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Study Histological changes and oxidative stress in the aorta resulting from induced hyperthyroidism in adult male rats

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***Abstract*—** This experiment was designed to identify indicators of oxidative stress and histological changes in the aortic wall of male albino rats with hyperthyroidism. The increasing prevalence of hyperthyroidism worldwide, and its direct association with higher morbidity and mortality rates when left untreated, has made it a highly important focus of histological and pathological studies. twelve adult male white rats, were used and divided into a control group, which had free access to food and water, and an experimental group, which was given 1.5 mg/kg of body weight levothyroxine (L-T4) orally. The experiment lasted 30 days, after which the animals were sacrificed, blood was drawn to examine indicators of oxidative stress, the aortas was also excised and prepared for examination There is a decrease in the concentration of antioxidants (SOD, GSH,CAT) and an increase in the level of (MDA), as well as an increase in thyroid hormones (T3,T4) and a decrease in (TSH) hormone. There are clear histological changes in the layers of the aortic wall in the groups affected by hyperthyroidism, The most important changes are an increase in the thickness of the arterial wall, degeneration of smooth muscle cells, and enlargement of the nuclei in the case of routine staining. However, the characteristic changes in the case of Masson's trichrome staining are an increase in the deposition of collagen fibers in the middle and outer tunica.

Keywords **—** Thyroid gland, Levothyroxine , Hyperthyroidism ,Oxidative stress , Aorta , Histopathology , Masson’s trichrome.

INTRODUCTION

The thyroid gland is one of the most important endocrine glands in the human body and mammals in general, as it plays an important role in regulating metabolic processes, thermal balance, growth, and the functions of vital organs through the secretion of the hormones thyroxin (T4) and triiodothyronine (T3). The activity of this gland depends on an integrated axis that begins from the hypothalamus and then the anterior pituitary gland via the thyroid-stimulating hormone (TSH) (1,2)

An increase in the levels of these hormones leads to hyperthyroidism, one of the most common endocrine disorders. This condition accelerates metabolism and disrupts the function of many systems, including the cardiovascular, nervous, reproductive, and skeletal systems. It is accompanied by a range of symptoms such as an increased basal metabolic rate, weight gain, rapid heartbeat, and elevated levels of anxiety and nervous tension. High levels of thyroid hormones also contribute to increased cellular oxygen consumption and higher production of free radicals, leading to oxidative stress. This oxidative stress is a major factor in the pathological changes associated with hyperthyroidism, which in turn is a primary cause of tissue damage in blood vessels (3,4).

The aorta, the largest arterial vessel in the circulatory system, has a wall composed of three main layers: the inner tunica (Intima), made up of flat epithelial cells; the middle tunica (Media), rich in smooth muscle cells and elastic fibers; and the outer tunica (Adventitia) , made up of connective tissue and vessels that nourish the artery. Thyroid hormones have a direct effect on the cardiovascular system, as an increase in them leads to an increase in metabolic activity and oxygen consumption, which increases the production of free radicals inside the vessels, leading to a defect in the formation of these blood vessels (5,6).

The function of the aorta depends on this tissue structure to maintain vascular elasticity and resist circulatory pressure. Therefore, any disturbance in the composition of the layers leads to a defect in vascular elasticity and an increase in arterial stiffness, by increasing collagen deposition (7). Aim of Study: Study of oxidative stress markers and histological changes of the aortic wall using hematoxylin-eosin and Masson’s

trichrome stains in male albino rats induced with hyperthyroidism.

MATERIALS AND MTHODS

Experimental Animals

Twelve mature male Swiss albino rats about 10-13 weeks, weighing 200-250 gm. were obtained from The College of Pharmacy, University of Karbala , Iraq , , aged about 10-12 weeks. The animals were maintained under laboratory conditions at a temperature of (22±5) light exposure time and darkness (12/12) hours, and relative humidity (50-55%), inside metal –covered plastic cages measured (40×20×20 cm) with sawdust flooring. Rats were included in the experiment after two weeks of adaptation and ensure that they were clear of diseases.

Induction of Hyperthyroidism

The German-made drug Levothyroxine (L-T4), produced by Berlin-Chemie AG was used to induce hyperthyroidism. The animals were administered via a gavage tube using a syringe, at a dose of 1.5 mg/kg for 30 days, with the drug tablets being dissolved in water according to the animal's weight (according to the initial experiment). After two weeks of dosing, blood samples were drawn to confirm the occurrence of hyperthyroidism.

Study Design

First Experiment: Estimating the Effective Dose of Levothyroxine

The results of the current study, when evaluating the effectiveness of a levothyroxine dose in inducing hyperthyroidism and its effect on several physiological parameters among three effective concentrations of the drug (1.50, 1.25, and 1) mg/kg of body weight for thirty days, showed significant differences at the level (P≤0.05). Specifically, there was a significant increase (P≤0.05) in the levels of the hormones T3 and T4 in the second and third efficacy groups (G4 and G3) at concentrations of 5.26 and 19.31 mg, respectively, compared to the control group (G1). However, the third group (G4), which represents the dose group of 1.5 mg/kg of body weight, showed statistically higher levels compared to the second prevention group (G3). Meanwhile, there were slight significant differences (P≤0.05) in the levels of the hormones T4 and T3. In the first activity group (G2) compared to the control group (G1).

The highest significant increase (P≤0.05) in MDA activity was observed in the third activity group (G4), representing a dose of 1.5 mg/kg of body weight, reaching 55.20 compared to the control group (G1).

In addition, a significant decrease (P≤0.05) in SOD enzyme levels was observed in all three activity groups compared to the control group. The third activity group (G4) recorded the largest significant decrease (approximately 43.11) compared to the control group (G1). The results in Table 1 below can be used as an indicator for selecting the effective drug dose.

Table 1. shows the effect of three concentrations of levothyroxine on the activity level of malondialdehyde (MDA), SOD enzyme, and thyroid hormones (T3) and (T4) in the blood serum of male albino rats.

	T3 moI/L	T4 g/dL	MDA nmol/l	SOD U/ml
Control group	1.44 ±0.01 D	5.58 ±0.15 D	41.6 0.60± C	54.28 ±0.55 A
Group 1 active ingredient (1 mg/kg)	2.61 ±0.18 C	8.86 ±0.13 C	45.07 ± 1.37 B	50.51 ±0.32 B
Group 2 active ingredient (1 .25 mg/kg)	3.94 ±0.01 B	15.10 ±0.45 B	5.124 0.25± B	46.88 ±1.54 C
Group 3 active ingredient (1.5 mg/kg)	5.26 ±0.27 A	19.31 ±0.40 A	55.20 ±1.08 A	43.11 ±0.15 D
L.S.D 0.05	0.55	.0588	.0547	.7485

The animals were randomly divided into two equal groups(N = 12).

Control group (N=6): did not undergo any procedures.

Hyperthyroidism group (N=6): Orally administered a dose of 1.5 mg /gm of body weight of levothyroxine (L-T4) dissolved in water for 30 days.

Blood was drawn from the above-mentioned experimental animals directly from the heart. After the animals fasted overnight before blood collection began, the blood was placed in tubes without anticoagulant. The blood was then separated by centrifugation at 3000 (RPM) for 15 minutes. The serum was stored in the refrigerator at 4ºC to measure the following parameters: SOD,GSH,CAT,MDA,T3,T4,TSH

Thyroid hormones T3, T4 and TSH were measured using the MiniVidas device and following the instructions attached to the testing kit (8). MDA levels were estimated using the modified method (9).

The SOD level was measured using the photochemical reaction method (10) ,and the GSH level was measured using the method, and catalase activity was assessed using the colorimetric method (11).

Tissue Preparation

At the end of the experiment, the animals were sacrificed. The thoracic cages were opened through a midline incision of the body, then the heart and aorta were dissected and washed well with water before being placed in formalin. The tissue sections were prepared and stained with H&E stain, as well as Mason's trichrome stain (12,13), and then the slides were examined with a light microscope.

STATISTICAL ANALYSIS

The results of the study were analyzed using the SPSS version 26 program in order to find out the significant differences between the averages, and the arithmetic mean

and standard error were calculated using One-way analysis of variance (ANOVA) with the calculation of the least significant difference (LSD), and the significant differences between the averages were determined at the probability level ($P \leq 0.05$).(14, 15).

RESULT AND DISCUSSION

The effect of hyperthyroidism on antioxidants and MDA. The results of the current study showed clear significant differences, as we observed a significant decrease ($p<0.05$) in the level of (SOD, GSH, CAT) concentrations in the hyperthyroidism group G2 compared to the control group G1. We also note a significant increase($p<0.05$) in the level of MDA concentration in the G2 compared to the first control group G1. (Table 2).

Table 2.shows oxidative stress biomarkers of Levothyroxine

Variables Groups	SOD U/mI	GSH µg/L	CAT KU/L	MDA nmoI/L
Control group (G1)	55.40 ±0.98 A	48.87 ±0.78 A	30.48 ±0.42 A	41.90 0.35 B
Hyperthyroidism group (G2)	42.84 ±0.66 B	40.05 ±0.99 B	25.00 ±0.51 B	56.21 ±0.46 A

Different letters within column indicating of significant differences ($p<0.05$)

Hyperthyroidism induced by administering levothyroxine to animals at a dose of (1.5 mg/kg) leads to an increase hormone secretion T3 and T4, which in turn increases the rate of cellular metabolism and oxygen consumption, leading to an increase in the production of free radicals (ROS), and thus an imbalance in oxidative stress (16). The decrease in (SOD,GSH CAT) levels, resulting from an imbalance between free radicals and antioxidants, is due to the inability of the antioxidant system to remove all free radicals and prevent their accumulation. Elevated MDA levels are a clear indicator of lipid peroxidation in cell membranes and reflect the severity of oxidative stress , affecting blood vessel function and thus causing structural changes in the aortic wall. These results are consistent with a studies (5 , 17,18)

The effect of hyperthyroidism on thyroid hormones and (TSH).

The results of the current study showed significant differences in the level of thyroid hormones, as we observed a significant increase ($p<0.05$) in the level of T3 and T4 in the hyperthyroidism group G2compared to the control group G1. In contrast, we observed a significant decrease ($p<0.05$) in the level of TSH in the hyperthyroidism group G2 compared to the control group G1(Table.3).

Table 3. Effect of Levothyroxine on thyroid hormones and TSH

Variables Groups	T3 NmoI/L	T4 µg/dL	TSH µIU/mL
Control group (G1)	1.43±0.03 B	5.56±0.01 B	1.21±0.11 A
Hyperthyroidism group (G2)	5.29±0.13 A	19.80±0.38 A	0.05±0.00 B

Different letters within column indicating of significant differences ($p<0.05$)

The results of the current study showed that the significant increase in thyroid hormones and the decrease in TSH are indicative of the successful induction of hyperthyroidism in experimental animals based on several physiological mechanisms. Administering the drug levothyroxine daily to healthy animals for one month led to an increase in the levels of T3 and T4 hormones, which in turn inhibited the secretion of thyrotropin-releasing hormone (TRH) from the hypothalamus. Consequently, it leads to a decrease in the secretion of TSH hormone from the pituitary gland as a result of the negative feedback through which the hypothalamic-pituitary-thyroid axis regulates the secretion of thyroid hormones. This explains the decrease in this hormone in the second group of hyperthyroidism (19,20).

This physiological mechanism is for maintaining hormonal balance in the body, and the increase in metabolic activity and oxidative stress explains the accompanying biochemical and tissue changes. These results are consistent with a studies (21,22).

The effect of hyperthyroidism on the tissue structure of the aortic wall.

The Tissue sections were stained with H&E from the control group showed a typical normal structure of the aortic wall, characterized by the regularity of its three layers. The inner tunica (Intima) appeared to consist of a single layer of simple squamous epithelial cells arranged regularly over a thin basal lamina. The middle tunica (Media) the thickest and main component of the wall was characterized by parallel elastic lamina and regular collagen fibers between healthy smooth muscle cells, reflecting intact vascular elasticity. The outer tunica (Adventitia) contained wavy connective tissue rich in collagen and elastic fibers with regular nuclei and small vessels (vasa vasorum), without any pathological changes observed (Figs.1 A,B).

The aortic wall consists of basic components including elastin, collagen, and smooth muscle. These elements give the artery its distinctive mechanical properties and elasticity. The viscoelastic properties of the artery wall are a result of the interaction between the passive components of connective tissue, such as elastin and collagen fibers, and the active components resulting from the contraction of smooth muscle. The balance between these elements is necessary to maintain the

expansion and contraction of the artery wall during blood circulation (23).

The aorta is considered a flexible artery containing a high percentage of elastin fibers compared to muscular arteries, which allows it to absorb the energy produced by the heart's contraction, store it, and then release it during relaxation to maintain a regular and continuous blood flow (24,25).

Recent studies have shown that any disturbance in the ratio of elastin fibers to collagen or in smooth muscle activity can lead to pathological changes in the aortic wall, such as aneurysms or atherosclerosis) 26,27)

While The Tissue sections were stained with H&E from the levothyroxine(L-T4)-induced hyperthyroidism group showed marked pathological changes in the structure of the aortic wall compared to the control group. These changes included a significant increase in the thickness of the middle layer, hypertrophy of smooth muscle cells, widening of the spaces between their fibers, changes in the nucleus shape, and irregularity of the elastic fibers compared to the control group.(Figs 2 A,B).

This indicates that hyperthyroidism increases cardiac output and blood flow rate, and this exposes the aortic wall to continuous mechanical stress, stimulating the developmental adaptation of smooth muscle cells by increasing their hypertrophy to withstand this additional pressure. This is consistent with what was mentioned in the study(28, 29.(

Hyperthyroidism raises the rate of cellular metabolism and the production of free radicals, which leads to these tissue changes. A study (30) confirmed that hyperthyroidism is associated with a significant increase in oxidative stress and damage to vascular cells.

Studies in the major arteries have confirmed that hyperthyroidism causes deterioration in the integrity of elastic fibers, swelling of endothelial cells and increased permeability, indicating the beginning of endothelial dysfunction, an early step in vascular diseases and increased susceptibility to inflammation (4).

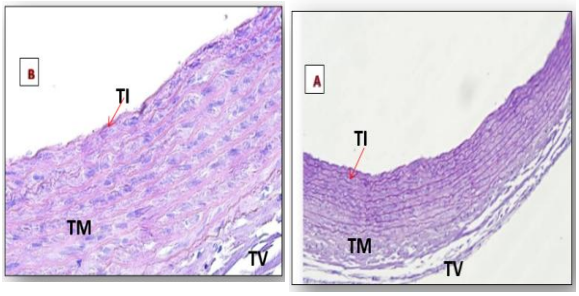

Figure 1. Histological sections the wall of aorta from the control group showing the three layers of wall : innermost thin tunica intima (TI) , in the middle the thick tunica media (TM) , in the outside tunica adventitia(TV) has a connective tissue resembling a wave.(H&E stain: A.100X,B.400X) .

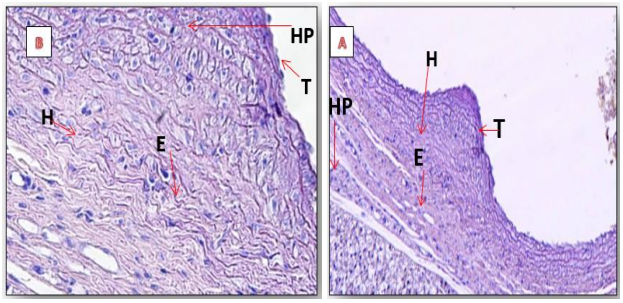

Figure 2. Histological sections of the aortic wall from the hyperthyroidism group treated with levothyroxine 1.5) mg/kg /day) , showing marked Thickening (T) of the arterial wall in tunica Intima and Hypertrophy in endothelial cell (H), irregularity in wall structure, Nuclei Enlargement(E), Hyperplasia of smooth muscle cells (HP) in tunica media , H&E stain: A.100X,B.400X)

Histological sections of the aorta stained with Massons' trichrome from the control group showed a normal vascular structure characterized by the regularity and integrity of the inner tunica (TI), free from any fibrosis or inflammation, in addition to the regularity of the middle tunica (TM) , which consists of layers of smooth muscle, elastic lamellae, and limited amounts of collagen fibers. The outer tunica (TV) is characterized by the presence the collagen fibers (31, 32).(Figs 3 A,B)

While, the tissue sections stained with levothyroxine-induced Massons' Trichrome stain revealed an increased deposition of collagen fibers within the middle layer and in adjacent areas of the outer tunica. This appeared as a dense blue discoloration and thick collagen bundles, indicating the beginning of the local fibrosis process (fibrotic remodeling) of the arterial wall. This accumulation is accompanied by the separation of the smooth muscle bundles and a change in their regularity (33).(Figs.4 A,B)

This reflects the effect of oxidative stress and high hemodynamic pressure associated with hyperthyroidism, which stimulate collagen production and activate inflammatory pathways that lead to increased arterial wall stiffness and reduced elasticity, changes that are associated with an increased risk of cardiovascular complications (34,35).

Thus, the Massons' trichrome stain is considered an indicator that levothyroxine-induced hyperthyroidism stimulates aortic fibrosis via mechanical, histological, and oxidative mechanisms (35,36).

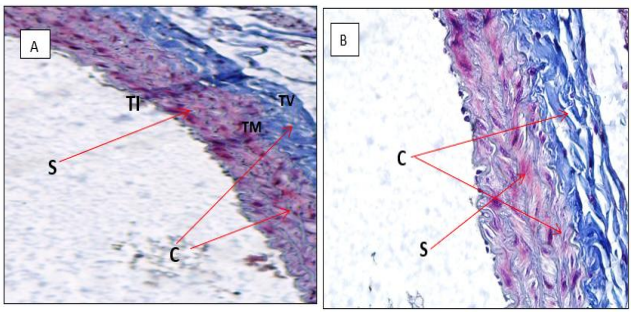

Figure 3. Histological sections the wall of aorta from the control group shows the three layers of the aorta, where we shows collagen fibers (C) colored blue in small quantities in the tunica Media with red smooth muscle fibers (S) also shows the collagen fibers in the tunica Adventitia.(Massons'trichrome stain : A. 100x,B.400x)

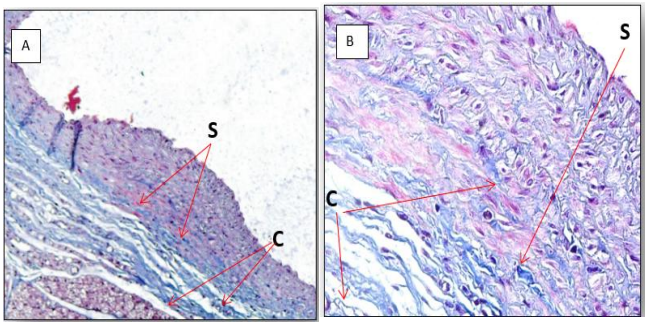

Figure 4. Histological sections the wall of aorta from the group treated with levothyroxine (1.5 mg/kg)/day, An increase in collagen fibers (C)is shows in the media and adventitia tunica, along with a decrease in smooth muscle(S).)Massons'trichrome stain : A. 100x,B.400x)

CONCLUSION

Histological results showed that levothyroxine-induced hyperthyroidism led to clear structural changes in the aortic wall, characterized by thickening of the vascular wall and disturbance of the artery structure in the case of H&E stain. Massons' trichrome dye also revealed an increase in the deposition of collagen fibers inside the wall, which indicates the occurrence of vascular fibrosis. Also ,Changes in the aortic wall were associated with thyroid gland hormones imbalance in male rats, as increased T3 and T4 hormones increase oxidative stress and free radical formation.

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