

Hypothyroidism in Male Rats of Different Ages Exposed to Nitrate Polluted Drinking Water.

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Abstract: The effect of nitrate pollution on the thyroid hormonal levels, body weight and other health indices was studied in male rats of different ages. The tested rats were divided into young (3 weeks old) and adult (12 weeks old) groups exposed to pollution via sodium nitrate (NaNO_3) intake in drinking water in concentrations (100 mg/L \approx 8.7 mg/Kg, 250 mg/L \approx 21.7 mg/Kg and 550 mg/L \approx 47.7 mg/Kg) daily for four months. The study revealed a dose dependent decrease in serum levels of thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) accompanied by increased levels of thyroid stimulating hormone (TSH) in all nitrate exposed rats, indicating development of hypothyroidism. A significant reduction of the body weight gain was also recorded in the young animals at different nitrate doses, but only in the adults with the high dose. Meanwhile, a significantly elevated nitric oxide (NO) level in the serum and urine were noticed that confined to the young animals with medium (250 mg/L) and high (550 mg/L) nitrate doses and the high dose adult animals. In addition, nitrate exposure resulted in a decreased serum concentrations of total proteins and protein fractions (albumin and globulin), accompanied by significant increases in the urea and creatinine levels in both serum and urine of the tested rats. This together with the above detected changes was found to exceed as the nitrate concentration increased and to be more pronounced in the young rats. Therefore, it can conclude that prolonged exposure to nitrate polluted drinking water is toxic to the thyroid hormonal activity and the other measured indices in both young and adult rats, but the young animals comprised the most age group susceptible to nitrate toxicity.

Key words: Nitrate pollution, hypothyroidism, nitric oxide.

INTRODUCTION

High consumption of nitrate polluted drinking water causes various health problems in human¹. The most well known are methemoglobinemia^[2,3], growth retardation⁴ and gastric cancer^[5].

Other targets for nitrate exposure, such as thyroid gland has been recently researched, however results obtained in this respect were contradictory. Some workers showed an increase in thyroid volume with signs of thyroid disorders when drinking water nitrate level is below^[1] or above^[6] the WHO standard of 50 mg/L^[7], while others failed to show such effect^[8]. This contradiction could be related to different factors including level of nitrate exposure as well as age of exposure^[1,9].

Therefore the present study was carried out on male rats of two ages; young (3 weeks-old) and adult (12 weeks-old) ingested sodium nitrate in drinking water at different concentrations (100, 250 and 550 mg/L) for continuous four months with the aim of evaluating nitrate effect on the thyroid hormonal status. Other health indices including body weight, total

protein, urea and creatinine as well as nitric oxide (as a marker of nitrate accumulation in the body) were also investigated.

MATERIAL AND METHODS

A-Animals: Healthy male Wistar albino rats (*Rattus rattus*) of two ages; young of 3-weeks old, weighing 30 ± 5 g and adult of 12-weeks old, weighing 175 ± 5 g were used in this experiment. All rats received standard diet and drinking water *ad libitum* during the entire experimental period.

B- Experimental design: Rats of each age were divided into four groups (each of 8 rats). The first one was the control group received tap water containing 4.9 ± 2 mg NO_3/L , and the other three groups received sodium nitrate in drinking water daily for four months at concentrations (100, 250, and 550 mg/L) to provide average daily doses of approximately 8.7, 21.7 and 47.7 mg/Kg^[10]. These doses were chosen based on the previous experimental model for evaluating nitrate toxicity^[9,11] as well as the recent ecological study carried by the present authors^[12].

C- Animals investigation:

1- Body weight gain: In order to follow the effect of nitrate on the body weight gain of rats, the body weight (g) was recorded at the start and end of the experiment for each tested group. After four months of nitrate exposure, rats were kept individually in metabolic cages for 24 hours. Urine was frequently collected, centrifuged and stored at -20 °C until analysis. The animals were then sacrificed and the blood samples were collected, centrifuged at 860 Xg for 15 minutes and non hemolysed sera were separated for biochemical and hormonal analysis.

2- Biochemical and Hormonal Analysis: Total protein and albumin concentrations were estimated using Diamond Diagnostic Kit as described by^[13,14], respectively. Globulin concentration was calculated by subtracting albumin concentration from the total protein value. Urea and creatinine levels were measured using Diamond diagnostic Kits based on the methods of^[15,16] respectively. Nitric oxide level was measured colorimetrically in both serum and urine using Diagnostic kit from ABC according to^[17]. In addition, serum thyroid hormones [triiodothyronin (T₃), thyroxine (T₄) and thyroid stimulating hormone(TSH)] level were measured by *in vitro* diagnostic radioimmunoassay with the immulite 2000 analyzer for the quantitative measurement as described by^[18,19,20] respectively.

3- Statistical Analysis: All data are represented as means + SE. One-way analysis of variance ANOVA was used to analyze the results. The level of statistical significant was set at P < 0.05. When significances between the means of treated and control groups were performed by Least Significant Difference (LSD) test^[21].

RESULTS AND DISCUSSION

1- Body Weight Gain: As shown in Table 1, the present data recorded a significant and dose-dependent decrease in the body weight gain of the young animals with different nitrate doses, besides the high dose adult animals if compared to their respective control groups.

2- Serum Analysis: As shown in Table 2, the chronic four months treatment by different nitrate doses induced significant and dose dependent reduction in serum T₃ and T₄ levels of all young and adult animal groups except for the low dose adult animals which showed non significant decrease compared to their respective control groups. In contrast, TSH showed increased serum levels that were significant with medium and high dose groups of both investigated ages. Meanwhile, by comparing the two studied age groups, all adult animals exhibited significant decreases in serum T₃ and T₄ levels compared to their respective young groups, while no significant differences were detected with the measured TSH level.

Moreover, the present biochemical analysis showed a dose dependent decreased serum levels of total protein, albumin and globulin accompanied with corresponding increases in serum urea and creatinine concentrations, which in all tended to be significant in the younger animals with medium and high nitrate doses and the adults with high dose only. It was also showed that all adult groups had significantly increased total protein, albumin, globulin, urea and creatinine values if compared to their respective young groups (Table 2).

Additionally, all nitrate exposed groups of both ages exhibited a dose-dependent increase in serum nitric oxide levels which were significant in the young rats with medium and high nitrate doses, as well as the high dose adult animals. Also, nitric oxide levels in all adult groups showed lower but non-significant values comparing to their respective young groups.

3- Urine Analysis: As shown in Table 3 obtained data indicated significant and dose dependent increase in urine total protein concentration of all nitrate exposed groups. Similarly, a dose dependent elevation in the urine levels of both urea and creatinine levels were observed, but this was only significant with both medium and high dose young groups, as well as high dose adult group. Moreover, all adult groups exhibited significant elevation in the urine total protein, urea and creatinine levels compared to their respective control groups.

With regard to nitric oxide, obtained data illustrated increased urine nitric oxide concentrations of all investigated groups, which were significantly observed with both medium and high dose young groups, as well as the high dose adult group. By comparing the two studied ages, urine nitric oxide markedly decreased in all adult animals compared to their respective young groups, but this decrease was non- significant, except for the high dose group.

Discussion: Body weight is one of the basic indices for assessment of the health status of an organism^[1,22]. In the present study, long term exposure to drinking water with different nitrate concentrations (100, 250, 550 mg/l) showed decreased body weight gain in the tested rats of different ages, with the most pronounced decrease being demonstrated among the younger animals, particularly with the high nitrate concentration. Similarly, other investigations indicated that nitrate exposure causes body weight reduction in a dose-dependent manner¹ that was greater in the young growing animals^[9]. In the present study, this reduced body weight can be explained by an increased protein catabolism revealed by the low serum total protein and protein fractions (albumin and globulin) along with high levels of urea and creatinine in both serum and urine of all nitrate exposed rats, however an increased urinary protein loss was also found. This protein loss probably resulting from nitrate-induced kidney dysfunction^[23] seemed to be additional factor affecting the body weight in the present study.

Table 1: Body weight gain (g) of young and adult male rats after 4 months exposure to different nitrate doses.

		Animal Groups									ANOVA
		Young Rats						Adult Rats			
		Control	Nitrate Exposed Groups			Control	Nitrate Exposed Groups				
			Low dose	Medium Dose	High dose		Low dose	Medium dose	High dose		
Initial body weight	Mean ± SE(N=8)	39.63± 1.17	38.63± 0.92	38.13± 0.79	38.50± 0.80	172.13± 0.79	172.38± 0.75	172.63±0.68	172.50±0.73	P < 0.001	
Final body weight	Mean± SE(N=8)	315.13± 8.66	279.88 ^a ± 5.11	223.00 ^a ± 6.65	171.50 ^a ± 6.87	330.63± 12.08	322.25 ± 10.27	319.00± 7.06	297.75 ^b ± 6.74	P < 0.001	
Body weight gain	Mean± SE(N=8)	275.5± 7.50	241.25 ^a ± 4.34	184.88 ^a ± 5.94	134.50 ^a ± 6.27	154.75± 9.07	149.88± 9.67	146.38± 6.48	125.25 ^b ± 6.19	P < 0.001	

Data are means ± SE, N= number of animals
a = significant difference between young nitrate exposed groups and their respective control.
b = significant difference between adult nitrate exposed groups and their respective control

Table 2: Serum biochemical parameters of young and adult male rats after four months exposure to different nitrate doses.

		Animal Groups									ANOVA
		Young Rats						Adult Rats			
		Control Group	Nitrate Exposed Groups			Control	Nitrate Exposed Groups				
			Low dose	Medium Dose	High dose		Low dose	Medium dose	High dose		
T ₃ (ng/dl)	Mean ± SE(N=5)	142.90± 6.08	118.60 ^a ± 7.76	104.20 ^a ± 1.88	88.00 ^a ± 2.15	40.05 ^a ± 0.539	38.00 ^a ± 1.41	24.60 ^b ± 1.33	21.40 ^b ± 1.33	P<0.001	
T ₄ (µg/dl)	Mean ± SE(N=5)	12.07± 0.679	10.06 ^a ± 0.334	8.53 ^a ± 0.767	7.11 ^a ± 0.838	4.38 ^a ± 0.593	3.93 ^a ± 0.198	3.34 ^b ± 0.331	2.86 ^b ± 0.178	P<0.001	
TSH(µIU/ml)	Mean ± SE(N=5)	0.050± 0.005	0.058± 0.003	0.063 ^a ± 0.003	0.065 ^a ± 0.004	0.043± 0.005	0.051± 0.005	0.056 ^b ± 0.003	0.058 ^b ± 0.004	P<0.05	
Total protein(g/dl)	Mean ± SE(N=8)	8.06± 0.102	7.69± 0.115	6.25 ^a ± 0.161	5.32 ^a ± 0.143	10.29 ^a ± 0.447	10.15 ^a ± 0.375	9.42 ^a ± 0.260	8.16 ^b ± 0.154	P<0.001	
Albumin(g/dl)	Mean ± SE(N=8)	5.12± 0.247	4.45 ± 0.141	3.15 ^a ± 0.151	3.06 ^a ± 0.050	6.25 ^a ± 0.215	6.17 ^a ± 0.258	5.92 ^a ± 0.173	4.41 ^b ± 0.301	P<0.001	
Globulin(g/dl)	Mean ± SE(N=8)	3.78± 0.206	3.44± 0.059	2.72 ^a ± 0.139	1.91 ^a ± 0.092	4.52 ^a ± 0.329	4.33 ^a ± 0.493	4.13 ^a ± 0.173	3.46 ^b ± 0.163	P<0.001	
Urea(mg/dl)	Mean ± SE(N=8)	48.70± 3.034	58.64± 3.500	69.24 ^a ± 3.139	85.21 ^a ± 2.747	80.33 ^a ± 2.792	88.67 ^a ± 2.834	93.01 ^a ± 3.492	100.95 ^b ± 3.645	P<0.001	
Creatinine(mg/dl)	Mean ± SE(N=8)	0.73± 0.024	0.81± 0.028	0.919 ^a ± 0.023	1.08 ^a ± 0.072	0.92 ^a ± 0.056	1.01 ^a ± 0.062	1.05 ^a ± 0.013	1.29 ^b ± 0.072	P<0.001	
NO(µM/dl)	Mean ± SE(N=5)	27.05± 2.57	29.60± 2.00	36.71 ^a ± 2.67	39.19 ^a ± 2.96	24.35± 2.49	25.37± 2.05	31.85± 2.57	34.95 ^b ± 3.12	P<0.05	

Data are means ± SE, N= number of animals
a = significant difference between young nitrate exposed groups and their respective control.
b = significant difference between adult nitrate exposed groups and their respective control
c = significant difference between different adult groups and their respective young groups

Table 3: Urine biochemical parameters of young and adult male rats after four months exposure to different nitrate doses.

		Animal Groups									ANOVA
		Young Rats						Adult Rats			
		Control Group	Nitrate Exposed Groups			Control	Nitrate Exposed Groups				
			Low dose	Medium Dose	High dose		Low dose	Medium dose	High dose		
Total protein(ng/dl)	Mean ± SE(N=8)	0.0013± 0.0002	0.240 ^a ± 0.025	0.430 ^a ± 0.021	0.540 ^a ± 0.027	0.0014± 0.0004	0.320 ^b ± 0.023	0.499 ^b ± 0.035	0.646 ^b ± 0.023	P<0.001	
Urea(mg/dl)	Mean ± SE(N=8)	60.02± 1.69	75.92± 3.08	81.04 ^a ± 3.26	96.09 ^a ± 3.57	96.93 ^a ± 2.94	127.94 ^a ± 4.78	130.39 ^a ± 4.46	167.18 ^b ± 5.96	P<0.001	
Creatinine(mg/dl)	Mean ± SE(N=8)	3.57± 0.274	3.92± 0.142	4.88 ^a ± 0.165	5.45 ^a ± 0.190	4.67 ^a ± 0.190	4.84 ^a ± 0.311	5.73 ^a ± 0.364	6.45 ^b ± 0.382	P<0.001	
NO(µM/dl)	Mean ± SE(N=5)	111.27± 6.8	158.28± 11.32	223.97 ^a ± 28.16	337.37 ^a ± 23.78	89.95± 16.66	143.77± 4.59	201.90 ^b ± 23.5	298.17 ^b ± 18.95	P<0.01	

Data are means ± SE, N= number of animals
a = significant difference between young nitrate exposed groups and their respective control.
b = significant difference between adult nitrate exposed groups and their respective control
c = significant difference between different adult groups and their respective young groups

Beside this, a number of investigations have focused on the influence of nitrate on the thyroid status. Some workers showed thyroid hypertrophy with decreased thyroid hormones in people using drinking water with nitrate concentrations below^[24] or above^[25] the WHO nitrate standard of 50 mg/l^[25,26]. Similarly, the animal studies by^[24,27] showed decreased serum levels of T₄ and T₃ with increased secretion of more TSH from pituitary to compensate for thyroid dysfunction in nitrate consumed rats. Such an effect is in keeping with the present study showing decreased serum levels of both T₄ and T₃ along with increased TSH in all tested rats, with the greatest effect being detected among the younger animals exposed to the high nitrate concentration.

Based on these findings together with the decreased body weight observed in this study, it can be said that nitrate exposure leads to a state of hypothyroidism with accompanying body weight loss, particularly in the younger animals whose exposure to nitrate started at an earlier age.

For evaluating these findings, it was suggested that nitrate-induced thyroid hormones deficiency could affect the body weight by the way of inhibiting food intake through its influence on the nervous regulation of feeding behaviour^[27] that was found to cause a decrease of growth hormone receptors within the liver, thus causing lack of plasma somatomedins^[28]. It was also found that hypothyroid patients have decreased

somatomedin levels and that the growth promoting effect of thyroid hormones might be mediated by somatomedins^[29]. Additionally, it was reported that thyroid hormones deficiency probably inhibits the synthesis of growth hormone releasing factor (GHRF) in the hypothalamus^[27] and that injection of T₄ in rats caused elevation of hypothalamic GHRF content^[30].

Regarding the mechanism by which nitrate causes thyroid hormones deficiency, several studies indicated that nitrate seems to exert its effect in the organism after their gastric reduction into nitrite, then nitric oxide known to alter the thyroid gland¹ as evidenced by: first the use of an inhibitor of NO synthesis reduces the vascular expansion observed in the human thyroid gland during goiter formation induced by a low iodine diet^[31], as NO is a biogenic messenger that converts guanosin triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) that induces vasodilatation^[1]. Second, the long-term exposure to NO donors (nitroprosside and S-nitroglutathione) significantly inhibits iodine transport and organification in cultured bovine thyroid cells and reduces the differentiation of these cells^[32]. This possible inhibition of iodine transport by NO would be exerted on Na⁺/I⁻ symporter (NIS), an intrinsic membrane protein that mediates the active transport of iodide into the thyroid cells^[33]. Additionally, it was explained that decreased accumulation of iodide in thyroid gland by nitrate might be responsible for the decreased activity of thyroid peroxidase, the enzyme that catalyzes the biosynthesis of thyroid hormones at different levels, including organification of iodide, iodination of tyrosine residues in thyroglobulin and coupling reactions for synthesis of T₄ and T₃^[4]. These data thus indicated that increased NO production by nitrate exposure probably inhibits iodide transport into thyroid gland that consequently causes thyroid hormones deficiency. The present study confirmed this as the nitrate induced decrease of thyroid hormones (T₃ and T₄) goes in parallel with the increased NO levels in both serum and urine of all tested rats of both ages.

In this concern, several researchers indicated that thyroid hormones are produced as thyroglobulin and stored in the thyroid follicles for months. The thyroglobulin store of thyroid gland is sufficient to meet the needs of the body for 1-3 months^[34]. For this reason, even if synthesis of thyroid hormones stops completely, a decrease in thyroid hormones levels in circulation and thus development of clinical insufficiency takes a long time^[34]. This may be the cause for the lack of nitrate effect in a number of studies with short term nitrate exposure^[35,36] in contrast to the long-term influence of nitrate, where the reserve iodide presumably became exhausted and thus resulted in a decreased serum levels of thyroid hormones, similarly as described in the present study and other earlier investigations^[1,24].

In conclusion: the present study demonstrated that prolonged ingestion of nitrate in drinking water represents a risk factor for the development of hypothyroidism in all tested rats that seems to exceed with increasing nitrate concentration and to be more obvious in the younger rats. So, sanitary activities should be applied for decreasing nitrate concentrations in public water supplies in the rural areas with high nitrate levels in their drinking water, with the aim of preventing incidence of hypothyroidism among population living in these areas, particularly the younger ones.

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