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The effects of irisin on the rat thoracic aorta: histological study

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ABSTRACT

Irisin, a polypeptide hormone that is released from skeletal muscle in response to exercise found to improve endothelial functions, protect against endothelial injuries and change blood pressure which also affected blood vessels. The aim of this study is to study the histological changes of the rat thoracic aorta in response to irisin injection. Thirty rats were used. Then divided into two groups; the control group without irisin injection, and the irisin injected group was subdivided into four subgroups with different irisin concentrations (20, 40 and 160 nM, respectively) twice a week for four weeks, the control group and each subgroup consisted of 6 rats each. After 4 weeks all rats were sacrificed, and the descending thoracic aorta was treated for histological evaluation. Sections were stained with Hematoxylin and Eosin (H and E) and orcein stains. Morphometric measurement included: intima-media thickness (IMT), number of elastic lamellae and number of smooth muscle cells nuclei. Histological study has shown that intraperitoneal injection of different concentrations of irisin (20, 40 and 160 nM) in rats has increased intima-media thickness, number of smooth muscle cell's nuclei, and increase the number of elastic lamellae in media layer of the thoracic aorta in a dose dependent manner. Irisin has significantly affected the morphology of the wall of the rat thoracic aorta indicating a role for irisin in influencing the growth factors of the thoracic aorta walls and activate smooth muscle cells in the thoracic aorta layers.

Key words: Irisin, hypertension, blood vessels, thoracic aorta, hematoxylin and eosin, orcein stain, morphometric measurement

INTRODUCTION

The aorta is considered the largest artery in the body and carries blood from the heart to the major vessels. It is considered an elastic artery with high elastic fibers in their tunics especially in tunica media. Like all other arteries, aorta is composed of 3 distinct layers; inner intimal layer, thick middle layer and outer adventitial layer. Each layer is separated from the other layer by an elastic lamina; the internal elastic lamina separates the tunica intima and media while the external elastic lamina separates the tunica media and adventitia [1]. The middle and thickest tunica media is composed mainly of smooth muscle cells and extracellular matrix (elastic fibers and collagen). The vascular smooth muscle cells (VSMCs) are responsible of contraction, production and secretion of collagen and elastic fibers [2]. The elastic fibers in tunica media are arranged in a concentric lamella, these fibers are responsible of distension, recoiling and normalize blood pressure in the aortic wall during pumping [3-5].

As known, the thickness and numbers of elastic lamellae depend on blood pressure and age. Age increases the arterial diameter, the thickness of intima and media layers, the number of sub-endothelial cells, and the medial calcifications in elastic lamellae [6]. However, it has also been shown that with age there is a decrease in smooth muscle cells in tunica media, an increase in the distance between elastic lamellae, an elastic fibers fragmentation and an extreme deposition of collagen [6-9]. Another aging change has also been reported in tunica intima and adventitia including thickening of these layers due to fibrosis [10].

The second factor affecting the aorta is blood pressure. It has been noted that there is an increase in the number and thickness of lamellae in hypertensive patients and rats [4, 11]. Under normal conditions, VSMCs are highly differentiated, have poor activity and exhibit low growth levels. On the other hand, under stress and pathological conditions (i.e. hypertension), VSMCs exhibit uncontrolled proliferation which leads to dedifferentiation and accumulation of smooth muscle cells in blood vessels' walls causing media thickening and vascular stiffness [12]. Stiffness occurs as a result of aortic elastin fibers fatigue and fragmentation [13]. Thus, hypertension seems to induce ultrastructural changes in the aorta, these changes include increase in the aortic wall, media thickness, elastic lamellae number

and thickness [11]. Proliferation of adventitial vasa vasorum also found to occur due to hypertension [14].

Irisin, a recently discovered myokine, that is released as a result of proteolytic activity of FNDC5 from skeletal muscles to the blood stream in response to exercise [15] found to have beneficial effects on regulation of the cardiovascular functions [16], enhance endothelial repair [17], protect against endothelial injury and improves atherosclerosis [18]. Moreover, irisin found to relax rats and mice mesenteric arteries through endothelium dependent and endothelium-independent mechanisms [16,19,20,21].

In this study, we hypothesized that irisin has structural effects on the thoracic aorta.

Therefore, we aimed to study the histological changes of the wall of the thoracic aorta in rats in response to irisin injection.

MATERIALS AND METHODS

Materials

Recombinant irisin (human, rat, mouse, canine) was obtained from phoenix pharmaceuticals, CA, USA., (Purity \geq 95%, Molecular Weight \sim 13 KDa). Irisin was dissolved in DMSO (the final DMSO concentration in the diluted working solution was 0.05%). Orcein, and Hematoxylin and Eosin (H&E) were obtained from SIGMA-ALDRICH, USA.

Animals

Twenty-four albino rats (120-230 g) were obtained from Jordan University of Science and Technology animal's house. All experiments have been performed on 5-7 weeks old rats, all rats were maintained in a standard condition (temperature of 20 °C) on a 12:12-h light-dark cycle, and fed a standard chow diet with a free access to water at The University of Jordan (JU) animal house. All experiments were approved by the university ethics committee for animal studies. Animals were randomly divided into 2 main groups: control group (n=6) and experimental or irisin injected group which was further subdivided into 3 subgroups (n=18, 6 in each group) according to the concentration of irisin injected into each rat (20, 40 and 160 nM, respectively or (1.3 mg/kg, 2.6 mg/kg, and 10.2 mg/kg), respectively. Rats were injected with irisin twice a week intraperitoneally (IP) for 4 weeks. After 4 weeks, all rats were sacrificed by cervical dislocation, and the descending thoracic aorta was excised

(scheme 1) and immersed in fixative (formaldehyde 10%) for histological evaluation. In a separate experiment, to eliminate the placebo effect, rats' (n=4) were injected with DMSO (0.05%). Then the thoracic aorta was taken for histological study. The results showed no histological changes in the rat thoracic aortic sections. Note: food consumption and rats' weight were measured prior and after every week of injection using a digital balance.

Light microscopy

Rats' thoracic aorta were immersed in 10% neutral-buffered formalin solution, and kept 48 hours in the fixative solution at room temperature. The samples routinely processed for paraffin sections. Sections of 5 μm thickness were mounted on glass slides and stained with (H&E) for general tissue morphology and orcein stain for elastic fibers detection. From each rat multiple sections of thoracic aorta was obtained (two ring and two longitudinal sections), refer to table 1. The staining procedure for H&E done according to leicabiosystems/staining overview guide [22]. The sections stained with newly prepared orcein stain (where 1 g orcein was dissolved in 100 ml 70% ethanol and 1 ml 25% HCl was added (according to Taenzer-Unna (Unna 1891) and as described by Romeis (1989)) [23, 24].

The prepared sections were examined and photographed using LEICA EC3 inverted light microscope equipped with LEICA CH, 9435, Switzerland camera connected to computer with LEICA application suite E2 version 1.8.0 software (LAS EZ).

Morphometric measurements

The stained sections with H&E and orcein were examined using LEICA application suite E2 version 1.8.0 software (LAS EZ) and image j software. Twenty four preparations in each stain were subjective to quantitative studies; intima-media thickness (IMT), number of elastic lamellae and number of smooth muscle cells' (SMCs) nuclei.

For the intima-media thickness measurements, the whole intima and media layer thicknesses were included in the H&E and orcein staining. The thickness was taken by drawing a line from the intimal layer (marked by luminal surface of intima) to the end of medial layer (marked by the external elastic lamina). Two lines were drawn from the luminal surface of intima to the external elastic lamina. The intima- media thickness was measured by taking the average of the two lines (in both ring and longitudinal sections) for each rat in each group and then the average of intima-media thickness of all rats was calculated and taken as final measurement. The measurements were calculated in scale bar =50 μm . The number of

elastic lamellae was measured manually by counting the elastic lamellae layers in the orcein stained segments. The total number of elastic lamellae were measured by taking the average of all elastic lamellae numbers from all segments of the same group. The number of SMCs nuclei was measured by using image j software where the section picture was opened and adjusted in a suitable threshold to be analyzed and counted.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5 for Windows (GraphPad Software, CA, USA). Statistical comparisons were obtained by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and student paired t test. A values of $P < 0.05$ were considered statistically significant. The quantitative data were examined by LEICA application suite E2 version 1.8.0 software (LAS EZ) and image j software.

RESULTS

General microscopic structure of rat thoracic aorta in control and experimental groups, as shown by using hematoxylin and eosin staining

H&E sections of the rat thoracic aorta of the control group have shown normal histological features for all layers. Tunica intima appeared as a continuous layer of endothelial cells with darkly stained nuclei. This layer was lying on an internal elastic lamina which appeared as a continuous lamina. Tunica media have shown normal, wavy multiple elastic lamellae which were concentrically arranged and normal spindle shaped SMCs seen between the concentric elastic lamellae. An external elastic lamina was also observed close to tunica adventitia as normal continuous lamina. Typical normal adventitial layer containing fibroblast, collagen and vasa vasorum has also been clearly seen (Figure 1A).

Sections of the rat thoracic aorta in all irisin-injected groups (20, 40 and 160 nM) have shown dose-dependent increase in the intima-media thickness (IMT) compared to that of the control group (from 78.3 μm in the control group, to 100.5, 103.2 and 115.4 μm , in irisin injected rats, respectively), where the increase in IMT was most evident in group 3 (160 nM). The increase was in the number of both elastic lamellae and SMCs. An increase in the number of SMCs nuclei also have been noticed (from 102 ± 20 in control group to 119 ± 33 ,

136 ± 34 and 144 ± 20 in irisin injected rats, respectively) where it was more evident in group 3 (160 nM), but this increasing is not significant (Figure 1 B, C and D), (Figure 2), (Table 2).

Histological structure of rat thoracic aorta in control and experimental groups, as shown by orcein staining

The orcein stained sections of the rat thoracic aorta of the control group have shown normal histological features for all layers. Tunica media have shown normal multiple elastic lamellae (Figure 3A). The orcein stained sections have shown dose-dependent increase in the elastic lamellae in irisin-injected groups (20, 40 and 160 nM) compared to that of the control group (8.6 layers in control rats). The increase in elastic lamellae was more evident in group 3 (dose 160 nM) with 10.5 layers. The increase was in the number of elastic lamellae (Figure 3 B, C and D), (Figure 2), (Table 2).

Body weight change and food intake results

Irisin injection did not affect food consumption in the rats. Body weights decreased in irisin injected groups and this change in rats' body weights were statistically significant (Figure 4). Where control group vs. 160 nM * $p < 0.05$, and *** $p < 0.001$ in control group vs. 30 and 40 nM).

DISCUSSION

The present study has investigated the possible histological changes of the wall of the thoracic aorta in rats injected with irisin intra-peritoneally (in vivo). Results from the study has demonstrated for the first time, an increase in the intima-media thickness (IMT) and an increase in the number of both SMCs nuclei and elastic laminae in the wall of rat thoracic aorta injected with irisin compared to non-injected (control) group under light microscope using H&E and orcein dyes.

Blood vessels are responsive to two major factors that can affect their development in healthy and pathological conditions; these factors are age and blood pressure. Age for example, increases the arterial wall diameter due to an increase in the thickness of the intima, media, and the elastic lamellae [6]. While high blood pressure causes changes in the walls of blood vessels which make them more vulnerable to develop cardiovascular diseases [6].

Elastin fibers of the aorta in hypertension become fatigued and fragmented which result in stiff aorta [13]. Thus, hypertension seems to induce ultrastructural changes in the aorta, these changes include increase in the aortic wall, media thickness, elastic lamellae number and thickness [11]. Proliferation of adventitial vasa vasorum also found to occur due to hypertension [14]. All the previously mentioned changes in response to increase in blood pressure make the heart work harder which may lead to cardiac hypertrophy [10].

Recently, irisin, an exercise-mediated polypeptide has attracted considerable attention due to its involvement in the treatment of cardiovascular and metabolic diseases [25] and its effect in lowering blood pressure [26].

A recent study has found that irisin lowered blood pressure in spontaneously hypertensive rats (SHRs) [26] indicating the possible involvement of irisin in the regulation of blood pressure. It is known that blood pressure can significantly modulate the structure of the blood vessels where an increase in the number and thickness of elastic lamellae in hypertensive patients and rats have been reported [4,11]. The present study showed by using light microscope and two different stains (H&E and Orcein for elastic tissue), the changes in the wall of thoracic aorta in response to irisin injection. The results showed a statistically significant dose dependent increase in intima-media thickness (IMT) in irisin injected groups. Also an increase in the number of elastic fibers and lamellae layers and hyperplasia of SMCs nuclei and condensation to their nuclei were also noticed in the rat thoracic aorta. Where its more prominent in rats who received the highest irisin dose 160 nM.

The increase in intima-media thickness (IMT) in irisin injected groups can be explained that irisin may have stimulated the endothelial cells of the rat thoracic aorta to produce growth factors which in return have stimulated the growth of VSMCs and hence increased in the production of elastic fibers [27-28]. It is well known that VSMCs are responsible for the secretion and production of collagen and elastic fibers in blood vessels [29]. Therefore, any changes in the number of elastic fibers should be attributed to the VSMCs. It has been reported that endothelial cells have been involved in the regulation of VSMCs through their secretion of growth factors such as PDGF-B [30-31].

It is worth mentioning that the morphological changes witnessed by irisin in this present study were not due to an increase of rat's age. The whole experiment lasted for a month which is not a lengthy period relative to the rat's lifespan which is estimated to be approximately 3 years [32]. In addition, there were no any morphological changes in the

control group indicating that the morphological changes in the wall of the rat thoracic aorta were most likely attributed to irisin.

It is also important to note that after irisin injection, rats did not show any notable changes in food or water intake. On the other hand, rats injected with irisin showed decreased body weights, which could be explained that irisin play an important role in fat metabolism [33] which may be a key in the future for obesity treatment.

Concluding remarks

This present study showed that irisin has significantly affected the morphology of the wall of the rat thoracic aorta by increasing its intima-media thickness, number of smooth muscle cells nuclei and the number of elastic lamellae in tunica media, these results indicated that irisin may has influenced the growth factors of the thoracic aorta walls and activate smooth muscle cells in the thoracic aorta layers.

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Conflict of interest

All authors declare no any conflict of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animal Welfare Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes and also adhered to ARRIVE guidelines.

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Scheme 1. A picture showing the place from where the thoracic aorta has been taken for staining (bold square, cut area)

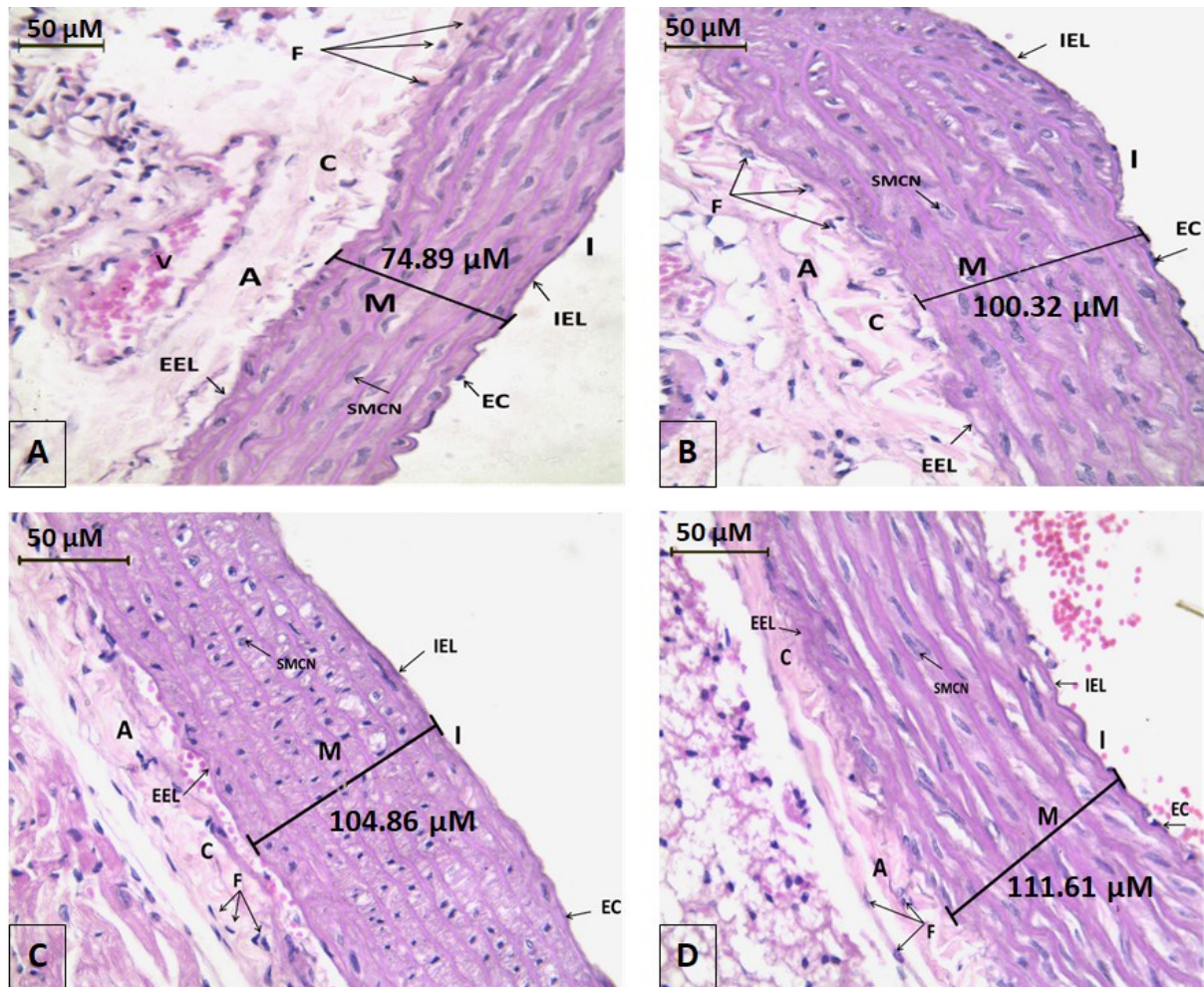


Figure 1. Histological sections of rat thoracic aorta wall stained with H&E ($\times 400$). A. Endothelial cells (EC, arrowhead) line the tunica intima (I), tunica media (M), and tunica adventitia (A) of the control group; B. An increase in the intima-media thickness, lamellae of elastic fibers and smooth muscle cell nuclei in irisin injected group 1; C. An increase in the intima-media thickness, lamellae of elastic fibers and hyperplasia of the smooth muscle cell nuclei in irisin injected group 2 (a longitudinal section); D. An increase in the intima-media thickness, lamellae of elastic fibers and hyperplasia of the smooth muscle cell nuclei in irisin injected group 3. Collagen (C), fibroblast cells (F, arrows) and blood vessels called vasa vasorum (V). The internal elastic lamina (IEL) separates the tunica intima and media, and the external elastic lamina (EEL) separates the tunica media and adventitia, small muscle cell nucleus (SMCN).

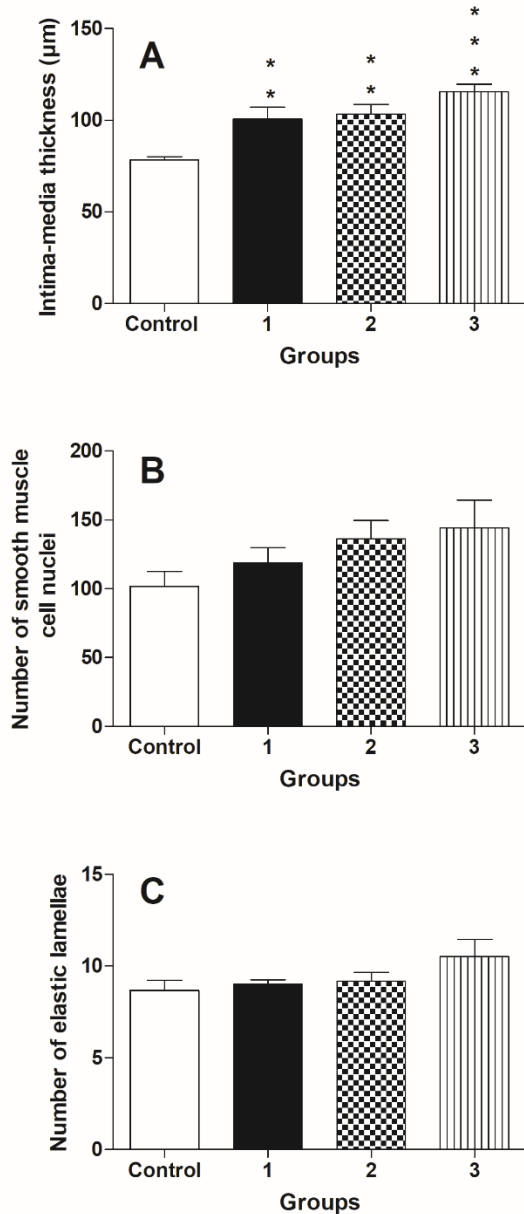


Figure 2. A bar graph representing aortic media components in different groups. A.

Represent the changes in intima-media thickness (IMT) between control group and different treatment groups of irisin. **B.** Represent the number of smooth muscle cell nuclei in control group and different treatment groups of irisin. **C.** Represent the number of elastic lamellae in control group and different treatment groups of irisin. Each bar represents the mean \pm SEM. * $P < 0.05$ vs. control, ** $P < 0.005$ vs. control, *** $P < 0.0001$ vs. control (one-way ANOVA followed by Dunnett's multiple comparison test, each group $n=6$). Groups 1, 2, and 3 received 20 nM (1.3 $\mu\text{g/Kg}$), 40 nM (2.6 $\mu\text{g/Kg}$), and 60 nM (10.2 $\mu\text{g/Kg}$), respectively.

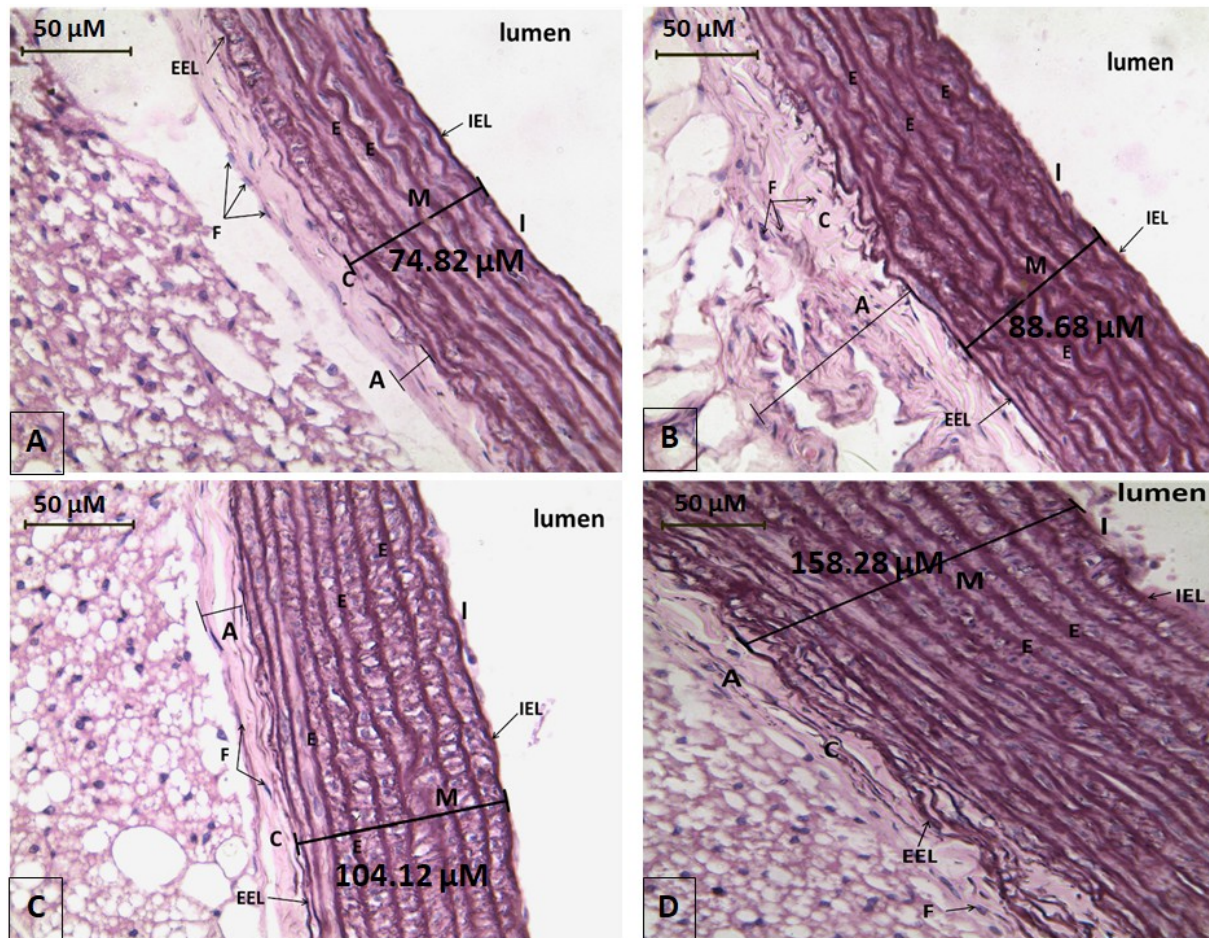


Figure 3. Histological sections of rat thoracic aorta wall stained with orcein stain ($\times 400$). Lamellae of elastic fibers (E) are shown for control group (A), irisin-injected group 1 (B), irisin-injected group 2 (C), and irisin-injected group 3 (D). Note the number of elastic lamellae in the experimental groups in comparison to control group. Tunica intima (I), tunica media (M), tunica adventitia (A), collagen (C), fibroblast (F), internal elastic lamina (IEL) and external elastic lamina (EEL).

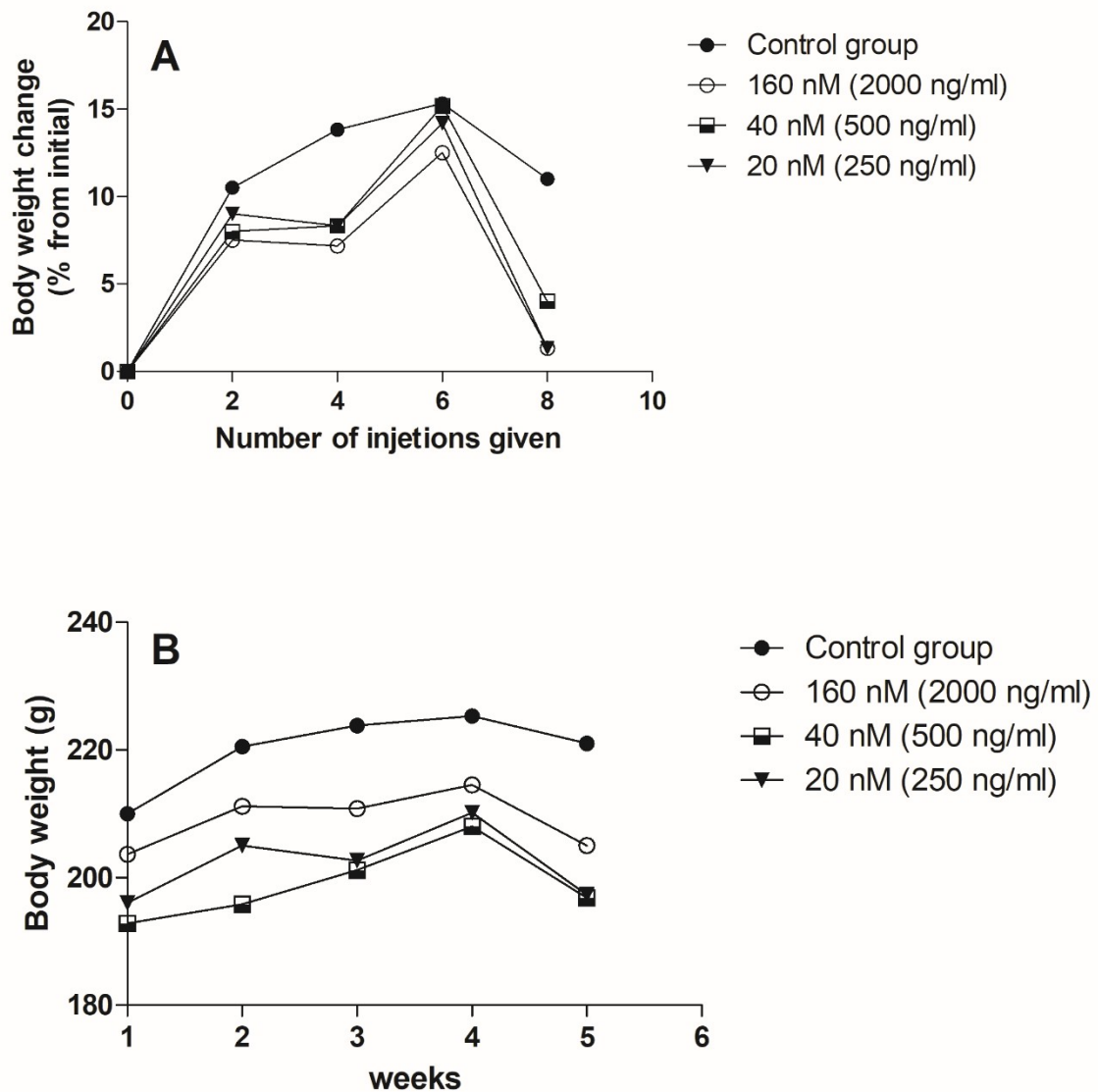


Figure 4. Body weight changes in control and experimental groups. A. A line graph showing body weights change (% from initial) in rats injected with irisin and without injection (control). **B.** Represent a line graph showing changes in body weight (g) for six animals in each group. The change in rats' weight is significant. (One-way ANOVA followed by Dunnett's multiple comparison test, each group n=6, *p < 0.05, **p < 0.01, and ***p < 0.001).

Table 1. Number of sections for H&E and Orcein stains per group

Groups	Ring section	longitudinal section
Control	12	12
20 nM	12	12
40 nM	12	12
160 nm	12	12

Table 2. Measurement of the aortic media components in different groups

	Control group	Irisin-injected group 1 (1.3 mg/kg)	Irisin-injected group 2 (2.6 mg/kg)	Irisin-Injected group 3 (10 mg/kg)
Intima-Media thickness (IMT) μm	78.3 \pm 1.6	100.5 \pm 6.3 **	103.2 \pm 5.3 **	115.4 \pm 3.9 ***
Number of elastic lamellae	8.6 \pm 0.55	9 \pm 0.25	9.1 \pm 0.47	10.5 \pm 0.95
Number of smooth muscle cell nuclei	102 \pm 11	119 \pm 11.5	136 \pm 13.4	144 \pm 20.2

Significant at *P<0.05 vs. control; **P<0.005 vs. control; ***P<0.0001 vs. control (one-way ANOVA followed by Dunnett's multiple comparison test), each group (n=6).