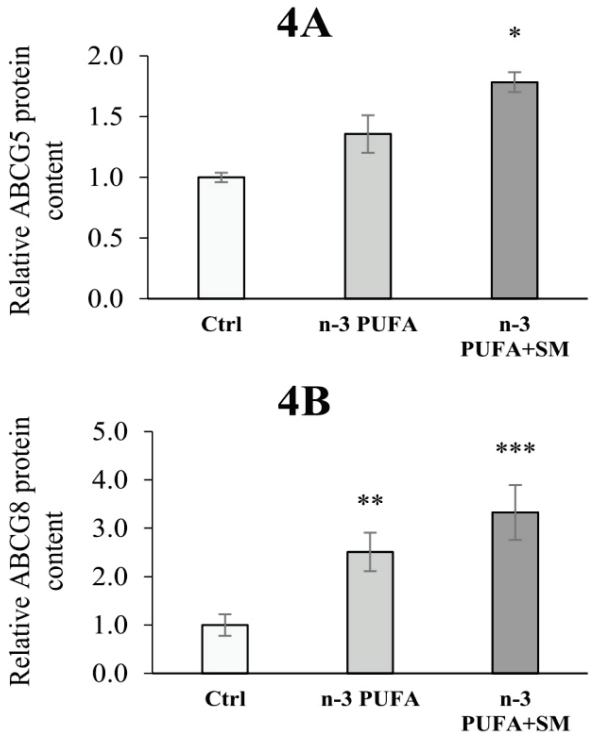

Fig. 4. Effect of n-3 PUFA and n-3 PUFA+SM on the protein content of ABCG5 (**A**) and ABCG8 (**B**) transporters in rat liver. Data are presented as mean ± SEM; n=6. *p<0.05, **p<0.01, ***p<0.001 vs. control group. ABCG5 and ABCG8 – ATP-binding cassette (ABC) cholesterol transporters G5 and G8, Ctrl. – control group, n-3 PUFA – experimental group of rats treated with n-3 polyunsaturated fatty acids, n-3 PUFA+SM – experimental group of rats treated with n-3 polyunsaturated fatty acids and micronized silymarin.

Parameters of antioxidant system and lipoperoxidation

Hypertriglyceridemia and ectopic lipid accumulation increase the production of reactive oxygen species; therefore, the effect of n-3 PUFA and their combination with SM on antioxidant enzymes activity was measured in the heart as well as in the liver and serum of control and experimental rats (Table 3).

In the liver, antioxidant parameters like an activity of SOD, CAT, and GSH-dependent enzymes GSH-Px and GST as well as the concentration of reduced form of glutathione were significantly increased in a group treated with the combination of n-3 PUFA and

SM compared to either untreated control or n-3 PUFA alone treated rats. Similar effects were also observed in blood serum where the activity of enzymes involved in the antioxidant system was increased in n-3 PUFA as well as n-3 PUFA+SM treated group compared to control animals. Antioxidant effects of n-3 PUFA were observed especially in heart tissue as alone in a diet or combined with SM.

Interestingly, while n-3 PUFA did not affect paraoxonase 1 (PON-1) activity, the combined diet of n-3 PUFA+SM caused a significant increase in its activity by 25 % (Table 3). In addition, n-3 PUFA or n-3 PUFA+SM administration ameliorated an oxidative stress because concentrations of lipoperoxidation products measured as TBARS in the liver, heart as well as in blood serum of experimental animals were lower in comparison to controls (Table 3).

Table 3. Parameters of antioxidant defence and lipoperoxidation in the liver, heart, and serum measured in control and experimental HHTg rats.

	Parameters	Control	n-3 PUFA	n-3 PUFA+SM
LIVER	*SOD (U/mg protein)*	0.104 ± 0.005	0.121 ± 0.012	0.129 ± 0.007*[2]
	CAT (μM H_2O_2 min/mg protein)	1362 ± 141	1864 ± 154*[1]	1834 ± 139*[2]
	GSH-Px (μM NADPH/min/g protein)	291 ± 9	358 ± 13**[1]	345 ± 15*[2]
	GST (nM CDNB/min/mg protein)	124 ± 11	152 ± 14	195 ± 14**[2,]*[3]
	GSH (μM/mg)	59.9 ± 1.0	68.9 ± 2.6*[1]	74.8 ± 2.9**[2,]*[3]
	TBARS (nM/mg protein)	1.50 ± 0.14	1.08 ± 0.01*[1]	1.01 ± 0.07*[2,3]
HEART	*SOD (U/mg protein)*	0.068 ± 0.003	0.096 ± 0.004***[1]	0.093 ± 0.006*[2]
	CAT (μM H_2O_2 min/mg protein)	524 ± 48	857 ± 45***[1]	810 ± 52*[2]
	GSH-Px (μM NADPH/min/g protein)	142 ± 13	192 ± 10*[1]	206 ± 17*[2]
	GST (nM CDNB/min/mg protein)	42.3 ± 3.2	54.5 ± 2.2*[1]	51.6 ± 1.9*[2]
	GSH (mM/g)	22.3 ± 2.9	31.7 ± 6.0*[1]	30.0 ± 3.0*[2]
	TBARS (nM/mg prot.)	0.658 ± 0.06	0.576 ± 0.03	0.431 ± 0.04*[2]
SERUM	*SOD (U/ml)*	1.277 ± 0.10	2.118 ± 0.22**[1]	2.073 ± 0.25*[2]
	CAT (μM H_2O_2 min/ml)	1658 ± 126	2072 ± 332*[1]	2248 ± 177*[2]
	GSH-Px (μM NADPH/min/ml)	215 ± 13	267 ± 16*[1]	257 ± 11*[2]
	GST (nM CDNB/min/ml)	5.42 ± 0.26	6.00 ± 0.41	4.90 ± 0.56
	GSH (nM/ml)	5.07 ± 0.47	7.19 ± 0.63*[1]	9.30 ± 0.30***[2;]*[3]
	PON-1 (μM/min/ml)	161 ± 11	155 ± 10	201 ± 15*[3]
	TBARS (nM/ml)	1.61 ± 0.22	1.27 ± 0.11	1.02 ± 0.09*[2]

Data are expressed as mean ± SEM; n=6. Data are statistical significant between groups: *p<0.05, **p<0.01, ***p<0.001. Statistical significant difference between groups: [1]n-3 PUFA vs. control, [2]n-3 PUFA+SM vs. control, [3]n-3 PUFA+SM vs. n-3 PUFA. U – one unit SOD is defined as the amount of the enzyme needed to exhibit 50 % dismutation of the superoxide radical, SOD – superoxiddismutase, GSH-Px – glutathione peroxidase, GST – glutathione transferase, CAT – catalase, GSH – glutathione, PON-1 – paraoxonase 1, TBARS – thiobarbituric acid reactive substances, n-3 PUFA – experimental group of rats treated with n-3 polyunsaturated fatty acids, n-3 PUFA+SM – experimental group of rats treated with n-3 polyunsaturated fatty acids and micronized silymarin.

Discussion

The diet containing n-3 PUFA, principally DHA and EPA, have been reported to have a good efficacy in the therapy of hypertriglyceridemia but findings from a meta-analysis showed that n-3 PUFA supplementation did not significantly affect major cardiovascular events (Rizos *et al.* 2012). Similarly, the positive influence on

hepatic steatosis, which is a part of metabolic syndrome, has not been clearly demonstrated (Sanyal *et al.* 2014). Therefore, the addition of micronized silymarin to n-3 PUFA supplementation was tested in this study as an efficient treatment for already developed metabolic disorders associated with genetically induced moderate hypertriglyceridemia with the use unique animal model – HHTg rat.

After four weeks supplementation of SM and n-3 PUFA, there was found the slightly lower body weight gain of rats (-5 %; $p<0.05$) compared to the control group. The decrease in body weight gain was partly caused by the loss of epididymal as well as perirenal adipose tissue (-18 % and -30 %, respectively) compared to the control group. However, data between n-3 PUFA diet and n-3 PUFA+SM diet was not statistically different. The body weight as well as the weight of epididymal and perirenal fat was also lower after n-3 PUFA supplementation but the decrease was not statistically significant in comparison to the controls.

Our results demonstrate that the supplementation with micronized SM increased triglyceride-lowering effect of n-3 PUFA (-45 % vs. -34 %, both $p<0.05$) and markedly decreased serum NEFA concentrations (-33 %) in HHTg rats. Cholesterol-lowering effect (-16 % or -18 %; $p<0.01$) was measured in group supplemented with n-3 PUFA without or with SM, respectively, compared to the controls. The hypolipidemic effects of SM are consistent with our earlier findings showing its effects on the reduction of serum TAG concentrations in HHTg rats and dietary-induced hypercholesterolemia (Orolin *et al.* 2007, Poruba *et al.* 2015). We did not test this effect of SM alone again. Our aim was to find out whether SM in the combination of n-3 PUFA improves a hypolipidemic effect of n-3 PUFA. This combined diet on animal and human health has not yet been published. As results show, micronized silymarin slightly improved hypolipidemic effect of n-3 PUFA; however, data between n-3 PUFA diet and n-3 PUFA+SM diet is not statistically significant.

Elevated levels of TAG in serum are related to their accumulation in non-adipose organs, especially liver, muscle tissue, and heart where it may cause a lipotoxicity and contribute to the pathogenesis of non-alcoholic fatty liver, muscle insulin resistance, or heart disorders (Divisova *et al.* 2002, Neuschwander-Tetri 2010). The supplementation of n-3 PUFA+SM caused the positive effect on ectopic triglyceride accumulation in skeletal muscle. The combination of n-3 PUFA+SM resulted in a TAG reduction by 35 % in this tissue, in contrast to the supplementation with n-3 PUFA alone. Lower triglyceride concentrations in skeletal muscle together with lower levels of circulating non-esterified fatty acids could be involved in the slightly improved glucose tolerance observed during OGTT.

Diet containing n-3 PUFA reduced lipid accumulation induced by high fat in the liver, contrary to many other studies. Different findings can be explained by the use of different animal models. Our animal model, HHTg rat, mimics the situation in humans where subjects with hepatic steatosis had more than 3-fold higher rates of *de novo* fatty acids synthesis than subjects without this illness (Aubert *et al.* 2011). However, the cholesterol content in the liver was significantly increased following n-3 PUFA treatment by 23 % ($p<0.05$) in comparison to the controls. The reason of this effect could be a consequence of the increased content of DHA (50 % DHA and 10 % EPA) in the supplementation. It has been reported that treatment with DHA, but not EPA, increases LDL-cholesterol levels (Allaire *et al.* 2016). Our novel and important finding is that this negative effect can be eliminated when silymarin was added to n-3 PUFA which is consistent with the known hepatoprotective effects of SM (Vargas-Mendoza *et al.* 2014).

To search for the molecular mechanisms responsible for effects mentioned above, mRNA expression of Abcg5 and Abcg8 genes as well as protein levels of ATP-binding cassette transporters ABCG5 and ABCG8 were measured in liver tissues. There was found out that the diet of n-3 PUFA+SM markedly increased mRNA expression and protein content of these transporters. These results are important because ABCG5/8 transporters are responsible for cholesterol efflux from the liver to the bile and thus represent an important elimination pathway for the cholesterol (Brown and Yu 2009). Hepatic ABCG5/8 overexpression leads to a decrease of apoB-lipoproteins and atherosclerosis in mice fed a “western” diet (Basso *et al.* 2007). To further clarify the mechanisms responsible for the role of n-3 PUFA and SM in the regulation of lipid metabolism, our study was focused on genes Abca1, Hmgcr, Ldlr, Cyp2e1, and Cyp7a1. Only a slight change in mRNA expression of abca1 was observed, as a gene coding ABCA1 protein important for the transport of free cholesterols and phospholipids through the plasma membrane into HDL particles (Wang and Smith 2014). A slight but statistically non-significant increase in abca1

mRNA expression was observed in the groups treated with n-3 PUFA or n-3 PUFA+SM compared to the controls. The effect of n-3 PUFA on Abca1 mRNA expression has been observed in only one study where supplementation with DHA/EPA in a ratio 1:1 slightly increased expression in Apo E-/- deficient mice (Liu *et al.* 2016).

The key role in the pathogenesis of hypertriglyceridemia-induced organ complications is ascribed to an oversupply of lipid metabolites which lead to subcellular stress, predominantly at the level of mitochondria, endoplasmic reticulum, and peroxisomes (Di Meo and Reed 2016). The result is then an increased production of free radicals and reactive oxygen species (ROS). An ineffective scavenging of ROS leading to oxidative stress may play an important role in the pathogenesis of NAFLD, heart failure, and skeletal muscle insulin resistance (DeFronzo 2010). All metabolic syndrome components including dyslipidemia and insulin resistance correlate with human oxidative stress levels (Hotamisligil and Davis 2016). To counteract this oxidative stress, cells have several antioxidant enzymes and a glutathione system. In the current study, n-3 PUFA with a high proportion of DHA increased the activity of SOD, CAT, and GSH-dependent enzymes GSH-Px and GST in the liver for the first time. The antioxidant effect of n-3 PUFA was potentiated by combined therapy with silymarin. An important finding of this study was that n-3 PUFA alone did not affect serum levels of PON-1 but the combination of n-3 PUFA+SM caused a significant increase in activity of this enzyme. Our findings are interesting because this enzyme can have anti-atherogenic effects by degrading lipid peroxides, decreasing HDL susceptibility to peroxidation, and increasing cholesterol efflux (Macharia *et al.* 2012). For beneficial effects of n-3 PUFA, both alone or combined with SM, there is an evidence that they are responsible for even the lower production of lipid peroxides as to be one of the main mechanisms leading to degeneration of cell membranes and the development of liver lesions.

From the point of view of cardiovascular disorders associated with hypertriglyceridemia, it is remarkable that beneficial effect of n-3 PUFA and their combination with SM was also evident in the myocardium. The enhanced activity of antioxidant enzymes, the higher glutathione concentration, and the reduced production of cytotoxic lipoperoxidation products were observed; therefore, these substances studied in HHTg rats ameliorated an oxidative stress in the heart tissue. It is noteworthy that observed protective effects of n-3 PUFA and silymarin were not associated with a decrease in triglyceride accumulation in the liver and the heart. The beneficial effects on the oxidative stress in tissues are probably mediated by mechanisms beyond ectopic lipid accumulation whose elucidation should be the focus of further studies.

In conclusion, the results of the current study demonstrated that the treatment with n-3 PUFA containing a higher proportion of DHA ameliorated metabolic disorders induced by hereditary hypertriglyceridemia. All amplified effects were achieved by the treatment with these n-3 PUFA in the combination with micronized silymarin. Serum levels of triglyceride, total cholesterol, and non-esterified fatty acids were significantly reduced by the combined action of n-3 PUFA+SM compared to the control group. Ectopic lipid accumulation in skeletal muscle was significantly reduced by the combined diet of n-3 PUFA+SM compared to the control group as well as to group with n-3 PUFA diet. Our results showed that these effects on dyslipidemia can be a consequence of the significant increase of genes important for lipid homeostasis as evidenced by an increase in Abcg5 and Abcg8 mRNA expression as well as of ABCG5 and ABCG8 protein level. In addition, the increased activity of antioxidant enzymes and amelioration of oxidative stress in the liver, heart as well as in blood serum were observed following n-3 PUFA treatment, and even more pronounced after application of the combination of n-3 PUFA+SM. For the first time, our results suggest that n-3 PUFA with a higher proportion of DHA and in the combination with silymarin could be a new effective therapy of hypertriglyceridemia-induced metabolic disorders.

Conflict of Interest

There is no conflict of interest.

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