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ASSESSMENT OF EFFECTS OF DIET COMPOSITION AND VITAMIN B SUPPLEMENTATION ON FREE RADICAL-RELATED PROCESSES IN THE BODY. ACTIVITY OF ANTIOXIDANT ENZYMES AND THE TOTAL ANTIOXIDANT STATUS OF RAT BLOOD

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Key words: supplementation, vitamins B, free radical-related processes

The study addressed effects of diet modification, involving substitution of whole grains with wheat flour and sucrose, and supplementation of the modified diet with selected B-group vitamins to compensate for, or exceed, the resultant deficiencies, on activities of: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) in the blood and liver, and on the total antioxidant status (TAS) of blood serum of female and male rats.

Diet modification was found to result, in females, in a significant increase of SOD and CAT activities both in the blood and in the liver, the increase being enhanced by supplementation. No effect of diet modification on GPx and GR activities was observed; it was only because of the supplementation that the activities of these enzymes in the blood were significantly suppressed.

Males, too, showed a significant increase in the SOD activity as a result of diet modification, the effect being enhanced by the supplementation applied. On the other hand, CAT and GPx activities in males were significantly affected by diet modification only, the supplementation affecting only the GR activity.

The supplementation was found to affect TAS which was decreased in both females and males.

INTRODUCTION

Studies on effects of diet composition and vitamin B supplementation, carried out for a number of years, have demonstrated that supplementation – applied to laboratory animals – not only affects the animals, but that the effects observed are not beneficial. The adverse effects were manifested as, *i.a.*, increased blood lipid and lipoprotein concentrations, accumulation of visceral fat tissue, and a change in the profile of fatty acids contained in that tissue [Friedrich & Sadowska, 2005]. In addition, the prooxidation-antioxidation equilibrium was disturbed, the disturbance involving a significant reduction of the concentration of non-enzymatic antioxidants and increased concentrations of lipid peroxidation products [Friedrich & Dolot, 2009].

Therefore, it was decided to follow, in an animal model, effects of changed diet composition (substitution of whole cereal grains by wheat flour and sucrose) and supplementation of the modified diet with selected B-group vitamins added in the amounts compensating or exceeding deficiencies resulting from diet modification, on the enzymatic antioxidant protection system, *i.e.*, activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) in the blood and liver, and on the total antioxidant status (TAS) of male and female rat blood serum.

MATERIALS AND METHODS

Following the consent of the Local Ethical Commission, the experiment was carried out in the vivarium of the Department of Human Nutrition Physiology, Agricultural University of Szczecin. The experiment involved males (60) and females (60) of the SPRD strain of laboratory rats aged 6-8 months. The initial individual weights of females and males were 225±25 and 325±25 g, respectively.

The rats were kept in metal cages placed in an air-conditioned room at 21±1°C, at the 12 h/12 h light/dark cycle.

Prior to the experiment, the rats were adapted to the vivarium conditions for a week. They were drinking pure water that had been left to stand some time before, and were fed a standard feed mixtures. Subsequently, they were divided into 4 treatment groups 15 individuals each: group I, fed the standard feed mixture; group II, fed the modified feed mixture; group III, fed the modified feed mixture supplemented with complementary amount of B-group vitamins; and group IV, fed the modified feed mixture supplemented with B-group vitamins in the amount exceeding (3-5 times) the deficiency brought about by the diet change.

The rats were fed *ad libitum* with pelleted, isocaloric, and isoproteinic feed mixtures manufactured from identical components by the Feed and Concentrate Manufacturers in Kcynia, following implementation of Procedure 5.14.5. (Cleaning

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of Machinery and Equipment). The standard feed mixture contained, *i.a.*, whole cereal grains. In the modified feed mixture, whole grains were replaced with Type 500 wheat flour, 50% of corn grains being replaced by sucrose. Other feed components were left unchanged. The composition of the feed mixtures used in the experiment is shown in Table 1.

The actual chemical composition of the feeds was determined based on assays performed as recommended by the appropriate Polish Standards [1975, 1994, 1996, 2000, 2005]. Prior to each experiment, feed samples were collected to be analysed for dry weight and contents of total lipids, total protein, and ash.

Dry weight was determined by drying a sample for 12 h at 100°C. Total lipids and protein were determined with the Soxhlet and Kjeldahl methods, respectively; the ash content was calculated from the weight loss on combustion carried out for 10 h at 550°C. Carbohydrate contents were calculated from differences between the dry weight and the sum of the remaining components, as recommended by Gawęcki & Jeszka [1986]. The proximate composition of the feed mixtures used in the experiment is shown in Table 2.

The total gross energy content of the feed mixtures used was calculated using physical energy equivalents: 4.15 kcal/g (17.4 kJ/g) for carbohydrates; 5.65 kcal/g (23.6 kJ/g) for protein; and 9.45 kcal/g (39.6 kJ/g) for lipids. The metabolic energy content of the feeds was calculated using Atwater's equivalents of 4.0 kcal/g (16.7 kJ/g) for carbohydrates; 4.0 kcal/g (16.7 kJ/g) for protein; and 9.0 kcal/g (37.6 kJ/g) for lipids.

TABLE 1. Percentage composition of feed mixtures.

Components	Basic feed mixture (%)	Modified feed mixture (%)
Wheat	36.4	6.0
Corn grains	20.0	10.0
Wheat bran	20.0	20.0
Soya-bruised grain	17.0	17.0
Dry milk whey	3.0	3.0
Phosphate 2-CA	0.8	0.8
Fodder chalk	1.5	1.5
Fodder salt	0.3	0.3
Premix LRM	1.0	1.0
Wheat flour (type 500)	—	30.4
Sucrose	—	10.0

TABLE 2. Chemical composition of feed mixtures used in the experiment.

Components	Basic feed mixture	Modified feed mixture
Protein (%)	19.16	18.48
Fat (%)	2.81	2.33
Carbohydrates (%)	63.76	65.46
Dry matter (%)	91.84	92.26
Ash (%)	6.09	5.98
Gross energy		
(kcal/g)	3.99	3.98
(kJ/g)	16.73	16.67
Metab. energy		
(kcal/g)	3.57	3.56
(kJ/g)	14.95	14.94

The contents of vitamins B_1, B_2, and B_6 in the feed mixtures applied (as determined using the HPLC technique at the Biotechnology Institute of the Agricultural and Feed Industry) as well as the content of vitamin PP, as calculated from relevant tables, are shown in Table 3.

Feed intake by the rats was calculated from the difference between the weight of the ration offered, the amount removed from the feeder, and the amount that fell down on the cage floor.

The rats of groups I and II were drinking pure water previously left to stand for a period of time. The rats of group III (compensatory supplementation) were offered, at the time of intensified activity, 30 mL of an aqueous vitamin solution, kept in dark bottles until used; the solution was prepared from the generally available vitamin formulas containing vitamin B_1 in the form of thiamine nitrate, B_2 in the form of riboflavin, B_6 in the form of pyridoxine chloride, and PP and nicotine amide, per unit feed weight consumed. The amounts of vitamins supplemented, *i.e.*, 1.33 mg thiamine, 0.38 mg riboflavin, 1.06 mg pyridoxine, and 11.22 mg nicotine amide per 1 kg feed were calculated from differences of their contents in the basic diet and in the modified one from which they were partly removed by component substitution. The rats of group IV (excess supplementation) were offered, at the time of intensified activity, 30 mL of an aqueous vitamin solution, in the amount exceeding (3-5 times) the deficiency brought about by the diet change, per unit feed weight consumed.

When calculating the vitamin doses to be administered in the aqueous solution, the animals' demand for vitamins was not considered. It was assumed that the vitamin dose in the basic diet was fully sufficient. In addition to the vitamin solution, the rats of both groups were drinking pure, stand water.

On each day of the experiment, the amount of vitamin solution drunk and the amount of feed consumed were calculated; the rats were weighed once a week. After 6 weeks, the rats were anaesthetised with Ketanest, following which the blood was drawn from the heart and the liver.

Activity of the enzymes in the blood was determined in erythrocytes (superoxide dismutase, with the adrenaline method as in Sykes *et al.* [1978] and catalase in reaction with hydrogen peroxide as in Aebi [1984]); in the full blood (glutathione peroxidase, using an appropriate Randox assay kit), and in erythrocyte haemolysate (glutathione reductase, using an appropriate Randox assay kit). In line with the accepted methodology, activities of the enzymes were expressed as per

TABLE 3. Contents of selected vitamin in 100 g of feed mixture.

Vitamin	Basic feed mixture (mg)	Modified feed mixture (mg)	Difference (%)
Thiamin (B_1)	0.225	0.092	59.1
Riboflavin (B_2)	0.080	0.042	47.4
Pyridoxine (B_6)	0.156	0.050	68.0
Nicotinate (PP)	1.684	0.562	66.6

gram haemoglobin the concentration of which was determined from its reaction with the Drabkin reagent.

The total antioxidant status was determined in the fresh blood serum, using an appropriate Randox assay kit.

Activities of the enzymes in the liver were determined in liver homogenates, using methods and reagents identical to those applied to blood assays. In line with the accepted methodology, activities of the enzymes were expressed as per gram protein the concentration of which in the homogenate was determined using the Lowry method [1951].

Changes in the absorbance of solutions were measured in a Metertech SP-8001 spectrophotometer.

The data obtained were checked for normality of distribution and subjected to statistical treatment with Statistica®6 software for Windows. The statistical treatment involved 2-way analysis of variance (sex x diet) and Duncan's multiple range test.

RESULTS

Feed consumption as well as changes in body weight and in visceral fat deposition in the rats (Table 4) examined were described in an earlier paper [Friedrich & Dolot, 2009].

The antioxidant enzyme activity (U) in the blood and liver of the rats turned out to have been significantly affected by diet composition and the supplementation applied.

The activities of superoxide dismutase and catalase in the blood of those females fed the modified feed were found to increase significantly, the increase being intensified by the supplementation, particularly that applied in excess (Table 5). On the other hand, diet modification exerted no significant effect on the activities of glutathione peroxidase and glutathione reductase; it was only when the supplementation was applied that the activities of the enzymes in the females' blood became significantly reduced.

TABLE 4. Effects of diet type (D) on diet intake, body weight increments and amounts of perivisceral fat tissue in female (n=60) and male (n=60) rats, depending on sex (S), $\bar{x}$ ±SD.

Examined trait	Feed mixture / Sex	Basic feed mixture (a)	Modified feed mixture (b)	Modified feed mixture + complementarily supplementation (c)	Modified feed mixture + redundant supplementation (d)	Significance of differences	Interaction
Diet intake (g)	Female	689±50	654±40	619±49	706±27	a-c**, b-d*, c-d**	D**, S**, DxS*
	Male	980±51	1021±63	973±57	1006±59	NS	
Diet intake (g/100 g body weight)	Female	265±9	259±10	234±18	267±15	a-c**, b-c**, c-d**	D**, S*, DxS*
	Male	257±15	246±7	244±8	247±11	a-b, c, d*	
Body weight (g)	Female	9.6±7.7	7.1±4.3	10.4±9.9	12.0±5.8	NS	D NS, S**, DxS*
	Male	43.5±13.6	63.2±13.2	48.5±13.5	53.0±15.4	a-b**, b-c*	
Body weight (g/100 g feed mixture)	Female	1.41±1.20	1,08±0,68	2.13±1.28	1.68±0.77	NS	D NS, D**, DxS*
	Male	4.46±1.44	6.18±1.19	4.49±1.37	5.36±1.71	a-b*	
Peri-intestinal fat (g/100 g body weight)	Female	0.913±0.228	1.142±0.151	1.227±0.168	1.111±0.226	a-b*, a-c**	D**, S**, DxS*
	Male	0.591±0.100	0.635±0.122	0.845±0.246	0.812±0.117	a-c, d**, b-c, d**	
Peri-intestinal fat (g/100g feed mixture)	Female	0.910±0.175	1.156±0.121	1.232±0.131	1.124±0.227	a-b, c**	D**, S**, DxS NS
	Male	0.224±0.042	0.257±0.048	0.344±0.097	0.328±0.048	a-c, d**, b-c, d**	

Differences found significant at: *p=0.05 and ** p=0.01.

TABLE 5. Effects of diet type (D) on diet intake and enzymes activity in blood of female (n=60) and male (n=60) ratd, depending on sex (S), $\bar{x}$ ±SD.

Enzyme activity	Feed mixture / Sex	Basic feed mixture (a)	Modified feed mixture (b)	Modified feed mixture + complementarily supplementation (c)	Modified feed mixture + redundant supplementation (d)	Significance of differences	Interaction
Superoxide dismutase (U/g prot.)	Female	2050±98	2277±190	2337±281	2586±144	a-b*, a-c*, a-d**, b-d**, c-d*	D**, S**, DxS**
	Male	1982±485	3034±574	3045±489	3687±526	a-b**, a-c**, a-d**, b-d*, c-d*	
Catalase (U/g prot.)	Female	16.3±6.7	23.8±7.9	29.3±6.2	30.7±6.8	a-b*, a-c**, a-d**	D**, S NS, DxS NS
	Male	18.8±4.6	27.5±6.0	27.2±5.4	28.8±3.7	a-b**, a-c**, a-d**	
Glutathione peroxidase (U/g prot.)	Female	64.1±14.4	56.3±9.5	10.7±1.2	9.7±1.4	a-c**, a-d**, b-c**, b-d**	D**, S**, DxS**
	Male	73.9±22.6	55.4±21.5	48.9±13.4	45.3±10.8	a-b*, a-c**, a-d**	
Glutathione reductase (U/g prot.)	Female	25.0±8.9	19.2±8.9	10.7±3.0	12.1±6.4	a-c**, a-d*	D**, S NS, DxS**
	Male	12.7±2.4	11.9±3.6	9.6±2.9	9.8±3.2	a-c*	

Differences found significant at: *p=0.05 and **p=0.01.

A similar effect of diet composition change and supplementation was observed in the activity of superoxide dismutase and catalase in the liver; the highest activities of both enzymes accompanied the supplementation (Table 6). On the other hand, analysis of changes in the activities of glutathione peroxidase and glutathione reductase in the liver showed the diet modification to have affected glutathione peroxidase only, the activity being significantly reduced, compared to the values typical of the females kept on both the basic diet and the supplemented modified diet. In the latter, the supplementation reverted the enzyme's activity to the level shown by the females kept on the basic diet. Despite the glutathione reductase activity reduction in the liver of the females fed the modified diet, the enzyme's activity remained unaffected by either the change in diet composition or its supplementation with the B-group vitamins used.

The change in diet composition was observed to bring about a significant increase in the superoxide dismutase activity in the blood of males (an effect observed also in the females); the effect was enhanced by the supplementation applied (Table 5). On the other hand, the activities of glutathione catalase and peroxidase in the blood were significantly affected by the change in diet composition only, the supplementation being a significant factor in the case of the glutathione reductase activity.

The activity of superoxide dismutase in the male liver was affected by the supplementation only (Table 6). The activity of catalase was stimulated by the change in diet composition, enhanced by the supplementation. On the other hand, the activities of glutathione peroxidase and reductase were significantly reduced by the modified diet consumed by males, the effect in glutathione reductase being significantly intensified by the supplementation.

Effects of the diet composition change and the vitamin B supplementation applied on the activities of superoxide dismutase and glutathione reductase were found to be significantly sex-dependent; the diet × sex interaction proved significant with respect to superoxide dismutase, glutathione peroxidase, and reductase (Table 5). Effects on the liver enzymes were found to be significantly sex-dependent with respect to superoxide dismutase and glutathione peroxidase; the diet × sex interaction was significant with respect to superoxide dismutase, glutathione peroxidase, and reductase (Table 6).

The change in diet composition had no effect on the total antioxidant status (TAS) of the blood serum in females and males, while the supplementation applied significantly affected TAS (Table 7). Under the influence of the supplementation, the female TAS was significantly reduced (compared to the TAS level in the non-supplemented group), the reduction being significant in the case of excess supplementation. A similar effect was recorded in males: only the excess supplementation exerted a significant effect on TAS.

No effects of sex or diet × sex interaction on the blood serum TAS were observed (Table 7).

TABLE 6. Effects of diet type (D) on diet intake and enzymes activity in liver of female (n=60) and male (n=60) rats, depending on sex (s), $\overline{x}$ ±SD.

Enzyme activity	Feed mixture / Sex	Basic feed mixture (a)	Modified feed mixture (b)	Modified feed mixture + complementarily supplementation (c)	Modified feed mixture + redundant supplementation (d)	Significance of differences	Interaction
Superoxide dismutase (U/g prot.)	Female Male	6538±1144 8602±2380	8438±1641 7355±1605	10191±1826 10743±1140	8838±1456 11321±1990	a-b*, a-c**, a-d**, b-c* a-c**, a-d**, b-c**, b-d**	D**, S**, DxS**
Catalase (U/g prot.)	Female Male	154.6±48.1 148.1±30.2	227.6±35.1 329.8±143.1	432.1±88.4 374.0±61.0	348.9±59.5 298.9±62.4	a-b**, a-c**, a-d**, b-c**, b-d**, c-d** a-b**, a-c**, a-d**, c-d*	D**, S NS, DxS**
Glutathione peroxidase (U/g prot.)	Female Male	2967±1584 1549±444	658±172 786±242	2578±808 587±157	3012±623 621±127	a-b**, b-c**, b-d** a-b**, a-c**, a-d**	D**, S**, DxS**
Glutathione reductase (U/g prot.)	Female Male	3089±1461 2709±556	1909±658 2164±344	1506±934 1324±507	2782±1456 1528±519	NS a-b**, a-c**, a-d**, b-c**, b-d**	D**, S NS, DxS NS

Differences found significant at: *p=0.05 and **p=0.01.

TABLE 7. Effects of diet type (D) on total antioxidant status (TAS – mmol Trolox/L) of blood serum in female (n=60) and male (n=60) rats, depending on sex (S), $\overline{x}$ ±SD.

Feed mixture / Sex	Basic feed mixture (a)	Modified feed mixture (b)	Modified feed mixture + complementarily supplementation (c)	Modified feed mixture + redundant supplementation (d)	Significance of differences	Interaction
Female	1.14±0.20	0.90±0.27	0.70±0.24	0.54±0.34	a-c*, a-d**, b-d*	D**, S NS, DxS NS
Male	1.09±0.15	1.12±0.28	0.87±0.22	0.56±0.28	a-d**, b-d**, c-d*	

Differences found significant at: *p=0.05 and **p=0.01.

DISCUSSION

In addition to low-molecular weight compounds, described in the previous paper [Friedrich & Dolot, 2009], the efficacy of body protection towards free-radical reactions depends also on the efficiency of the antioxidant enzyme system.

The change of diet resulted in a significant increase in superoxide dismutase both in the blood and in the liver of the animals examined. The strengthened enzymatic defence seems to be an adaptation to oxidative stress which accompanied the drop in reduced glutathione (GSH) and reduced sulphydryl groups (-SH), induced by the change of diet [Friedrich & Dolot, 2009]. In their study involving rats, Stroev *et al.* [2005] demonstrated an increase in the SOD synthesis gene expression during intensified free-radical processes.

The modified diet's effects could have also be an outcome of the high blood glucose level in the rats kept on the diet applied, as revealed in a previous study [Sadowska, 2002]. Appearance of increased amounts of glucose in the blood has already been demonstrated as inducing, in different metabolic pathways, intensification of glucose metabolism. Oxidative stress accompanying high glycaemia has been reported by numerous workers [Bemeur *et al*., 2004]. As observed by Ceriello [2000], post-prandial glycaemia plays a particularly important role in the process. Even a brief activation of metabolic pathways, induced by increased glucose availability, may lead to oxidative stress and to damages in biological macromolecules. In this context, analysis of vitamin B supplementation on the SOD increase suggests the increase to have been caused primarily by the vitamin B-induced enhancement of glucose transformations, and hence enhancement of reactive oxygen production, including the superoxide anion-radical (O) of a key importance to the reactions mediated by SOD. However, evidence of a potentially prooxidative role of vitamin B, so far reported from *in vitro* studies [Levine & Saltzman, 2002; Vijaya *et al*., 2005], cannot be disregarded.

Effects of sex on the SOD activity both in the blood and in the liver were found to be significant, as was the diet ×sex interaction. Inasmuch as the activity of the enzyme in males and females kept on the basic diet was comparable, the change in diet composition and the diet supplementation applied exerted a much stronger effect on the enzyme's activity in males than in females. The effect occurred despite estrogens and folliculotropic hormone stimulating the SOD gene expression [Strehlow *et al.,* 2003]. However, the main cause of the enzyme's activity being induced is the presence of oxidising factors [Röhrdanz *et al*., 2001]. It seems that the increased SOD activity in the males under study could have been related to the higher level of oxidative stress, found earlier [Friedrich & Dolot, 2009] and manifested as lower concentrations of reduced glutathione and total sulphydryl groups.

The experiment showed also a significant effect of the change in diet and its supplementation on the catalase activity.

It is now known that the highly oxidative hydrogen hyperoxide (H_2O_2) formed in SOD-catalysed reactions is disproportionated when affected by catalase or glutathione peroxidase. The relationship between the activities of SOD and CAT, resulting from the reactions they catalyse in the body, must have been involved in the effect on the observed increase in the CAT activity, as a result of the increased SOD activity and, hence, the H_2O_2 concentration.

On the other hand, the supplementation-induced increase in the CAT activity in the liver of females may be explained by the fact that CAT, to effectively play its catalytic role, needs reduced nicotinamide adenine phosphate (NADPH) dinucleotide the precursor of which is niacin available *via* supplementation. Why, however, did the supplementation stimulate the CAT activity only in females? Perhaps the effect was related to the high female tissues SOD activity and its immediate product, H_2O_2. The latter is capable of inhibiting CAT, and could have been produced by males in the amount inhibiting the enzyme, as demonstrated by Vijaya *et al.* [2005].

As shown by the analysis, the CAT activity was not significantly affected by sex, while a significant diet × sex interaction was revealed in the liver CAT activity. This effect could have been related to the increase in fat accumulation by the liver tissue, as observed earlier in supplemented females [Friedrich & Dolot, 2009]. Intensified lipogenesis in hepatocytes is one of the physiological sources of oxygen radicals in the body. Lipogenesis-related processes could have been significantly involved in the CAT activity in the animals under study.

The antioxidant protection of the system relies heavily also on glutathione enzymes, *i.e.*, glutathione peroxidase (GPx) and glutathione reductase (GR).

GPx catalyses reduction of H_2O_2 and organic hyperoxides. The high specificity of the enzyme towards electron donors (only GSH) results in its activity being depressed when the reduced glutathione concentration in the cell decreases.

The blood GPx activity in the experiment described was found to be significantly depended on diet composition in males and on the supplementation in females.

Considering the function of the enzyme discussed, it seems that the effect observed in males was directly related to the reduction of GSH concentration in the blood and liver. On the other hand, the absence of the effect in females could have been a result of the absolute GSH concentrations in the blood and liver being almost doubled, compared to those in males. Because, however, the female GSH concentration decreased due to the supplementation as well, at some point it could, perhaps, drop below the threshold necessary for the regular GPx activity.

This, however, does not explain changes in the enzyme's activity in the liver. In both sexes, the change in diet composition resulted in the reduced GPx activity; the supplementation applied, while producing no effect in males, increased the enzyme's activity in females to the levels shown by the rats kept on the basic diet.

The amount of feed consumed was one of the differences between males and females [Friedrich & Dolot, 2009]. In this context, it may be presumed that females, while consuming significantly more feed (per unit body weight), took up, *i.a*., more selenium, an element limiting the GPx activity [Payne & Southern, 2005]. Selenium-related enhancement of the GPx activity was reported by, *i.a.*, Flight &Clifton [2006] who fed whole grains to their experimental rats.

Both in the blood and in the liver, he activity of glutathione peroxidase was found to be significantly sex-dependent; the diet ×sex interaction in this relationship proved significant as well. Considering that the enzyme's activity in the blood of both sex-

es kept on the standard feed was comparable, the supplementation-induced differences seem to have appeared as a result of differences in the amount of feed consumed (and the consequent different uptakes of regulating substances) and peroxidation intensity rather than being a sex hormone effect.

Analysis of the glutathione reductase (GR) activity in tissues of the animals studied showed the change in diet composition to have resulted in a reduction of the GR activity in the male liver only. The decrease in concentration of reduced glutathione in animals kept on a modified diet, observed in an earlier study [Friedrich & Dolot, 2009] is, in general, a factor inducing an increase in the GR activity. Why, then, did such an increase not occur in this experiment? For GR to be effective, it requires two co-enzymes: FAD and NADPH, the precursors of which are riboflavin and niacin, respectively. Reduced amounts of these vitamins in the modified feed could then have contributed to inhibiting the GR activity increase.

Similarly to peroxidase, the GR activity was found to be significantly reduced in the supplemented rats. The effect is usually explained by reduction in oxidative stress, the reduction being brought about by vitamins B [Erat *et al*., 2007]. However, the reduction in the GR activity is then accompanied by an increase in the concentration of reduced glutathione. Considering the absence of such supplementation effect, the experiment discussed should rather result in the removal of oxidised GSH from the cells, leading to a decrease in the overall glutathione pool.

The diet ×sex interaction in the blood GR activity was found to be significant, but differences between males and females were significant only in the rats kept on the basic diet.

The higher GR values found in females could have been brought about as a result of female sex hormones controlling induction of reductase biosynthesis genes [Diaz- Flores *et al.*, 1999].

Changes in non-enzymatic and enzymatic antioxidant protection, found in an earlier study [Friedrich & Dolot, 2009] and in this experiment demonstrate unequivocally the presence of oxidative stress brought about not only by the natural antioxidant deficiency in the diet, but also by the supplementation applied.

The total antioxidant status (TAS) of the serum is a good indicator of protection of the blood (and, to some extent, of the entire system) from reactive oxygen. TAS expresses the net ability of the material to counteract a specific oxidation reaction and is a sum of activities of all antioxidants contained in the material in question [Ghiselli *et al*., 2000].

The experiment described showed no effect of the change in diet composition on the TAS value. This result may be taken as indication that, despite of a considerable impoverishment of the modified feed in numerous substances that occur naturally in the unchanged feed, mechanisms responsible for the equilibrium between oxidation and reduction continued to function efficiently in the experimental rats. This was perhaps due to the modified feed still containing a certain amount of vitamins and regulating components, additionally aided by increased food consumption, sufficient to maintain TAS at the level comparable to that observed in the animals kept on the basic diet.

The effect is interesting in the light of evidence showing the vitamin B deficient diets to produce a significant reduction of TAS [Taysi, 2005]. The deficiency of the modified diet was indicated, in an earlier study and in this experiment, by significant changes in the antioxidant protection components, *i.e.*, reduced glutathione, free sulphhydryl groups, superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase; however, the total antioxidant status remained unaffected. It follows then that response of the whole body is not only a sum of responses of individual parts, but the body has numerous additional possibilities to protect itself from adverse effects of external factors, including a deficient diet. However, the protection has its limits, as indicated by changes in TAS induced by the supplementation which, according to literature data, ought to strengthen the total antioxidant status [Matxain *el al.*, 2006; Raschle *et al*., 2007].

In this context, the significant TAS reduction observed as a consequence of the compensatory supplementation may suggest a direct prooxidative action of the vitamins applied. The effect was earlier described by Perry *et al*. [2004], who used high doses of vitamin B_6, and by Hu *et al*. [1995] who used thiamine.

The TAS reduction could have been also related to changes in metabolic pathways in which the vitamins used for supplementation act as co-enzymes. This is the case with, *i.a.*, enhanced biosynthesis of fatty acids and lipogenesis, observed in animals and manifested as accumulation of visceral adipose tissue and liver fat [Friedrich & Dolot, 2009], lipogenesis being a direct cause of enhancement of free-radical reactions [Olusi, 2002; Harrison *et al*., 2003].

No effect of sex and diet × sex interaction on the blood serum TAS was found.

Analysis of the changes taking place in the animals used in the experiment makes it difficult to unequivocally pinpoint a mechanism responsible for the effect of synthetic vitamins used in the supplementation applied. Did the mechanism involve catalysing of some reactions enhancing free-radical reactions (such as lipid accumulation)? Or perhaps the effects observed resulted from deficiency of other regulating compounds in the diet, the supplementation enhancing the imbalance? Or was the mechanism in question a result of direct prooxidative reactions found so far under *in vitro* conditions? Or was it the vitamins themselves, chemically synthesised under conditions favouring emergence of various analogues and vitamin-like compounds lacking vitamin properties, that were at the root of the effects?

In conclusion, it seems that the prevalent opinion regarding vitamin B supplementation as safe and beneficial for the body should be critically reviewed, particularly in the present situation of nutritional deficiencies, food product enrichment with various substances used by food producers, and the very pervasive diet supplementation.

CONCLUSIONS

1. The change in diet composition, whereby whole cereal grains were substituted by white flour and sucrose, acted in favour of disturbances in the prooxidation-antioxidation equilibrium by, *i.a.*, impoverishing the diet in numerous natural substances that act as regulators or antioxidants.

2. Counteraction of the resultant deficiencies by supplementing selected, synthetic B-group vitamins did not reverse the ad-

verse changes in the enzymatic antioxidant protection system in the body and even intensified those changes, as evidenced by changes in the activity of the enzymes assayed, although the serum's total antioxidant status (TAS) remained unaltered.

3. Even a small excess supplementation was found to affect the body's antioxidant protection status, the effect being manifested as intensification of changes in the enzymes' activity and a significant reduction in TAS, which may indicate a direct prooxidative action of the vitamins used in the experiment.

4. Enhancement of free radical reactions proceeding in the liver was not always correlated with the intensity of changes in the blood; this, together with mobilisation of adaptive mechanisms manifested as the absence of changes in TAS and highly individual body responses may lead to an erroneous perception of the true state of the organism, should only the TAS values be determined.

5. The responses of the body to the change in diet composition and to the supplementation applied was significantly sex-dependent.

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