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NITRITES AFFECT THYROID STATUS AND SERUM LIPOPROTEINS IN WISTAR RATS

Renata B. Kostogrys[1], Paweł M. Pisulewski[1], Anna Pecio[2]

[1]Department of Human Nutrition, Faculty of Food Science and Technology, Agricultural University, Kraków;
[2]Department of Comparative Anatomy, Faculty of Biology and Earth Sciences, Jagiellonian University, Kraków

Key words: rats, nitrites, thyroid gland, thyroid hormones, lipoproteins

The objectives of the present study were to estimate the effects of increasing levels of the dietary intake of nitrites on: (1) urinary iodine excretion, 2) changes in the morphology of thyroid follicles, and 3) thyroid gland hormonegenesis, in laboratory rats. Effects of nitrites on serum lipoproteins were examined as well. Feeding graded amounts of nitrites to rats resulted in: (1) decreased iodine absorption in the digestive tract as indicated by decreased urinary iodine excretion, (2) altered thyroid follicle morphology as indicated by both hyperplasia and hypertrophy of the follicular epithelial cells, (3) altered metabolism of thyroid hormones as indicated by decreased serum concentrations of fT_4 and tended to increase serum concentrations of TSH, and (4) decreased total cholesterol and HDL in serum and tended to increase serum triacylglycerols. Therefore, nitrite 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. In addition, dietary nitrite tended to elevate serum LDL-cholesterol and triacylglycerol concentrations, and to decrease serum HDL-concentrations, hence inducing an atherogenic lipoprotein profile.

INTRODUCTION

Nitrates and nitrites are present in a number of 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]. Toxicological studies have documented that nitrite is toxic to animals [Jensen, 1995]. The toxic effects of nitrites have been related to the formation of methaemoglobin, inducing hypertrophy of the adrenal zona glomerulosa in rats and the formation of cancerogenic nitrozoamines [Jensen, 1995; McKnight, 1999; WHO Technical Report Series, 1995]. Apart from the above known effects, nitrites 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; Zaki *et al*., 2004] and also humans [van Maanen *et al*., 1994; Gatseva *et al*., 1998; Vladeva *et al*., 2000]. In several studies, the development of goitre was accompanied by histomorphological changes of the thyroid gland [Horing *et al*., 1986] and a decrease in the secretion of thyroid hormones [Jahreis *et al*., 1991]. Interestingly, nitrates, in contrast to nitrites, are relatively nontoxic, but an elevated nitrate load may produce potential harmful effects *via* an endogeneous conversion of nitrates to nitrites [Jensen, 1995].

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

MATERIAL 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 116±5 g, were obtained from the Institute of Animal Production in Kraków, Poland. 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°C) and ambient humidity, with 12-h light-dark cycle. These rats were fed restricted amounts (15 g per day) of semi-purified AIN'93G diets [Reeves, 1993] and had free access to distilled water. The diets (I, II, III and IV; Table 1) supplied the following amounts of sodium nitrites (mg per kg of body weight): I – 0, II – 20, III – 80, and IV – 250 mg, and were fed for 3 weeks. This range of sodium nitrite administration included its no-observed effect level (NOEL = 20 mg per kg body weight). Food intake was measured daily and animal body weight was recorded weekly.

Urine and blood collection and thyroid preparation. Urine was collected quantitatively from each rat during the last 3 days (18–20 day) of the experiment. The pooled 3-day samples were stored at -20°C until analysis. At the end of the experiment (day 20), the rats were anaesthetised with thiopen-

Author's address for correspondence: Paweł M. Pisulewski, Department of Human Nutrition, Faculty of Food Science and Technology, The Agricultural University, ul. Balicka 122, 30-149 Kraków, Poland; tel.: (48 12) 662 50 18; e-mail: rrpisule@cyf-kr.edu.pl

TABLE 1. Composition of experimental diets (%).

	Group I	Group II	Group III	Group IV
Corn starch	63.25	63.25	63.25	63.25
Caseine	10	10	10	10
Sucrose	10	10	10	10
Soybean oil	7	7	7	7
Cellulose powder	5	5	5	5
Mineral mixture[a]	3.5	3.5	3.5	3.5
Vitamin mixture[b]	1	1	1	1
Choline	0.25	0.25	0.25	0.25
Tert-butylhydrochinon	0.0014	0.0014	0.0014	0.0014
Sodium nitrite (mg/kg b.w.)	0	20	80	250

[a]AIN-93G mineral mixture; [b]AIN-93G vitamin mixture

tal (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 x *g*, 15 min). The serum samples were stored at -20°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 spectrophotometric method as modified by Dunn *et al*. [1993]. Serum free thyroxine (fT_4) and serum thyroid stimulating hormone (TSH) concentrations were measured using a lumino-immunoassay LIA-mat F_4 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 respectivelly). The LDL+VLDL fraction of cholesterol was calculated as the difference between TC and HDL-C. Triacylglycerols were estimated enzymatically using a standard assay kit (BioVendor cat.-no 12805).

Thyroid follicle morphological examination. A part of trachea with thyroid gland on both sides was removed and fixed in Bouin's fluid for 3 days. Then, the tissues were dehydrated in alcohol, embedded in paraffin and sectioned serially at 7 μm. For histological evaluation the sections were stained with two methods: hematoxylin & eosin and trichrome [Kiernan, 1990]. The follicle colloid evaluation was made using the slides with PAS-positive reaction. The mean height of 30 epithelial cells of follicle was measured in 8 rats chosen randomly from each experimental group (2 rats per group).

Statistical analysis. The effect of nitrite 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 significant differences between treatment means.

RESULTS AND DISCUSSION

Body weight

No evident signs of dietary nitrite toxicity were observed in this study (Figure 1). In fact, the growth of the nitrite-fed rats (20, 80 and 250 mg per kg of body weight), over the period of 20 days, was comparable with that of the control animals.

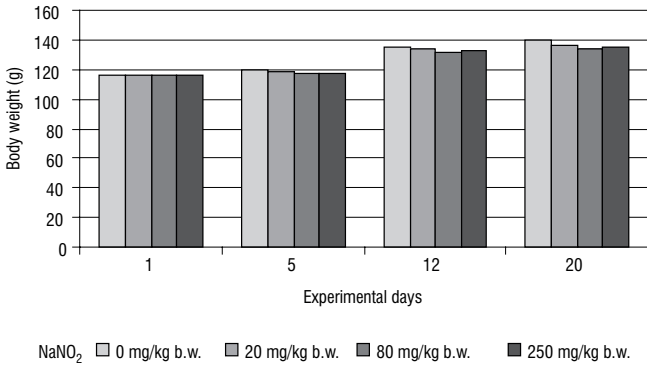

FIGURE 1. Body weight changes during the experiment (g).

The lack of negative effects of graded dietary nitrite concentrations on body weight of rats could have resulted from the short duration of the experimental period (20 days), during which the potential toxic effects of nitrite did not impair the growth of rats. In contrast to our findings, nitrite intoxication has been reported to largely decrease the growth of rats [Bilczuk, 1976; Fritsch *et al*., 1980; Chow *et al*., 1980]. However, the above experiments were conducted for much longer periods of time (6–14 months). The possible causes of these effects were either a reduction of food and water intake or an increase in protein catabolism or decreased plasma T_3 and T_4 concentrations impairing the growth of rats.

Urinary iodine concentration

Generally, the nitrite-fed rats showed significantly lower 24-h urinary iodine concentrations (μg/dL, $p<0.001$), compared with the control animals (Table 2). Urinary iodine concentrations were decreased with increasing nitrite intakes (25.44 μg/dL in Group I *vs*. 16.76 μg/dL in Group IV, $p<0.001$).

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

	Iodine (μg/dL)
Group I	25.44±1.29[a]
Group II	25.98±1.75[a]
Group III	21.61±1.18[ab]
Group IV	16.76±1.52[b]

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

The finding that increasing dietary nitrite intake significantly decreased urinary iodine concentrations (Table 2) could be explained by decreased iodine absorption in the digestive tract. This negative effect of nitrite could be either indirect *i.e.* by inhibiting Na^+/K^+ ATP-ase complex and energy generation for iodine transmembrane transport [Grudziński, 1998] or direct *i.e.* by inhibiting sodium-iodide

symporter Na^+/I^- [Kotani *et al*., 1998; Dohan *et al*., 2000; Chung, 2002; Szokeova *et al*., 2001], both involved in iodine trapping in gastric mucosa.

Thyroid follicle morphology

The nitrite-fed rats showed histological changes in the thyroid gland (Figures 2, 3; Table 3). The height of the epithelial follicle cells was significantly higher in the nitrite-fed rats, 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 nitrite-fed animals (Groups II, III, and IV, Figure 4). The most apparent effects of nitrite feeding on thyroid follicular cell hyperplasia and hypertrophy were observed in rats fed the highest level of nitrite, *i.e.* 250 mg per kg b.w. Interestingly, these changes in thyroid tissue were observed even at the NOEL level of sodium nitrite administration. Finally, we found that the vascularity of the thyroid tissue from nitrite-fed animals was much more developed, compared with the control ones (Figure 2).

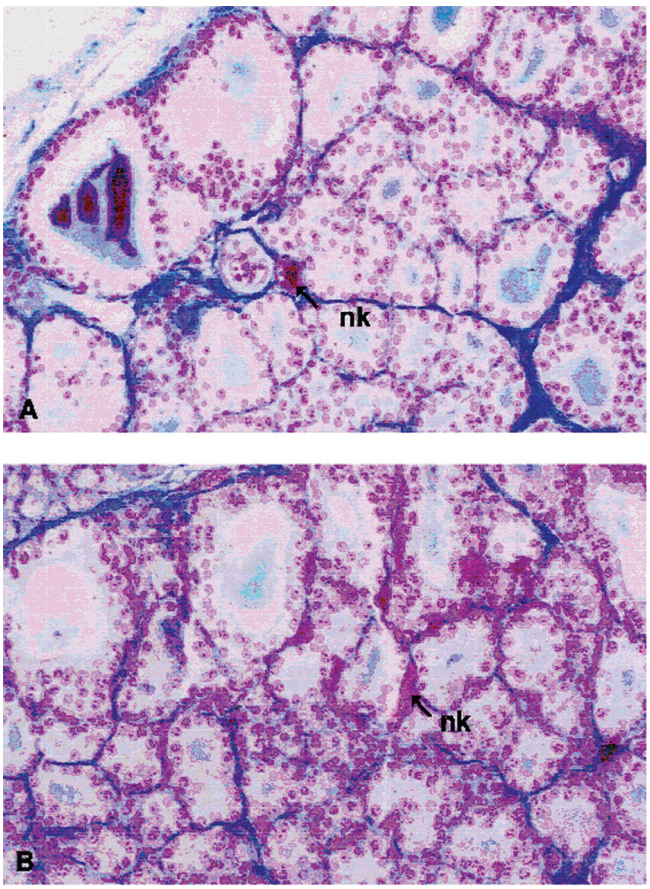

FIGURE 2. Histology of the thyroid.
A. Group I (nitrite 0 mg/kg b.w.), B. Group IV (nitrite 250 mg/kg b.w.); nk – blood vessels.

TABLE 3. Thyroid follicular activity in nitrite-fed rats.

	Epithelial cell height (μm)
Group I	8.97 ± 0.37^a
Group II	14.67 ± 0.53^b
Group III	15.52 ± 0.39^b
Group IV	20.66 ± 0.64^c

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

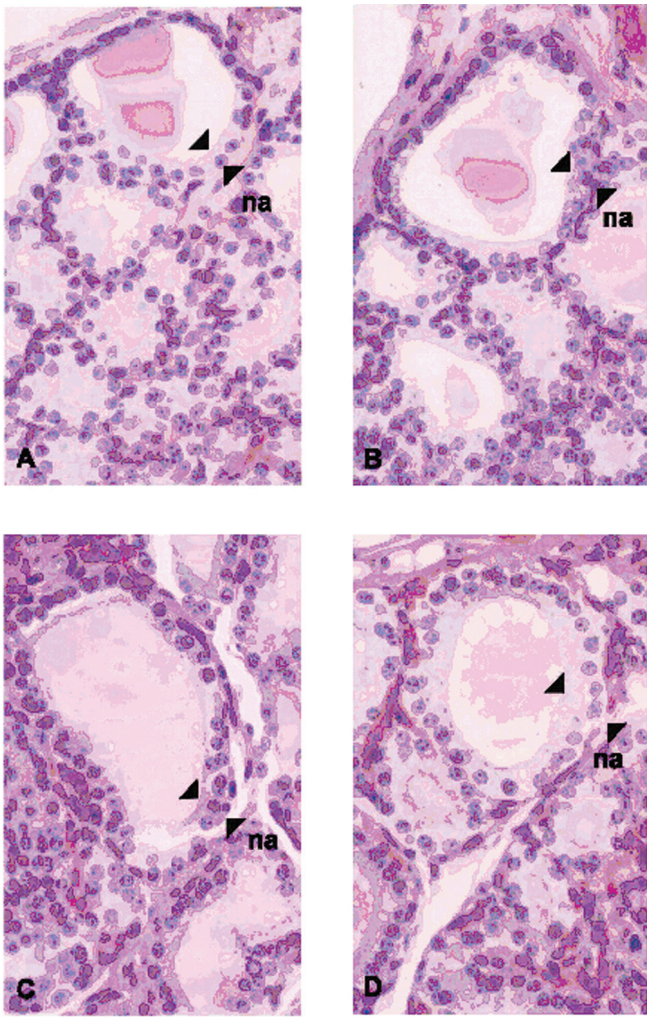

FIGURE 3. Thyroid follicles in rats.
A. Group I (0 mg/kg b.w.), B. Group II (20 mg/kg b.w.), C. Group III (80 mg/kg b.w.), D. Group IV (250 mg/kg b.w.); na – epithelial cells.

As indicated above, nitrite administration led to 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 of the amount of follicular colloid was observed, in the nitrite-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 caused follicular hyperplasia and hypertrophy in rats [Kanno, 1992], similar to that observed in our studies.

Thyroid metabolism hormones (fT_4 & TSH)

The dietary nitrite levels of 20, 80 and 250 mg per kg b.w., caused a highly significant decrease (37.9%, 46.3% and 49.4.%, respectively) in circulating levels of serum fT_4 (pmol/L) ($p<0.001$), and even the NOEL level of dietary nitrite had a significant negative effect on free tyroxine concentrations. In contrast, serum TSH levels, determined on day 20, tended to be increased in nitrite-fed rats (Table 4). The dietary nitrite levels of 20, 80 and 250 mg per kg b.w., increased TSH concentrations by 7.1%, 41.7%, and 40.2%, respectively. This tendency was, however, not significant ($p>0.05$).

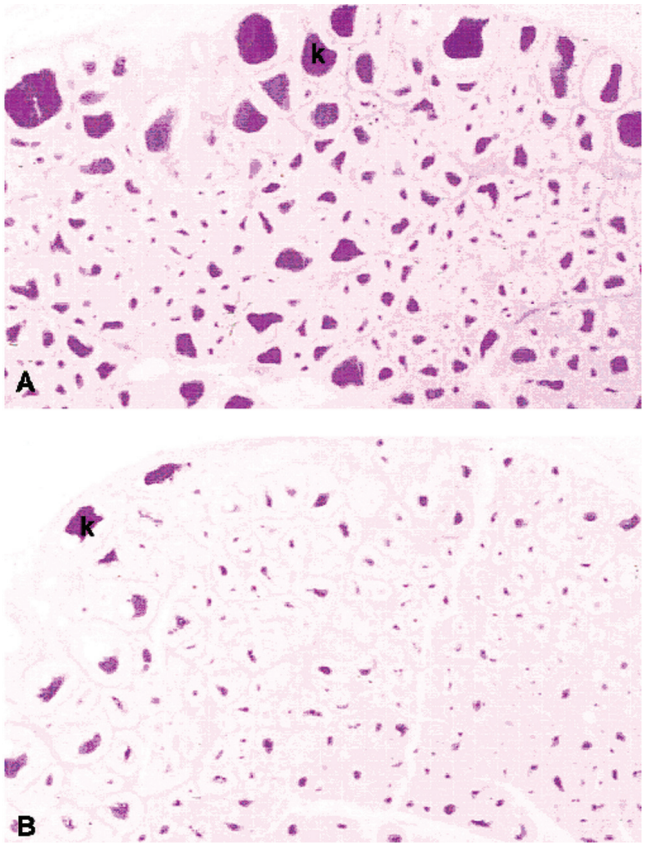

FIGURE 4. Histology of the thyroid.
A. Group I (nitrite 0 mg/kg b.w.), B. Group IV (nitrite 250 mg/kg b.w.); k – colloid.

TABLE 4. Serum fT_4 and TSH levels in rats after treatment with nitrite in food.

	fT_4 (pmol/L)	TSH (ng/dL)
Group I	22.51± 2.42[a]	2.66± 0.33
Group II	13.97± 1.60[b]	2.85± 0.49
Group III	12.09± 1.95[b]	3.77± 0.37
Group IV	11.40± 1.67[b]	3.73± 0.70

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

The observed decreased secretion of fT_4 could be due to the inhibition of iodine transmembrane transport by a competitive iodine inhibitor, *i.e.* nitrite, to thyroid epithelial cells. As discussed above, the iodine binding may be blocked by nitrite 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 circulating fT_4 thyroid hormone concentrations 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].

Plasma lipid profile

Increasing dietary nitrite levels tended to depress total serum cholesterol and HDL-cholesterol concentrations. At the highest dietary nitrite level, the concentrations of these fractions decreased significantly ($p<0.05$), by 22.6% and 32.5%, respectively. In contrast, feeding incremental doses of nitrite to rats did not result in any significant changes in serum LDL-cholesterol and triacylglycerol concentrations (Table 5). Compared to the control animals, however, the concentrations of these fractions, increased in the rats fed the highest dietary nitrite level (*i.e.* 250 mg per kg of b.w.), by 30% and 42.4%, respectively, but not statistically significant.

According to Duntas [2002] and Luboshitzky [2002], sub-clinical hypothyroidism (SH) and altered concentrations of thyroid metabolism hormones (decreased fT_4 and increased TSH concentrations) are associated with normal or slightly elevated total cholesterol levels, increased LDL-cholesterol and lower HDL-cholesterol concentrations. Also in our study, increasing dietary nitrite levels led to a significantly decreased serum HDL-cholesterol and tended to increase serum LDL-cholesterol and triacyglycerols, thus confirming in part the results of the earlier experiments [Duntas, 2002, Luboshitzky *et al*., 2002]. Surprisingly, serum total cholesterol was significantly reduced. In view of the above, it might be noted that elevated serum triacylglycerol concentrations (Table 5) are an independent risk factor for cardiovascular disease. This effect could be related to a reduced removal rate of triacyglycerols from plasma in hypothyroidism [Duntas, 2002]. In addition, the development of hypertriglyceridemia associated with sub-clinical hypothyroism was reported in humans by Luboshizky [2002].

CONCLUSIONS

The present study indicated that feeding graded amounts of nitrite to rats resulted in: (1) decreased iodine absorption in the digestive tract as indicated by decreased urinary iodine excretion, (2) altered thyroid follicle morphology as indicated by both hyperplasia and hypertrophy of the follicular epithelial cells, (3) altered metabolism of thyroid hormones as indicated by decreased serum concentrations of fT_4 and increased serum concentrations of TSH, and (4) decreased total cholesterol and HDL in serum and increased serum triacylglycerols. Therefore, nitrite may be considered as a competitive iodine inhibitor, affecting the thyroid-pituitary hor-

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

	Total cholesterol	LDL – cholesterol	HDL – cholesterol	Triacylglycerol
Group I	2.43±0.13[a]	0.40±0.07	2.03±0.12[a]	0.59±0.10
Group II	2.37±0.13[a]	0.38±0.17	1.99±0.21[a]	0.54±0.06
Group III	2.27±0.12[ab]	0.48±0.08	1.79±0.14[ab]	0.87±0.36
Group IV	1.88±0.05[b]	0.52±0.04	1.37±0.05[b]	0.84±0.15

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

monal axis in a way similar to that of iodine deficiency, thus acting as a goitrogen. In addition, dietary nitrite tended to elevate serum LDL-cholesterol and triacylglycerol concentrations, and to decrease serum HDL-concentrations, hence inducing an atherogenic lipoprotein profile.

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

Renata B. Kostogrys[1], Paweł M. Pisulewski[1], Anna Pecio[2]

[1]Katedra Żywienia Człowieka, Wydział Technologii Żywności, Akademia Rolnicza, Kraków;
[2]Zakład Anatomii Porównawczej, Uniwersytet Jagielloński, Kraków

Celem pracy było zbadanie wpływu dodatku azotanów(III) w diecie na wydalanie jodu, morfologię tarczycy oraz poziom hormonów tarczycy u szczurów laboratoryjnych. Dodatkowo badano wpływ azotanów(III) na profil lipidowy. Dodatek azotanów(III) w diecie powodował obniżenie stężenia jodu w moczu (tab. 2). Dodatkowo zaobserwowano zmiany w morfologii tarczycy (hipertrofia i hiperplazja komórek głównych tarczycy). Poziom hormonu fT_4 w surowicy szczurów otrzymujących wzrastające dawki $NaNO_2$ obniżył się, natomiast stężenie TSH w surowicy miało tendencję wzrostową (tab. 3). Dodatek azotanów(III) istotnie statystycznie ($P<0.05$) obniżył poziom cholesterolu całkowitego oraz cholesterolu frakcji HDL. Zaobserwowano nieistotny statystycznie wzrost cholesterolu frakcji LDL oraz trójglicerydów (tab. 5).

Azotany(III), kompetencyjny inhibitor jodu, powodując zaburzenie działania osi przysadkowo-podwzgórzowej, zmieniają poziom hormonów TSH i fT_4 oraz histologię tarczycy. Świadczy to o wolotwórczym działaniu azotanów(III), które dadatkowo wpływając na metabolizm lipidów indukują aterogenny profil lipidowy u szczurów laboratoryjnych.