

**NITRATES AFFECT THYROID STATUS AND SERUM TRIACYLGLYCEROLS IN WISTAR RATS***Renata B. Kostogryś<sup>1</sup>, Paweł M. Pisulewski<sup>1</sup>, Anna Pecio<sup>2</sup>*<sup>1</sup>*Department of Human Nutrition, Faculty of Food Science and Technology, The Agricultural University, Kraków;*<sup>2</sup>*Department of Comparative Anatomy, Faculty of Biology and Earth Sciences, The Jagiellonian University, Kraków*

Key words: nitrates, nitrites, thyroid hormones, lipoproteins

The effect of increasing levels of sodium nitrate on urinary iodine excretion, changes in the morphology of thyroid follicles, and thyroid gland hormonogenesis were studied. In addition, effects of nitrates on serum lipoproteins were examined. Twenty-four, 5-week old, growing male rats of Wistar strain were randomly assigned to four experimental groups (I – 0, II – 500, III – 1500, and IV – 3000 mg NaNO<sub>3</sub> per kg body weight). For 3 weeks the rats were fed restricted amounts of AIN<sup>93</sup>G diets and had free access to distilled water. Body weight of the animals was recorded weekly. No external signs of dietary nitrate toxicity were observed in this study. However, urinary iodine concentrations tended to decrease with increasing nitrate intakes (13.19 µg/dL in Group I vs. 7.55 µg/dL in Group IV). The nitrate-fed rats showed histological changes in thyroid follicles. The thyroids were diffusely hyperplastic with small follicles. The epithelial follicle cells were also significantly higher in the nitrate-fed rats. In addition, mild to moderate irregularity of hypertrophic follicular cells and decreased amount of colloid were observed in the nitrate-fed animals (Groups II, III, and IV). Finally, we found that the vascularity of the thyroid tissue from nitrate-fed rats was much more developed, compared with the control animals. Serum fT<sub>4</sub> levels (pmol/L), tended to be decreased in the nitrate-fed rats. On the other hand, the dietary nitrate doses of 1500 and 3000 mg/kg body weight, caused highly significant increases (65% and 300%, respectively) in circulating levels of serum TSH (p<0.001). Feeding incremental doses of nitrate to rats did not result in significant increases in serum lipoproteins (total cholesterol and LDL-cholesterol and HDL-cholesterol). In contrast, the highest dietary nitrate level increased significantly (p<0.05) serum triacylglycerol concentrations by 45.5%, compared to the control group of rats. Therefore, nitrate may be considered as a competitive iodine inhibitor, affecting the thyroid-pituitary hormonal axis, in a way similar to that of iodine deficiency, thus acting as a goitrogen.

**INTRODUCTION**

Nitrates and nitrites are frequently present in foods. They are used as food additives, being added as preservatives and colour fixatives to some processed foods (particularly cured meats), fish and cheese [WHO Technical Report Series, 1995]. Additionally, nitrites and nitrates are produced endogenously [Jensen, 1995].

According to available evidence, nitrites are toxic to animals [Jensen, 1995]. In contrast, nitrates are relatively non-toxic, but an elevated nitrate load may produce potential harmful effects *via* an endogeneous conversion of nitrate to nitrite [Jensen, 1995]. The major toxic effects of nitrites have been related to their ability to oxidize hemoglobin to methemoglobin and to the potential role of nitrites in the formation of mutagenic and carcinogenic compounds [Jensen, 1995; McKnight, 1999; WHO Technical Report Series, 1995].

Apart from the above known effects, nitrates can also interfere with normal iodine thyroid metabolism by inhibiting iodine uptake by the thyroid gland, thus leading to the development of goitre in laboratory animals, *e.g.* rats [Bloomfield, 1961; Horing *et al.*, 1986; Jahreis *et al.*, 1991]

and also in humans [van Maanen *et al.*, 1994; Gatseva *et al.*, 1998; Vladeva *et al.*, 2000]. In several studies, the development of goitre was accompanied by histological modifications of the thyroid gland [Horing *et al.*, 1986] and a decrease in the secretion of thyroid hormones [Jahreis *et al.*, 1991].

However, the above studies are lacking information on the effects of nitrates on urinary iodine excretion as a marker of the iodine status. Moreover, they do not provide detailed morphological evidence of nitrate effects on the thyroid tissue (*i.e.* thyroid follicles) nor information on the secretion of the thyroid stimulating hormone (TSH) involved in the thyroid metabolism.

In this context, the major objective of the present study was to estimate the effect of increasing levels of the dietary intake of nitrates on: (1) urinary iodine excretion, (2) changes in the morphology of thyroid follicles, and (3) thyroid gland hormonogenesis in laboratory rats. In view of the evidence that insufficient secretion of thyroid hormones may adversely alter the composition and transport of lipoproteins [Duntas, 2002], effects of nitrates on serum lipoproteins were examined as well.

## MATERIALS AND METHODS

**Animals, diets and experimental design.** All experimental procedures complied with the Polish Ethical Standards. Twenty-four, 5-week old, growing male rats of Wistar strain, weighing initially  $113 \pm 7$  g, were obtained from the Institute of Animal Production in Kraków. They were randomly assigned to four experimental groups, 6 animals each, and housed individually in screen-bottomed stainless steel cages, in an isolated room with controlled temperature ( $25^\circ\text{C}$ ) and ambient humidity, with 12-h light-dark cycle. These rats were fed restricted amounts (15 g per day) of semi-purified AIN<sup>93</sup>G diets [Reeves, 1993] and had free access to distilled water. The diets (I, II, III and IV; Table 1) supplied the following amounts of nitrates expressed as sodium nitrate ( $\text{NaNO}_3$ ) and were fed for 3 weeks.  $\text{NaNO}_3$  was added to the diet as a water solution in the amounts required to achieve the following doses: Group I – 0 mg  $\text{NaNO}_3/\text{kg}$  b.w.; Group II – 500 mg  $\text{NaNO}_3/\text{kg}$  b.w.; Group III – 1500 mg  $\text{NaNO}_3/\text{kg}$  b.w.; and Group IV – 3000 mg  $\text{NaNO}_3/\text{kg}$  b.w. This range of nitrate administration included its no-observed-effect-level (NOEL = 500 mg of sodium nitrate per kg body weight) [Walton, 1999]. Food intake was measured daily and animal body weight was recorded weekly.

TABLE 1. Composition of the experimental diet.

Components	(%)
Corn starch	63.3
Caseine	10
Sucrose	10
Soybean oil	7
Cellulose powder	5
Mineral mixture <sup>a</sup>	3.5
Vitamin mixture <sup>b</sup>	1
Choline	0.25
<i>tert</i> -butylhydrochinon	0.0014
Sodium nitrate (mg/kg b.w.)	0

<sup>a</sup>AIN-93G mineral mixture; <sup>b</sup>AIN-93G vitamin mixture; Before feeding the animals, a water solution of  $\text{NaNO}_3$  was added to the diet in the amounts required to achieve the following doses: Group I – 0 mg  $\text{NaNO}_3/\text{kg}$  b.w.; Group II – 500 mg  $\text{NaNO}_3/\text{kg}$  b.w.; Group III – 1500 mg  $\text{NaNO}_3/\text{kg}$  b.w.; and Group IV – 3000 mg  $\text{NaNO}_3/\text{kg}$  b.w.

### Urine and blood collection and thyroid preparation.

Urine was collected quantitatively from each rat in the last 3 days (16–18 d) of the experiment. The pooled 3-day samples were stored at  $-20^\circ\text{C}$  until analysis. At the end of the experiment (18 d), the rats were anaesthetised with thiopental (Biochemie GmbH, Austria; 25 mg/100 g body weight). Blood was rapidly collected by cardiac puncture, transferred to centrifuge tubes with no anticoagulant, and serum was separated by low-speed centrifugation ( $1500 \times g$ , 15 min). The serum samples were stored at  $-20^\circ\text{C}$  until analysis. After bleeding, thyroid glands were carefully excised and fixed in Bouin's fluid [Kiernan, 1990].

**Analyses.** Iodine in urine was determined after digestion with chloric acid solution (potassium chlorate and perchloric acid, 70%) using the Sandell-Kolthoff spectrophotomet-

ric method as modified by Dunn *et al.* [1993]. Serum free thyroxine ( $\text{fT}_4$ ) and serum thyroid stimulating hormone (TSH) concentrations were measured using lumino-immunoassay LIA-mat F<sub>4</sub> kit (Byk-Sangtec Diagnostica GmbH&Co KG) and The IMMULITE Rat TSH Application kit (DPC Biermann GmbH), respectively. Serum total cholesterol (TC) and its HDL fraction were analysed enzymatically with standard kits (BioVendor cat.-no 10851 and BioVendor cat.-no 10855 respectively). The LDL+VLDL fraction of cholesterol was calculated as the difference between TC and HDL-C. Triacylglycerol content was estimated enzymatically with standard kits (BioVendor cat.-no 12805).

**Thyroid follicle morphological examination.** A part of trachea with thyroid gland on both sides were removed from all rats and fixed in Bouin's fluid for 3 days. Then the tissues were dehydrated in alcohol, embedded in paraffin and sectioned serially at  $7 \mu\text{m}$ . For histological evaluation the sections were stained with two methods: hematoxylin and eosin and trichrome [Kiernan, 1990]. The follicle colloid evaluation was made using the slides with PAS-positive reaction. The mean height of 50 epithelial cells of follicle was measured in 8 rats chosen randomly from each experimental group (2 rats per group).

**Statistical analysis.** The effect of nitrate treatments was analysed by one-way ANOVA generated by the STATISTICA version 6.1 package (StatSoft, Tulsa, OK.). Where appropriate, treatment means were compared by the Tukey's multiple range test and p values  $<0.05$  were considered as showing a significant difference between treatment means.

## RESULTS

### Body weight

No external signs of dietary nitrate toxicity were observed in this study (Figure 1). In fact, the growth of the nitrate-fed rats (500, 1500 and 3000 mg per kg of body weight), over the period of 18 days, was comparable with that of the control animals.

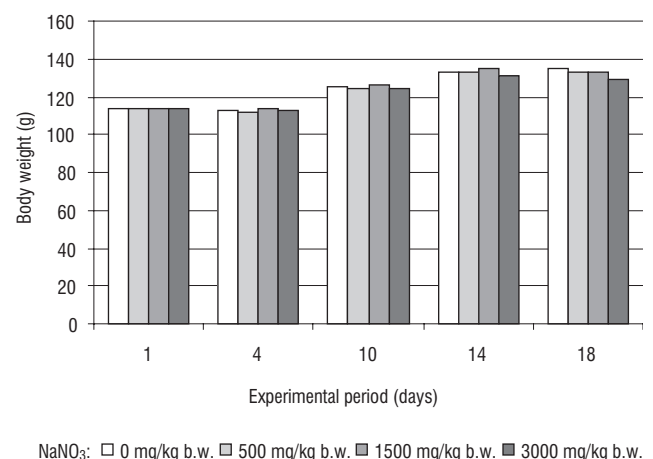


FIGURE 1. Body weight changes during the experiment (g).

### Urinary iodine concentration

Generally, the nitrate fed-rats showed lower 24-h urinary iodine concentrations ( $\mu\text{g/dL}$ ), compared with the control animals, yet the differences were not statistically significant ( $p>0.05$ ) (Table 2). Urinary iodine concentrations tended also to decrease with increasing nitrate intakes ( $13.19 \mu\text{g/dL}$  in Group I vs.  $7.55 \mu\text{g/dL}$  in Group IV).

TABLE 2. Urinary iodine concentrations in nitrate-fed rats.

	Iodine (mg/dL)
Group I	$13.19 \pm 2.27$
Group II	$14.25 \pm 2.84$
Group III	$9.55 \pm 2.49$
Group IV	$7.55 \pm 0.34$

Values are means  $\pm$  SEM, means are not significantly different

### Thyroid follicle morphology

The nitrate-fed rats showed histological changes in the thyroid gland (Figures 2, 3 and Table 3). The height of the epithelial follicle cells was significantly higher in the nitrate-fed rats than in the control group, thus indicating increased follicle activity (Table 3, Figure 3). In addition, mild to moderate irregularity of follicles and decreased amount of colloid were observed in the nitrate-fed animals (Groups II, III, and IV). The most apparent effects of nitrate feeding on thyroid follicular cell hyperplasia and hypertrophy were observed in rats fed the highest level of nitrate, *i.e.* 3000 mg

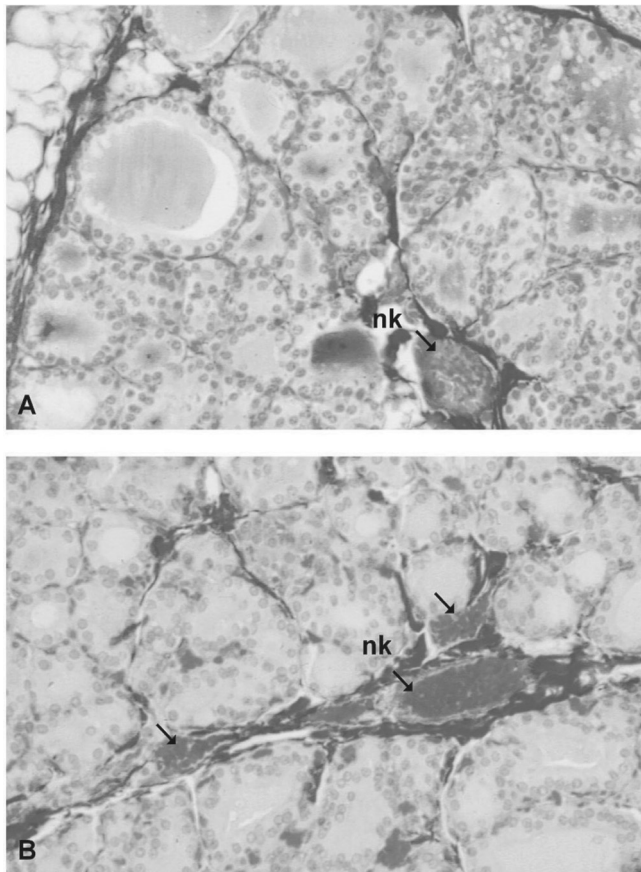


FIGURE 2. Histology of the thyroid. (A) Group I (nitrate 0 mg/kg b.w.); (B) Group IV (nitrate 3000 mg/kg b.w.); nk – blood vessels.

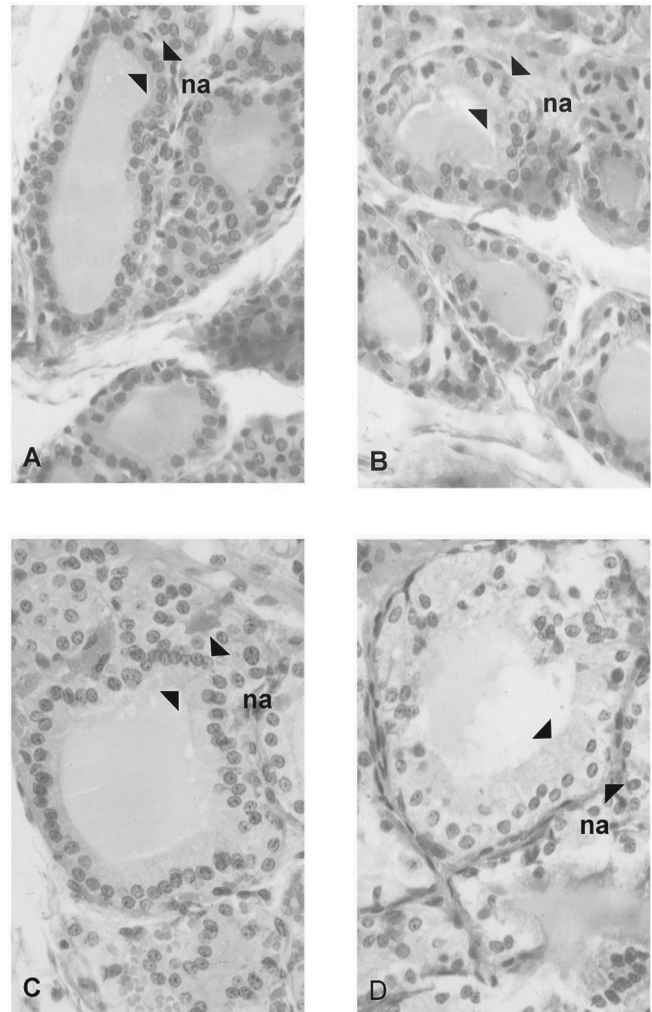


FIGURE 3. Thyroid follicles in rats. (A) Group I (0 mg/kg b.w.); (B) Group II (500 mg/kg b.w.); (C) Group III (1500 mg/kg b.w.); (D) Group IV (3000 mg/kg b.w.); na – epithelial cells.

per kg body weight. Interestingly, these changes in thyroid tissue were observed even at the NOEL of nitrate administration. Finally, we found that the vascularity of the thyroid tissue from the nitrate-fed rats was much more developed, compared with the control animals (Figure 2).

TABLE 3. Thyroid epithelial cell height in nitrate-fed rats.

	Epithelial cell height ( $\mu\text{m}$ )
Group I	$7.59 \pm 1.6^a$
Group II	$15.84 \pm 2.09^b$
Group III	$20.27 \pm 2.71^c$
Group IV	$30.58 \pm 4.53^d$

Values are means  $\pm$  SEM; Means followed by different letters are significantly different at  $p<0.001$

### Serum free thyroxine ( $\text{fT}_4$ ) and serum thyroid stimulating hormone (TSH) concentrations

Serum  $\text{fT}_4$  levels (pmol/L), determined on day 18, tended to decrease in the nitrate-fed rats (Table 4). For example, the dietary nitrate doses of 1500 and 3000 mg per kg body weight, decreased  $\text{fT}_4$  concentrations by 5.1% and 21.7%, respectively. However, this tendency was not significant ( $p>0.05$ ). On the other hand, the dietary nitrate doses of

TABLE 4. Serum fT<sub>4</sub> and TSH levels in rats after treatment by nitrate in diet.

	fT <sub>4</sub> (pmol/L)	TSH (ng/dL)
Group I	23.65±1.73	2.17±0.31 <sup>a</sup>
Group II	25.42±2.97	2.11±0.31 <sup>a</sup>
Group III	22.45±1.18	3.59±0.57 <sup>a</sup>
Group IV	18.52±3.25	6.22±0.87 <sup>b</sup>

Values are means ± SEM; Means followed by different letters are significantly different at p<0.001

3000 mg per kg body mass, caused highly significant increases (300%) in circulating levels of serum TSH (p<0.001). These increases were significantly correlated with graded levels of dietary nitrate intake (r=0.8; p<0.05). The NOEL of dietary nitrate had no effect on the concentration of thyroid hormones.

#### Plasma lipid profile

Total cholesterol and LDL-cholesterol concentrations were increased in the rats fed the highest dietary nitrate dose (*i.e.* 3000 mg per kg of body weight) by 7% and 28% respectively, compared with the control animals, however no significant effects of dietary nitrate on total cholesterol, LDL-cholesterol and HDL-cholesterol concentrations were noted (Table 5). In contrast, the highest dietary nitrate level increased significantly (p<0.05) serum triacylglycerol concentrations by 45.5%, compared to the control group of rats.

#### DISCUSSION

No negative effects of graded dietary nitrate concentrations on body weight of rats were evidenced in the present experiment. It was likely to result from a short experimental period (18 d), during which the potential toxic effects of nitrate did not impair the growth of rats. In contrast to our findings, nitrate intoxication may largely decrease the growth of rats [Chow *et al.*, 1980; Fritch *et al.*, 1980; Ogur *et al.*, 2000; Zaki *et al.*, 2004]. However, the above experiments were conducted for much longer periods of time (2–14 months). The potential causes of these effects were either a reduction in food and water intake or an increase in protein catabolism or decreased plasma T<sub>3</sub> and T<sub>4</sub> concentrations that impaired the growth of rats, as suggested by Zaki *et al.* [2004].

We have used the urinary iodine as a biochemical marker of iodine deficiency. It was expressed as the urinary iodine concentration (μg/dL), as recommended by Dunn *et al.* [1993].

The finding that an increasing dietary nitrate intake tended to decrease urinary iodine concentrations (Table 2)

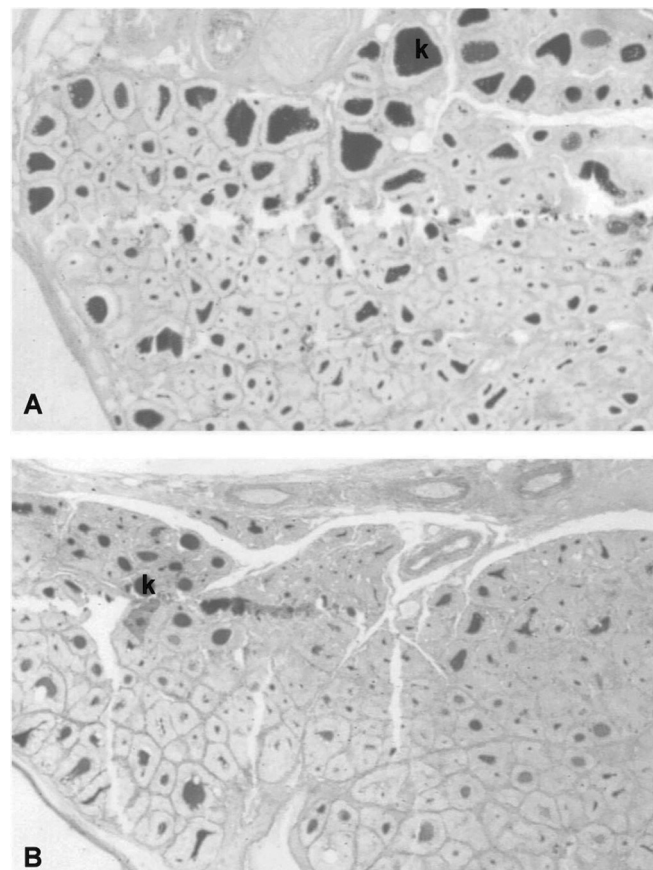


FIGURE 4. Histology of the thyroid. (A) Group I (nitrate 0 mg/kg b.w.); (B) Group IV (nitrate 3000 mg/kg b.w.); k – colloid.

could be explained by decreased iodine absorption in the digestive tract. This negative effect of nitrate could be either indirect, *i.e.* by inhibiting Na<sup>+</sup>/K<sup>+</sup> ATP-ase complex and energy generation for iodine transmembrane transport [Grudziński, 1998] or direct, *i.e.* by inhibiting sodium-iodide symporter Na<sup>+</sup>/I<sup>-</sup> [Kotani *et al.*, 1998; Dohan *et al.*, 2000; Chung, 2002; Szokeova *et al.*, 2001], both involved in iodine trapping in gastric mucosa.

Feeding graded amounts of nitrate to rats led to changes in thyroid gland morphology (Figures 2, 3, 4 and Table 3). In fact, nitrate administration resulted in both hyperplasia and hypertrophy of the thyroid gland. Moreover, the height of the epithelial follicle cells was significantly increased, mild to moderate irregularity of follicle was found and a decrease in the amount of follicular colloid was observed, in the nitrate-fed animals. These changes were essentially the same as in severe iodine deficiency in animal models. For example, long-term administration of a low iodine diet has been reported to cause follicular hyperplasia and hypertrophy in rats [Kanno, 1992], similar to that observed in our

TABLE 5. Effect of nitrate on serum lipoprotein fractions in experimental rats.

	Cholesterol (TC) (mmol/L)	LDL-cholesterol (mmol/L)	HDL-cholesterol (mmol/L)	Triacylglycerol (mmol/L)
Group I	2.10±0.12	0.32±0.06	1.78±0.11	0.44±0.02 <sup>a</sup>
Group II	2.18±0.16	0.46±0.07	1.73±0.15	0.42±0.04 <sup>a</sup>
Group III	2.02±0.08	0.44±0.04	1.58±0.08	0.50±0.04 <sup>a</sup>
Group IV	2.25±0.15	0.41±0.07	1.85±0.14	0.64±0.09 <sup>b</sup>

Values are means ± SEM; Means followed by different letters are significantly different at p<0.05

studies. Thus, the observed effects suggested the same negative changes in thyroid morphology as produced by iodine deficiency [Kanno *et al.*, 1992]. Nitrate may be considered as an iodine-binding competitive inhibitor, affecting the thyroid-pituitary hormonal axis and changing thyroid morphology, in a way similar to that of iodine deficiency. In contrast to our observations, nitrate administration to rats in drinking water (50, 150 and 500 mg/L) by Zaki *et al.* [2004] flattened follicular epithelium (by 50%) and increased the amount of follicular colloid, compared to the control animals. The potential causes of these morphological differences could involve the duration of nitrate administration in the respective experiments, *i.e.* 18 days in the reported experiment and 5 months in that of Zaki *et al.* [2004] as well as the level of nitrate intoxication.

In the present study, nitrate administration altered thyroid hormone metabolism by decreasing (insignificantly) serum  $fT_4$  and increasing ( $p < 0.01$ ) serum TSH levels. Similarly, nitrate administration in drinking water significantly decreased plasma  $T_3$  and  $T_4$  levels [Zaki *et al.*, 2004]. The decreased secretion of  $fT_4$  could be due to the inhibition of iodine transmembrane transport by a competitive iodine inhibitor, *i.e.* nitrate, to thyroid epithelial cells. As discussed above, the iodine binding may be blocked by nitrate either indirectly, *i.e.* by inhibition of  $Na^+/K^+$  ATP-ase complex or directly *i.e.* by inhibition of sodium-iodide symporter  $Na^+/I^-$  [Chung, 2002; Dohan & Carrasco, 2003], both involved in iodine trapping by these cells. The increased serum TSH concentrations observed in our studies could be expected. Namely, in a number of experiments, feeding animals with iodine-deficient diets decreased the concentrations of circulating  $fT_4$  thyroid hormone and increased the release of TSH from the pituitary gland. Thus, the observed effects suggested the same negative feedback mechanism, involving the thyroid-pituitary hormonal axis, similar to that produced by iodine deficiency [Kanno *et al.*, 1992].

In view of the earlier findings that the composition and transport of lipoproteins are seriously disturbed in human thyroid diseases, we studied serum lipid profile in the nitrate-fed rats (Table 5). According to Duntas [2002] and Luboshitzky [2002], sub-clinical hypothyroidism (SH) and altered concentrations of hormones (decreased  $fT_4$  and increased TSH concentrations) are associated with normal or slightly elevated total cholesterol levels, increased LDL, and lower HDL concentrations. In contrast to the above findings we were not able to show apparent significant effects of dietary nitrate treatment on serum total cholesterol or its fractions, *i.e.* LDL and HDL. On the other hand, serum triacylglycerol concentrations (Table 5), an independent risk factor for cardiovascular disease, were significantly elevated in the nitrate-fed rats. This effect could be related to a reduced removal rate of triacylglycerols from plasma in hypothyroidism [Duntas, 2002]. In addition, the development of hypertriglyceridemia associated with sub-clinical hypothyroidism was reported in humans by Luboshitzky [2002].

## CONCLUSIONS

In conclusion, the present study clearly indicates that

feeding graded amounts of nitrate to rats (1) decreases iodine absorption in the digestive tract as indicated by decreased urinary iodine excretion, (2) alters thyroid follicle morphology as indicated by both hyperplasia and hypertrophy of the follicular epithelial cells, (3) alters metabolism of thyroid hormones as indicated by decreased serum concentrations of  $fT_4$  and increased serum concentrations of TSH, and (4) increases serum triacylglycerols. Therefore, nitrate may be considered a competitive iodine inhibitor, affecting the thyroid-pituitary hormonal axis, in a way similar to that of iodine deficiency, thus acting as a goitrogen.

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## WPŁYW AZOTANÓW(V) NA FUNKCJONOWANIE TARCZYCY ORAZ PROFIL LIPIDOWY U SZCZURÓW RASY WISTAR

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Celem pracy było zbadanie wpływu dodatku NaNO<sub>3</sub> w diecie na wydalanie jodu, budowę morfologiczną tarczycy oraz poziom hormonów tarczycy u szczurów. Dodatkowo badano wpływ azotanów(V) na profil lipidowy.

Badania przeprowadzono na dwudziestu czterech szczurach – 5-cio tygodniowych samcach szczepu Wistar. Przez okres 18 dni zwierzęta karmiono dietą półsyntetyczną wg AIN (1993) z dodatkiem NaNO<sub>3</sub> (I grupa – 0, II grupa – 500, III grupa – 1500, IV grupa – 3000 mg/kg m.c.). Przyjęto ograniczony poziom żywienia tj. 15 g diety półsyntetycznej/zwierzę/dzień. Zwierzęta miały nieograniczony dostęp do wody. Szczury ważono w odstępach tygodniowych.

W przeprowadzonym doświadczeniu nie stwierdzono istotnych zmian w przyrostach masy ciała. Dodatek azotanów w diecie powodował obniżenie się stężenia jodu w moczu o charakterze tendencji ( $p > 0.05$ ) (13,19  $\mu\text{g/dL}$  w grupie kontrolnej; 7,55  $\mu\text{g/dL}$  w grupie IV). Negatywny wpływ azotanów na metabolizm jodu i funkcjonowanie tarczycy potwierdziły obserwacje mikroskopowe gruczołu. Dodatek azotanów(V) w diecie powodował zwiększenie ilości małych pęcherzyków. Zaobserwowano różnice w komórkach głównych tarczycy, które wyłącznie w kontroli tworzyły jednowarstwowy nabłonek sześcienny, natomiast w pozostałych grupach przypominały stany aktywnej wydzielniczo komórki, stając się bardziej zbliżony do nabłonka walcowatego. Dodatkowo zaobserwowano nieregularność pęcherzyków oraz zmniejszoną ilość koloidu w pęcherzykach (tab. 3, rys. 2, 3). Podawanie azotanów(V) wpłynęło również na unaczynienie gruczołu tarczycy. Wykazano zwiększenie zarówno ilości, jak i rozmiarów naczyń krwionośnych w porównaniu z grupą kontrolną. Poziom hormonu fT<sub>4</sub> w surowicy szczurów otrzymujących wzrastające dawki NaNO<sub>3</sub> obniżył się, nie było to jednak statystycznie istotne. Natomiast stężenie TSH w surowicy szczurów karmionych dietą z dodatkiem NaNO<sub>3</sub> wzrosło istotnie statystycznie ( $p < 0.001$ ) (tab. 4). Azotany(V) nie miały istotnego wpływu na frakcję cholesterolu LDL oraz HDL. Jednak dodatek azotanów istotnie statystycznie ( $p < 0.05$ ) zwiększył poziom trójglicerydów w surowicy krwi (o 45% w porównaniu z grupą kontrolną) (tab. 5).

Dodatek azotanów V w diecie szczurów powoduje zmiany poziomu hormonów TSH i fT<sub>4</sub> oraz obrazu histologicznego tarczycy, co sugeruje zakłócenie metabolizmu jodowego. Azotany(V), kompetencyjny inhibitor jodu, powodują zaburzenie działania osi przysadkowo-podwzgórzowej. Azotany(V) są związkami o pośrednim działaniu wolotwórczym.