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EVALUATION OF THE EFFECT OF DIET COMPOSITION AND B-GROUP VITAMINS SUPPLEMENTATION ON SELECTED CALCIUM METABOLISM PARAMETERS IN FEMALE RATS

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Background. The aim of the study was to observe the effects of a modified diet, in which whole grains of cereals had been isocalorically substituted with wheat flour (type "500") and saccharose, and supplementation with B-group vitamins, on selected calcium metabolism parameters in female rats.
Material and methods. The experiment was carried out on 36 female rats aged 6 months. Animals were fed two different diets. Groups I and II received clean tap water to drink, while group III had water solution of group-B vitamins.
Results. An analysis of the outcomes of the diet modifications allowed concluding that the females fed on the modified feed, either supplemented or non-supplemented, excreted more calcium with urine and exhibited its lower concentrations in blood plasma, as compared with the females fed on the standard feed. No significant differences, however, were observed in plasma ionized calcium concentrations in the studied animals, which implies that the regulation mechanism of its bio-active form concentrations is preserved. It has been found that the applied supplementation of the modified diet promoted bone calcium release inducing plasma alkaline phosphatase activity in this group of animals. Supplementation was also accompanied by a shift in calcium distribution manifested by its increased concentrations in erythrocytes.
Conclusions. Change in diet composition and supplementation were found to significantly affect calcium metabolism of the rats examined. Observed intracellular calcium accumulation may have been an underlying cause of an increased adipose tissue accumulation in B-group vitamin supplemented animals, which had been observed in previous studies.

Key words: supplementation, B-vitamins, calcium metabolism, rat

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INTRODUCTION

The results of the studies indicate that only a small fraction of the population are aware of the principles of proper nutrition and are concerned to follow them on a day-to-day basis [Brzozowska 2001]. However, due to a growing awareness of nutritional issues, there is an increasing percentage of people who struggle to change their diet into a better one. Such changes rarely lead to diet improvement in terms of its composition, while mineral and/or vitamin supplementation of the diet is a more commonplace behaviour. A survey by the National Food and Nutrition Institute carried out in Poland in 2000 revealed that 20% of the population applied diet supplementation [Szponar et al. 2004]. In France or the United Kingdom supplementation is more popular, up to 50% of adults take at least one dietary supplement [Harrison et al. 2004, Touvier et al. 2006]. Supplementation is perceived by consumers as an easy way to preserve health, as well as to stay both physically and mentally fit.

These findings underlie our motivation to undertake studies on an effect of diet composition and its supplementation with B-group vitamins on the lipid/carbohydrate metabolism. The experiments, carried out using an animal model in our Department of Human Nutritional Physiology, have revealed that B-group vitamin supplementation promoted a growth in visceral adipose tissue, as well as in the fat of the liver and muscles [Friedrich and Goluch-Koniuszy 2007, Friedrich and Sadowska 2005 b, 2008]. Supplementation also led to a reduced feed consumption and, in consequence, to a lower intake of many important nutritional components, such as calcium [Friedrich and Sadowska 2005 b].

Numerous epidemiological studies have revealed a linkage between obesity incidence and dietary calcium intake. Such associations were first observed by McCarron et al. [1984] in the NHANES-I study. Similar findings were reported by Carruth et al. [1999], Skinner et al. [1999], and Teegarden et al. [1999]. There has been no explanation given, however, to the observed effects; no conclusions have been drawn and the information was announced as a peculiarity only.

Studies by Zemel et al. [2000], who investigated the role of intracellular calcium and the regulation of its concentration, were first to provide the physiological grounds for explaining the relationship between calcium intake and body weight (adipose tissue accumulation).

Hence in the presented study, we experiment on model animals to learn about the effects of diet composition and its B-group vitamin supplementation on selected calcium metabolism parameters.

MATERIAL AND METHODS

Materials

Rats were obtained from the animal husbandry of Chair and Department of Toxycology, Poznań University of Medical Sciences, Poland. Spectrophotometric assays were performed using Cormay (Lublin, Poland) reagents and the SP-8001 spectrophotometer (Metertech Inc., Taiwan). Atomic absorption spectrometry was done using Merck (Darmstadt, Germany) reagents on AAnalyst 400 (Perkin Elmer, Massachusetts, USA).

Methods

The experiment, approved by the Local Ethical Committee in Szczecin (permit no. 3/2006), was carried out in the vivarium of the Department of Human Nutritional Physiology, on 36 SPRD-strain female rats aged 6-8 months, of initial body weight 248 ±19.4 g. Following a week long conditioning in the vivarium environment (temperature 21-22°C, 12 h/12 h light/dark cycle), the animals were randomised and sorted into three (n = 12) 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 percentage of the remaining components was unchanged (Table 1). All diets were based on the balanced modification of the AIN-93 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
Soya-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	0	30.4
Saccharose	0	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 metabolic energy was calculated using commonly applied energy equivalents (Table 2).

The contents of vitamins B_1, B_2, and B_6 in the feeds applied (as determined HPLC methods), contents of vitamin PP (as calculated from relevant tables), as well as the contents of calcium (as determined by atomic absorption spectrometry), are shown in Table 3.

Table 2. Chemical composition of feeds used in the experiment

Component	Basic feed	Modified feed
Total protein, %	18.1	17.5
Crude fat, %	2.10	2.19
Carbohydrates, %	65.8	66.2
Dry matter, %	92.1	91.6
Total ash, %	6.08	5.69
Brutto energy		
$kcal \cdot g^{-1}$	3.95	3.94
$kJ \cdot g^{-1}$	16.5	16.5
Metabolic energy		
$kcal \cdot g^{-1}$	3.54	3.54
$kJ \cdot g^{-1}$	14.8	14.8

Table 3. Content of vitamins B and calcium in 100 g of diet

Component	Basic feed, mg	Modified feed, mg
Thiamin (B_1)	0.225	0.092
Riboflavin (B_2)	0.080	0.042
Pyridoxine (B_6)	0.156	0.050
Nicotinate (PP)	1.684	0.562
Calcium	1 110	1 050

Groups I and II received tap water to drink, while group III had water solution of group-B vitamins (B_1, B_2, B_6, and PP) obtained from available pharmaceutical products (Polfa, Kutno, Poland). The supplemented vitamins were administered in 3- to 5-fold higher quantity compared to the difference between their content in the standard and the modified feeds (3.249 mg thiamine, 1.083 mg riboflavin, 2.694 mg pyridoxine, and 9.58 mg nicotinic acid amide per 1 kg of feed), calculated in relation to amount of feed consumed by the animals. The rats received drinking tap water surplus ad libitum.

Used scheme of the experiment partly reflected the change of the diet composition and supplementation used by people observed in last decades.

The experiment lasted for seven weeks; the amount of feed consumed, water solutions of vitamins and water drunk by the animals were recorded daily, whereas once a week the animals were weighed. During the 6th week of the experiment, the animals were placed in metabolic cages; following a 48-hour conditioning, a 24-hour urine collections were done in order to determine the concentrations of creatinine (kinetic spectrophotometry) and calcium (atomic absorption spectrometry).

On the completion of the experiment, the animals were fasted overnight, anesthetized (with Ketanest, Pfizer Ireland Pharmaceuticals), and blood was drawn from the heart to measure the hematocrit, which was done by centrifuging blood in capillary tubes in the hematocrit centrifuge (MPW 52, MPW Med. instruments, Warsaw, Poland).

Blood hemolysates were obtained through addition of 9 ml of deionized water to 1 ml of blood. The remaining blood was centrifuged, at 2000 g for 10 min at 5°C (MPW 350-R, MPW Med. instruments, Warsaw, Poland).

Calcium concentrations were measured in the resulting plasma and whole blood hemolysate by means of atomic absorption spectrometry. Calcium concentrations in the red cells were calculated indirectly, by method based on the concentrations law, which required determining the packed cell volume index, as well as calcium concentrations in both plasma and whole blood [Duda and Przybyszowski 1977, Kozielec et al. 2006]. Blood plasma was also assayed for the concentrations of albumins (spectrophotometry), alkaline phosphatase – ALP (kinetic spectrophotometry), gamma-glutamyl transpeptidase – GGTP (kinetic spectrophotometry), as well as ionized calcium (using a ion selective electrode).

The right femoral bones were collected from the animals as well. The bones were dry-mineralized, and calcium concentration was determined using atomic absorption spectrometry.

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 2005].

RESULTS AND DISCUSSION

Analysis of the results revealed a significant effect of the diet composition on the feed intake by the females. The modifications in the composition of the diet significantly reduced the amount of feed consumed as expressed per 100 g of body weight (Table 4). The water consumption was comparable among groups of animals (Table 4).

Table 4. Effect of diet type and supplementation on feed and water consumption in rats ($\bar{x}$ ±SD; n = 36)

Trait	Basic feed	Modified feed	Modified feed + supplementation
Feed consumption g/100 g body mass/7 weeks	283 ±11.6 b	265 ±13.2 a	255 ±7.2 a
Water consumption ml/100 g body mass/day	11.3 ±1.52 a	10.5 ±2.0 a	10.9 ±1.38 a

a, b – means denoted with the same letters are not significantly different, $p < 0.05$.

Glucose blood concentration represents one of the key factors that take part in the physiology-level regulation of hunger and satiation. The modified feed applied in the experiment, containing white flour and saccharose, which are easily digestible and rapidly release larger quantities of glucose to bloodstream, boosted the feeling of satiation. Visceral adipose tissue, which increased due to the B-group vitamin supplementation [Friedrich and Sadowska 2005 b], may have also been a feed intake-inhibiting factor, since it is not only a source of free fatty acids readily released to blood, but also informs the system about its energy resources [Archer et al. 2002]. Reduced consumption of the

modified feeds, which due to altered composition also becomes short of some vitamins and minerals, is an adverse effect, as the intake of not only basic nutritional components, but also of those of regulatory character, drops considerably.

Reduced feed consumption, containing besides less quantity of calcium, may have been one of the causes underlying the observed lower blood plasma calcium levels in the rats fed on the modified, either supplemented or non-supplemented feed (Table 5).

The calcium concentration did not, however, fall outside the physiological range, which for these animals is 1.33-3.25 mmol/l [Carpenter et al. 2001].

A lower plasma calcium level in the supplemented animals may have also resulted from a lower concentration of albumins observed in this group (Table 6).

Table 5. Effects of diet type and supplementation on chosen indicators of calcium metabolism in rats ($\bar{x}$ ±SD; n = 36)

Indicator	Basic feed	Modified feed	Modified feed + supplementation
Plasma calcium concentration, mmol·l^{-1}	2.67 ±0.08 b	2.51 ±0.09 a	2.43 ±0.08 a
Ionised plasma calcium concentration, mmol·l^{-1}	0.84 ±0.04 a	0.86 ±0.03 a	0.86 ±0.1 a
Erythrocyte calcium concentration, µmol·l^{-1}	4.71 ±0.35 a	4.98 ±0.42 a	5.71 ±0.28 b
Bone calcium content, mg·g d.m.$^{-1}$	241 ±12.3 a	246 ±11.6 a	238 ±6.5 a
Urinary calcium excretion, mmol·µmol creat.$^{-1}$·24 h^{-1}	0.604 ±0.13 a	0.890 ±0.15 b	0.840 ±0.13 b
Plasma alcaline phosphatase concentration, u·l^{-1}	87.6 ±6.02 a	119 ±9.0 c	99.0 ±4.12 b

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

Table 6. Effects of diet type and supplementation on plasma albumin concentration and plasma GGTP concentration in rats, ($\bar{x}$ ±SD; n = 36)

Indicator	Basic feed	Modified feed	Modiffied feed + supplementation
Plasma albumin concentration, g·l^{-1}	45.8 ±3.33 b	43.1 ±2.01 b	40.5 ±2.09 a
Plasma GGTP concentration, u·l^{-1}	11.0 ±3.15 a	10.4 ±2.54 a	9.31 ±1.32 a

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

Albumins act as non-specific transport agents also for calcium, and a drop in the concentration of these proteins results in a decrease in plasma total calcium [Labriola et al. 2009]. However, with variable levels of albumins, the level of plasma ionized calcium most often remains unchanged. Also in the presented studies, we have not found significant differences in blood plasma ionized calcium concentrations in the studied animal (Table 5). Ionized calcium is the only bio-active form of calcium, therefore its concentration undergoes very rigorous regulation which involves calciotropic hormones as well as other mediators, affecting primarily the osseous tissue metabolism.

A decrease in blood plasma calcium concentration contributes to the release of parathormone, which enhances osteolysis and induces the synthesis of vitamin D3, which in turn stimulates small intestine calcium absorption. The presented studies revealed a lower, though statistically non-significantly, bone calcium concentration in the females fed on the modified and vitamin-supplemented feed, in which plasma total calcium was lowest (Table 5). In the plasma of the animals that received the modified, either non-supplemented or supplemented feed, we observed also a higher activity of alkaline phosphatase, as compared with that observed in the animals fed on the standard feed (Table 5).

The total activity of alkaline phosphatase involves several isoenzymes, which hydrolyse phosphates in an alkaline environment. The highest ALP concentrations occur in osteoblasts and hepatocytes; hence, its elevated level accompanies enhanced bone remodelling or hepatic disorders. The distinguishing factors in terms of the origin of plasma alkaline phosphatase include the plasma concentration of gamma-glutamyl transpeptidase, which increases along with ALP activity growth observed in liver diseases [Zilva and Pannall 1979]. In the presented studies, no changes in GGTP activity were recorded (Table 6), which implies an increase in the activity of the bone-originating fraction of plasma alkaline phosphatase of the studied animals. Some of the analyses indicate also that the hepatic-origin fraction in blood plasma of rats represents a small percentage of ALP, therefore it has been accepted that a vast majority of ALP activity in blood is of bone origin [Gołębiewska et al. 2000, Koyama et al. 1998].

In juvenile, growing organisms, an increase in the bone fraction of alkaline phosphatase activity is attributed to the development and growth of the skeletal system; in adult forms, however, this effect is associated with a disturbed balance of bone remodelling processes. If resorption processes prevail over osteogenesis, the mineral density of bone decreases, which is accompanied by an increase in repairing activities of the osteoblasts, which in turn is reflected in an increased activity of alkaline phosphatase [Rahnama et al. 2002]. Consequently, the increased activity of alkaline phosphatase reflects acceleration of bone formation as a result of its enhanced resorption which possibly leads to osteomalacia [Marcinkowska-Suchowierska et al. 1992, Romagnoli et al. 1998]. Consequences of enhanced osteolysis should include an increased blood plasma calcium concentration. In the discussed experiment, however, we have not observed such an effect. What has been confirmed, however, is the enhanced urinary excretion of calcium in the animals fed on the modified, either B-group-vitamins supplemented or non-supplemented feed (Table 5), which may have also contributed to the observed lower plasma calcium concentrations in these animals.

The amount of urinary excreted calcium is a trade-off between glomerular filtration and tubular reabsorption. Under normal conditions, 99% of calcium filtered in the renal glomeruli is reabsorbed back, primarily in the proximal convoluted tubule. In the distal tubule, calcium reabsorption is regulated hormonally by parathormone, calcitonin, the active form of D_3 vitamin and by glucocorticoids. In the presented studies, it was glucocorticoids that may have been responsible for calciuria. In their previous studies on modified diets and their effects on steroid hormones concentrations, Friedrich and Sadowska [2007] observed an increased blood plasma concentration of corticosterone in female rats fed on a modified, non-supplemented feed. A similar effect of easily assimilated carbohydrates on cortisol concentrations in cattle neonates was reported by Friedrich [1995].

Glucocorticoids affect the functioning of many organs, including the kidney. Glucocorticoids stimulate glomerular filtration and diuresis, whereas renal tubular reabsorption of calcium decreases, which leads to increased calciuria. Glucocorticoids also affect calcium management through reduction of its small intestine absorption, inhibiting the synthesis of the calcium-binding protein (CaBP) [Doga et al. 2008]. This activity is independent from vitamin D_3. On the other hand, the increased urinary excretion of calcium observed in vitamin-supplemented females is difficult to explain. It may have been due to a change in the composition of fatty acids in the visceral adipose tissue, as observed by Friedrich and Sadowska [2005 a], which occurs as a result of vitamin supplementation of the diet. The mentioned authors observed an increase in the level of arachidonic acid present in the visceral fat of group B vitamin-supplemented animals. This acid inhibits the transformation of cholesterol into aldosterone, which in consequence leads to an increased urinary excretion of sodium [Goodfriend et al. 1995, Elliott and Goodfriend 1993]. The increased amount of excreted sodium is one of the factors determining an elevated excretion of calcium in urine [Kleeman et al. 1964], especially if the diet lacks calcium [Nordin and Policy 1987]. This effect could have been intensified by the oxidative stress, which had been observed during group B vitamins supplementation by Friedrich et al. [2005], and which reduces the affinity of mineralocorticoid receptors to aldosterone stimulating urinary loss of calcium [Fiebeler and Luft 2005].

We have also found a significant effect of the applied supplementation on an increase in calcium concentration in erythrocytes (Table 5). Calcium is characterised by a sharp gradient of transmembrane concentration; it concentrates mainly in the extracellular spaces and cells contain only a small fraction of calcium that can be found outside. This makes calcium an ideal messenger, which serves as a quick cell activator. A vast majority of intracellular calcium is found in the organelles (mitochondria and microsomes). A deficiency or excess in intracellular calcium is not always reflected by a distinct clinical symptom typical for calcium metabolism disorders. Slowly developing disturbances trigger compensation mechanisms which enable long term maintenance of homeostasis. Research shows, however, that an increase in intracellular calcium of erythrocytes is linked with an elevated risk of hypertension [Orlov et al. 1988]. Calcium, through affinity to some kinases or specific proteins, may induce also many metabolic processes inside the cell. The intracellular increase in calcium concentration may lead to insulin resistance, accelerates the rate of lipogenesis and accumulation of triaglycerols in both adipose tissue and muscles [Zemel 1995]. Therefore, calcium management disorders may have been a possible cause underlying the increased accumulation of visceral and intramuscular adipose tissue, found in previous studies under group-B vitamins supplementation of animal diet.

CONCLUSIONS

The analysis of the results allowed concluding that:

1. The applied modification of diet composition had an effect on calcium management in female rats, leading to a decrease in its blood plasma concentration and increased calciuria.

2. No changes in blood plasma ionized calcium concentrations have been found in the studied animals, which implies that regulatory mechanisms of its bio-active form were preserved.

3. The applied supplementation of the diet with B-group vitamins promoted calcium storing in erythrocytes, which may lead to arterial hypertension and insulin resistance; however, explanation of the mechanisms of the observed changes in calcium distribution require further studies.

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OCENA WPŁYWU SKŁADU DIETY I ZASTOSOWANEJ SUPLEMENTACJI WITAMINAMI Z GRUPY B NA WYBRANE WSKAŹNIKI GOSPODARKI WAPNIEM U SAMIC SZCZURA

Wstęp. Celem badań była ocena wpływu zmiany składu diety, w której pełne ziarna zbóż zostały zastąpione izokalorycznie mąką pszenną (typ 500) i sacharozą, i jej suplementacji witaminami z grupy B na wybrane wskaźniki metabolizmu wapnia u samic szczura.
Materiał i metody. Badania przeprowadzono na 36 samicach szczura w wieku 6 miesięcy. Zwierzęta żywiono dwiema paszami o różnym składzie. Do picia grupy I i II otrzymywały czystą wodę, grupa III otrzymywała roztwór witamin z grupy B.
Wyniki. Analizując wpływ zmiany składu diety, stwierdzono, że samice żywione paszą zmodyfikowaną, zarówno suplementowaną, jak i niesuplementowaną, wydalały większe ilości wapnia z moczem, przy mniejszym jego stężeniu w osoczu krwi w porównaniu z samicami żywionymi paszą podstawową. Nie stwierdzono jednak istotnych różnic w stężeniu wapnia zjonizowanego w osoczu badanych zwierząt, co wskazuje na zachowanie mechanizmu regulacji stężenia jego formy biologicznie aktywnej. Stwierdzono, że zastosowana suplementacja diety zmodyfikowanej sprzyjała uwalnianiu wapnia z kości, wpływając na wzrost aktywności fosfatazy zasadowej w osoczu krwi zwierząt tej grupy. Po zastosowaniu suplementacji obserwowano także zmiany dystrybucji wapnia, manifestujące się wzrostem jego stężenia w erytrocytach.
Wnioski. Zmiana składu diety i zastosowana suplementacja wywarły istotny wpływ na gospodarkę wapniem u badanych samic. Stwierdzone wewnątrzkomórkowe gromadzenie wapnia mogło być przyczyną, obserwowanego we wcześniejszych badaniach, zwiększonego gromadzenia tkanki tłuszczowej u zwierząt suplementowanych witaminami z grupy B.

Słowa kluczowe: suplementacja, witaminy z grupy B, metabolizm wapnia, szczur

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