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Impaired vascular function induced by aging: Role of irisin and perivascular adipose tissue

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Received: 24 January 2023 / Revised: 27 February 2023 / Accepted: 01 March 2023

ABSTRACT: The aim of the study is to explain the possible effects of exercise-induced novel peptide irisin on endothelial function accompanied by perivascular adipose tissue (PVAT) in aortas of both natural aging and D-galactose (D-Gal) aging mimetic rats. Female Sprague-Dawley rats were randomly divided into 6 groups (n=10/per group): sedentary young group (SY), exercised young group (EY), sedentary aging group (SA), exercised aging group (EA), sedentary D-Gal (300 mg/kg/day, i.p., 9 weeks) induced aging group (S+D-Gal), exercised D-Gal induced aging group (E+D-Gal). Cardiac and aortic samples were collected for biochemical and histopathological examinations. The aortas were taken for contractile response studies in the absence or presence of perivascular adipose tissue (PVAT-/+). The levels of irisin in both plasma and PVAT were detected. Regardless of the models the vaso-relaxant effects of irisin were shown alone and age-related vascular dysfunction has also been recorded compared to the SY group these age-related impaired irisin responses were found to be improved by regular exercise. The plasma and PVAT irisin levels were decreased by aging and exercise reversed this decrement which is verified by the immune expression of irisin in aortic and PVAT tissues. Our results show the differences in vascular function and PVAT in aging and the contribution of PVAT to the response of irisin with exercise in two different aging models.

KEYWORDS: exercise; aging; vascular dysfunction; irisin; PVAT

1. INTRODUCTION

A large body of evidence from human as well as animal studies suggests that endothelial dysfunction can be attenuated by regular physical activity [1, 2]. Aerobic exercise in aging can reduce or prevent increases in large elastic artery stiffness, and improve autonomic-cardiovascular function and endothelium-dependent dilation [3], and these benefits are attributed largely to the suppression of oxidative stress and chronic low-grade inflammation. Aging is a major risk factor that increases the prevalence of cardiovascular diseases. The capacity of macroscopic and microscopic arteries to dilate in response to endothelium-dependent vasodilators is significantly impaired in humans and experimental animals with advanced age. Impaired endothelial phenotype is characterized by alterations in endothelial barrier function, nitric oxide (NO) bioavailability, reactive oxygen metabolite (ROM) biology, leukocyte and platelet adhesions [4].

Recently a novel myokine, adipokine, and a hormone, irisin, discovered which has been proposed to mediate some of the beneficial effects of aerobic exercise. Exercise induces the activation of peroxisome proliferator-activated receptor-gamma coactivator-1α (PGC-1α) from skeletal muscle that increases the expression of fibronectin type III domain containing 5 (FNDC5) gene and a cleavage product of FNDC5 protein known as irisin [5]. It has been suggested that the browning of white adipose tissue in response to exercise involves the activation of this pathway. Irisin may increase uncoupling protein-1 (UCP-1) expression that leads to increased thermogenesis [6]. Furthermore, increased expression of irisin potently increases energy expenditure, reduces body weight, and improves diet-induced insulin resistance in mice [5]. The remarkable roles of irisin were documented in many metabolic syndrome models through its impacts on cardiovascular disease and endothelial function [7] which provide evidence that irisin pretreatment improves endothelial dysfunction in

How to cite this article: Ozdemir-Kumral ZN, Bülbül Aycı N, Cilingir-Kaya OT, Kurtel H. Impaired vascular function induced by aging: Role of irisin and perivascular adipose tissue. J Res Pharm. 2023; 27(2): 733-752.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.356

animal models of obesity and diabetes. However, its role in endothelial dysfunction associated with aging and the effect of exercise intervention were not investigated before.
It has been suggested that the phenotype of adipose tissue content around the large arteries (PVAT) changes with advanced age [6]. The anticontractile effect of healthy PVAT to various agonists is attenuated with old age as well as in metabolic conditions like obesity [8, 9]. Several studies have shown that altered PVAT phenotype is associated with increased expression of inflammatory genes [10], local production of cytokines/adipokines, and increased immune cell infiltration [11, 12]. Although a role for irisin in regulating PVAT function has been implicated in obese mice, whether irisin is present in PVAT remains to be demonstrated. To understand better aging-induced alterations in vascular function, it is important to know if exercise-induced browning in white adipose tissue also includes PVAT and if so, whether irisin is involved in this mechanism. The major objectives of the present study were 1) to describe the role of irisin and PVAT on aging-induced vascular dysfunction and 2) to understand whether beneficial effects of exercise normally observed on vascular function are associated with alterations of irisin responses to vascular function and PVAT composition.

2. RESULTS

2.1. Body weight, body fat ratio (BFR), and amount of PVAT

At the beginning of the experiment, the animals were 3 months old, and the body weights were monitored monthly till the end of the protocol. Age-induced changes were demonstrated with the presence of increased BFR levels in the SA group ($p<0.001$) but not in the S+D-gal group (Table.1) as compared to SY. That was accompanied by an increase in the amount of PVAT in the SA ($p<0.001$) group when compared to the SY group. Age induction with D-gal did not affect PVAT volume (2.4 ± 0.5). At the end of the experiments, blood glucose, triglyceride, and cholesterol levels were significantly increased in SA ($p<0.001$) and S+D-gal ($p<0.01$) groups as compared to the SY group and exercise significantly decreased these parameters ($p<0.05$-0.001) except triglyceride levels (Sup.1).

Table 1. Body fat ratio (BFR), body weight (BW), perivascular adipose tissue (PVAT) amount, blood glucose, triglycerides and total cholesterol levels are presented. Data are expressed as mean ± SEM. * p<0,05, ** p<0,01, *** p<0,001 compared to SY group, + p<0,05, ++ p<0,01, +++ p<0,001 compared to respective sedentary groups.

	SY	SA	EY	EA
Glucose (nmol/g)	97.4 ± 4.1	236.5 ± 16.2***	86.9 ± 3.4**	168.0 ± 12.9+
Triglycerides (□mol/g)	83.1 ± 3.4	104.9± 7.5*	76.6 ± 4.4*	86 ± 6.1+
Total cholesterol (U/mg protein)	80.9 ± 2.5	165 ± 6.9***	69.3 ± 3.4***	105.5 ± 9.1++
BFR (%)	2.9 ± 0.3	6.5 ± 0.6***	2.6 ± 0.2**	4.3 ± 0.3++
BW (g)	302.4 ± 9.3	365.7 ± 12.2***	296.3 ± 6.3***	314.4 ± 3.1+++
PVAT amount (g/100 mm tissue)	2.3 ± 0.2	3.5 ± 0.3***	2.0 ± 0.2	3.1 ± 0.3

Sup.1. Summary of metabolic changes in sedantary; Sedentary+Young (SY), Sedentary+Aged (SA), Sedentary+D-Galactose (S+D-Gal) and exercise, Exercise+D-Galactose (E+D-Gal) groups. MPO: Myeloperoxidase, MDA: Malondialdehyde, GSH: Glutathione, SOD: Superoxide dismutase, CAT: Catalase, TNF-α: Tumor necrosis factor- alpha * p<0,05, ** p<0,01, *** p<0,001 compared to SY group, + p<0,05, ++ p<0,01, +++ p<0,001 compared to respective sedentary groups.

Parameters	SY	SA	S+D-Gal	E+D-Gal
BW (g)	302,4±9,3	365,7±12,2***	314,8±7,6	294,7±5,9

Total cholesterol ***(U/mg protein)***	80,9±2,5	165±6,9**	114,8±7,2**	91,7±7,4
Triglycerides ***(µmol/g)***	83,1±3,4	104,9±7,5*	102,1±7,7	82±3,7
Fasting blood glucose ***(nmol/g)***	97,4±4,1	236,5±16,2***	134,6±14,4*	94,6±5,3+
Body Fat Ratio % ***(fat weight -g / body weight -g)***	2,9±0,3	6,5±0,6***	3,2±0,4	2,7±0,2
PVAT amount ***(g/100 mm aortic tissue)***	2,3±0,2	3,5±0,3***	2,4±0,5	2,1±0,1
Irisin levels ***(plasma, ng/ml)***	110,7±7	46,1±6,7***	69,8±8,2*	101,1±9,4+
Irisin levels ***(PVAT, ng/ml)***	2,2±0,4	0,7±0,1***	1,2±0,2*	1,7±0,3
Cardiac *MPO activity* ***(U/g tissue)***	7,3±2,4	27,3±5,2**	31,3±5,8**	19,4±2,7+
Cardiac *MDA levels* ***(nmol/g tissue)***	15,3±3,6	61,9±9,8*	36,1±9,4*	25,9±3,8+
Cardiac *GSH levels* ***(µmol/g tissue)***	2,4±0,4	1,1±0,3*	1,4±0,3	2,4±0,5+
Cardiac *SOD levels* ***(U/mg protein)***	1,9±0,1	0,9±0,2***	1,5±0,1**	1,6±0,1+
Cardiac *CAT levels* ***(U/mg protein)***	267,2±9,1	111,2±7,1***	149,6±15,8***	172,8±12,4
Aortic *SOD levels* ***(U/mg protein)***	29,7±1,7	16±1,7***	20,1±2,8*	22,1±2,3
Aortic *CAT levels* ***(U/mg protein)***	4,4±0,5	1,9±0,1***	2,6±0,3*	4,2±0,5+
Serum *TNF-a levels* ***(pg/ml)***	51,7±0,9	74,6±2,9***	66,2±2,9***	57,4±2,6+

2.2. Oxidative stress parameters and TNF-α in cardiac and aortic tissues

As a consequence of aging, cardiac MPO and serum TNF-α activities were significantly elevated in the SA group as compared to the SY group ($p<0.05$-0.01). Significantly suppressed TNF-α activity but not MPO ($p<0.05$) was recorded in the EA group. Decreased antioxidant (SOD, CAT, and GSH) levels in the heart and aorta of SA rats ($p<0.01$-0.001) as compared to SY rats concomitant with decreased inflammatory parameters. The SA rats presented with elevated MDA levels ($p<0.05$) indicated that the balance between the oxidant versus antioxidant system is altered with age favoring the oxidant stress (Table.2). In a separate group of experiments application of the age-mimetic model, D-gal represented similar results, causing a depleted antioxidant level in the cardiac and aortic tissues accompanied by increased inflammatory markers. However, exercise was not effective in increasing antioxidant enzymes in this mimetic model (Sup.1).

Table 2. Oxidative stress and inflammatory parameters of the experimental groups. SOD; superoxide dismutase, CAT; catalase, GSH; glutathione, MDA; malondialdehyde, MPO; myeloperoxidase. Data are expressed as mean ± SEM. * p<0,05, ** p<0,01, *** p<0,001 compared to SY group, + p<0,05, ++ p<0,01, +++ p<0,001 compared to respective sedentary groups.

		SY	SA	EY	EA
SERUM	**TNF-α (pg/ml)**	51.8 ± 0.9	74.6 ± 2.9***	52.8 ± 1.9	62.9 ± 3.5+
HEART	**SOD (U/mg protein)**	1.9 ± 0.1	0.9 ± 0.2***	1.9 ± 0.2	1.5 ± 0.1+
	CAT (U/mg protein)	267.2 ± 9.1	111.2 ± 7.1***	267.7 ± 18.7	152.7 ± 12.3+
	GSH (mmol/g tissue)	2.4 ± 0.4	1.0 ± 0.3*	3.2 ± 0.5	1.3 ± 0.3+
	MDA (nmol/g tissue)	15.3 ± 3.6	61.9 ± 9.8	20.2 ± 2.9	33.8 ± 4.03
	MPO (U/g tissue)	7.3 ± 2.4	27.3 ± 5.2	6.9 ± 2.1	19.7 ± 2.2
AORTA	**SOD (U/mg protein)**	26.7 ± 1.7	16.1 ± 1.7***	33.2 ± 2.5	23.2 ± 1.9+
	CAT (U/mg protein)	4.4 ± 0.5	1.9 ± 0.1***	5.8 ± 0.6	3.8 ± 0.5+++

2.3. Plasma and PVAT irisin levels

As shown in Table 3, the plasma and PVAT irisin levels significantly decreased in SA (p<0.001) and S+D-gal groups (Sup.1) as compared to the SY group. The diminished irisin levels in plasma and PVAT significantly increased with exercise in the EA group (p<0.05). These observations indicated that aging is associated with alterations in PVAT biology. Whether these alterations have a phenotypic meaning requires attention since exercise intervention was also effective in increasing plasma and PVAT irisin levels. To further address this issue, we investigated vascular responses to irisin in myography set up in the presence and absence of PVAT (Fig. 1, 2, and 3). These results were consistent with histological observations as immune reactivity of irisin in PVAT and the endothelial layer of thoracic aortas was found to be elevated in the EA group (Fig. 4).

Table 3. Serum Tumor necrosis factor-alpha (TNF-α), plasma and PVAT irisin levels. Data are expressed as mean ± SEM.

		SY	SA	EY	EA
PLASMA	**Irisin(ng/ml)**	110.7 ± 7.1	46.1 ± 6.6***	134.3 ± 19.2	100.7 ± 15.4+
PVAT	**Irisin (ng/mg tissue)**	2.2 ± 0.4	0.8 ± 0.1*	2.1 ± 0.2	1.6 ± 0.2++
SERUM	**TNF-α (pg/ml)**	51,7±0,9	74,6±2,9***	52,8±1,9	62,9±3,5+

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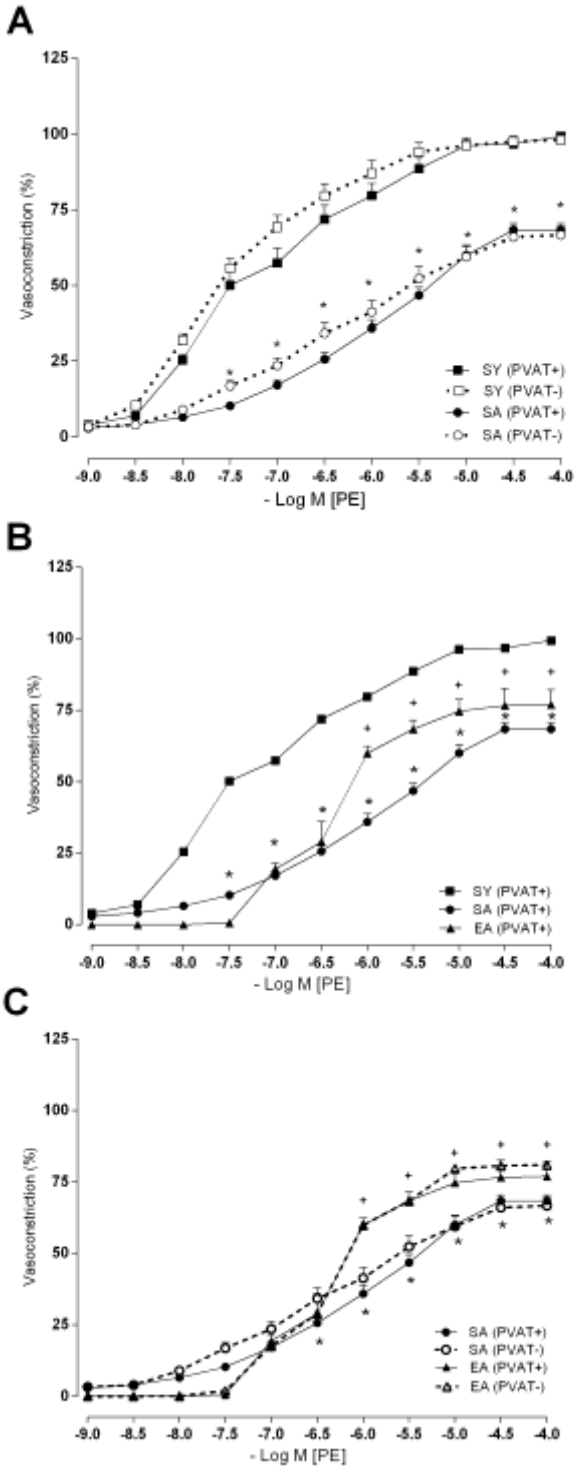

Figure 1. Cumulative concentration-response curves (10-9 M - 10-4 M) for the PE-induced contraction of abdominal aortic rings of sedentary young (SY) and sedentary aged (SA) rats with (+) or without (-) perivascular adipose tissue (PVAT). Points indicate the percentage of contraction induced by 124 mM KCl. The values shown are the mean±SEM of five to eight animals per group. *p<0.05 compared to aortic rings of SY rats within the same group (one-way ANOVA).

http://dx.doi.org/10.29228/jrp.356
J Res Pharm 2023; 27(2): 733-752

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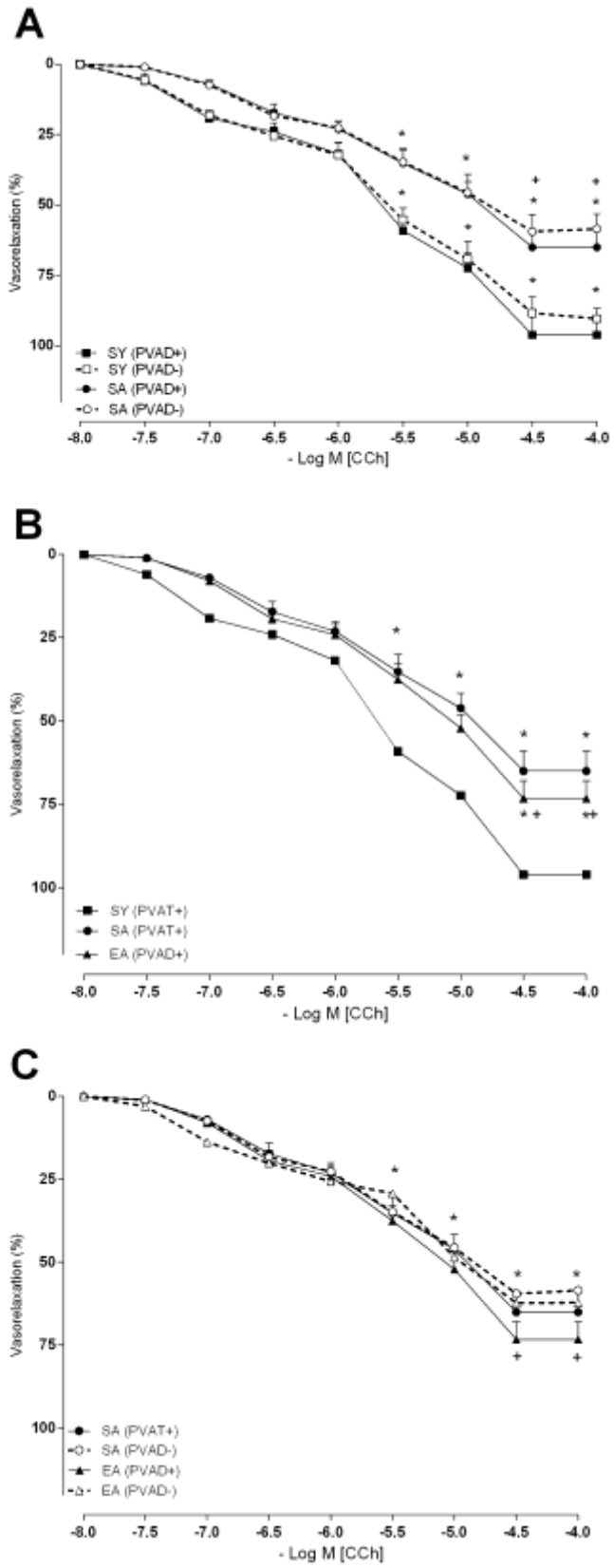

Figure 2. Concentration response curves obtained by cumulative addition of CCh to rat abdominal aortic rings pre-contracted with 10-5 M PE. The values shown are the mean±SEM of five to eight animals per group. *p<0.05 compared to aortic rings of SY rats within the same group (one-way ANOVA).

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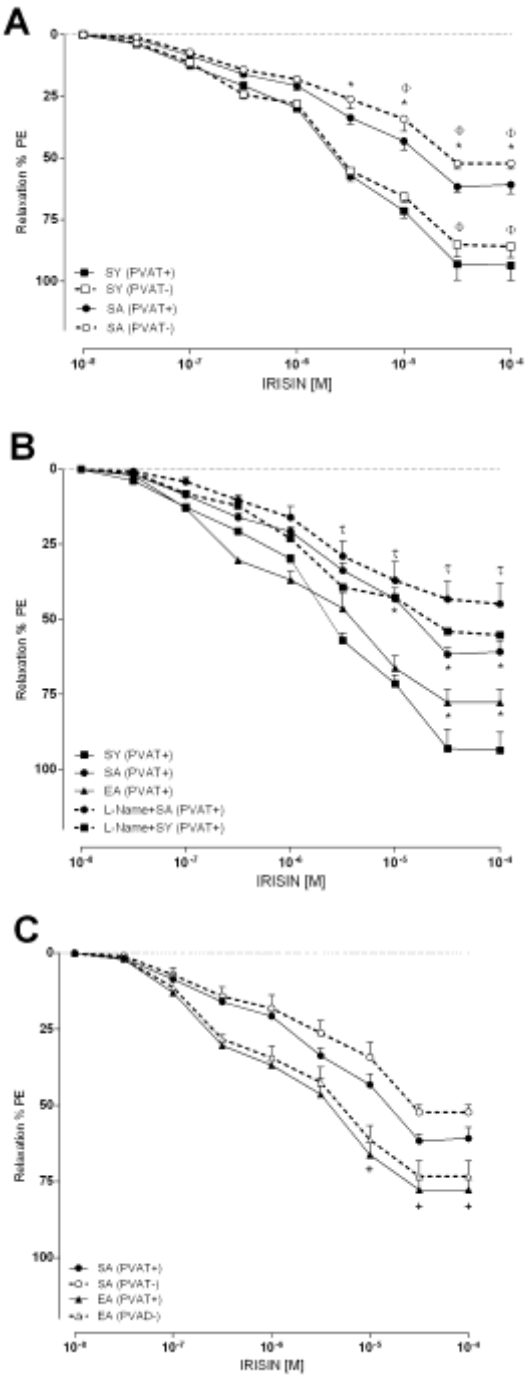

Figure 3. Concentration response curves obtained by cumulative addition of irisin (10-9 M - 10-4 M) to rat abdominal aortic rings pre-contracted with 10-5 M PE.

2.4. Effect of aging with exercise on vascular functions of the thoracic aortas

The effect of aging on vascular function was assessed in rats by measuring PE-mediated vasoconstriction (Fig. 1, EC values added in the supplement section). Fig 1A shows PE responses in the presence and absence of PVAT in SY and SA groups. The results demonstrate that PE-induced contraction is weakened ($p<0.05$) in SA rats (in a range of 10-7 M to the highest concentrations). Weakened responses to PE significantly improved with exercise (Fig. 1B). Exercise-induced improvements did not alter by the absence of PVAT (Fig 1C) indicating that PVAT is not involved in PE contractions. Similar results were obtained with the D-gal treatments (Sup.l).

As shown in Fig. 2, significant ($p<0.05$) differences in relaxation responses of aortic segments were noted between the SY and SA groups. Furthermore, the presence of PVAT did not affect any of the CCh-induced relaxation responses (Fig. 2A). Exercise training significantly improved this impaired relaxation response to CCh (Fig. 2B, $p<0.05$) without PVAT possession privilege.

D-gal-induced aging was associated with a changed relaxation response to the highest concentrations of CCh but PVAT was not found to be related to this attenuated relaxation (Table 4).

Endothelium-independent relaxations were also evaluated in aortic rings by testing SNP-induced relaxations in all groups. Like previous vascular responses, SA rats showed impaired relaxations to SNP (Table 4). Taken together aorta of aged animals represented not only weakened endothelium-dependent but also endothelium-independent relaxations plus disrupted contractions (Fig 1.) indicating a vascular dysfunction including the smooth muscle tissue. The attenuation of dose-dependent SNP dilation in aging was slightly reversed by exercise (Table 4). It should be noted that D-gal-induced aging was not associated with altered relaxation responses to SNP (Table 4).

In Fig. 3, aortic responses to irisin following pre-contraction with PE were shown. Irisin was effective in dilating the aortic rings dose-dependently pointing to the presence of vasorelaxant machinery for irisin in the vasculature (Fig. 3A). These relaxations were significantly impaired ($p< 0.05$) in SA animals compared to SY. Interestingly, irisin-induced relaxations were impaired when PVAT was removed from both young and aged animals indicating a modulatory role for PVAT. In Fig. 3B, the role of NO in irisin-induced relaxations was investigated by incubating the rings with L-NAME. Our results showed that relaxations to irisin were significantly attenuated with L-NAME indicating that NO was partially responsible. Exercise significantly improved irisin responses in aged animals (Fig. 3C) independent of PVAT existence. It is interesting to note that while the absence of PVAT further attenuated age-induced responses to irisin (Fig. 3A) beneficial effects of exercise did not alter by PVAT as observed with other agonists used (Fig. 1 and 2) indicating that in vitro application of irisin and following vasodilatation was not related with PVAT. In D-Gal experiments, as shown in the supplementary irisin responses were attenuated in a NO-dependent manner and exercise intervention was partly beneficial.

Table 4. Vascular responses to phenylephrine (PE), carbachol (CCh), sodium nitroprusside (SNP), irisin and L-Name (10-5 M) incubated irisin in thoracic aortas with (+) or without (-) perivascular adipose tissue (PVAT) from sedentary young; SY, sedentary aged; SA, exercised aged; EA, sedentary D-galactose aged; S+D-gal and exercised D-galactose aged; E+D-gal rats. * p<0,05, ** p<0,01, *** p<0,001 compared to SY group, + p<0,05, ++ p<0,01, +++ p<0,001 compared to respective sedentary groups. PVAT (Perivascular adipose tissue)

Groups (n)		PE		CCh		SNP		Irisin		L-Name+Irisin	
		- $LogEC_{50}$	E_{max}	- $LogEC_{50}$	E_{max}	- $LogEC_{50}$	E_{max}	- $LogEC_{50}$	E_{max}	- $LogEC_{50}$	E_{max}
SY (10)	PVAT +	7.33 ± 0.07	91.75 ± 1.65	5.59 ± 0.08	98.14 ± 3.47	7.68 ± 0.10	89.79 ± 2.31	5.60 ± 0.06	95.66 ± 2.68	5.85 ± 0.13+	54.35 ± 2.80
	PVAT -	7.58 ± 0.05	93.67 ± 1.30	5.63 ± 0.08	90.81 ± 3.18	7.65 ± 0.08	92.09 ± 2.10	5.67 ± 0.07	86.59 ± 2.58	5.78 ± 0.13	54.18 ± 2.99
SA (9)	PVAT +	5.98 ± 0.05*	67.27 ± 1.46*	5.55 ± 0.11*	66.02 ± 3.29	7.24 ± 0.13*	65.26 ± 2.44	5.52 ± 0.07*	62.45 ± 2.07	5.52 ± 0.14	41.05 ± 2.63

	PVAT-	6.40 ± 0.08*	63.06 ± 1.72*	5.70 ± 0.12*	59.04 ± 2.96	7.21 ± 0.15*	61.20 ± 2.59	5.42 ± 0.12*	53.85 ± 2.87	5.51 ± 0.16	38.89 ± 2.83
EA (10)	PVAT+	6.73 ± 0.08+	71.35 ± 1.97+	5.48 ± 0.09+	75.51 ± 3.18	7.66 ± 0.10+	74.58 ± 2.07	5.90 ± 0.09+	76.29 ± 2.52	5.74 ± 0.17	45.05 ± 3.05
	PVAT-	6.34 ± 0.06	81.74 ± 1.89+	5.48 ± 0.13	64.51 ± 3.56	7.48 ± 0.13	72.36 ± 2.49	5.86 ± 0.10	71.99 ± 2.77	5.57 ± 0.18	42.09 ± 3.36
S+D-gal (8)	PVAT+	6.27 ± 0.08	83.75 ± 2.39	5.63 ± 0.12	77.57 ± 3.62	7.66 ± 0.09+	77.56 ± 1.98	5.49 ± 0.08	73.99 ±2.64	5.81 ± 0.17	39.81 ± 2.62
	PVAT-	6.78 ± 0.09	80.27 ± 2.28	5.70 ± 0.11	69.48 ± 3.01	7.61 ± 0.10	81.78 ± 1.94	5.35 ± 0.12	58.84 ± 3.47	5.89 ± 0.21	37.06 ± 2.96
E+D-gal (9)	PVAT+	6.40 ± 0.05	103.5 ± 1.60	5.68 ± 0.12	79.71 ± 3.78	7.63 ± 0.11+	81.73 ± 2.23	5.55 ± 0.08	82.95 ± 2.94	6.16 ± 0.15	48.79 ± 2.52
	PVAT-	6.48 ± 0.04	86.62 ±1.39	5.36 ± 0.11	78.75 ± 4.16	7.68 ± 0.12	79.87 ± 2.54	5.63 ± 0.11	62.32 ± 2.86	6.10 ± 0.18	45.52 ± 2.84

2.5. Histological examination

In the light microscopic evaluations, regular longitudinal cardiac fibers and the dispersed connective tissue among these fibers with normal morphology were observed in the cardiac tissues of the SY and EY groups (Fig. 4). In the SA group, the connective tissue enlargement between the heart muscle bundles and slight vascular congestion revealed. When sedentary groups were compared to each other, cytoplasms with regular morphology in the SY group were seen whereas degeneration in the cytoplasms and dilated connective tissue between the heart muscle bundles were observed in DY and D-Gal group (sup.2). There was a decrease in the degeneration in D-Gal group after the exercise treatment. In the DY group, connective tissue showed regular morphology however, severe cytoplasmic degeneration was seen. The integrity of the connective tissue between cardiomyocytes was still preserved in the exercise group (Fig. 4D).

The light microscopic evaluation of the SY aorta demonstrated a regular contour of the internal elastic lamina (Fig. 5A). The EY group showed similar morphology as the SY group (Fig 5B). In the SA group, the regular contour of the internal elastic membrane was disrupted and there was some degeneration revealed in the tunica intima and tunica media (Fig 5C). In the EA group, however, the disruption of the internal elastic lamina was partially present, it preserved its integrity (Fig 5D). The regular endothelial structure was observed in the SY group, whereas there was an increase in PVAT in the EY group compared to the SY group. White adipose (WA) tissue was dominant in the SA group compared to the SY group, while brown adipose (BA) tissue increased in the EA group. In D-gal groups there was no clear damage in endothelial structure whereas BA tissue density prominently increased in the E+D-gal group (sup.2). Actually, an increase in the density of BA tissue in all exercise groups was observed.

As shown in demonstrative light micrographs of the immunohistochemical labeling in aortic tissues to determine the index of irisin, the intensity of brown stained areas originating from DAB dye was calculated with a quantitative measurement in the ImageJ program (Fig. 6G). The total staining score was revealed significantly (Fig. 6F, $p<0.05$) decreased in the SA group (3.5 ± 0.8) compared to the SY group (5.7 ± 0.8) but not in the S+D-gal group (4.5 ± 0.7). It was shown that this reduction tended to increase in the endothelial layer of the EA group (5.4 ± 0.7). There was a significant (Fig. 6E, $p<0.05$) increment in the PVAT irisin immunoreactivity of the EA group (1.6 ± 0.2) compared to the SA group (0.9 ± 0.01). In the S+D-gal group (1.8 ± 0.4) immunoreactivity of irisin slightly increased but did not change significantly with exercise (2.7 ± 0.03). As calculated with Image J the total staining score was found significantly (Fig. 6F, $p<0.001$) decreased in the SA group (7.4 ± 1.9) compared to the SY group (89.4 ± 10.9) and the reduction was significantly ($p<0.001$) prevented in EA group (39.2± 12.9).

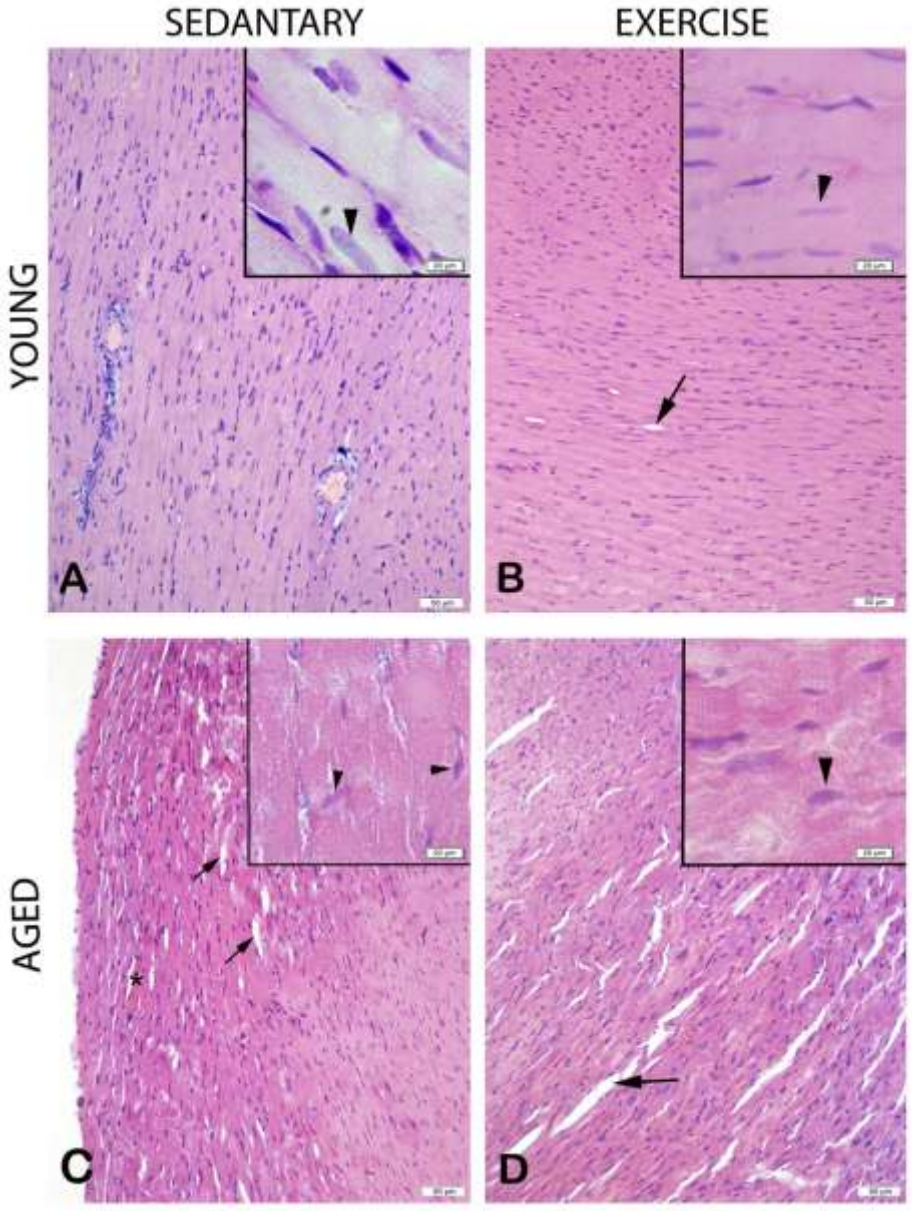

Figure 4. Demonstrative light micrographs of cardiac tissues from experimental groups. Arrowhead: Cardiocyte with regular morphology. Arrow: Degeneration in connective tissue between the cardiocyte. Asterisk (*): Vascular congestion. Hematoxylin and Eosin stain.

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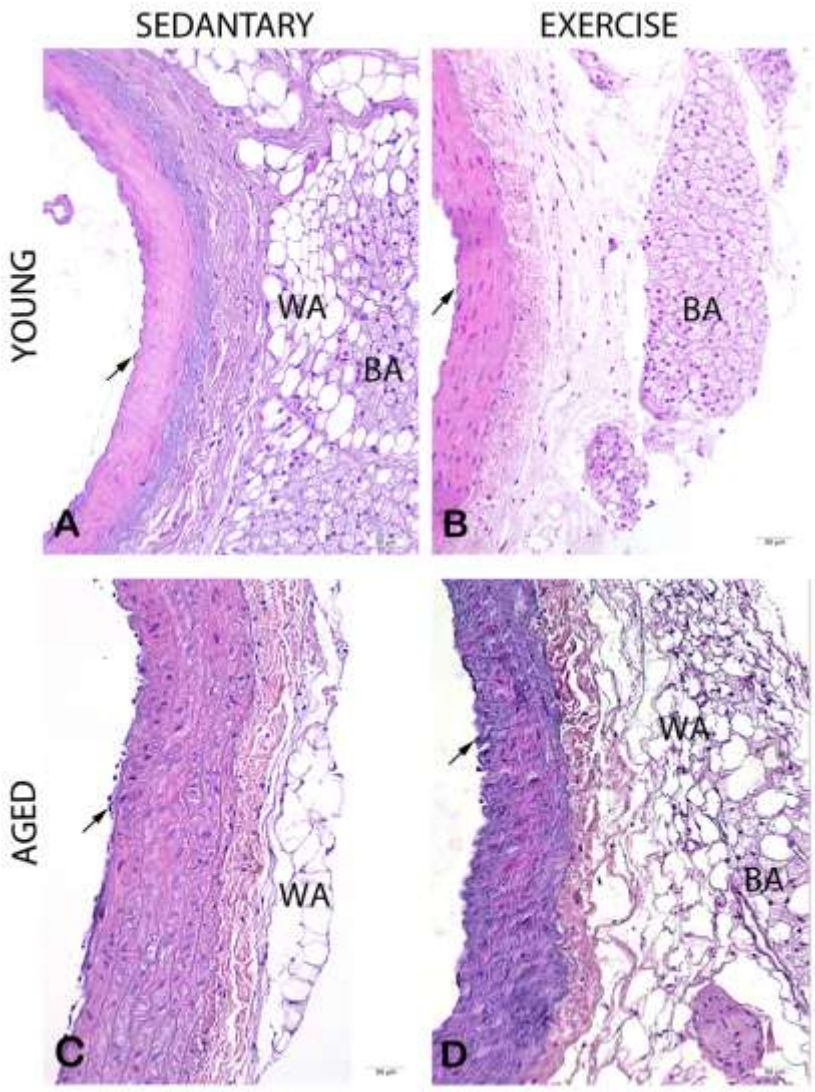

Figure 5. Demonstrative light micrographs of aort tissues from experimental groups. Arrow: Endothelial cell. BA: Brown adipose tissue. WA: White adipose tissue. Hematoxylin and Eosin stain.

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J Res Pharm 2023; 27(2): 733-752

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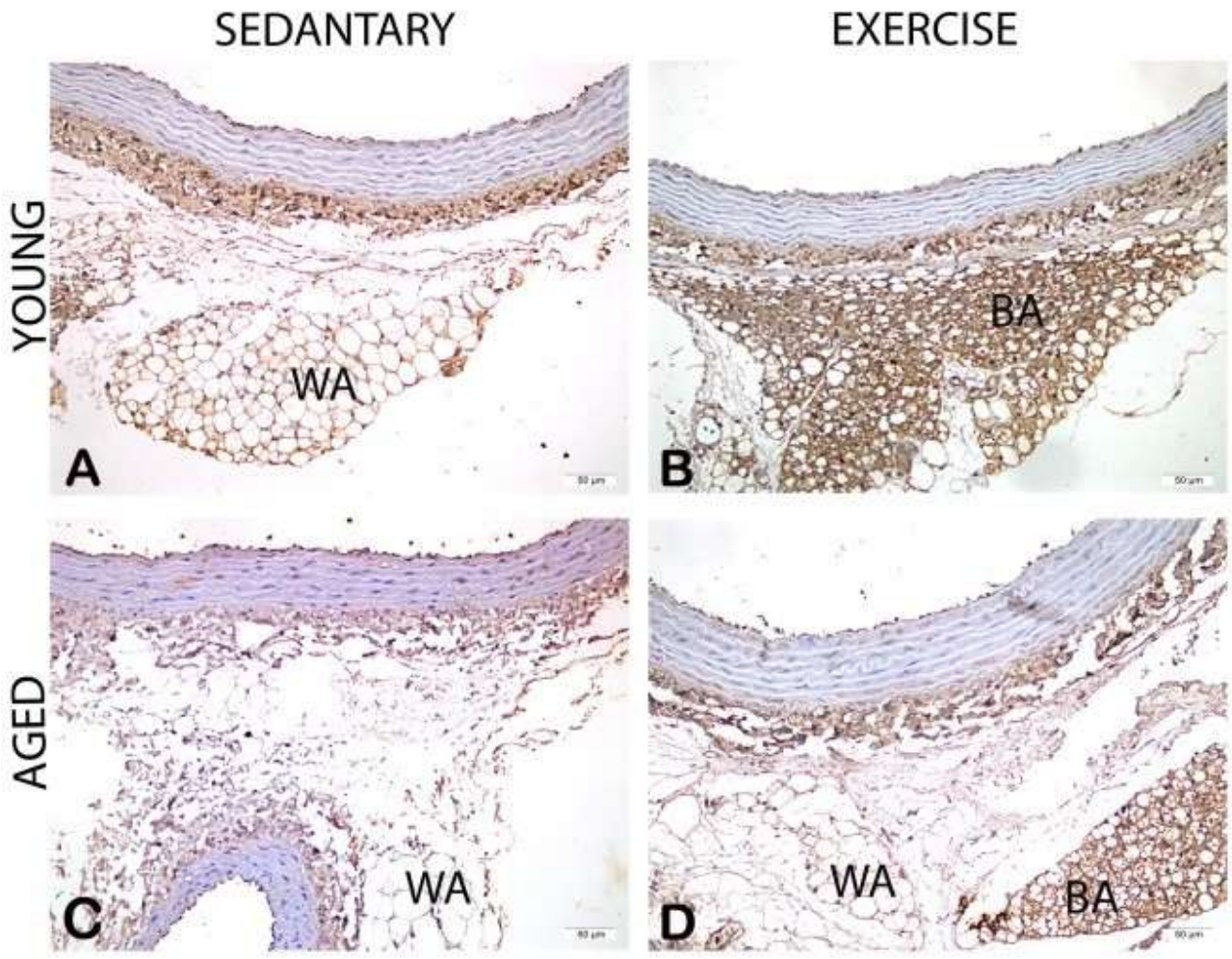

Figure 6. A. SY, B. EY, C. SA, D. EA. Demonstrative light micrographs of irisin immune-labelling on aortic tissues from experimental groups. BA: Brown adipose tissue. WA: White adipose tissue. Brown-stained regions indicate the irisin immunoreactivity.

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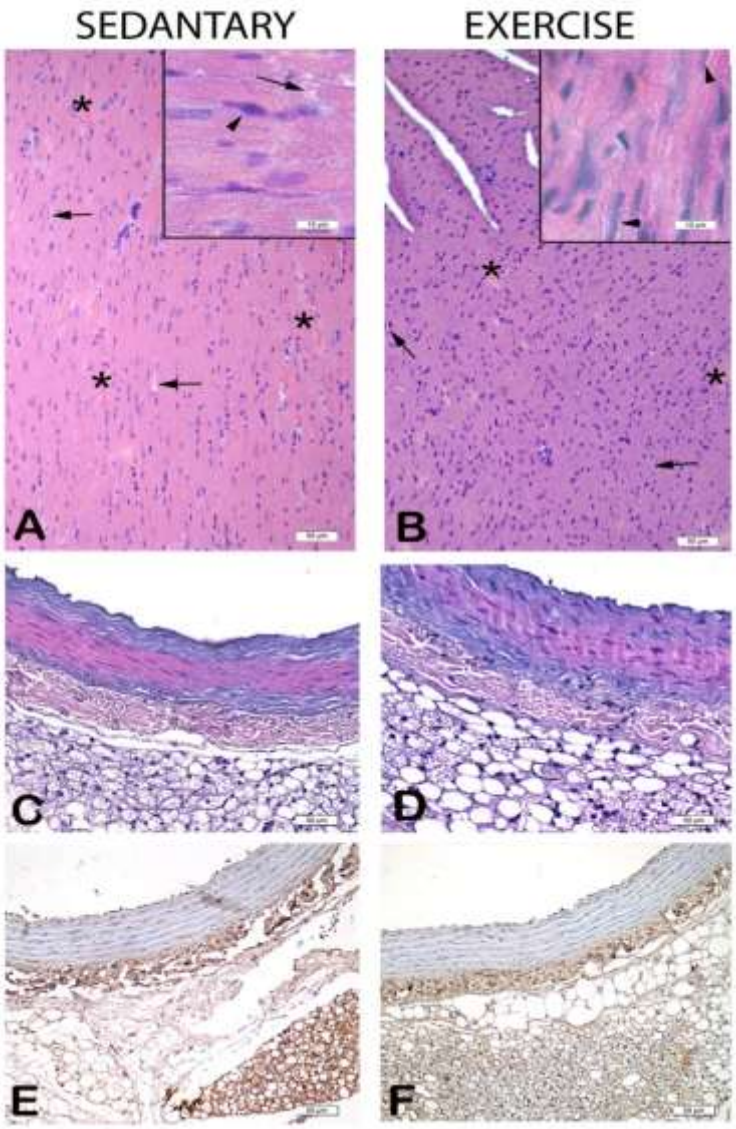

Sup.2 D-Gal: Demonstrative light micrographs of D-Gal groups. A. Cardiac tissue of SY group. B. Cardiac tissue of EY group. Arrow: Dilation of connective tissue between fibers. Asterisk (*): Vascular congestion. Arrowhead: Nucleus of cardiac muscle fiber. C. Aorta tissue of SY group. D. Aorta tissue of EY group. Hematoxylin and Eosin stain. E. Aorta tissue for immune-labelled for irisin of SY group. F. Aorta tissue for immune-labelled for irisin of EY group.

3. DISCUSSION

The present study is focused on the relationship between cardiovascular aging and the adipose/muscle-driven molecule irisin. Natural aging and D-galactose aging mimicked animals were used to study this notion. Although D-galactose administration mimicked some of the naturally occurring aging phenotypes important differences were observed in body weights, BFR, and the amount of PVAT. Therefore, we focused on the results of the natural aging model and for the sake of simplicity, the term “aging” is used to mean natural aging in the manuscript. Our findings revealed that aging is associated with inflammatory changes and vascular dysfunction with a significant increase in aortic plaque and hypertrophic areas in cardiac tissues. Additionally, significant alterations in BFR, hypertrophy index (Heart weight / Body weight), serum cholesterol, glucose, and TNF-α levels as well as reductions in tissue antioxidant capacity have been observed. Our results show that exercise intervention has an important role in reducing age-associated cardiovascular pathology since better vascular responses and improved biochemical and histologic outcome has been documented following exercise. Although some of the beneficial effects of exercise are well documented in the literature, the information on aged animal models is scarce and our study provides a piece of important additional information about the effects of exercise in an animal model.

Syslová [19] and Odden [20] reported that age is associated with increased levels of markers of oxidative stress and inflammation which are important risk factors for cardiovascular diseases. We have observed increased MDA levels and MPO activity in the cardiac tissues of aged groups whereas exercise attenuated these elevations. Furthermore, reduced SOD, CAT, and GSH levels observed in cardiac and aortic tissues of SA rats significantly increased with exercise demonstrating an improvement in antioxidant capacity. Additionally, the circulatory level of TNF-α was increased with aging and this elevation was prevented with exercise.

Available literature indicates that age-associated inflammatory changes involving oxidant-antioxidant imbalance increased cytokine production and vascular dysfunction. To this end, we investigated the possible participation of the vascular component in the aged and exercised groups. Contraction induced by KCl in the thoracic aortas showed a declining profile in aged rats but no significant differences were found in the responses (data not shown). On the other hand, there was a gradient in the effect of aging on the PE response namely both Emax and sensitivity was reduced, and these results are in good agreement with the findings reported by Takayanagi et al. [21]. Our results demonstrate that the weakened responses to PE significantly improved with exercise consistent with previous studies [22, 23]. The presence of PVAT on aortic rings had no effect on the vasoconstrictor responses to the α1-adrenoreceptor agonist PE in SY and SA rats.

Previous studies show that aging reduces not only contractions but also relaxations elicited by some vasodilators such as NO from endothelial cells. In the present study; the endothelium-dependent relaxation responses were induced by CCh whereas, endothelium-independent relaxations were produced by SNP. Relaxation responses to CCh and SNP significantly reduced with aging indicating endothelium-dependent and independent dysfunction. Exercise training predominantly reversed CCh responses of aged animals while SNP responses only slightly improved.

Many studies in animals have shown that alterations in PVAT phenotype with aging play an important role in cardiovascular disease [24, 25]. These alterations involve an increase in WAT content and enlargement of the adipocytes [26]. It has been suggested that aged-induced impairment in vascular responses to various agonists is associated with PVAT alterations. In the present study, aging exhibited an increase in PVAT weight, but exercise did not change it. Our results showed that although the amount of PVAT did not alter with exercise there was a significant alteration in its morphology. The dominance of WAT was observed in the PVAT under a light microscope of SA animals and this condition was significantly changed in favor of BAT after exercise. While differences occurred in the biochemical and morphological structure of PVAT in aged rats, endothelial responses also changed. In brief, the impaired contractile responses improved by exercise however these changes did not alter by the absence of PVAT. Furthermore, exercise training also improved the impaired relaxation responses without PVAT possession privilege. The imbalance in PVAT-derived adipokines with aging has been shown to have a direct effect on vascular contractility in the progression or regression of cardiac diseases by changing the local inflammatory environment and promoting VSMC proliferation or migration [27] but not been seen in our study. Vascular studies both in vivo and in vitro have demonstrated an association between increased PVAT mass and decreased anti-contractile effects of PVAT which can be thought to be the result of an imbalance in adipokine secretion and activation of inflammatory and oxidative stress pathways [25, 28-30] but it also provides beneficial protective effects in physiological conditions such as aging [31] which can be said in our study in the name of vaso-responses. Many factors affecting the inflammatory process, such as dietary changes and exercise, also change the effects of PVAT with side effects of these changes as macrophage activation [32]. In some conditions, PVAT becomes hypertrophied and eventually causes inflammation and oxidative stress, which, although morphologically attenuated, can be irreversible in terms of bioavailability effects [27, 33, 34]. In our study, the presence of PVAT did not affect any of the CCh-induced relaxation responses (in a range of 10-6 M to the highest concentrations) and exercise training significantly improved the impaired relaxation response to CCh without PVAT possession privilege. As previously reported exercise contributes to the release of substances responsible for affecting vascular tone from PVAT and it is also supported by other studies which demonstrated that exercise training reduces PVAT inflammation [35, 36] and macrophage infiltration [37]. We showed that exercise increased SOD and catalase and prevented neutrophil infiltration and lipid peroxidation in cardiac tissue which can be directly mentioned as factors that alter vascular reactivity. However, there is conflicting evidence concerning the morphology of the adipose tissue surrounding the human aortas with some studies finding BAT shows different effects on vascular activity

profile [10, 38]. In the present study, moderate growth in adipose tissue mass due to D-gal-induced aging did not improve the anticontractile properties of PVAT indicating that both quantitative and qualitative changes of PVAT are important when considering its participation in vascular tone in D-gal. Our results show that the presence of PVAT did not modify the cumulative concentration responses to SNP in natural aging [39]. Similarly, VSM cells are directly stimulated by SNP via stimulation of soluble guanylyl cyclase and induction of hyperpolarization [40], and aging did not result in altered relaxation to SNP in aortic rings with or without PVAT thus, sensitivity to NO was unaltered. Furthermore, we have demonstrated that pre-constricted and NOS-inhibitor (L-NAME) added aortic rings were still able to relax fully to baseline (Table 4). And these findings are in line with evidence from studies conducted on aortic rings of rodents and humans which reported that VSM sensitivity to NO is uninjured by aging and reduced endothelial-derived NO bioavailability with aging contributes to vascular pathologies [41, 42].

In this study, although the PE, CCh, and SNP responses of PVAT (-) tissues are not different from those of PVAT (+) ones, an increase in age-related PVAT, decreased serum and PVAT levels of irisin (supported by decreased immunoreactivity with age) and increased levels of irisin in PVAT with exercise made us ask the following questions. Does irisin affect vascular function? If so, what is the role of the age factor? Could irisin play a role in the possible ameliorative effect of exercise on vascular responses? Are irisin responses affected by PVAT? To answer these questions, vascular responses were investigated at different irisin doses in myography experiments. The present study showed that irisin is a vasoactive molecule that relaxes the aortic segments, and this is related to NO. Interestingly, we showed that the relaxant effect of irisin was affected by the absence of PVAT since relaxation increased in the presence of PVAT. Vascular dysfunction is a hallmark that is associated with atherosclerosis and occurs in chronic inflammation-related disorders and aging. In this regard, it is possible that irisin which has been reported to have beneficial effects on energy metabolism and insulin resistance can play an important role in the regulation of vascular function because metabolic dysfunction is considered a major risk factor for vascular diseases that are often seen in aging. Several studies in humans propose that irisin may regulate vascular endothelial function [43, 44]. A study reported that irisin relaxes the endothelium in a dose-dependent manner even in endothelium-dependent and independent pathways [45]. Irisin may play a role in the therapeutic effects of exercise on vascular responses because improved vascular responses with exercise are accompanied by increased serum and PVAT levels of irisin. Our study also revealed that NO mediated the anti-contractile effect of irisin in the SA group and PVAT denudation had no further effect, which may be indicative of a decrease in PVAT-derived NO in aging. There are acute exercise protocols that have been shown to represent strong stimuli for irisin release if characterized by sufficient intensity and/or duration [46]. Circulating irisin concentrations of exercised subjects are shown higher than that of sedentary in line with our study [47]. It is interesting to note that increased BAT in exercised groups compared to their sedentary groups was accompanied by increased irisin immun expressions in PVAT. Increased white adiposity combined with ROS production and altered adipokine profile promotes age-related PVAT dysfunction and exercise partially prevents this ongoing profile. Although an elevated amount of PVAT was recorded in aged rats the morphology shifted towards BAT with exercise which may be a source of irisin.

Many observational studies offer findings that clearly influence the relationship between exercise and inflammatory biomarkers [23]. In the present study, elevated serum TNF-α levels as well as increased cardiac lipid peroxidation and MPO activity in aged rats significantly decreased in the EA group.

The study was performed only in female rats. More soon, a higher accumulation of cardiovascular disease risk factors has been shown in middle-aged women during the postmenopausal period. Exercise plays an essential role in combating the physiological decline associated with aging. Sex differences have been observed in oxidative stress generation [48]. Female rats have an intrinsically higher antioxidant capacity, which resulted in increased levels of GSH, and the adaptation to altered antioxidant capacity, induced by physical activity, appeared to be affected by gender differences. So, this study gives another opportunity to see the unique changes in only one sex which is mostly not preferred in studies because of called as its "disadvantages". Human studies revealed that low-dose of physical activity which refers to moderate exercise in rats attenuates cardiovascular disease [49].

4. CONCLUSIONS

Overall present studies help us to answer the previously asked questions notably, ruined vascular responses in aging were significantly improved in exercise, and some of these responses are mediated by irisin that involves adipose tissue phenotype Considering that the absence of effective therapy for aging and related diseases, the evidence presented here could be a step forward in understanding the value of irisin as a key agent. Future experiments need to define further the mechanisms underlying age-related PVAT dysfunction and the effects of exercise and exercise-derived peptides.

5. MATERIALS AND METHODS

5.1. Animals and chemicals

Female Wistar albino rats (250-300 g, 14 weeks old, n = 60), supplied by the MU Animal Center (DEHAMER), were housed in a humidity (65–70 %) and temperature-controlled room (22 ± 2°C) with standardized light/dark (12/12 hour) cycles until they reached 6 or 21 months old. Rats were fed with standard rat pellets and tap water ad libitum. All experimental protocols and procedures were approved by the Marmara University (MU) Animal Care and Use Committee (approval code: 07.2015.mar.) and were in compliance with international standards, principles and guidelines developed by the New York Academy of Sciences and Turkish law on the use of animals in experiments. Animals were randomly divided into 6 groups (n=10/per group); sedentary young group (SY, 6 months old), exercised young group (EY, 6 months old), sedentary aging group (SA, 21 months old), exercised aging group (EA, 21 months old), sedentary D-Gal (300 mg/kg/day, i.p., 9 weeks) induced aging group (S+D-Gal, 6 months old), exercised D-Gal induced aging group (E+D-Gal, 6 months old).

5.2. D-Galactose induced aging model

To investigate the underlying mechanisms of the aging, recurrent D-Galactose (300 mg/kg, i.p., Sigma Chemical, St. Louis, MO) injection for 9 weeks is an acceptable aging model [13].

5.3. Exercise protocol

A modified moderate load swimming exercise model was selected in the exercise groups and rats were acclimated to swimming for 5 days with increasing duration from 10 minutes on the first day to 30 minutes by the 5th day [1]. The exercise was performed for 30 minutes (min) per day, 5 days per week for 3 weeks for the following nine weeks' exercise sessions were performed for 40 minutes per day. Sedentary groups were put on their feet in a separate pool filled with 5 cm warm water while exercise groups swam.

The decapitation was performed within 24 hours following the last exercise protocol. Animals were anesthetized with a mixture of ketamine (100 mg/kg) and chlorpromazine (0.75 mg/kg, i.p.) and blood was obtained by cardiac puncture. The serum and plasma were collected, and stored at –80°C.

5.4. Measurement of blood glucose, total cholesterol, and triglyceride levels

Fasting blood glucose levels were measured at the beginning of the experiment, at 5 months following the experiment, at 12 months (data not shown) and on the last experiment day (15 months) by a micro-autoanalyzer (Accutrend Plus Glucose, Accutrend Total Cholesterol, and Accutrend Triglycerides, respectively; Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Total cholesterol and triglyceride measurements were made on the day of decapitation.

5.5. Vascular reactivity studies

After preparation of the appropriate (approximately 2-3 mm wide) abdominal aortic samples mounted into the organ bath (Biopac MP35 Systems, Inc. COMMAT Ltd., Turkey) [1]. All activities measured in PVAT + or PVAT – aortic tissues. Contractile responses to PE are expressed as a percent of the maximal contraction induced by 120 mM KCl. Endothelium-dependent relaxation responses to CCh were expressed as a percent of the contraction caused by submaximal PE while endothelium-independent relaxation responses were

evaluated by a nitric oxide donor sodium nitroprusside (SNP, 10–9 - 10–5 M). To determine the direct vasoactivity of the irisin, the same aortic rings were exposed to increasing concentrations (10–9 - 10–4 M) after pre-contraction with submaximal PE. In some experiments, vascular reactivity to irisin was assessed in the presence (20 min pre-incubation) of L-NG-Nitroarginine methyl ester (L-NAME, 10–5 M, Sigma Aldrich) to determine the contribution of NO to the vaso-relaxation response.

5.6. Measurement of TNF-α levels in serum, irisin levels in plasma and PVAT

Circulating irisin levels have been quantified on plasma samples and PVAT irisin levels measured by a specific competitive enzyme immunoassay kit (Cat. No. EK-067-029 from Phoenix Pharmaceuticals, Karlsruhe, Germany). Serum levels of TNF-α were quantified according to the manufacturer's instructions and guidelines (Thermo Fisher Scientific, San Diego, CA, USA) using enzyme-linked immunosorbent assay (ELISA, Cat. No. BMS622) kits.

5.7. Measurement of cardiac myeloperoxidase activity, malondialdehyde (MDA) and glutathione (GSH) levels

In cardiac tissue samples, MPO activity was determined in order to evaluate the accumulation of polymorphonuclear leukocytes in the cardiac tissue. The method used to determine MPO activity in the cardiac tissues was similar to that previously described [14]. The results were given as nmol MDA/g tissue. The GSH levels were determined with a spectrophotometric measurement based on the modified Ellman procedure in cardiac tissues. The results are expressed in µmol GSH/g tissue [15].

5.8. Measurement of superoxide dismutase (SOD) and catalase (CAT) activity in the cardiac and aortic tissues

To determine the cardiac and aortic tissue levels of SOD and CAT tissue samples were homogenized with saline and aliquoted. Respectively SOD activity was measured according to the Mylroie et al. [16] and CAT activity was measured according to the method of Aebi [17].

5.9. Histopathological Evaluation

For light microscopic investigations cardiac and thoracic aorta tissues were fixed with 10% formalin and the sections (3 µm) were taken and stained with hematoxylin and eosin (H&E) for general histopathological evaluations after routine paraffin embedding processes. All tissue sections were examined under a photomicroscope (Olympus BX51, Tokyo, Japan) for the evaluation of histopathological changes by three experienced histologists (NB, OTCK, NO) who were blind to the groups of the study. The histological score of the organs was calculated as the sum of the scores (0–3) given for each criterion [14].

5.10. Immunohistochemical analysis of irisin in the aorta and PVAT

Aorta and surrounding perivascular adipose tissues from all groups were fixed in a 10 % formaldehyde solution and washed in tap water for 2 hours. Then the tissues were dehydrated with increasing concentrations (70, 90, 96 and 100%) of ethanol and cleared with xylene. Paraffin-embedded sections that were cut at 3-µm thickness were de-paraffinized with xylene and rehydrated with ethanol and water. Antigen retrieval was accomplished by Decloacking chamber (Bicare Medical DC2008) in a citrate diva buffer (DV Sitogen 2004 LX, MX pH 6.2) for 40 min. at 110°C. Peroxidase (HRP) conjugated polymeric immunohistochemistry staining method was used to evaluate irisin-immunoreactivity in aorta and PVAT together. The endogenous peroxidase activity was blocked with 3% H2O2 (ScyTek ACA 125) for 15 min at room temperature and later rinsed with phosphate buffered saline (PBS). Sections were pretreated with citrate buffer (pH 6.0) in a 200 W microwave oven for 20 min. for antigen recovery. The slides were cooled at room temperature for 20 min. and washed in two separate phosphate buffer solutions (pH 7.4) for 5 min. To prevent nonspecific staining, tissues were subjected to a 10-min protein blockade (EXPOSE Rabbit specific HRP/DAB detection IHC kit, Abcam, Cambridge, UK). The blocking solution was removed and the primary antibody of the rabbit anti-Irisin (H-067-17, Phoenix Pharmaceuticals, Inc., CA, USA) was dropped to the sections at a dilution of 1: 300 and incubated for 1 night at 4 ° C. HRP-Polymer (EXPOSE Rabbit specific HRP

/ DAB detection IHC kit, Abcam, Cambridge, UK) was applied to the sections washed for 5 min with two separate PBS and incubated for 15 min. After incubation, sections were washed again with two separate PBS for 5 minutes and incubated in 3,3'-diaminobenzidine (DAB) chromogen for 5 min. Nuclear counterstaining was performed with Mayer Hematoxylin for 1 min and slides were dehydrated with 96% ethanol. The slides which were closed with the covering material were evaluated under a light microscope. In order to detect specific staining, skin tissue was used as positive and negative controls simultaneously with aortic tissues. While all the method steps were applied to the cross-section of the positive control tissue, the primary antibody stage in the negative control tissue was omitted. Slides with immunohistochemically staining were observed under a light microscope and were photographed with a light microscope (Leica 390-CU, Germany). In a number of 6 to 8 slides in each experimental group and 5 randomly selected areas in each preparation were evaluated at x40 magnification. Microphotographs of all groups were independently studied by two blind expert histologists (OTCK, NOY) and analyzed by using the Image-J program [18].

5.11. Data analysis

Statistical analysis was carried out using GraphPad Prism 9.0 (GraphPad Software, Inc. La Jolla, CA, USA). Percentage reversal of the PE contraction (with or without L-NAME) was the basis to present the relaxation response of all protocols in the thoracic aorta rings. The maximal relaxation response was presented as Emax, concentration producing 50% of the maximal response was presented as EC50 to vasoactive agents, and the determination of the data pD2 = log EC50 was expressed as sensitivity or potency. All data are expressed as means ± S.E.M. Groups of data were analyzed using one-way ANOVA followed by Tukey's multiple comparison tests or Student's t test where appropriate. Values of $p<0.05$ were regarded as significant.

Acknowledgements: The authors are grateful to histologist Dr. Naziye Ozkan Yenal for her helps in histopathological evolutions. This work was supported by a grant from the Marmara University Research Fund, Istanbul, Turkey, SAG-C- DRP-130515- 0160 (received by H.K.).

Data Availability

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

Author contributions: Z.N.O-K, H.K., designed the study, and performed the study. O.T.C-K., performed and evaluated the histological studies; Z.N.O-K, H.K., performed data analysis, and wrote the article. All authors reviewed and approved the final version of the article.

Conflict of interest statement: The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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