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EFFECT OF DIET COMPOSITION AND MIXTURE OF SELECTED FOOD ADDITIVES ON THE ERYTHROCYTIC SYSTEM AND IRON METABOLISM IN PERIPHERAL BLOOD OF MALE RATS

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Background. Metabolic processes of food additives which are "exogenous xenobiotics" are catalysed, primarily, by enzymes located in microsomes of hepatocytes affiliated to P-450 cytochrome superfamily, containing iron. The aim of the study was to investigate the effect of diet composition and selected food additives on the erythrocyte system and iron metabolism in peripheral blood of male rats.
Material and methods. The experiment was carried out on 30 male rats sorted into three equinumerous groups. For drinking animals received pure, settled tap water, animals from group III were receiving additionally an aqueous solution of sodium (nitrate), potassium nitrite, benzoic acid, sorbic acid and monosodium glutamate.
Results. Ascertained a significant effect of changes in diet composition on the increase in hematocrit marker value and the count of red blood cells in blood of animals examined. Used food additives diminished hemoglobin concentration, hematocrit value and red blood cell count, diminishing also iron concentration in serum, the total iron binding capacity and transferrin saturation with iron.
Conclusions. Analysis of the results allowed ascertain adverse changes in values of the erythrocytic system markers, occurring under the influence of the applied mixture of food additives. Used food additives change the iron metabolism, most likely from the necessity of applied xenobiotics biotransformation by heme-containing monoxygenases of P-450 cytochrome.

Key words: food additives, iron metabolism, rat

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INTRODUCTION

A contemporary change in lifestyle facilitates improper nutritional choices by modifying consumer's attitude to food available on the market. When selecting food products, many people are driven chiefly by their appearance, extended shelf life, as well as easiness and short time of their further preparation. In response to demands of the market, producers apply a variety of pre-treatment processes of raw material, protecting and improving parameters of the resultant food products with various food additives. Over the last few years, a considerable increase has been observed in Poland in the assortment of highly-processed food products, the production of which involves both novel technological processes, as well as a number of additive substances [Górska-Warsewicz 2007]. Components commonly added to food include nitrates and nitrites, sorbic acid and its salts, benzoic acid and monosodium glutamate.

In Poland, the use of food additives is stipulated in respective regulations of the Minister of Heath [Rozporządzenie... 2008]. Thus, their use is legally permitted, yet within provisions determining contents of individual additives in selected food products that are safe for consumer health. Nonetheless, can the common consumption of various food products containing food additives exert no effect on human body?

The above-mentioned food additives are acknowledged as the so-called "exogenous xenobiotics" whose metabolism proceeds in liver. Metabolic processes are catalysed, primarily, by enzymes located in microsomes of hepatocytes affiliated to P-450 cytochrome superfamily, containing iron. They catalyse the conjugation of hydroxylated or otherwise transformed compounds with glucuronic, sulfuric, and acetic acids, and with glutathione.

Liver is additionally a site where many compounds are subject to biosynthesis, including iron-transporting transferrin and copper-transporting ceruloplasmin, being responsible for release of iron reserves.

In view of the above, the objective of this animal model study was to investigate the effect of diet composition and selected food additives on the erythrocyte system and iron metabolism in peripheral blood.

MATERIAL AND METHODS

The experiment, approved by the Local Ethical Committee in Szczecin (Approval no. 31/2006), was carried out in the vivarium of the Department of Human Nutritional Physiology, on 30 SPRD-strain male rats aged 5-7 months, of initial body weight 429 ±31.5 g. Rats were obtained from the animal husbandry of the Chair and Department of Toxycology, Poznań University of Medical Sciences, Poland.

Following a week long conditioning in the vivarium environment (temperature 21--22°C, 12 h/12 h light/dark cycle), the animals were randomized and sorted into three equinumerous groups of equal body weight, fed *ad libitum* on pelleted feeds composed of the same components, besides those differentiating, produced by the Feeds and Concentrates Plant in Kcynia, Poland, after having implemented the procedure 5.14.5. "Cleaning of machines and devices". Group I was fed standard feed (Labofeed H), while groups II and III received modified feed, in which 83.5% of wheat was substituted with wheat flour (type "500"), and 50% of corn grain was substituted with saccharose. The percent-

age of the remaining components was unchanged (Table 1). All diets were based on the balanced modification of the AIN-93 M diet formulation [Reeves et al. 1993].

Table 1. Component composition of feeds used in the experiment

Component	Basic feed, %	Modified feed, %
Wheat	36.4	6
Corn grain	20	10
Wheat bran	20	20
Dry whey	3	3
Fodder salt[1]	0.3	0.3
Soy-bean grain	17	17
Fodder chalk[2]	1.5	1.5
Phosphate 2-CA[3]	0.8	0.8
Premix LRM[4]	1	1
Wheat flour	–	30.4
Saccharose	–	10

[1]Mainly NaCl.
[2]Mainly $CaCO_3$.
[3]$CaHPO_4$.
[4]Vitamin-mineral composition used in animals feeds.

In order to establish the chemical composition of the feeds, basic chemical assays were carried out. We measured the concentration of total nitrogen, converted to quantity of protein, raw fat, dry matter, and ash. The content of carbohydrates was derived from the difference between dry matter and the remaining solid components. The brutto and metabolic energy was calculated using commonly applied energy equivalents (Table 2).

Table 2. Chemical composition of feeds used in the experiment

Component	Basic feed	Modified feed
Total protein, %	19.2	18.5
Crude fat, %	2.81	2.33
Carbohydrates, %	63.8	65.5
Dry matter, %	91.8	92.3
Total ash, %	6.09	5.98
Brutto energy		
kcal·g^{-1}	3.99	3.98
kJ·g^{-1}	16.7	16.7
Metabolic energy		
kcal·g^{-1}	3.57	3.56
kJ·g^{-1}	14.9	14.9

For drinking, animals from group I and II were provided pure, settled tap water. Whereas animals from group III were receiving, in the period of intensified activity, 5 ml of an aqueous solution of selected food additives, i.e.: sodium nitrite (E 250) at a dose of 0.07 mg/kg body weight (BW), potassium nitrate (E 252) at a dose of 5.07 mg/kg BW, benzoic acid (E 210) at a dose of 1.39 mg/kg BW, sorbic acid (E 200) at a dose of 0.51 mg/kg BW, and monosodium glutamate (E 621) at a dose of 17.65 mg/kg BW. These doses corresponded to an average intake of these food additives by men, converted per body mass kilogram, reaching: 11.7% of ADI for sodium nitrite, 137% of ADI for potassium nitrate, 27.8% of ADI for benzoic acid, and 2.0% of ADI for sorbic acid [Traczyk et al. 2003, Wawrzyniak et al. 2008]. Having drunk the solutions of additives, the animals were re-drunk with pure, settled tap water.

After 7-week experiment, the animals were anaesthetized with intramuscular injection of Ketanest administered at a dose of 10 mg/kg BW. Next, blood was sampled from their heart to tubes with EDTA/K2 and to the so-called "clot" tubes, centrifuged in an MPW – 350R laboratory centrifuge with cooling, at a temperature of 4^{o}C, and the speed of 3500 rpm for 20 min.

Full blood was assayed for basic markers of the erythrocytic system. The concentration of hemoglobin (Hb) was determined with the colorimetric, cyanmethemoglobin method. Hematocrit (HCT), red blood cell count (RBC), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were determined with the method based on electric impedance measurement with a Hitachi hematology analyser.

Blood serum obtained after clot centrifugation was assayed for the concentration of iron (Fe) with the colorimetric method with ferrene, and for the total iron binding capacity (TIBC). The unsaturated iron binding capacity (UIBC) was then computed by subtracting the value of iron concentration in blood serum from the TIBC value. Additional calculations were made for transferrin saturation (TfS) defined as the percentage ratio of iron concentration to the total iron binding capacity [Krawiec et al. 2007].

All colorimetric calculations were carried out using reagents by Aqua-Med company on a Metertech spectrophotometer.

The resulting data were tested for normality of distribution and processed statistically by means of the Statistica software package, using the Duncan test at the significance level $\alpha = 0.05$ [Statsoft 2009].

RESULTS AND DISCUSSION

An analysis of the results achieved demonstrated a significant effect of a change in diet composition and food additives applied on feed intake by animals and their body weight gains (Table 3). A mixture of food additives applied in the diet were found to affect its increased intake, which however was not reflected in body weight gains. In the case of animals administered diet with food additives, the body weight gains were significantly lower as compared to the animals fed the basal feed mixture.

When analysing values of selected hematological markers indicative of the health status of the test animals, it should be emphasized that they were fitting within physiological norms [Carpenter et al. 2001]. Nevertheless, the analysis of results indicated the change in diet composition to have a significant effect on values of hematocrit concentration and RBC count in blood of the animals examined (Table 4). The animals receiving

Table 3. Effect of diet type and mixture of selected food additives on feed intake and body weight gain of male rats ($\bar{x}$ ±SD; n = 30)

Examined trait	Basic feed	Modified feed	Modified feed + food additives
Feed consumption, g/100 g body mass/7 weeks	239 ±6.0[a]	239 ±11.3[a]	253 ±13.1[b]
Body weight gain, g/7 weeks	63.6 ±13.1[b]	48.7 ±13.9[ab]	42.2 ±10.4[a]
Body weight gain, g/100 g feed	5.36 ±1.01[b]	4.25 ±1.10[ab]	3.55 ±0.73[a]

a, b – means in line denoted with the same letters are not significantly different, $p \leq 0.05$.

Table 4. Effect of diet composition and selected food additives on values of selected markers of the erythrocytic system of peripheral blood in male rats ($\bar{x}$ ±SD; n = 30)

Examined trait	Basic feed	Modified feed	Modified feed + food additives
Hemoglobin, mmol·l^{-1}	8.63 ±0.37[ab]	8.81 ±0.33[b]	8,44 ±0.32[a]
Hematocrit	0.42 ±0.016[a]	0.43 ±0.019[b]	0.41 ±0.012[a]
RBC, 10^{12}·l^{-1}	8.44 ±0.35[a]	8.78 ±0.42[b]	8.45 ±0.13[a]
MCV, fl	49.2 ±1.09[a]	49.3 ±1.42[a]	48.9 ±1.29[a]
MCH, pg	17.4 ±0.33[a]	17.2 ±0.48[a]	17.4 ±0.49[a]
MCHC, g·dl^{-1}	35.3 ±0.49[ab]	34.9 ±0.43[a]	35.6 ±0.43[b]

a, b – means in line denoted with the same letters are not significantly different, $p \leq 0.05$.

the modified feed mixture were characterised by higher values of HTC marker and a higher number of erythrocytes in blood unit as compared to both animals fed the basal feed mixture and these receiving the modified diet with additives. Values of the other markers of the erythrocytic system were similar to those recorded for the animals on the basal feed mixture.

Erythrocytes are the basic morphotic constituents of blood and carriers of oxygen-binding hemoglobin. Their proper number and saturation with hemoglobin are a prerequisite for providing the appropriate amount of oxygen to cells. Enhanced synthesis of erythrocytes is observed as a result of tissue anoxia or increased body demand for oxygen. Taking into account the lower body weight gains of the animals fed the modified diet per feed intake unit, it may be speculated that the rate of catabolic transformations in tissues of the investigated animals was increased, which was reflected in the increased oxygen demand. This was likely to be one of the causes of the noticed higher RBC count in blood of the animals fed the modified diet.

Considering the composition of the modified diet and duration of experiment, it may be assumed that saccharose occurring in the diet may facilitate the increase in blood glucose level and enhance the non-enzymatic glycosylation of hemoglobin [Chmielewska et al. 1996]. The glycated hemoglobin displays a lower capability for binding and donating oxygen, which may be compensated for with an increasing red blood cells

count in blood [Błażucka et al. 2006]. This theory is corroborated by the increased number of erythrocytes, at the unchanged hemoglobin concentration, in blood of the experimental animals fed the modified diet as compared to these administered the basal feed mixture.

Incorporation of aqueous solutions of mixture selected food additives to diets of rats was found to exert a significant effect on selected markers of the erythrocytic system of their peripheral blood. A statistically significant decrease, in respect of the values determined for the animals fed the modified diet, was noted in the case of: hemoglobin concentration, value of the hematocrit marker and red blood cells count, whereas an increase was demonstrated in the mean corpuscular hemoglobin concentration (MCHC). In the case of animals fed diet with mixture of food additives, the values of erythrocytic system markers were comparable to these observed in the animals fed the basal feed mixture.

The number of red blood cells is affected by factors that stimulate the synthesis of erythrocytes as well as by the rate of their degradation that may be inhibited by protective factors. The latter factors known to stabilize cellular membranes of not only erythrocytes include, i.a. vitamin A whose diminished concentration in liver of rats administered sodium nitrite was observed by Bilczuk [1980]. This may be due to the oxidative effect of nitrites on vitamin A in the digestive tract even before it is absorbed from diet and may facilitate the enhanced degradation of erythrocytes [Philips 1966].

Some works report also on the direct toxic effect of nitrites on erythrocytes and hemoglobin contained in them [Dudka et al. 1998, Stepuro et al. 1994, Tyburczyk et al. 1991]. Dudka et al. [1998] demonstrated that the concentration of hemoglobin in blood of rats administered sodium nitrite was significantly lower as compared to that noted for control animals. This phenomenon has been shown to be a result of both nitrites-induced disturbances in heme biosynthesis as well as transformation of hemoglobin into methemoglobin [Dudka et al. 1998, Lhuissier et al. 1976].

The present study demonstrated also an increase in the mean curpuscular hemoglobin concentration (MCHC) in the case of animals receiving a diet with mixture of food additives. The increase in MCHC is usually observed in hyperchromic anemia, spherocytosis or in intracellular dehydration.

Two of the applied additives were sodium salts. Under physiological conditions, the quantity of sodium excreted with urine is equal with its intake. Renal regulation of sodium excretion is affected by a variety of factors, yet in this case the homeostatic mechanisms may be disturbed by sodium excess in diet [Knypl 2002]. Kuchlewska [2010] reported that the additives used in the experiment were enhancing water retention in the body of experimental animals, thus increasing its accumulation in vascular bed, which was indicated by the observed change in hematocrit marker value. Sodium is an extracellular cation and its accumulation in this space may facilitate intracellular dehydration.

Considering the mixture of food additives applied, their complex and diversified effect on red blood cell markers should further be discussed. Nitrates and nitrites trigger destruction of B-group vitamins [Lhuissier et al. 1976, Saint-Blanquat 1980], thereby promoting anemia. They additionally enhance free-radical reactions that may affect the shape and permeability of a cellular membrane of erythrocytes. Oxidation of heme iron, occurring during methemoglobin synthesis from hemoglobin under the influence

of nitrites, is a source of reactive oxygen species that initiate the chain of oxidative processes in a red blood cell. The oxidative stress results in enhanced peroxidation of lipids and aggregation of proteins of erythrocyte membrane. These processes affect changes in the permeability of the cellular membrane, and diminish erythrocyte deformability, leading in this way to earlier removal of erythrocytes from circulation, thus shortening their life cycle [Bartosz 2004].

Changes in erythrocyte markers occurring as a result of changes in diet composition and food additives applied might have been also due to altered iron metabolism. The analysis of the results demonstrated the change in diet composition to affect a reduction in iron concentration, as well as in total and latent capacity for its binding in blood serum. The food additives applied were found to intensify this effect (Table 5). In contrast, the change in diet composition did not affect the degree of transferrin saturation with iron, whereas a decrease in its saturation was observed when the animals were fed diets with mixture of food additives.

Table 5. Effect of diet composition and selected food additives on selected markers of iron metabolism in blood serum of male rats ($\overline{x}$ ±SD; n = 30)

Examined trait	Basic feed	Modified feed	Modified feed + food additives
Fe, μmol·l^{-1}	30.0 ±1.43^{c}	28.3 ±3.01^{b}	23.2 ±1.06^{a}
TIBC, μmol·l^{-1}	114 ±2.09^{c}	104 ±1.99^{b}	97.0 ±6.81^{a}
UIBC, μmol·l^{-1}	84.2 ±2.31^{b}	75.0 ±2.88^{a}	74.2 ±7.01^{a}
TfS, %	26.3 ±1.02^{b}	27.2 ±2.51^{b}	23.9 ±2.52^{a}

a, b, c – means in line denoted with the same letters are not significantly different, $p \leq 0.05$.

Iron is an element which occurs in all cells of a body incorporated into heme proteins, including hemoglobin, myoglobin, flavoproteins and cytochromes. In blood serum it is transported having been first bound with transferrin, the concentration of which may be assayed by the intermediate method through determining the total iron binding capacity (TIBC). Its concentration is significantly correlated with iron content of bone marrow, which in turn is incorporated into hemoglobin [Milman et al. 1993]. Taking into account the lower total iron binding capacity and the lower transferrin saturation with iron observed in blood serum of the animals fed the modified diet with food additives, it may be speculated that also iron content of the bone marrow would be lower in that group of animals, which has indirectly been confirmed by the results obtained for erythrocytic system markers.

The mixture of food additives applied in the experiment are xenobiotics. Their metabolism is regulated by liver, namely by the system of isoenzymes belonging to the P-450 cytochrome superfamily functioning therein [Riddick et al. 2004]. These enzymes include over 50 proteins containing heme, with iron being its constituent. A key enzyme regulating the biosynthesis of heme is synthase of δ-aminolevulinic acid. The metabolism of xenobiotics involves increased utilization of heme by P-450 cytochrome, which results in a reduced intracellular concentration of heme. This, in turn, enhances the activity of δ-aminolevulinic acid synthase and significantly accelerates heme synthesis

accordingly to cell's demand [Furuyama et al. 2007, Nebert and Gonzalez 1987]. In view of the above, it may be speculated that the metabolism of applied xenobiotics is likely to enforce iron shifting to liver to incorporate it into heme being a constituent of cytochromes.

Peters and Teel [2003 a, b] demonstrated that also saccharose addition to diet affected the activity of hepatic enzymes from the P-450 cytochrome superfamily. The changes in values of iron metabolism markers observed in this study under the influence of changes in diet composition were not significant from the viewpoint of physiology, and perhaps it was the incorporation of food additives to diet which was so exhausting to hepatic metabolism that it finally triggered a significant change in iron distribution.

The food additives as a mixture used in the experiment are permitted by law, and their individual effects have been thoroughly investigated and determined as safe. In view of the results obtained, however, it would be advisable to reconsider investigating the effect of these additives administered together, likewise they occur in an everyday diet, on the systemic metabolism taking into account also other changes in diet composition, including e.g. its increasing processing.

CONCLUSIONS

Analysis of the results allowed drawing the following conclusions:

1. A significant effect of changes in diet composition on the increase in hematocrit marker value and the red blood cell count in blood of animals examined, maybe as a result of catabolic transformation and increased body demand for oxygen or the diminution of the possibility of the transportation of oxygen.

2. Adverse changes in values of the erythrocytic system markers, occurring under the influence of the applied mixture of food additives, possibly through disorders of erythrocyte synthesis, enhanced hemolysis and water shifting to the extracellular compartment.

3. A change in iron metabolism as a consequence of changes in diet composition and food additives applied, resulting most likely from the necessity of applied xenobiotics biotransformation by heme-containing monoxygenases of P-450 cytochrome.

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WPŁYW SKŁADU DIETY I MIESZANINY WYBRANYCH DODATKÓW DO ŻYWNOŚCI NA UKŁAD CZERWONOKRWINKOWY I GOSPODARKĘ ŻELAZEM WE KRWI OBWODOWEJ U SAMCÓW SZCZURA

Wstęp. Procesy przemian ksenobiotyków, do których są zaliczane dodatki do żywności, katalizują enzymy grupy cytochromu P-450, w skład których wchodzi żelazo. Celem pracy było określenie, na modelu zwierzęcym, wpływu składu diety i wybranych dodatków do żywności na układ czerwonokrwinkowy i gospodarkę żelazem we krwi obwodowej.
Materiał i metody. Doświadczenie przeprowadzono na 30 samcach szczura szczepu Wistar, podzielonych na trzy grupy. Do picia zwierzęta otrzymywały wodę, a grupie III dodatkowo podawano wodny roztwór azotynu sodu, azotanu potasu, kwasu benzoesowego, kwasu sorbowego i glutaminianu sodu.
Wyniki. Stwierdzono istotny wzrost wartości wskaźnika hematokrytowego oraz ilości krwinek czerwonych we krwi badanych zwierząt pod wpływem zmiany składu diety. Zastosowane dodatki do żywności istotnie zmniejszały stężenie hemoglobiny, wartość wskaźnika hematokrytowego oraz ilość krwinek czerwonych we krwi badanych zwierząt, zmniejszając także stężenie żelaza w surowicy krwi, całkowitą zdolność wiązania żelaza i stopień wysycenia transferyny żelazem.
Wnioski. Analizując uzyskane wyniki, stwierdzono niekorzystne zmiany wartości wskaźników układu czerwonokrwinkowego, zachodzące pod wpływem zastosowanej mieszaniny dodatków do żywności. Zastosowane dodatki zmieniały metabolizm żelaza, najprawdopodobniej ze względu na konieczność biotransformacji zastosowanych ksenobiotyków przez monooksygenazy cytochromu P-450 zawierające hem.

Słowa kluczowe: dodatki do żywności, metabolizm żelaza, szczur

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