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The long-term effect of a protein-deficient-diet enriched with vitamin B6 on the blood parameters in unexercised and exercised rats

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ABSTRACT

Protein undernutrition affects *inter alia* blood parameters and immune system. These negative effects may be improved by the addition of vitamin B6 to the diet. Here, we evaluated the effect of vitamin B6 supplementation and physical exercise in rats fed a protein-deficient diet. Rats were divided into six groups: exercised or unexercised – control, protein deficient, protein deficient with vitamin B6 supplementation. Sixty days of protein malnutrition caused significant changes in the rat body weight, haematological parameters (mainly red blood cells parameters), immunological parameters (NK and NKT cells) and biochemical parameters (total protein and albumin concentration and activity of AST). The rat exercise did not intensify the negative effects of protein malnutrition. The vitamin B6 supplementation in protein-deficient groups significantly improved body weight and red blood cell parameters. These results indicate that vitamin B6 should be considered as a valuable supplement for individuals suffering from protein malnutrition.

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Rats; protein deficiency diet; vitamin B6; exercise; blood parameters

Introduction

According to the definition by the World Health Organization (2017), the malnutrition includes deficiencies, excesses or imbalances in the intake of energy and/or nutrients. The malnutrition is divided into two main groups: overnutrition (which causes obesity) and undernutrition (which causes kwashiorkor). The term "malnutrition" is also often used in scientific and everyday language as the undernutrition caused by calories, protein or micronutrients' deficiency (Bharadwaj et al., 2016), and this is how we define "malnutrition" in this study. Malnutrition is currently one of the biggest problems in developing countries where over 850 million people are undernourished. The better understanding

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of the effects of malnutrition may help to extenuate negative consequences of poor and unbalanced diet (Odermatt, 2011) and will be helpful in choosing controlled and limited nutrition for treatments of various disorders, such as metabolic disorder or cancer.

One such nutrition-deficient diets is the protein-deficient diet. This diet may be successfully used in patients suffering from kidney failure, such as chronic renal failure or liver failure such as hepatic encephalopathy (Ambühl, 2011). It had been reported that the low-protein diet improves the quality of life of patients suffering from these diseases and slows down the progression of diseases (Merli & Riggio, 2009). However, the long-term malnutrition caused negative health effects, such a reduced body weight, reduced cell number in tissues and organs, modified fatty acids' content and affected selected blood parameters, especially in young, growing subjects (Lewicka et al., 2017; Prestes-Carneiro et al., 2006). In addition, the long-term physical activity may increase negative consequences of the protein-deficient diet (Fontana, Klein, & Holloszy, 2006).

One of the factors which could diminish negative effects of protein malnutrition is vitamin B6. It is well known that vitamin B6 (pyridoxamine) is involved in protein metabolism. Vitamin B6 is a coenzyme of several amino decarboxylases (e.g. aromatic acid, glutamate, histidine) and transaminases (e.g. GABA transaminase, kynurenine aminotransferase, branched chain amino acid 2-oxoglutarate aminotransferase) (Clayton, 2006). As a coenzyme of phosphorylases, vitamin B6 affects glycogenesis and glycogenolysis in the muscles. Pyridoxamine reduces glycation of proteins and therefore is beneficial in preventing the adverse effects of poor glycemic control in diabetes (Onorato, Jenkins, Thorpe, & Baynes, 2000). It also plays an important role in immune cell function and blood-forming processes (Cheng, Chang, Lee, Lin, & Huang, 2006).

We previously described that B6 supplementation exhibited a protective effect in growing rats fed for a short period of time (30 days) with the protein-deficient diet (Lewicka et al., 2012). Vitamin B6 improved body weight and food intake and selected morphological parameters in rat blood. Therefore, in the present work, we decided to extend the time of the experiment and evaluate the effect of long-term feeding (for 60 days) with the protein-deficient diet supplemented with vitamin B6 on the selected parameters of rat blood in conjunction with physical exercise. The haematological, immunological and biochemical parameters of the blood were examined.

Materials and methods

Animals

The experiment lasted 60 days. The study was performed on 70 male Wistar rats, with an initial body weight of 127 ± 7 g. The experimental protocol was approved by the IV Local Ethic Committee for Animals Studies in Warsaw. Animals were kept in metal cages (5 rats per cage) in an air-conditioned room in standard conditions (12-hour light cycle, temperature 23°C). Animals were fed *ad libitum* with a semisynthetic isocaloric diet (energy value 350 kcal/100 g) for 60 days. Rats were divided into six groups: control (I, 10 rats), control + exercise (II, 10 rats), protein deficient (III, 10 rats), protein deficient + exercise (IV, 10 rats), protein deficient with B6 supplementation (V, 15 rats) and protein deficient with B6 supplementation + exercise (VI, 15 rats). Exercise groups were subjected to physical exercise 5 days a week. Exercise consisted of 1-hour running on a treadmill belt at a

speed of 20 m/min. Body weight of rats and food intake were examined two or three times a week. Blood samples (about 500 µL) were collected from the tail after 60 days of experiment.

Diets

We used three types of diet: (1) control diet – containing 20% energy from proteins, (2) experimental diet – containing 4.5% energy from protein and (3) experimental diet containing 4.5% of energy from proteins, supplemented with vitamin B6 (300% of the norm). In the control diet, 20% of energy derived from the protein, 65% from the carbohydrates and 15% from the fat, of which approximately 2% were the essential fatty acids (EFAs). To prevent a difference in calorie content between groups, the remaining energy was derived from the carbohydrates. Our experimental diet had 4.5% of energy derived from proteins, 80.5% from carbohydrates and 15% from the fat, of which approximately 2% were the EFAs. Diets were supplemented with mineral salts and vitamins, in accordance with the guidelines for the rat (National Research Council (US) Subcommittee on Laboratory Animal Nutrition and Nutritional Requirements of Laboratory Animals, 1951). The components of the diets are shown in Table 1.

Blood samples

Blood samples were collected into tubes containing 0.05% EDTA in PBS (haematological or immunological analysis) or "on clot" to obtain serum (biochemical analysis). After 1 h clotting at room temperature, serum was separated by centrifugation at 2000 *x* g for 20 min and stored at −70°C until further analysis.

Haematological analysis

A haematological analysis was performed in a haematological analyzer (Exigo Boule Medical AB, Stockholm, Sweden) according to the manufacturer's protocol. We evaluated

Table 1. Diet composition.

Diet component	20% energy from protein g/100 g	4.5% energy from protein g/100 g
Sunflower oil	0.41	0.55
Lard	5.45	5.31
Casein	18.97	4.56
Egg powder	1.61	0.20
Wheat flour	19.43	19.43
Wheat starch	30.00	30.00
Potato starch	9.14	11.36
Sugar	10.00	23.59
Mineral mix[a]	4.00	4.00
Vitamin mix[b]	1.00	1.00

[a]100 g mineral mixture contains: 32.2 g $KHPO_4$, 30 g $CaCO_3$, 16.7 g NaCl, 10.2 g $MgSO_4$, 7.5 g $CaHPO_4$, 2.75 g $FeC_6P_5O_7$, 0.51 g $MnSO_4$, 0.08 g KJ, 0.03g $CuSO_4$, 0.025 g $ZnCl_2$, 0.005 g $CoCl_2$.

[b]100 g vitamin mixture (in potato starch) contains: 54500IU vit. D3, 0.1 g vit. K, 0.003 g vit. B12, 1 g choline chloride, 0.101 g folic acid, 0.003 g biotin, 1.0 g inositol, 1.0 g PABA, 125000IU vit. A, 0.15 g vit. B6*, 0.25 g vit.E, 0.5 g vit. B1, 2.5 g vit. C, 0.5 g vit. PP, 0.25 g vit. B2, 2.5 g calcium panthotenate. *B6 supplemented diet –0.45 g B6/100 g vitamin mixture.

the following parameters: WBC (white blood cells), RBC (red blood cells), HGB (hemoglobin), HCT (haematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration) and PLT (platelets). Results are presented as the mean with ± standard error.

Flow cytometry analysis

Phenotyping of T lymphocyte populations was performed by direct immunofluorescence staining. Rat T/B/NK Cell Antibody Cocktail containing: anti-Rat CD3 APC, anti-Rat CD45RA FITC, anti-Rat CD161 PE (first vial) and anti-Rat CD3 APC, anti-Rat CD8a FIT, anti-Rat CD4 PE were purchased from Becton Dickinson, Poland. The immunofluorescence staining was performed as described previously (Lewicki et al., 2014). Cytometric analysis was performed using FACS Calibur Flow Cytometer equipped with CellQuest Software (BD Biosciences). First, the WBC populations were identified by morphological parameters (FSC/SSC). Subsequently, the lymphocytes gate (R1) was chosen and the lymphocytes subpopulations within R1 gate were analysed. Additional phenotypic determination of WBC population (lymphocytes, monocytes, neutrophils, eosinophils) was made using FSC/SSC parameters. Results are presented as % of total WBC count.

Biochemical analysis

Blood samples were collected on "clot" and subsequently centrifuged at 2000 *x* g for 20 min. at 4°C. Supernatants were frozen at −80°C until further analysis. The concentration of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein and albumin in the serum were evaluated using diagnostic test (Pointe Scientific). Absorbance of each sample was measured using FLUOstar Omega (BMG Labtech). All serum samples were tested in duplicates. Aminotransferases activities are presented in UI/L, total protein concentration in g/L and albumin concentration in g/L (Pointe Scientific) ± SEM.

Statistical analysis

Results are presented as means ± SEM. Statistical evaluation of the results was performed with one-way ANOVA and Bartlett's test for equal variances, and the significance of differences between the groups was verified with a Bonferroni Multiple Comparison Post Test (Graph Pad Prism). The p-value < .05 was considered as significant.

Results

Body weight and diet consumption

The average food consumption and the final body weight of rats fed experimental diets (I-VI) measured at the 60th day of experiment are shown in Table 2.

The average body weight of rats fed control diet (20% of protein, group I) at 60th day of experiment was 503.7 g. There was no statistical difference between the body weight of this group, and the weight of control exercised group (20% of protein + exercise, group II).

Table 2. The effect of protein-deficient diet unsupplemented or supplemented with B6 on average consumption and body weight of rats at the 60th day of experiment in exercised or unexercised rats.

	20% of protein (I)	20% of protein + training (II)	4.5% of protein (III)	4.5% of protein + training	4.5% of protein + vit. B6 (V)	4.5% of protein + training + vit. B6 (VI)
Body weight (g)	503.7 ± 16.1^{a}	502.0 ± 31.9^{a}	199.2 ± 7.9^{c}	219.6 ± 9.5^{c}	294 ± 10.6^{b}	268.3 ± 7.3^{b}
Daily consumption (g)	25.4 ± 0.7^{a}	26.4 ± 0.4^{a}	17.6 ± 1.0^{c}	21.0 ± 0.3^{b}	21.6 ± 1.3^{b}	22.3 ± 0.8^{b}
n	*n*–10	*n*–10	*n*–10	*n*–10	*n*–15	*n*–15

Notes: Rats were fed for 60 days with: control diet (20% energy from protein, group I and II), low-protein diet (4.5% energy from protein, group III and IV) or low-protein diet (4.5% energy from protein) additionally supplemented with 300% of B6 group (V and VI). a,b,c: differences level between groups, $p < .05$.

Protein-deficient diet caused a significant reduction in body mass of rats. There was about 2.6 times decrease in the weight of rats from group III at the 60th day of experiment in comparison to the control group (I). We did not observe a significant difference between exercised and unexercised rats fed protein-deficient diet (group IV and III), however, exercised rats exhibited a slight increase in weight (about 10% at 60th day of experiment). Vitamin B6 addition to the diet resulted in a significant increase of the body mass of rats from the malnourished groups. These rats weighted about 40% more at the 60th day of experiment. Similar to the control diet, exercise did not significantly affect the weight of rats fed a protein-deficient diet supplemented with vitamin B6.

The highest value of the food consumption in both exercised and unexercised control groups was 26.4 and 25.4 g/rat/day, respectively. Rats fed protein-deficient diets (III–VI group) consumed on average approximately 75% (~21 g/rat/day) at 60th day of what the control group consumed. The lowest consumption value was noted for group III (unexercised, protein malnourished rats) – 17.6 (g/rat/day).

Haematological analysis

Results of blood haematological analysis of rats fed with control (I, II), protein-deficient diet (III–IV) and protein-deficient diet supplemented with vitamin B6 (V–VI) are presented in Table 3.

Table 3. The effect of protein-deficient diet unsupplemented or supplemented with B6 on haematological parameters at 60th day of experiment in exercised or unexercised rats.

Haematological parameters	20% of protein (I)	20% of protein + exercise (II)	4.5% of protein (III)	4.5% of protein + exercise (IV)	4.5% of protein + B6 (V)	4.5% of protein + B6 + exercise (VI)
RBC (10^6/μL)	8.9 ± 0.2^{a}	8.4 ± 0.2^{a}	9.0 ± 0.2^{a}	8.5 ± 0.2^{a}	9.6 ± 0.1^{b}	9.8 ± 0.1^{b}
HGB (g/dL)	13.2 ± 3.3^{a}	12.8 ± 1.6^{a}	12.1 ± 1.9^{b}	11.7 ± 3.7^{b}	13.3 ± 2.4^{a}	13.2 ± 2.5^{a}
HCT (%)	37.8 ± 1.1^{a}	37.1 ± 0.8^{a}	36.6 ± 1.0^{a}	35.2 ± 1.5^{a}	39.9 ± 0.7^{ab}	39.2 ± 0.8^{b}
MCV (fL)	42.6 ± 0.6^{ab}	44.5 ± 0.7^{a}	40.8 ± 0.6^{b}	41.2 ± 0.7^{ab}	41.9 ± 0.7^{ab}	40.0 ± 0.7^{b}
MCH (pg)	15.1 ± 0.4^{a}	15.3 ± 0.3^{a}	13.5 ± 0.4^{b}	13.6 ± 0.1^{b}	13.7 ± 0.2^{b}	13.5 ± 0.2^{b}
MCHC (g/L)	350 ± 10.9^{a}	348 ± 6.8^{a}	332 ± 5.8^{a}	333 ± 5.0^{a}	333 ± 6.6^{a}	337 ± 4.0^{a}
PLT (10^3/μL)	875 ± 39.7^{a}	941 ± 53.4^{a}	861 ± 94.4^{a}	762 ± 68.9^{a}	943 ± 25.1^{a}	840 ± 31.0^{a}
	n–10	*n*–10	*n*–10	*n*–10	*n*–15	*n*–15

Notes: Rats were fed for 60 days with: control diet (20% energy from protein, group I and II), low-protein diet (4.5% energy from protein, group III and IV) or low-protein diet (4.5% energy from protein) additionally supplemented with 300% of B6 group (V and VI). a,b: differences level between groups, $p < .05$.

For all tested haematological parameters, there was no significant difference between exercised and unexercised rats fed with control, protein deficient or protein-deficient diet with vitamin B6. Protein deficiency diet did not affect red blood cells (RBC) and platelets' number in the blood. There was a significant increase in RBC in both (exercised and unexercised rats) protein-deficient groups supplemented with vitamin B6 ($p < .05$). Hemoglobin level in protein-deficient rats was significantly lower, approximately 15% in unexercised, and 10% in exercised ($p < .05$) rats. Vitamin B6 supplementation reduced the negative impact of protein deficiency on the hemoglobin concentration and haematocrit in the blood. Low-protein diet slightly decreased, and vitamin B6 addition slightly increased HCT level; however, the difference was not significant. Generally, rats fed control diet (both exercised and unexercised) had a higher level of MCV, MCH and MCHC in comparison to rats fed protein-deficient diets (groups III–VI). However, significant changes were noticed only in MCH (between I, II group and III–VI group; $p < .05$) and MCV level (between I and VI, $p < .05$).

Cytometric analysis

Results of WBC analysis in rats fed control (I, II), protein-deficient diets (III–IV) and protein-deficient diets supplemented with vitamin B6 (V–VI) are presented in Table 4.

Mean WBC cell number in blood of rats fed control diet (20% of protein) amounted to $9.8 \times 10^3/\mu L$ in unexercised and $10.7 \times 10^3/\mu L$ in exercised rats. There was a significant increase in WBC in groups of exercised rats fed protein-deficient diet (IV, mean $16.6 \times 10^3/\mu L$, $p < .05$) in compression to the other groups (I, II, III, V, VI). There was no difference in the percentage of lymphocytes, neutrophils, eosinophils and monocytes; however, the low-protein diet (III–VI) caused a slight decrease in average percentage of lymphocytes and an increase of percentage of neutrophils. Vitamin B6 supplementation did not affect immunological parameters.

The phenotyping of lymphocytes from rats fed control (I, II) and experiment diets (III–VI) are presented in Table 5.

The analysis of lymphocytes populations revealed some significant differences between tested groups. Rats fed low-protein diet and additionally exercised had decreased percentage of $CD8^+$ T cells in comparison to both exercised and unexercised control group.

Table 4. The effect of protein-deficient diet unsupplemented or supplemented with vitamin B6 on white blood cells (WBC) at 60th day of experiment in exercised and unexercised rats.

WBC parameters	20% of protein (I)	20% of protein + exercise (II)	4.5% of protein (III)	4.5% of protein + exercise (IV)	4.5% of protein + B6 (V)	4.5% of protein + B6 + exercise (VI)
WBC ($10^3/\mu l$)	9.8 ± 0.7	10.7 ± 0.6	9.5 ± 0.5	**16.6 ± 1.0***	12.4 ± 1.2	11.5 ± 0.9
Lymphocytes (%)	67.9 ± 2.8	71.5 ± 1.9	62.4 ± 3.6	58.9 ± 4.0	62.4 ± 2.9	60.7 ± 2.6
Neutrophils (%)	24.2 ± 2.4	19.9 ± 4.1	30.9 ± 3.7	34.9 ± 3.5	28.5 ± 2.5	30.1 ± 2.6
Eosinophils (%)	2.6 ± 0.2	2.5 ± 0.7	2.6 ± 0.4	1.5 ± 0.2	1.6 ± 0.2	1.5 ± 0.2
Monocytes (%)	5.2 ± 0.8	6.1 ± 1.0	4.1 ± 0.5	4.7 ± 0.9	7.5 ± 1.0	7.7 ± 1.0
	n–10	*n*–10	*n*–10	*n*–10	*n*–15	*n*–15

Note: Rats were fed for 60 days with: control diet (20% energy from protein, group I and II), low-protein diet (4.5% energy from protein, group III and IV) or low-protein diet (4.5% energy from protein) supplemented with 300% of B6 (group V and VI). *Significant differences ($p < .001$).

Table 5. The effect of protein-deficient diet unsupplemented or supplemented with vitamin B6 on the percentage of lymphocytes populations at 60th day of experiment in exercised and unexercised rats.

Lymphocyte populations	20% of protein (I)	20% of protein + exercise (II)	4.5% of protein (III)	4.5% of protein + exercise (IV)	4.5% of protein + B6 (V)	4.5% of protein + B6 + exercise (VI)
Lymphocytes T ($CD3^+$) (%)	58.0 ± 3.6^a	49.7 ± 3.2^a	55.7 ± 3.8^a	47.3 ± 1.3^a	50.2 ± 2.6^a	45.7 ± 4.1^a
Lymphocytes B ($CD45RA^+$) (%)	24.4 ± 1.5^a	25.4 ± 1.9^a	22.4 ± 2.4^a	26.8 ± 1.4^a	22.8 ± 1.6^a	25.0 ± 1.2^a
Lymph T ($CD4^+$) (%)	36.8 ± 2.3^a	37.6 ± 2.6^a	42.0 ± 3.3^a	38.4 ± 1.3^a	41.7 ± 1.6^a	36.9 ± 1.9^a
Lymph T ($CD8^+$) (%)	14.2 ± 0.7^a	13.8 ± 0.5^a	12.7 ± 1.0^{ab}	10.4 ± 0.4^b	13.2 ± 0.8^{ab}	11.5 ± 0.5^{ab}
CD4/CD8 ratio	2.6 ± 0.3^a	2.7 ± 0.2^a	3.3 ± 0.3^{ab}	3.7 ± 0.3^b	3.2 ± 0.3^{ab}	3.2 ± 0.2^{ab}
NK ($CD161^+CD3^-$) (%)	9.4 ± 0.8^a	7.6 ± 0.8^{ab}	4.8 ± 0.3^b	6.7 ± 0.6^{ab}	5.6 ± 0.6^b	7.0 ± 1.0^{ab}
NKT ($CD161^+CD3^+$) (%)	3.9 ± 0.4^a	3.1 ± 0.3^a	1.4 ± 0.2^b	1.4 ± 0.1^b	1.9 ± 0.3^b	1.7 ± 0.1^b
	n–10	*n*–10	*n*–10	*n*–10	*n*–15	*n*–15

Notes: Rats were fed for 60 days with: control diet (20% energy from protein, group I and II), low-protein diet (4.5% energy from protein, group III and IV) or low-protein diet (4.5% energy from protein) supplemented with 300% of B6, (group V and VI). a,b: differences level between groups, $p < .05$.

Similar results were observed in a CD4/CD8 ratio ($p < .05$). Protein malnutrition resulted in a decrease in NK percentage (regardless of vitamin B6 supplementation) in comparison to control group, but only in unexercised groups (III and V). Interestingly the NK percentage in exercised rats (irrespective of the type of diet) showed no differences. Protein deficiency also caused an abnormal percentage of NKT cells. A significant decrease of NKT percentage in rats fed with all experimental diets was observed in comparison to the control group ($p < .001$); however, the higher differences in the percentage of these cells were found in groups without vitamin B6 supplementation. There was no significant difference in the average percentage of T and B cells in all exercised groups (II, IV and VI). Interestingly, we noticed some similar trends in all exercised groups: the decrease of the percentage of T cells and increase of the percentage of B cells.

Biochemical analysis

Results of aminotransferases activity (ALT, AST, UI/L), total protein and albumin concentration (g/L) in the serum of rats fed control (I, II), protein deficiency diet (III, IV) and protein deficiency diet supplemented with vitamin B6 are presented in Table 6.

The average activity of ALT did not differ between studied groups (I–VI), while the AST activity revealed some changes. Unexercised rats fed control diet (I) exhibited the lowest AST activity in comparison to other tested groups. Exercise caused an increase of AST activity in all studied groups (II, IV, VI; $p < .001$) when compared to the control (I). Unexercised rats fed low-protein diet had an elevated AST activity (100.9 IU/L, $p < .05$) and the vitamin B6 addition insignificantly reduced AST activity (85.1 IU/L).

The total protein and albumin concentration in serum, between exercised and unexercised group fed the same diet type was similar. Low-protein diet intake resulted in the decrease in total protein concentration in serum. The vitamin B6 supplementation significantly improved this parameter especially in the exercised group (III versus VI, $p < .01$). Albumin concentration was less variable than total protein concentration. There was a significant reduction of albumins ($p < .05$) only in the group IV and V when compared to the control groups (I, II).

Table 6. The effect of protein-deficient diet unsupplemented or supplemented with vitamin B6 on serum aminotransferases activity, total protein and albumin concentration at 60th day of experiment in exercised or unexercised rats.

	20% of protein (I)	20% of protein + exercise (II)	4.5% of protein (III)	4.5% of protein + exercise (IV)	4.5% of protein + B6 (V)	4.5% of protein + B6 + exercise (VI)
Alanine aminotransferase (UI/L)	17.3 ± 2.3^{a}	17.5 ± 2.1^{a}	18.4 ± 2.2^{a}	24.1 ± 3.4^{a}	19.1 ± 2.7^{a}	18.7 ± 2.5^{a}
Aspartate aminotransferase (UI/L)	69.0 ± 3.5^{a}	104.2 ± 8.2^{c}	100.9 ± 2.0^{c}	99.2 ± 1.3^{bc}	85.1 ± 4.3^{b}	90.3 ± 2.3^{bc}
Total protein (g/L)	81.1 ± 2.3^{a}	81.4 ± 2.2^{a}	56.3 ± 2.7^{b}	49.1 ± 2.4^{b}	59.1 ± 1.4^{bc}	64.1 ± 1.2^{c}
Albumine concentration (g/L)	38.4 ± 2.1^{a}	39.5 ± 1.9^{a}	32.9 ± 1.2^{ab}	30.5 ± 1.3^{b}	31.7 ± 1.3^{b}	35.2 ± 1.1^{ab}
	n–10	*n*–10	*n*–10	*n*–10	*n*–15	*n*–15

Notes: Rats were fed for 60 days with: control diet (20% energy from protein, group I and II), low-protein diet (4.5% energy from protein, group III and IV) or low-protein diet (4.5% energy from protein) a supplemented with 300% of B6 (group V and VI). a,b,c: differences level between groups, $p < .05$.

Discussion

We examined the effect of protein deficiency diet (4.5% of protein) on selected biochemical, haematological and immunological blood parameters in rats subjected or not subjected to the physical exercise. The negative influence of protein malnutrition on blood parameters (mainly biochemical and haematological) is basically well known (Araújo, Sant'Ana, Molinari, & Miranda Neto, 2005); however, in our study, we additionally evaluated the effect of vitamin B6 supplementation (300% of norm) as a potential factor to reduce adverse effect of protein-deficient diet.

The body weight and food consumption of rats fed for 60 days with protein-deficient diet were significantly decreased in comparison to the control rats. These results are in alignment with other studies showing that protein malnutrition reduces body mass in both humans and animals (Bray et al., 2012; Lewicki et al., 2014). The addition of vitamin B6 (300% of norm) inhibits the negative influence of protein malnutrition. These data are consistent with our previously published study (Lewicka et al., 2012). Rodríguez-Rodríguez et al. (2008) showed that vitamin B6 lessens the negative effect of hypocaloric diet and helps maintaining fat-free mass in overweight/obese women. A similar correlation was observed by Debski, Bertrandt, Klos, and Gralak (2007). They showed that in rats fed with protein-restricted diet (9% of protein), the addition of vitamin B6 decreased body weight. In contrast, a study of Debski, Bertrandt, Klos, and Gralak (2006) showed that vitamin B6 enrichment of low-protein diet (4.5% of protein) did not affect significantly body weight and food consumption in rats. Taking together, we believe that the vitamin B6 supplementation may have an opposite effect on the body weight depending on the organism health status: in severe protein malnutrition may increase body weight, and in normal nutrition, or less severe malnutrition may decrease body weight.

It is well known that protein malnutrition affects several morphological parameters in the blood. This was shown not only in mammals but also in birds and fish. In the research conducted on rats, feeding protein-deficient diet (9.5% of protein) for 80 days significantly

reduced hemoglobin, MCV and MCH concentration and increased MCHC level (Prestes-Carneiro et al., 2006). Protein deprivation (from 24% to 13% of crude protein) caused the decrease of RBC number and MCV level and increased the MCH and MCHC in fingerlings of common carp (*Cyprinus Carpio*) (Al-Sraji & Nasir, 2013). Similarly, Mohamed, Ali, Malik, and Yousif (2012) observed a reduction of MCV and MCH level in birds fed lower protein content diet (21% in comparison to 23% of crude protein). In the present study, protein malnutrition (4.5% of protein) had a negative impact on hemoglobin, MCV and MCH parameters (all of them were lower). The results are consistent with the work of other researchers and with our previous study (Lewicka et al., 2012). Pyridoxine is involved in the proper functioning of the haematological system. Vitamin B6 deficiency significantly reduced MCV, hemoglobin and haematocrit in mice; however, the addition of pyridoxine for 3 days increased HGB levels and HCT, MCV and MCH parameters (Tangjarukij, Navasumrit, Zelikoff, & Ruchirawat, 2009). In our study, the addition of vitamin B6 caused a significant increase in the number of red blood cells and the percentage of haematocrit (in comparison to the control and to the protein-deficient groups) and hemoglobin content (when compared to the protein-deficient group). Therefore, in our opinion, the vitamin B6 supplementation could be used as a protective agent against negative effects of protein malnutrition diet on haematological system.

The immune system protects organisms against a potential microbe threat. The WBC (lymphocytes, granulocytes and monocytes) play a major role in the immune response. Malnutrition is the primary factor of immunodeficiency worldwide and profoundly alters cell-mediated immune responses in both human and experimental animals (Katona & Katona-Apte, 2008), and affects different levels of immune response. It has been shown that protein deficiency reduces IgA immune response in mouse and rat models (McGee & McMurray, 1988; Sullivan, Vaerman, & Soo, 1993). Protein-energy malnourished mice were more susceptible to influenza infection (Taylor et al., 2013), and had decreased cytokine production (Iyer et al., 2012). The protein-deficient guinea pigs had reduced bactericidal activities of the neutrophils (Sobrado et al., 1983). Results obtained in the present work did not reveal any significant impact of low-protein diet on white blood cell number, or on the percentage or distribution of leukocytes in peripheral blood of rats. There was also no effect on the percentage of B and T cells. Our results are in agreement with Huang and Fraker (2003), who observed that consumption of a moderately low-protein diet (about 6% of protein) does not alter haematopoietic processes in young adult mice (both CD4+ and CD8+ cells).

NK cells exhibit cytotoxic properties and survey the body for aberrant expression of MHC class I molecules and stress markers on the autologous cells (Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008). NKT cells play mainly immunoregulatory role, but they are also able to eliminate some pathogens through direct cytotoxity (Godfrey, Stankovi, & Baxter, 2010). In the present work, we noticed a reduction of NK and NKT percentage in rats fed with low-protein diet (4.5%). Similar trends were also found in other studies (Lewicki et al., 2014; Li et al., 2004). The reduction of the percentage of NK and NKT cells in peripheral blood observed in our present study is not a good prognosis for the protein malnutrition organisms. It has been demonstrated that the decreased percentage of NK and NKT cells in the peripheral blood of patients is associated with autoimmune diseases, such as multiple sclerosis, systemic lupus

erythematosus, Sjögren's syndrome, rheumatoid arthritis and psoriasis (Poggi & Zocchi, 2014; Wu & Van Kaer, 2009). Moreover, a lower percentage of NK cells is associated with a severe disease in patients with common variable immunodeficiency (Ebbo et al., 2016).

The protein malnutrition reduces a content of most proteins in the body, including enzymes and hormonal proteins (Wykes et al., 1996). Protein malnutrition in conjunction with a sufficient supply of energy from fat and carbohydrates results in a hepatic steatosis, and in the advanced stages, in fibrosis (Benjamin, Kushwah, Sharma, & Katiyar, 2006). We also showed that protein malnutrition diet caused a significant decrease in serum total protein concentration (about 35%, $p < .01$). The vitamin B6 supplementation did not affect total protein concentration in the serum. These results are in agreement with other researchers' results (Mezey, 1982). Interestingly, we observed a slight, but not significant, increase of the total protein concentration in serum of the exercised groups.

Albumin is a protein produced by the liver. Daily production of the protein provides about 5% of total serum albumin. The major function of albumin is to carry/transfer various minerals, hormones, enzymes and fatty acids in the organism (Garcia-Martinez et al., 2013). Physicians frequently rely on albumin levels to assess a patient's nutritional status (Bharadwaj et al., 2016). In the present study, all protein-deficient groups exhibited a significantly lower concentration of albumin in the serum. These results are in agreement with Prenner et al. (2014), who found that in the cardiac transplant recipients, albumin concentration is a better predictor of underlying malnutrition than the body mass index and the subjective global assessment. Also Cross, Yi, Thomas, Garcia, and Della Valle (2014) postulated that albumin concentration is a better prediction factor in the evaluation of malnutrition in patients undergoing elective orthopaedic surgery. In contrast, Baron, Hudson, and Steele (2010) showed that serum albumin is not useful as a marker for malnutrition in scleroderma paradigm and should not be considered to be a useful marker in other chronic diseases. A lower concentration of albumin may be also related to the damage of liver function (Lee, 2012). Therefore, in the present study, we also examined markers of liver failure such as the activity of alanine and ASTs. These enzymes are also involved in the metabolism of proteins in the organism. We observed that unexercised protein-deficient groups of rats had a significantly higher activity of AST than the control group. These results are disturbing and may indicate liver steatosis, which was found in rats fed with a diet deficient in protein (5%) by van Zutphen et al. (2016). The important finding is that the addition of vitamin B6 lessened this negative effect. A high level of AST activity observed in all exercised group may be the result of long-term gruelling exercise (Pettersson et al., 2008). What is promising is that the exercise did not intensify the negative effects of protein depletion. In summary, all these results suggest that in protein-deficient organisms, Vitamin B6 supplementation may act as a liver protector.

Conclusions

Sixty days of protein malnutrition caused significant changes in the organism of rats. These changes were not intensified by moderate but prolonged exercise. Moreover, the exercise lessened some of the negative effects of malnutrition on the immune system.

Vitamin B6 supplementation exhibits protective role against body weight loss and negative effects on the haematological system. These are promising results, and therefore, vitamin B6 should be considered as a useful supplement for individuals suffering from protein malnutrition.

Disclosure statement

No potential conflict of interest was reported by the authors.

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