

# Influence of protein deficient diet, vitamin B<sub>2</sub> supplementation and physical training on serum composition of polyunsaturated fatty acids (PUFAs) in rats

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## Abstract

**Introduction.** Prolonged shortages of protein in the diet significantly alter the composition and content of polyunsaturated fatty acids (PUFA) in tissues and body fluids. One of nutritional factors which may reduce negative effects of protein malnutrition might be vitamin B<sub>2</sub> due to its influence on lipids metabolism.

**Objective.** The aim of the study was to investigate the influence of low protein (LP) diet enriched with vitamin B<sub>2</sub> on the content and composition of PUFA in the blood serum of rats treated with dosed physical exercise.

**Materials and method.** The experiment was carried out for 3 months on 72 growing male Wistar rats divided into 5 groups. Animals were fed *ad libitum* on a diet with an energy value of 350 kcal/100 g, in which 4.5% of the energy was provided by protein. In the control diet, 20% of the energy was provided by protein. Two groups were fed the diet enriched with vitamin B<sub>2</sub>. The two groups of tested animals were trained for 5 days a week.

**Results.** LP diet caused a decrease in  $\alpha$ -linolenic acid (ALA) after 30 days, and a decrease in docosahexaenoic acid (DHA) after 60 days of experiment, compared with rats fed the control diet. After 60 and 90 days of the experiment, a significant decrease was noted in arachidonic acid (AA) in serum of trained rats, compared with sedentary rats fed the LP diet. Physical activity increased LA (mainly on day 30), EPA (on day 90) and reduced AA content (on day 90) in serum of rats fed the LP diet. B<sub>2</sub> supplementation in the trained LP group did not change the EPA and AA dependence; however, there was a decrease in LA content in comparison to the non-supplemented trained group.

**Conclusions.** Results of this study suggest that all investigated factors (protein deficiency, physical exercise and supplementation of vitamin B<sub>2</sub>) have significant impact on PUFA composition of serum in rats.

## Key words

Protein malnutrition, vitamin B<sub>2</sub>, training, PUFA

## INTRODUCTION

Nutritional deficiencies and food restrictions have been the objectives of investigations for several decades. Prolonged shortages of protein in the diet significantly alter the composition and content of polyunsaturated fatty acids (PUFA) in tissues and body fluids, and are also the cause of disorders of metabolism in body lipids. Inhibition of activity of certain enzymes involved in the synthesis of CoA or disorders in fatty acids absorption from food substantially affect their composition and content in tissues and body fluids [1]. Reduction of PUFA content was found among malnourished children, the elderly and people suffering from fatty liver disease [2, 3]. Similar effects were observed in malnourished rat models in experiments performed by Bertrandt et al. Limitation of consumption of a full value diet to 50%, combined with dietary addition of B<sub>2</sub>, B<sub>6</sub> and folic acid, resulted in PUFA changes in serum of rats after only 30 days of feeding (decrease of linoleic (LA) and alpha-

linolenic (ALA), as well as an increase in arachidonic (AA) and docosahexaenoic (DHA) acids) [4]. Rats fed a protein deficient diet (9%) additionally supplemented with vitamin B<sub>6</sub> had increased ALA, as well as DHA and decreased LA content in serum. Significant changes of PUFA composition in the liver of these rats were also noticed [5]. An additional reduction in energy (by 50% reduction of daily intake) in protein-malnourished rats enriched with vitamin B<sub>6</sub> resulted in greater changes in PUFA content [6]. Interestingly, there was not only protein deficiency but also an increased level of protein in the diet (40%) combined with B<sub>6</sub> deficiency modified PUFA content in rats liver. Pregnotato et al. have shown a complex interaction among tested nutritional factors with a prevailing influence of dietary proteins on lipid content of liver, and low EFA intake and vitamin B<sub>6</sub> on PUFA metabolism [7].

The results of many experiments show that physical activity significantly affects the volume of lipid indices, causing, e.g. a reduction in total cholesterol in plasma, as well as reduction of triacylglycerols [8]. It was noticed that physical training also affects some haematological parameters. An increase in mean corpuscular haemoglobin (MCV) and decrease in mean corpuscular haemoglobin concentration (MCHC) in rats was also observed [9]. Dosed physical exercise, especially

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with an appropriately chosen diet, additionally affected the composition and content of fatty acids (FA) in body fluids and tissues. It has been found in humans that a high level of physical fitness is associated with elevated  $\omega$ 3-PUFA (DHA, 22:6 $\omega$ 3) concentration, and decrease in  $\omega$ 6-PUFA (18:2 $\omega$ 6) in plasma [10]. Physically active people require a higher intake of vitamins because they have increased enzyme activity and secretion via sweat and urine. Malara et al. reported that physically active people were prone to vitamin B6 and B2 deficiency [11].

One of the nutritional factors which can partly ameliorate the effects of long-lasting use of protein deficient diet might be vitamin B2. It has been shown that vitamin B2 supplementation affects lipids metabolism and increases beta-oxidation of FA. The beta-oxidation process reducing the level of FA in tissues indirectly influences the level and composition of FA in serum [12]. Furthermore, acetyl-CoA formed in the beta-oxidation pathway provides the substrates necessary for the synthesis of PUFA.

## OBJECTIVE

The aim of the study was to evaluate the impact of vitamin B2 supplementation and training in rats fed a low protein (LP) diet for 3 months in the context of composition and content of PUFA in blood serum.

## MATERIALS AND METHOD

The study was performed on 72 growing male Wistar breed rats, initially weighting 140.0 $\pm$ 7.7g. Animals were kept in stainless steel cages, maximum 5 animals in each cage, in a room with controlled temperature, humidity and lighting (12-hour day cycle). Rats were fed *ad libitum* a semi-synthetic, isocaloric diet with an energy value of 350 kcal/100 g (1,466.5 kJ/100g) for 90 days. In the control diet, 20% of the energy was provided by protein, and in experimental diet only 4.5%. Half of animals received the protein-restricted diet enriched with vitamin B2 to a level 3 times higher than in the control diet (7.5 mg of riboflavin/100g diet). In all the diets, 15% of the energy came from fat, including approx. 2% from essential fatty acids (EFA). Sunflower oil was a source of PUFA. Diets were enriched with mineral salts and vitamins in accordance with the guidelines for rats. The energy value

**Table 1.** Diet composition

Diet component	20% energy from protein		4.5% energy from protein	
	g/100g	kcal	g/100g	kcal
Sunflower oil	0.41	3.69	0.55	4.95
Lard	5.45	48.91	5.31	47.57
Casein	18.97	60.70	4.56	14.59
Egg powder	1.61	9.33	0.20	1.16
Wheat flour	19.43	67.61	19.43	67.61
Wheat starch	30.00	120.00	30.00	120.00
Potato starch	9.14	-	11.36	-
Sugar	10.00	39.90	23.59	94.12
Mineral mix <sup>1)</sup>	4.00	-	4.00	-
Vitamin mix <sup>2)</sup>	1.00	-	1.00	-

<sup>1)</sup>1000 g mineral mixture contains: 322g KHPO<sub>4</sub>, 300g CaCO<sub>3</sub>, 167g NaCl, 102g MgSO<sub>4</sub>, 75g CaHPO<sub>4</sub>, 27.5g FeC<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 5.1. MnSO<sub>4</sub>, 0.8g KJ, 0.3g CuSO<sub>4</sub>, 0.25g ZnCl<sub>2</sub>, 0.05g CoCl<sub>2</sub>

<sup>2)</sup>1000 g vitamin mixture contains: 545000 IU vit. D<sub>3</sub>, 1.0 g vit. K, 30 $\mu$ g vit. B<sub>12</sub>, 10 g choline chloride, 1.01g folic acid, 0.03g biotin, 10.0g inositol, 10.0g PABA, 1250000 IU vit. A, 1.5g vit. B<sub>6</sub>, 2.5g vit. E, 5.0g vit. B<sub>9</sub>, 25g vit. C, 5.0g vit. PP, 2.5g vit. B<sub>2</sub>, 25.0g calcium pantothenate

of the low-protein diet was supplemented with carbohydrates (Tab. 1). Half of the rats fed the protein deficient diet (with and without B2 supplementation) were trained for 5 days a week. Training consisted of 1-hour runs on a treadmill with a speed of 20 m/min.

All animals and containers with food were weighed twice a week, after which an average weight and consumption of individual diet per rat was calculated (Tab. 2).

Blood samples were collected after 30, 60 and 90 days of the study from tail vein of rats. Rats were sacrificed by cervical displacement while unconscious (orbital anaesthesia). The study protocol was approved by the Local Ethics Committee for Animal Studies in Warsaw.

**Gas Chromatograph (GC) analysis.** Blood samples were centrifuged for 20 min. at 2,000g and serum collected. Serum samples were stored at -20°C until analysis. Lipids from the serum were extracted by modified Folch methods [13]. Extracted fatty acids were saponificated and esterificated using 3N HCl in methanol [14]. The separation of methyl esters of fatty acids were performed using the Perkin Elmer gas chromatograph with the Omegawax 250 capillary column (30m x 0.25 mm I.D., the 0.25  $\mu$ m). Total time of analysis was 57 min. Identification of PUFA was based on comparison of retention times with 'Supelco 37 Component FAME Mix', used separately or in mixture standards using C-15 fatty acid as the internal standard. Content of the following acids was assessed: linoleic acid (LA),  $\alpha$ -linolenic acid (ALA), arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Quantitative evaluation was calculated based on the received spike area of analyzed fatty acids in the sample, compared with the analogical area of standard (Sigma), and expressed as a % of total fatty acids. Results were presented as means  $\pm$  SD. Obtained results were statistically verified using the one-way ANOVA and Tukey parametric test (GraphPad Software, Inc., San Diego, CA). Differences of means at p $\geq$ 0.05 were considered significant.

## RESULTS AND DISCUSSION

The study aimed to assess the impact of nutrition on sedentary or trained rats fed a protein-deficient diet with/without enrichment of vitamin B2 on PUFA composition in blood serum.

Daily feed intakes were lower in rats given a diet restricted in proteins compared to the rats fed the control diet. Body weight was also lower in rats fed the deficient diet, compared to animals fed a control diet (Tab. 2).

Protein shortages in a diet may affect the composition of fatty acids by inhibiting the activity of some desaturases, as well as influencing enzymes involved in the biosynthesis of such fatty acids as acetyl-CoA synthetase and beta-hydroxybutyric dehydrogenase. Decrease in beta-hydroxybutyric dehydrogenase activity hampers CoA synthesis which is necessary for fatty acids biosynthesis [15]. Protein-deficient diets are known to increase the intramuscular content of saturated fatty acids in pigs, probably by tissue specific activation of lipogenic enzymes [16]. Also, in a study by Marin et al. (2003), performed on monkeys, it was shown that long-lasting protein deprivation (24 months) reflected in liver lipids composition by lowering the proportion of unsaturated to saturated FA [17]. In the presented study, the sum of PUFA

**Table 2.** Average daily feed intakes (n=3) and final body weights of rats

	20% of protein (I) n-15	4.5% of protein (II) n-15	4.5% of protein + training (III) n-14	4.5% of protein + vit. B <sub>2</sub> (IV) n-15	4.5% of protein + training + vit. B <sub>2</sub> (V) n-13
Daily feed intake (g)	22.08±2.81a	19.00±2.59b	20.71±3.01a	18.80±2.74b	19.74±2.84a,b
Body weight (g)	583.1±45.96a	354.33±35.95b	364.75±73.11b	347.47±58.31b	373.33±34.94b

was slightly lower only in rats fed the low protein diet for 3 months, but a significant difference appeared only in the group of trained animals given the B<sub>2</sub>-enriched diet (Tab. 5). Changes in fatty acid content in the blood serum of rats fed a diet with limited amounts of protein may not only result from changes in its metabolism but also as a result of disturbances in the absorption of fatty acids from food. This is significant particularly in the case of LA and ALA acids, which are the parent acids for the synthesis of other acids [18]. The protein-deficient diet caused a significant decrease in ALA acid after 30 days, and a decrease in DHA acid after 60 days of the

study, compared to the control rats (Tab. 3, 4). In a previous study performed by Bertrandt et al., a significant decrease in DHA was also shown after 90 days use of a protein-deficient diet in rats [5]. In this study the content of DHA was lower, but the difference was not significant because of a relatively high variation among animals.

In extreme conditions, a 3-months use of a protein-free diet Gerson (1974), an increase was observed of LA content, which thereby reduced the quantity of AA and DHA acids in the external mitochondrial membranes of rats' liver [15].

**Table 3.** PUFA content (in % of total FA) in blood serum of rats fed 30 days with the diet supplying 20% or 4.5% energy provided from protein, with or without vitamin B<sub>2</sub> enrichment in sedentary or physically trained animals

Fatty acid	Day 30				
	20% of protein (I) n-15	4.5% of protein (II) n-15	4.5% of protein + training (III) n-14	4.5% of protein + vit. B <sub>2</sub> (IV) n-15	4.5% of protein + training + vit. B <sub>2</sub> (V) n-13
18:2n-6 (LA)	12.94±1.03a	11.73±2.31a	16.96± 2.21b	12.53 ± 2.65a	15.59±3.09a,b
18:3n-3 (ALA)	0.27±0.22b	0.09±0.10a	0.1±0.17a	0.11±0.16a	0.07±0.12a
20:4n-6 (AA)	8.81±2.46a,b	6.91±3.31a	8.77±1.82a,b	11.31 ± 3.44b	6.71±2.98a
20:5n-3 (EPA)	0.09±0.14	0.18±0.12	0.1 ± 0.11	0.11±0.17	0.13± 0.21
22:6n-3 (DHA)	1.22±1.04	1.51±1.07	1.54±1.87	0.69±1.07	1.35±0.85
Σ estimated fatty acids	23.33±2.48a,b	20.42±5.63a	27.47 ±2.80b	24.75±4.78a,b	23.85±4.40a,b

Means ± SD, n= animal number per group  
Means with unlike superscript differ at p≤0.05.

**Table 4.** PUFA content (in % of total FA) in blood serum of rats fed 60 days with the diet supplying 20% or 4.5% energy provided from protein, with or without vitamin B<sub>2</sub> enrichment in sedentary or physically trained animals

Fatty acid	Day 60				
	20% of protein (I) n-15	4.5% of protein (II) n-15	4.5% of protein + training (III) n-14	4.5% of protein + vit. B <sub>2</sub> (IV) n-15	4.5% of protein + training + vit. B <sub>2</sub> (V) n-13
18:2n-6 (LA)	11.88±0.72a	13.87±2.44a,b	14.94 ± 3.54b	14.07±0.82a,b	15.27±2.03b
18:3n-3 (ALA)	0.13±0.07	0.14±0.10	0.18±0.10	0.12±0.11	0.09±0.07
20:4n-6 (AA)	9.79±0.90a,b	12.02±0.99a	7.61±1.36b	10.88± 1.79a,b	10.85±2.99a,b
20:5n-3 (EPA)	0.16±0.07	0.09±0.09	0.13±0.10	0.17±0.15	0.25±0.14
22:6n-3 (DHA)	1.92±0.38a	1.29±0.87b	2.32±1.68a	1.34±0.53a,b	1.05±0.90 b
Σ estimated fatty acids	23.88±1.18	27.41±3.55	25.18±2.65	26.58±2.68	27.51±5.40

Means ± SD, n= animal number per group  
Means with unlike superscript differ at p≤0.05

**Table 5.** PUFA content (in % of total FA) in blood serum of rats fed 90 days with the diet supplying 20% or 4.5% energy provided from protein, with or without vitamin B<sub>2</sub> enrichment in sedentary or physically trained animals

Fatty acid	Day 90				
	20% of protein (I) n-15	4.5% of protein (II) n-15	4.5% of protein + training (III) n-14	4.5% of protein + vit. B <sub>2</sub> (IV) n-15	4.5% of protein + training + vit. B <sub>2</sub> (V) n-13
18:2n-6 (LA)	13.23±1.22a	12.02±1.26a	15.92± 2.02b	11.53±3.05a,b	11.36±4.34 a
18:3n-3 (ALA)	0.19±0.10a,b	0.09±0.04a	0.12±0.16a	0.19± 0.14a,b	0.36±0.21b
20:4n-6 (AA)	8.00±1.12a	8.82± 2.69a	4.48±1.29b	7.57± 4.25a	4.92±3.20b
20:5n-3 (EPA)	0.07±0.09a	0.09±0.14a	0.19±0.09a,b	0.29±0.15a,b	0.53±0.47 b
22:6n-3 (DHA)	1.73±0.56b	1.25± 0.94a,b	0.51 ± 0.35a	0.94±0.61a,b	1.29±0.96a,b
Σ estimated fatty acids	23.22±1.17b	22.27±4.26a,b	21.22±4.36a,b	20.52±4.90a,b	18.46±4.05a

Means ± SD, n= animal number per group  
Means with unlike superscript differ at p≤0.05

It is suggested that physical training can act selectively on the biosynthesis of certain fatty acids. A statistically significant decrease in AA acid in rats of the trained group was observed on day 60 and day 90 of the current study (Tab. 4, 5). It is probable that the period of prolonged physical exercise diminished conversion of AA from LA, which is consistent with the observation of increased content of LA – differences statistically significant on day 30 and day 90 (Tab. 3, 5). It was found that physical training increased the process of beta-oxidation in rats, which is associated with increased activity of mitochondrial enzymes in skeletal muscles [19]. Research carried out by Terada (2004) on the activity of enzymes involved in fatty acids oxidation in skeletal muscles, revealed an increase in FA oxidation enzyme efficiency through an increase in 3- $\beta$  hydroxyacyl-CoA dehydrogenase (HAD) activity, in the case of high intensity intermittent physical training, as well as low intensity prolonged training (6h), compared to the control group [20]. Anttila et al. (2010) suggested another mechanism for the observed fatty acids reduction in serum [21]. This reduction of free fatty acid concentration in plasma of trained fish was explained by an increased capacity of fatty acids uptake from plasma.

It is possible that the decrease of AA acid in the trained groups may be related with synthesis of pro-inflammatory prostaglandins, the precursor of which is AA acid [22]. Beneficial effects of physical training on the body were examined by combining it with supplementation with polyunsaturated fatty acids. Supplementation of men and women with 2 g DHA and 3 g EPA per day for 6 weeks, during physical training, caused an increase in stroke volume and cardiac output [23], while supplementation with fish oil rich in n-3 fatty acids decreased cardiac contractions frequency during exercise [24].

Physical exercise increases the need for certain vitamins taking part in antioxidant reactions and also in metabolic pathways producing energy. Active individuals consuming a low energy diet, or making poor dietary choices (e.g. protein restriction), are at greater risk of poor vitamin B status including riboflavin (B2) [25]. Riboflavin deficiency is associated with disturbances in energy production via oxidative phosphorylation of the electron transport chain with FMN and FAD as cofactors. Beta-oxidation of FA is also dependent on these flavins. Malara et al. examined the nutritional status of vitamin B1, B2, and B6 with respect to dietary intake and activity coefficients of the enzymes associated with these vitamins (erythrocyte enzymes transketolase, glutathione reductase, and aspartic aminotransferase) [11]. The authors concluded that the men and women consumed insufficient quantities of vitamins B1 and B6, and were prone to vitamin B2 deficiency. Furthermore, Tanabe et al. reported that controlled dietary antioxidant vitamin intake level (vitamin B2, C and E) may have little influence on oxidative stress or antioxidant capacity at rest and after an acute cycle of ergometric exercise measured in middle-aged and elderly individuals subjected to physical activity [26]. The addition of vitamin B2 to the protein-deficient diet of trained rats minimised the decrease of AA in rat serum, but such an effect was observed only after 2 months of the study with a protein-deficient diet (Tab. 4).

Vitamin B2 in the group of untrained rats fed the diet with a limited supply of protein had a stimulating effect on PUFA biosynthesis, which is proved by a statistically significant increase in AA acid on day 30 of the experiment (Tab. 3). In

subsequent months, in this group no increase was observed of this acid, which might be the result of long-term protein malnourishment. The stimulating effect of vitamin B2 on the synthesis of certain PUFA may be associated with the provision of acetyl-CoA originating from beta-oxidation processes, or from fatty acids transportation with carnitine, for which vitamin B2 participation is also essential [12].

When trained rats were fed the LP diet enriched with vitamin B2, a decline in DHA acid on day 60 (Tab. 4) and decrease of LA day 90 of the study was observed (Tab. 5). The statistically significant decrease in LA acid could be due to increased beta-oxidation because of the intensified vitamin B2 supply. There is strong evidence that B2 participates in lipids metabolism in the body. A hindering of fatty acids oxidation processes was found, which depended on the degree of riboflavin deficiency [27]. Riboflavin deficiencies cause an increase in LA and decrease in AA acids in liver phospholipids [12, 28] and in mitochondria and microsomes phosphatidylcholine [29]. Acetyl-CoA, which is produced as a result of beta-oxidation caused by the increased amount of vitamin B2, may also constitute a substrate to biosynthesis of fatty acids, in this case, AA acid.

The protein-deficient diet affected changes in polyunsaturated fatty acids in rat blood serum, causing a decrease in ALA and DHA content. Training caused a decrease in the content AA acid, whereas vitamin B2 affected the increase in AA acid. A statistically significant increase in LA acid was observed after 90 days in the group of LP trained rats. Most likely, the physical activity of the rats fed the protein-deficient diet caused increased mobilization of PUFA, mainly LA acid, from tissue reserves, or the greater bioavailability of this acid from the diet. Interestingly, after 90 days of the study, an increase was observed in ALA acid in the trained group with the diet enriched with vitamin B2, compared with the other groups. Both factors may cause increased absorption of this acid from feed, or increased release from adipose tissue into the serum. The decrease in the content of omega-6 in the trained group supplemented with vitamin B2 and increase in the content of omega-3 fatty acids is a very promising result. It is well known that a diet enriched with omega-3 acids (mainly DHA and EPA) decreases the risk of dyslipidaemia and hypertension, and may affect insulin resistance [30]. It also may be beneficial for body mass reduction in obese people [31].

Plasma FA are good markers of dietary intake, and are also considered biomarkers for long-term essential FA intake because their changes of concentrations are parallel with changes of FA composition of adipose tissue [32]. The results of the presented study also suggest that protein deficiency, physical exercise and supplementation of vitamin B2 have significant impact on the PUFA composition of serum in rats.

## CONCLUSIONS

The use of a low protein diet for 3 months significantly reduced and changed the level of PUFA in the serum of trained rats subjected to vitamin B2 supplementation. Consumption of an LP diet enriched with vitamin B2 in sedentary subjects did not affect serum FA composition in rats. Physical activity led to an increase in LA (mainly on day 30 of the study), EPA (on day 90 of the study) and a reduction in AA content on day 90 of the study in the serum of rats fed LP. B2 supplementation

in the trained LP group did not change the EPA and AA dependence. However, a decrease in LA content was noted on day 90 of the study, which decreased the omega 6/3 ratio. Therefore, in the opinion of the authors of the current study, B2 supplementation should be considered in physically active people who consume diets deficient in protein.

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