

# The effect of $^{131}\text{I}$ -induced hypothyroidism on the levels of nitric oxide (NO), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF- $\alpha$ ), total nitric oxide synthase (NOS) activity, and expression of NOS isoforms in rats

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## ABSTRACT

Accumulating evidence has shown that hypothyroidism affects the cardiovascular system, significantly increasing the incidence of cardiovascular diseases. In the present study we investigated the effect of radioactive iodine ( $^{131}\text{I}$ )-induced hypothyroidism on several parameters of vascular function, such as nitric oxide (NO), total nitric oxide synthase (NOS) activity and expression of NOS isoforms, as well as on interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- $\alpha$ ) as indicators of inflammation, in rats. A dose of 150  $\mu\text{Ci}$  of  $^{131}\text{I}$  was determined as optimal for establishing the model of hypothyroidism in rats. After administration of  $^{131}\text{I}$ , at the end of month 1, 2, and 4 ( $n = 3$  for each time point), NO, IL-6, and TNF- $\alpha$  in the serum and total NOS activity in the aorta were determined in 150  $\mu\text{Ci}$  group, compared to controls. The mRNA and protein expression of endothelial, neuronal, and inducible NOS (eNOS, nNOS, and iNOS) in the rat aorta was also estimated, using quantitative reverse transcription polymerase chain reaction and Western blot, respectively. The levels of IL-6 and TNF- $\alpha$  increased in 150  $\mu\text{Ci}$  group; the results were significant at the end of month 2 and 4 for IL-6, and at all time points for TNF- $\alpha$ . The levels of NO decreased significantly at the end of month 2 and 4 in 150  $\mu\text{Ci}$  group. The total NOS activity increased significantly in 150  $\mu\text{Ci}$  group, at all three time points. Significant changes in the mRNA and protein expression of all three NOS isoforms were observed in 150  $\mu\text{Ci}$  group compared to controls. NO, IL-6, TNF- $\alpha$  levels and NOS activity and expression are altered in hypothyroid state, and the underlying mechanism should be further investigated.

KEY WORDS: Hypothyroidism; I-131; radioactive iodine; aorta; nitric oxide; NO; nitric oxide synthase; NOS; eNOS; nNOS; iNOS; inflammatory mediators; interleukin; IL-6; TNF- $\alpha$

DOI: <http://dx.doi.org/10.17305/bjbms.2018.2350>

*Bosn J Basic Med Sci.* 2018;18(4):305-312. © 2018 ABMSFBH

## INTRODUCTION

In many countries, radioactiveiodine ( $^{131}\text{I}$  or I-131) has been utilized for more than 60 years; for example in China, I-131 treatment has been in use since 1958 [1]. Moreover, I-131 is the first choice for the treatment of hyperthyroidism in adults in European countries and the USA. Hypothyroidism is a major complication of I-131 treatment. The cumulative incidence of hypothyroidism, in I-131 treated patients with hyperthyroidism caused by Graves' disease, has been gradually increasing each year, reaching around 80% in adults and

95% among children and adolescents [2-4]. Hypothyroidism is a relatively common endocrine disease. In a general population, the occurrence of hypothyroidism is 10% [5]; more than 95% of the affected individuals have primary hypothyroidism, most commonly caused by I-131 treatment of hyperthyroidism. Accumulating evidence has shown that hypothyroidism seriously affects the cardiovascular system, significantly increasing the incidence of cardiovascular diseases [6, 7]. Both subclinical hypothyroidism and hypothyroidism decrease the cardiac output, increase the peripheral resistance, and impair the endothelial function [6,8]. Impaired endothelial function is associated with decreased vasodilation [9]. The vascular endothelium, as the interface between the blood and vessel walls, plays a major role in regulating vascular structure and function [10].

Impaired nitric oxide (NO) bioavailability, due to reduced production of NO by nitric oxide synthase (NOS) or its

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increased breakdown by reactive oxygen species (ROS), is commonly linked to endothelial dysfunction [11]. Other studies confirmed that NO is one of the most important factors of vasodilation. NO biological half-life is only 4 to 8 seconds, and it participates in the function of cardiovascular system in the following ways: regulation of vascular tension, anti-oxidative, anti-inflammatory, and anticoagulant effect, fibrinolytic action, inhibition of leukocyte adhesion and migration, inhibition of proliferation and migration of smooth muscle cells, and inhibition of platelet aggregation and adhesion [10].

Three isoforms of NOS are known in mammals, encoded by different genes: 1) endothelial NOS (eNOS or NOS<sub>3</sub>) is mainly present in the endothelium, and is encoded by the *NOS3* gene located on 7q35-36; 2) neuronal NOS (nNOS or NOS<sub>1</sub>), encoded by the *NOS1* gene located on 12q24.2, is expressed predominantly in nervous tissue and skeletal muscle; and 3) inducible NOS (iNOS or NOS<sub>2</sub>) encoded by the *NOS2* gene (17q11.2) has a role in the immune and cardiovascular system. Increased NO production is induced by intracellular antigens, some tumor cells, and microbial products as well as in abnormal physiological conditions, such as heart failure and inflammation. Moreover, all three NOS isoforms are expressed in other tissue/cell types, and many tissues express more than one isoform. For example, nNOS has also been identified in the spinal cord, sympathetic ganglia, adrenal glands, epithelial cells of different organs, kidney macula densa cells, pancreatic islet cells, and vascular smooth muscle. eNOS was also detected in platelets, some neurons in the brain, human placenta, and in kidney tubular epithelial cells. In addition, the three NOS isoforms are expressed in myocardial cells and play a critical role in the cardiovascular system [12,13].

Inflammatory mediators, such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF- $\alpha$ ), have been associated with hypothyroidism [14]. IL-6, a pleiotropic cytokine released by fibroblasts, T lymphocytes, endothelial cells, and monocytes, has multiple biological activities in different target cells. Furthermore, IL-6 has a crucial role in vascular inflammation. Also, a previous study showed that the concentration of IL-6 is increased in hypothyroid rats [14,15]. TNF- $\alpha$ , a cytokine mainly expressed by monocyte-macrophage cells, is activated in patients with thyroid dysfunction; a high concentration of TNF- $\alpha$  has been demonstrated in patients with hypothyroidism [16].

In the present study, we investigated the effect of <sup>131</sup>I-induced hypothyroidism on NO, total NOS activity and expression of NOS isoforms as parameters of vascular function, and on IL-6 and TNF- $\alpha$  as indicators of inflammation, in rats. Rats were intraperitoneally (i.p.) injected with <sup>131</sup>I to establish a model of hypothyroidism. According to the literature, the dosage of <sup>131</sup>I ranged from 50-450  $\mu$ Ci and the longest exposure lasted 5 months [17-20]. In our experiments, the rats

in different groups were i.p. injected with 75  $\mu$ Ci, 150  $\mu$ Ci, 300  $\mu$ Ci, or 450  $\mu$ Ci <sup>131</sup>I. The weight of the animals, thyroid uptake (counts per minute [CPM]), and thyroid function at different time points were compared between the groups, to determine the optimal <sup>131</sup>I dose for the model. In the selected rat model of hypothyroidism, we assessed the level of NO, IL-6, and TNF- $\alpha$  in the serum and total NOS activity in the aorta at different time points. Finally, we estimated the mRNA and protein expression of eNOS, nNOS, and iNOS in the rat aorta, using qRT-PCR and Western blot, respectively.

## MATERIALS AND METHODS

### Reagents

The enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and TNF- $\alpha$  were obtained from Neobioscience Co., Ltd. (Guangdong, China). Kits for the detection of total NOS activity and NO were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-eNOS, anti-iNOS, and anti-nNOS antibodies were obtained from Bioss Biotechnology Co., Ltd. (Beijing, China). TRIzol reagent was obtained from Tiangen Biotechnology Co., Ltd. (Beijing, China). PrimeScript™ RT reagent kit with gDNA Eraser, SYBR Premix Ex Taq™ II (Tli RNaseH Plus), ROX plus, and DL2000 DNA marker were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Primers for NOS genes (Table 1) were synthesized by Invitrogen (Shanghai, China). Rat anti-NOS monoclonal antibody and horseradish peroxidase (HRP)-conjugated anti-rat secondary antibody were obtained from Zhongshan Golden Bridge Biotechnology Co., Ltd (Beijing, China). Triiodothyronine (T<sub>3</sub>), thyroxine (T<sub>4</sub>), and thyroid stimulating hormone (TSH) kits were obtained from Beijing kit-guide Bo High Biological Technology Co., Ltd. (Beijing, China).

### Experimental groups and treatment

Forty-five male Sprague-Dawley rats, aged 4 weeks and weighing 80-100 g, were obtained from the Department of Laboratory Animal Center of the Chongqing Medical

**TABLE 1.** Primers sequences for nitric oxide synthase (NOS) genes

Genes	Primers sequence
<i>NOS3</i>	F: ATCCTGGCAGCCCTAAGACCTATGT R: GAGTCCGAAAATGTCCCTCGTGGTAG
<i>NOS1</i>	F: GCTCCTCTGGCTCAACAGAATACA R: CACGGATTTCCTCTTTGTGTTGG
<i>NOS2</i>	F: CAGCATCCACGCCAAGAACG R: CACAGTTTGGTCTGGCGAAG
<i>GAPDH</i>	F: TTCCTACCCCAATGTATCCG R: CCACCCTGTTGCTGTAGCCATA

NOS3 or eNOS: endothelial NOS; NOS 1 or nNOS: neuronal NOS; NOS2 or iNOS: inducible NOS; GAPDH: glyceraldehyde 3-phosphate dehydrogenase

University. The guidelines of Chongqing Medical University for the care and use of animals, followed in this study, were approved by the Chongqing science and technology commission (SYXK2007-0001, SCXK2007-0001, and SCXK2007-0002). The animals were housed and fed for 14 days under a 12-hour light/dark cycle at an appropriate temperature and humidity. The rats were randomly assigned to 5 groups ( $n = 9$  divided at three time points for each  $^{131}\text{I}$  dose): control group, and groups with 75  $\mu\text{Ci}$ , 150  $\mu\text{Ci}$ , 300  $\mu\text{Ci}$ , and 450  $\mu\text{Ci}$  of  $^{131}\text{I}$  injected i.p. Following the administration of  $^{131}\text{I}$ , the rats were euthanized (at the end of month 1, 2, and 4;  $n = 3$  for each time point) for biochemical analysis of serum, aorta, and thyroid glands.

### Weight measurement

Before the euthanization, the rats were weighed to determine the difference in weight between different time points in each group.

### Blood and tissue samples

At each time point, an appropriate amount of chloral hydrate was injected i.p. in rats as an anesthetic. The blood was collected by puncture of the left ventricle, left for 30 minutes without anticoagulant and followed by centrifugation for 15 minutes at 3000 r/min to obtain the serum. The serum samples were stored at  $-80^\circ\text{C}$ . After collecting the blood, the right atrium was punctured to clean immediately the blood. Then, the aortas (from the ascending to the abdominal aorta) were collected and stored at  $-80^\circ\text{C}$ . Finally, the thyroid glands were collected.

### Measurement of thyroid radioactivity

Following the collection of samples, the thyroid radioactivity was detected using a scintillator detector. The distance between the detector and tissue was 10 cm and the detection time was 1 minute. The results were recorded as CPM.

### Assessment of thyroid function, IL-6, TNF- $\alpha$ , and NO

The levels of T<sub>3</sub> and T<sub>4</sub> were determined in the plasma using the radioimmunoassay kits. TSH was measured by a solid-phase competitive chemiluminescent enzyme immunoassay using the IMMULITE 2000 analyzer (Siemens Healthcare Diagnostics, USA). Serum IL-6, TNF- $\alpha$ , and NO were measured by ELISA, according to the kit instructions. All analyses were performed in triplicate, and the mean of the three measurements was used for the statistical analysis.

### Total NOS activity

The total NOS activity was measured by the detection kit. The tissue from aorta (0.15 g) was homogenized in 1.35 g

normal saline to make 10% tissue homogenate, and centrifuged at 2500 r/min for 10 minutes. The 50- $\mu\text{L}$  supernatant was used to measure the NOS activity, protein content, and total NOS activity, according to the kit instructions.

### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The aorta tissue was ground to powder in a pre-cooled mortar. The total cellular RNA was extracted from the cells using TRIzol reagent. The samples were then purified with 75% ethanol, and the purity and concentration of RNA were determined at 260 nm with an ND-2000 NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized using a PrimeScript<sup>™</sup> RT reagent Kit with gDNA Eraser for RT-PCR. qRT-PCR was performed in a 20- $\mu\text{L}$  reaction, including 0.5  $\mu\text{L}$  forward primers and 0.5  $\mu\text{L}$  reverse primers, 2  $\times$  SYBR Green reaction mix, and 2  $\mu\text{L}$  cDNA. The PCR protocol was as follows: initial denaturation at  $94^\circ\text{C}$  for 30 seconds; 40 cycles of denaturation at  $95^\circ\text{C}$  for 15 seconds, annealing at  $60^\circ\text{C}$  for 30 seconds, and extension at  $72^\circ\text{C}$  for 30 seconds; 1 cycle at  $72^\circ\text{C}$  for 10 minutes. The melting curves were measured at the end of the amplification. All target genes (eNOS, nNOS, and iNOS) and  $\beta$ -actin gene were amplified in triplicate in a 96-well plate. Data were analyzed using the  $2^{-\Delta\Delta\text{CT}}$  method.

### Western blot analysis

The protein expression of eNOS, nNOS, and iNOS was assayed at the three time points ( $n = 3$  for each time point). Briefly, the aorta was dissected, total protein was isolated using RIPA buffer, and the protein concentration was estimated. The proteins (30  $\mu\text{g}$ ) were denatured by a standard method, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked for 1 hour at room temperature with gentle agitation. Subsequently, they were incubated overnight at  $4^\circ\text{C}$  with monoclonal antibodies against nNOS, eNOS, and iNOS at a dilution of 1:1000. Then, the membranes were washed and incubated with the secondary antibody at 1:5000, for 1 hour at room temperature in the dark, with gentle agitation. Lastly, the immunoreactive bands were developed using detection reagents.

### Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics for Windows, Version 19.0. (IBM Corp., Armonk, NY). Quantitative data were expressed as mean  $\pm$  standard deviation (SD). The statistical significance of the treatments was evaluated using Student's *t*-test and two-way ANOVA.

**TABLE 2.** Body weight of rats treated with  $^{131}\text{I}$  (n=3 per each time point, mean $\pm$ SD)

Month	Control group (g)	75 $\mu\text{Ci}$ (g)	150 $\mu\text{Ci}$ (g)	300 $\mu\text{Ci}$ (g)	450 $\mu\text{Ci}$ (g)
1	260.33 $\pm$ 17.21	281.67 $\pm$ 11.93	369.76 $\pm$ 20.01*	119.67 $\pm$ 11.68*	126.54 $\pm$ 22.48*
2	358.23 $\pm$ 17.67	370.33 $\pm$ 16.20	460.00 $\pm$ 22.91*	129.67 $\pm$ 16.09*	127.67 $\pm$ 16.07*
4	396.33 $\pm$ 6.66	407.00 $\pm$ 17.58	513.31 $\pm$ 8.89*	138.94 $\pm$ 7.55*	140.19 $\pm$ 9.64*

\* $p < 0.05$  vs. control group

The differences were considered statistically significant at  $p < 0.05$ .

## RESULTS

### Rat body weight

As shown in Table 2, no differences were observed in the body weight of rats between control and 75  $\mu\text{Ci}$  group. Compared to control group, the body weight of rats in 150  $\mu\text{Ci}$  group was significantly increased ( $p < 0.05$ ), while the weight was decreased in 300  $\mu\text{Ci}$  and 450  $\mu\text{Ci}$  groups ( $p < 0.05$ ).

### Thyroid radioactivity in $^{131}\text{I}$ -treated rats

The thyroid radioactivity gradually increased from the low-dose to high-dose group after the  $^{131}\text{I}$  administration at the end of month 1, and was markedly decreased in each dose group at the end of month 2 and 4; most of the radioactivity values being similar to the natural background value (Table 3).

### Thyroid function in $^{131}\text{I}$ -treated rats

The thyroid function did not differ between control and 75  $\mu\text{Ci}$  group. In the other groups, serum T<sub>3</sub> and T<sub>4</sub> levels decreased gradually with increasing doses of  $^{131}\text{I}$ , and the results were statistically significant compared to control group ( $p < 0.05$ ). On the contrary, serum TSH significantly increased with the increasing doses of  $^{131}\text{I}$ , compared to control group [ $p < 0.05$ ] (Table 4). According to our results, 150  $\mu\text{Ci}$  dose of  $^{131}\text{I}$  was optimal and used for further experiments.

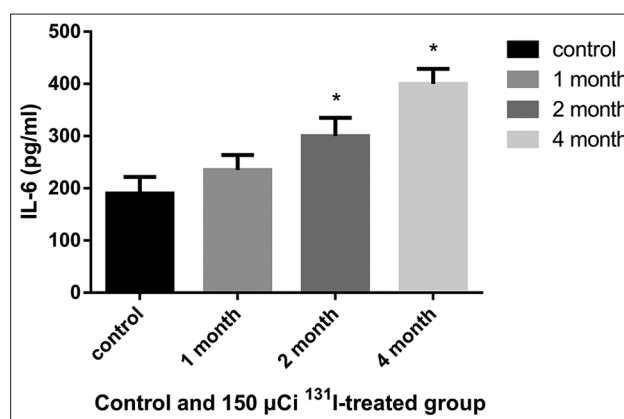
### Serum IL-6 and TNF- $\alpha$ levels in $^{131}\text{I}$ -treated hypothyroid rats

Figures 1 and 2 indicate the serum levels of IL-6 and TNF- $\alpha$ , respectively, of hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$ . Compared to control group, the increase in IL-6 levels was significant in the treated hypothyroid rats ( $p < 0.05$ ), at the end of month 2 and 4; in addition, the prolonged exposure to  $^{131}\text{I}$  led to higher levels of IL-6. The levels of TNF- $\alpha$  were also significantly increased in the treated hypothyroid rats compared to control group ( $p < 0.05$ ), at all three time points; the levels of TNF- $\alpha$  at the end of month 2 and 4 were higher compared to those at the end of the 1<sup>st</sup> month.

**TABLE 3.** Thyroid radioactivity (counts per minute [CPM]) of rats treated with  $^{131}\text{I}$  (n=3 per each time point, mean $\pm$ SD)

Month	75 $\mu\text{Ci}$	150 $\mu\text{Ci}$	300 $\mu\text{Ci}$	450 $\mu\text{Ci}$
1	1956.33 $\pm$ 62.28	4529.00 $\pm$ 89.63	8999.66 $\pm$ 328.56	12712.72 $\pm$ 646.274
2	-*	-	576.83 $\pm$ 14.53	832.33 $\pm$ 250.14
4	-	-	-	-

\*The radioactivity was not detectable



**FIGURE 1.** Levels of serum interleukin 6 (IL-6) in hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$ . Compared to control group, the increase in IL-6 levels was significant in the treated hypothyroid rats, at the end of month 2 and 4; moreover, the prolonged exposure to  $^{131}\text{I}$  led to higher levels of IL-6. \* $p < 0.05$  vs. control group.

### Serum NO levels and total NOS activity in the aorta of $^{131}\text{I}$ -treated hypothyroid rats

Figure 3 shows that the serum levels of NO decreased significantly at the end of month 2 and 4 in hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$ , compared to control group ( $p < 0.05$ ); however, at the end of month 1, the NO levels were distinctly higher in the treated hypothyroid rats compared to control group ( $p < 0.05$ ). Figure 4 indicates that the total NOS activity in the aorta increased significantly in hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$ , compared to control group ( $p < 0.05$ ), at all three time points. The total NOS activity was higher with the prolonged exposure to  $^{131}\text{I}$ .

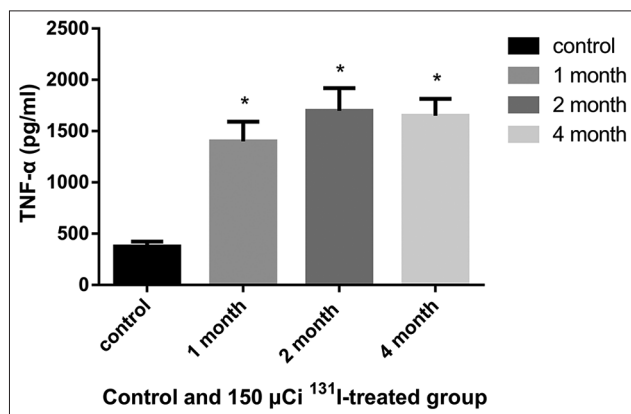
### eNOS, nNOS, and iNOS gene expression in the aorta of $^{131}\text{I}$ -treated hypothyroid rats

Compared to control group (all  $p < 0.05$ ), the mRNA expression of eNOS increased in 150  $\mu\text{Ci}$  group at the end of month 1 (Figure 5A), but decreased at the end of month 2 and 4; the eNOS gene expression was the lowest after 4 months. However,

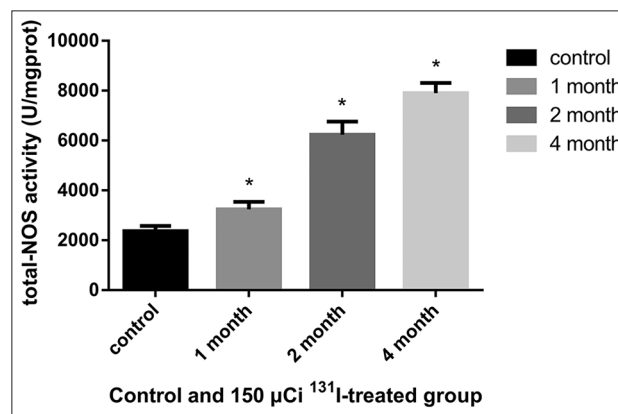
**TABLE 4.** Thyroid function of rats treated with  $^{131}\text{I}$  (n=3 per each time point, mean $\pm$ SD)

Thyroid hormone	Month	Control group	75 $\mu\text{Ci}$	150 $\mu\text{Ci}$	300 $\mu\text{Ci}$	450 $\mu\text{Ci}$
T3 (ng/mL)	1	1.15 $\pm$ 0.11	1.04 $\pm$ 0.30	0.83 $\pm$ 0.03*	0.58 $\pm$ 0.70*	0.49 $\pm$ 0.04*
	2	1.21 $\pm$ 0.11	1.21 $\pm$ 0.82	0.65 $\pm$ 0.15*	0.38 $\pm$ 0.09*	0.31 $\pm$ 0.02*
	4	1.17 $\pm$ 0.10	1.16 $\pm$ 0.14	0.58 $\pm$ 0.08*	0.35 $\pm$ 0.21*	0.32 $\pm$ 0.05*
T4 (pmol/L)	1	13.82 $\pm$ 0.28	12.55 $\pm$ 1.34	9.23 $\pm$ 2.11*	7.73 $\pm$ 0.93*	5.90 $\pm$ 1.22*
	2	13.36 $\pm$ 0.57	12.77 $\pm$ 0.98	7.01 $\pm$ 1.05*	6.24 $\pm$ 1.31*	5.35 $\pm$ 0.10*
	4	13.57 $\pm$ 0.54	12.92 $\pm$ 0.83	6.05 $\pm$ 0.31*	5.62 $\pm$ 0.82*	5.39 $\pm$ 0.16*
TSH (mIU/L)	1	7.72 $\pm$ 0.32	9.14 $\pm$ 0.58*	15.04 $\pm$ 2.02*	19.13 $\pm$ 4.01*	20.91 $\pm$ 3.20*
	2	8.03 $\pm$ 0.93	8.34 $\pm$ 1.56	19.12 $\pm$ 2.25*	21.55 $\pm$ 0.56*	23.46 $\pm$ 2.62*
	4	8.12 $\pm$ 0.23	8.19 $\pm$ 0.53	19.61 $\pm$ 0.79*	21.90 $\pm$ 2.68*	23.52 $\pm$ 4.65*

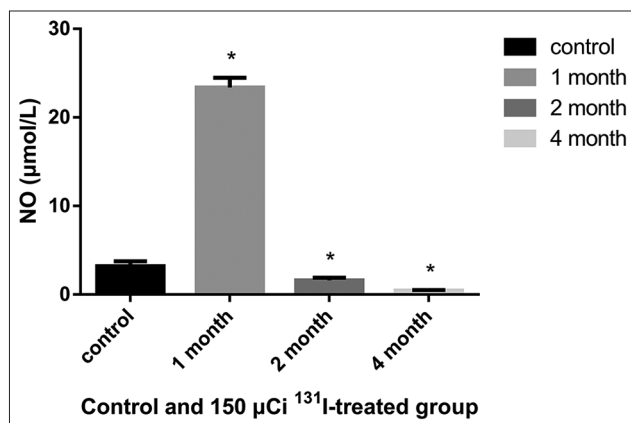
\*  $p < 0.05$  vs. control group. T3: Triiodothyronine; T4: Thyroxine; TSH: Thyroid-stimulating hormone



**FIGURE 2.** Levels of serum tumor necrosis factor alpha (TNF- $\alpha$ ) in hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$ . The levels of TNF- $\alpha$  were significantly increased in the treated hypothyroid rats compared to control group, at all three time points; the levels of TNF- $\alpha$  at the end of month 2 and 4 were higher compared to the levels at the end of month 1. \* $p < 0.05$  vs. control group.



**FIGURE 4.** Total nitric oxide synthase (NOS) activity in the aorta of hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$ . The total NOS activity in the treated hypothyroid rats increased significantly compared to control group. \* $p < 0.05$  vs. control group.



**FIGURE 3.** Serum levels of nitric oxide (NO) in hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$ . The levels of NO decreased significantly at the end of month 2 and 4 in the treated hypothyroid rats, compared to control group; however, at the end of month 1, the NO levels were significantly higher in the treated hypothyroid rats compared to controls. \* $p < 0.05$  vs. control group.

we obtained completely the opposite results for nNOS (Figure 5B). The nNOS mRNA expression was decreased at the end of month 1 and increased at the end of month 2 and 4 in 150  $\mu\text{Ci}$  compared to control group (all  $p < 0.05$ ). After 4 months, the expression of nNOS was the highest. The expression of iNOS increased with the prolonged exposure to  $^{131}\text{I}$  (Figure 5C),

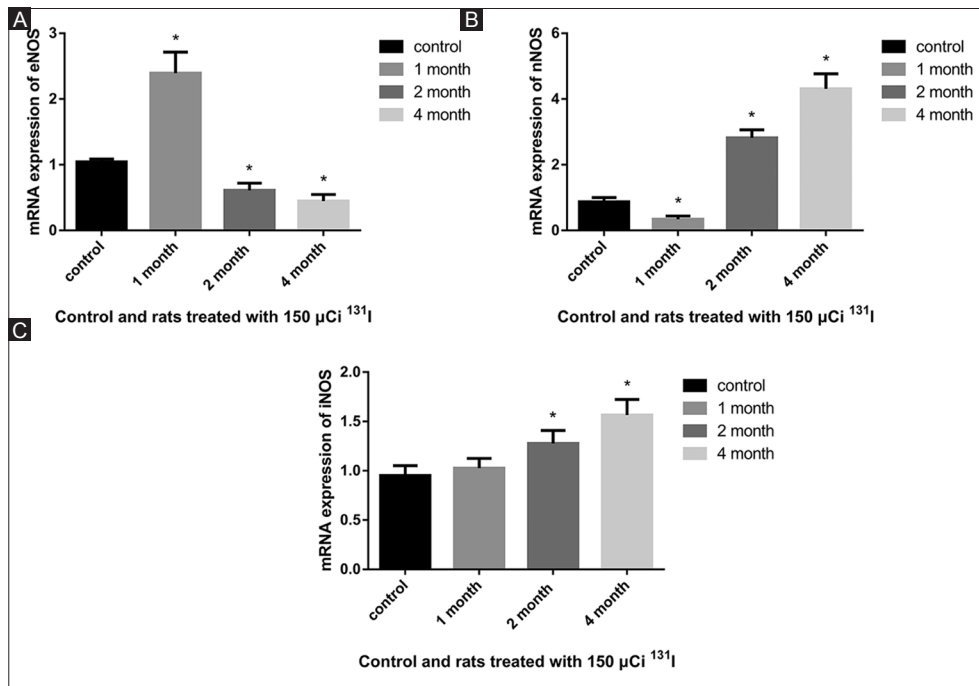
and it was significantly higher in 150  $\mu\text{Ci}$  group at the end of month 2 and 4, compared to control group (all  $p < 0.05$ ).

eNOS, nNOS, and iNOS protein levels in the aorta of  $^{131}\text{I}$ -treated hypothyroid rats

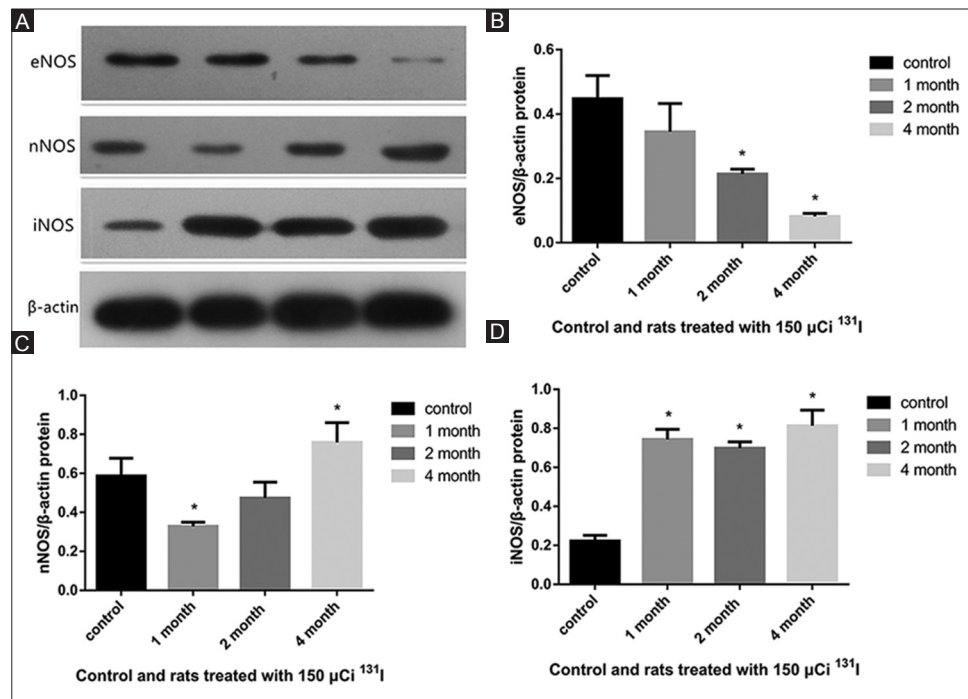
The results of Western blot analysis of eNOS, nNOS, and iNOS in hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$  are shown in Figure 6A. The eNOS protein levels decreased in 150  $\mu\text{Ci}$  group compared to control group (Figure 6B); the results were significant except for the group at the end of month 1 ( $p > 0.05$ ). Compared to control group, the protein levels of nNOS in 150  $\mu\text{Ci}$  group were markedly decreased at the end of month 1 (Figure 6C). The levels of nNOS were gradually increased at the end of month 2 and 4, compared to the 1<sup>st</sup> month, and the differences were significant at the end of month 1 and 4 in relation to control group ( $p < 0.05$ ). As shown in Figure 6D, the protein levels of iNOS in 150  $\mu\text{Ci}$  group were significantly increased compared to control group at all time points ( $p < 0.05$ ).

## DISCUSSION

In previous studies, chronic hypothyroidism was induced using antithyroid drugs (e.g., methimazole [MMI] and



**FIGURE 5.** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of mRNA expression of endothelial nitric oxide synthase [eNOS] (A), neuronal NOS [nNOS] (B), and inducible NOS [iNOS] (C) in the aorta of hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$  at different time points. Compared to control group, the mRNA expression of eNOS increased in 150  $\mu\text{Ci}$  group at the end of month 1, but decreased at the end of month 2 and 4; the eNOS gene expression after 4 months was the lowest. The nNOS mRNA expression was decreased at the end of month 1 and increased at the end of month 2 and 4 in 150  $\mu\text{Ci}$  compared to control group. After 4 months, the expression of nNOS was the highest. The expression of iNOS increased with the prolonged exposure to  $^{131}\text{I}$ , and it was significantly higher in 150  $\mu\text{Ci}$  group at the end of month 2 and 4, compared to control group. \* $p < 0.05$  vs. control group.



**FIGURE 6.** Western blot analysis of endothelial nitric oxide synthase [eNOS], neuronal NOS [nNOS], and inducible NOS [iNOS] in the aorta of hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$  at different time points (A). The eNOS protein levels decreased in 150  $\mu\text{Ci}$  compared to control group (B). The results were significant except for the group at the end of month 1. Compared to control group, the protein levels of nNOS in 150  $\mu\text{Ci}$  group were markedly decreased at the end of month 1 (C). The levels of nNOS were gradually increased at the end of month 2 and 4, compared to the 1<sup>st</sup> month, and the differences were significant at the end of month 1 and 4 in relation to control group. The protein levels of iNOS in 150  $\mu\text{Ci}$  group were significantly increased compared to control group, at all three time points (D). The NOS protein expressions were compared to  $\beta$ -actin. The results are presented as mean  $\pm$  SD ( $n = 3$  per group). \* $p < 0.05$  vs. control group.

propylthiouracil [PTU]), which reduce the production of thyroid hormones [21-23], while acute hypothyroidism was induced

by thyroid resection [24]. Comparable to the above-mentioned chronic model, in this study, we induced hypothyroidism in rats

by  $^{131}\text{I}$ , and a similar approach is used in clinical setting in the treatment of hyperthyroidism with  $^{131}\text{I}$ . The radioactive iodine is taken up by thyroid cells which leads to their death and decreased levels of thyroid hormone. However, hypothyroidism as well as radiation from  $^{131}\text{I}$  also affect the expression of NOS in those patients. Several studies reported the dosage of  $^{131}\text{I}$  ranging from 50  $\mu\text{Ci}$  to 450  $\mu\text{Ci}$ , and the most effective doses ranged between 75-277  $\mu\text{Ci}$  [17-20]. In this study, we tested 75  $\mu\text{Ci}$ , 150  $\mu\text{Ci}$ , 300  $\mu\text{Ci}$ , and 450  $\mu\text{Ci}$  dose of  $^{131}\text{I}$ , and 150  $\mu\text{Ci}$  was selected as the optimal dose for establishing the model of hypothyroidism in rats.

Similar to our results, a correlation between hypothyroidism and low-grade inflammation was indicated previously [14,25]. Levothyroxine (L-T4) treatment of hypothyroid rats markedly decreased the elevated serum levels of TNF- $\alpha$  and IL-6 [1]. Marfella *et al.* [26] also observed significantly lower plasma TNF- $\alpha$  and IL-6 levels in patients with subclinical hypothyroidism treated with L-T4 compared to the untreated individuals [26]. However, only a few studies observed those changes over a prolonged period. As shown in Figures 1 and 2, compared to control group, the levels of IL-6 and TNF- $\alpha$  increased with longer duration of the  $^{131}\text{I}$  treatment. Although an increase of TNF- $\alpha$  and IL-6 was also induced by the exposure of rats to gamma radiation [27], we showed that at the end of month 2 and 4, when the CPM was similar to the natural background value, the levels of TNF- $\alpha$  and IL-6 were still continually increasing, indicating that the elevated levels of the pro-inflammatory cytokines are probably associated with hypothyroidism. In addition, increased levels of TNF- $\alpha$  and IL-6 have been suggested as a risk factor for adverse cardiovascular events [14,28].

In our study, the hypothyroid rats had lower serum NO levels at the end of month 2 and 4 compared to controls, while the NOS activity in the aorta was increased at all three time points; moreover, the prolonged exposure to  $^{131}\text{I}$  led to higher NOS activity. Lower NO levels in hypothyroid rats were also shown in another study [29], while other authors reported that radiation could increase the level of NO [30-32]. A markedly higher NO level observed in our study after 1 month of  $^{131}\text{I}$  treatment may be due to the radiation injury induced by  $^{131}\text{I}$ . Namely, the effect of  $^{131}\text{I}$  is stronger at 1 month and it gradually weakens in the subsequent period. In line with our results, higher NOS activity in the aorta of hypothyroid rats was demonstrated in another study [33], but several other studies showed lower NOS activity in hypothyroid rats [22-24]. This discrepancy may, at least partially, be related to the age and strain of animals, methodological differences, changes in the expression of the NOS isoforms, or to the altered NOS activity in subcellular fractions [23,33,34]. Nevertheless, in this study, we did not find a significant correlation between NO levels and NOS activity.

After the administration of  $^{131}\text{I}$  in our study, the gene expression of eNOS decreased in hypothyroid rats, except in the first

month, the expression of nNOS was the opposite to eNOS, while the gene expression of iNOS continuously increased over the three time points. The protein levels of eNOS decreased significantly in the hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$ , compared to control group. The nNOS protein levels in the treated hypothyroid rats were drastically decreased at the end of month 1, then gradually increased at the end of month 2 and 4. The protein levels of iNOS in 150  $\mu\text{Ci}$  group were significantly increased compared to control group, at all three time points. The few differences between our qRT-PCR and Western blot results may be attributable to the method of hypothyroidism induction, hypothyroid state, or radiation effect. Only a few other studies investigated the eNOS, iNOS, and nNOS levels in the aorta of hypothyroid rats, using qRT-PCR and Western blot, with variable results. In agreement with our results, two studies showed decreased levels of eNOS in the aorta of hypothyroid rats [22,35]. However, one of those studies [35] showed the opposite results for iNOS levels. In the study of Sarati *et al.* [33] adult hypothyroid rats had higher eNOS and iNOS protein levels compared to control animals, while no difference was observed in nNOS protein levels between the two groups [33]. Moreover, McAllister *et al.* observed decreased protein expression of nNOS in their hypothyroid rats [22], contrary to our results at the end of month 2 and 4.

Overall, we observed a similar trend for NO and NOS levels based on qRT-PCR results in hypothyroid rats treated with 150  $\mu\text{Ci}$   $^{131}\text{I}$ , but some differences were noticed between the NO and eNOS at protein level, and this should be further investigated.

## CONCLUSION

Previous studies indicated the effect of thyroid hormones on the cardiovascular system. Some of our results agree with that observation, while other differ. The underlying mechanisms of the effect of hypothyroidism on the cardiovascular system need further clarification.

## DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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