Involvement of SIRT1-eNOS Axis in Vascular Senescence Responses to Ursolic Acid and Exercise Training in Aged Type 2 Diabetes Model of Rats

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Research Article

Abstract

Background

Ursolic acid (UA) mediates the vasorelaxant activity via nitric oxide (NO) release, and up-regulation of eNOS in endothelial cells in disease conditions with increased oxidative stress. The present study aimed to reflect on the impact of eight weeks of a combination of ursolic acid (UA) supplementation and resistance/endurance training in old male Wistar rats having high-fat diet and/or low-dose streptozotocin-induced type 2 diabetes (HFD/STZ-induced T2D), with an emphasis on sirtuin 1 (SIRT1)-endothelial nitric oxide synthase (eNOS) axis and oxidative stress (OS) indices in their aortic tissues.

Methods

A total number of 56 21-month-old male Wistar rats with HFD/STZ-induced T2Dwere randomized into seven groups (n = eight animals per group): (1) sedentary old non-diabetic (C); (2) sedentary HFD/STZ-induced T2D (D); (3) sedentary HFD/STZ-induced T2D plus UA (UA) (DU); (4) endurance-trained HFD/STZ-induced T2D (DE); (5) resistance-trained HFD/STZ-induced T2D (DR); (6) endurance-trained HFD/STZ-induced T2D plus UA (DEU); and (7) resistance-trained STZ-diabetic plus UA (DRU) rats.

Results

The study results established no significant interaction between the UA supplementation and the resistance/endurance training with regard to the levels of glucose (p = 0.534), insulin (p = 0.327), high-density lipoprotein cholesterol (HDL-c) (p = 0.960), cholesterol (p = 0.107), SIRT1 (p = 0.640), and eNOS (p = 0.151). However, the resistance/endurance training plus the UA consumption could partially reverse the levels of malondialdehyde (MDA) (p = 0.001), nitric oxide (NO) (p = 0.009), as well as total antioxidant capacity (TAC) (p = 0.016).

Conclusions

In general, the UA supplementation combined with the resistance/endurance training did not affect vascular aging biomarkers. To develop novel practical nutritional strategies involving UA intake, further studies are thus needed to clarify how chronic consumption of UA with/without resistance/endurance training reverses vascular aging process in old male Wistar rats with HFD/STZ-induced T2D.

Introduction

Both aging and diabetes are two well-known risk factors associated with vascular aging/senescence, so that diabetes increases aging process through augmenting the severity of the loss of autonomic functions [1, 2]. In this sense, vascular aging/senescence refers to a particular kind of organic aging

related to age-associated modifications in the structure and the function of endothelial cells (ECs) [3]. Vascular aging also shapes the severity of diverse cardiovascular diseases (CVDs), which make it a serious risk factor and lead to high mortality rates in the elderly population [1, 4]. The cellular-molecular mechanisms underlying vascular aging as well as diabetes in older adults are complicated and entail numerous signaling pathways and parameters [5]. The majority of the inflammatory mechanisms associated with vascular aging are thus correlated with advancing age, which overlap the mechanisms present in diabetes [6].

It has been additionally well-established that sirtuin 1 (SIRT1) is implicated in diet and age-induced pathogenesis of type 2 diabetes (T2D) [7, 8]. Besides, SIRT1-endothelial nitric oxide synthase (eNOS) axis [9], oxidative stress (OS), malondialdehyde (MDA), lipid profile, as well as total antioxidant capacity (TAC) and superoxide dismutase (SOD) [10] are among the indices of endothelial-vascular function impairment due to aging and diabetes [6]. However, the mechanisms underlying the changes in the SIRT1-eNOS axis mediated by aging and diabetes in ECs remain obscure [11]. It seems that the SIRT1-eNOS axis can account for the above-mentioned mechanisms in case of aging and diabetes coexistence and influence vascular functions [9, 11]. Moreover, it has been suggested that increased vascular expression and function of SIRT1 can protect early vascular senescence and regulate vasorelaxant responses [12].

As caloric restriction interventions alter cellular redox status and subsequently make changes in gene expression and activity of SIRT1 [13, 14], it seems that the same modifications caused by resistance/endurance training as an intervention may show similar effects. Recently, evidence has illustrated the impact of resistance/endurance training on the SIRT1-eNOS axis and OS indices [15, 16]. For example, Ferrara et al. had reported that six weeks of an endurance-type training had elevated SIRT1 activity in the heart of aged rat models [17].

In this context, several experimental and epidemiological findings had further underlined the role of natural bioactive compounds and synthetic substances in the prevention of CVDs and aging-related endothelial dysfunction, since they could be directly associated with vascular protection via different cellular-molecular pathways [18–20], especially some SIRT1-mediated anti-aging natural compounds in ECs [21].

Scientists have also suggested the potential synergism of resistance/endurance training with other natural supplements [22]. Moreover, it has been evidenced that chronic training interventions may increase cellular, molecular, and physiological adaptations combined with natural supplements that will not be possibly achieved in response to training and pharmaceuticals alone. Thus, multimodal interventions (namely, training plus supplement intake) may boost the efficacy of training programs for less-sensitive individuals such as aged cases with diabetes.

Ursolic acid (UA, 3 β-hydroxy-urs-12-en-28-oic acid) is a major component of various traditional Chinese medicinal herbs, plants, fruits and foods such as Fructus Mume, Gardeniae Fructus, Fructus Ligustri Lucidi, Hedyotis diffusa Willd and apple peel [23, 24], and is well known to have a wide range of biological functions, including antioxidant, neuroprotection, hepatoprotection, regulating blood glucose,

and anti-inflammatory activities [23, 25, 26]. In this regard, it has been shown that UA mediates the vasorelaxant activity via nitric oxide (NO) release in the aorta tissue [27], and up-regulation of eNOS and down regulation of Nox4 in human endothelial cells in disease conditions with increased oxidative stress [28]. Schwaiger et al., illustrated the anti-inflammatory effects of UA by inhibiting TNFα-stimulated expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) and support the use of traditional herbal food is rich in UA, for the treatment of chronically inflammatory processes [29]. Recently, Bakhtiari et al. had revealed that UA had elevated SIRT1 activities in molecular docking and experimental studies [30].

Several lines of evidence have further referred to enhanced SIRT1 content following an increase in the rate of training [31–35]; nonetheless, various outputs have failed to demonstrate changes in the gene expression of SIRT1 based on training modality [36]. Moreover, variations have been observed in training intensity, volume, duration, SIRT1 content measurement, as well as respective tissues. In this line, Chan et al. had reflected on the expression of SIRT1 in aortic ECs after a 14-week endurance training program. Chronic exercises had thus shown a descending trend in the protein expression level of SIRT1 in hyperhomocysteinemia-induced aortic endothelial oxidative injury [37]. To the best of authors' knowledge, there was no research on SIRT1 expression in vascular tissues with regard to the use of chronic resistance/endurance training combined with UA supplementation in aged Wistar rats with diabetes. Accordingly, this study aimed to determine the impact of eight weeks of a combination of resistance/endurance exercise training and UA supplementation in old Wistar rats with diabetes based on the SIRT1-eNOS axis and OS indices.

With regard to the pivotal roles of SIRT1 in vascular senescence, it was hypothesized that that a combination of resistance/endurance training and UA supplement, compared with no training/placebo in a control group, would differently change the SIRT1-eNOS axis as well as OS biomarkers. To develop pharmacological treatments and physical activity interventions for the amelioration of vascular aging and prevention of age-related vascular pathologies, this study planned to investigate the effects of eight weeks of a combination of endurance/resistance training and UA supplementation on old male Wistar rats with high-fat diet and or low-dose streptozotocin-induced type 2 diabetes (HFD/STZ-induced T2D) with regard to the SIRT1-eNOS axis and oxidative stress biomarkers.

Materials And Methods

Animal

The local Ethics Committee for Laboratory Animals of Shahrekord University (Shahrekord, Iran) approved the study. All methods were performed in accordance with the relevant guidelines and regulations. The experiment was conducted in accordance with the ARRIVE guidelines (Animal Research) [38] for the care and use of research animals.

A total number of 56 21-month-old male Wistar rats, weighing 427±44 g, were accordingly purchased from Pasteur Institute, Tehran, Iran, and then maintained at a temperature-controlled facility (20-22°C)

with 40-70% humidity, 12-h light/dark cycle, as well as free to access to a commercial standard pellet chow diet and water during the experiments. As the rats became familiarized with the laboratory conditions, they were randomized into seven groups (n=eight animals per group): (1) sedentary old non-diabetic (C); (2) sedentary HFD/STZ-induced T2D (D); (3) sedentary HFD/STZ-induced T2D plus UA (DU); (4) endurance-trained HFD/STZ-induced T2D (DE); (5) resistance-trained HFD/STZ-induced T2D (DR); (6) endurance-trained HFD/STZ-induced T2D plus UA (DEU); and (7) resistance-trained STZ-diabetic plus UA (DRU) rats.

HFD/STZ-Induced T2D

As demonstrated in the protocol presented by Zhang et al. [39] and Liu et al., HFD/STZ-induced T2D was stimulated [40]. Therefore, the animals in the sham group were fed with a standard rodent-chow diet (sham; energy from fats [10%], carbohydrates [75%], and protein [15%]=3.8 kcal/g), whereas the animals in the HFD groups were placed on a high-fat diet; that is, HF; energy from fats (55%), carbohydrates (31%), as well as protein (14%)=5.2 kcal/g. Then, an eight-week diet was considered for maintaining the two groups. In the course of the fourth week, the HFD/STZ-induced T2D group was treated with low-dose STZ (Sigma-Aldrich, St Louis, MO, USA). In the next step, the low-dose STZ, that is, 30 mg/kg dissolved in 0.1 M sodium-citrate buffer at a pH of 4.4, was injected to the animals intraperitoneally. The level of blood glucose was also tested after the first week with a blood glucose meter. Accordingly, the animals with blood glucose levels ⬚16.7 mmol/l were injected with STZ (30 mg/kg) for the second time. After that, the sham group was injected with citrate-buffer (as a vehicle) (0.25 ml/kg). It is notable that the mentioned diets were maintained at the post-injection stage. After four weeks, each rat with the blood glucose concentration ⬚16.7 mmol/l was regarded to be diabetic and thus was chosen for additional investigations [41, 42].

Subsequently, HFD/STZ-induced T2D old male Wistar rats were fed with the high-fat diet (55% fats, 31% carbohydrates, and 14% protein). The sham animals also received a standard diet (10% fats, 75% carbohydrates, and 15% protein) throughout the study. The HFD plus UA (250 mg UA per kg of body weight rat/day) was further prepared by mixing 500 mg of UA per kg of HFD (0.5% UA plus HFD, Royan Company, Isfahan. Iran.) [43] (figure 1). It should be noted that the high-fat diet plus UA was prepared at the three-day interval to avoid the oxidation of fat or other compounds (Knowledge-Based Company, Healthy-Aging Supplement 9870, Tehran, Iran). The daily mean amount of the food intake was calculated as a difference between the amount of the remained food and the total one provided, divided by the number of days and rats in cages. Since the energetic values between the diets differed, the use of food in grams was converted into the caloric intake [44] and finally weekly body weight for each rat was measured during the investigation [45].

Exercise testing and exercise training

To evaluate vVO$_{2max}$, a ten three-min phase running test on a rodent treadmill was used. According to Leandro et al., initial running speed test was equal to 0.3 km/h and then speed elevated by 0.3 km.h^{-1} per

3 min (the slope was equal to 0%). Therefore, in case of the rats' inability to continue the running in all phases of the experiment, the speed at that phase was considered as vVO$_{2max}$ [46].

To evaluate, determination of maximum voluntary carrying capacity (MVCC) the animals were let to be familiarized with a vertical climbing model (110 cm, 2-cm grid, 85° incline) without overloading [47]. Following a 72-h familiarization, all the animals were also tested to assess the respective MVCC. However, all of them carried a load of 75% of the body weight to the top of the ladder (viz. a house chamber) for the first climb. Then, the rats were allowed to rest for 120 s. After that, some weight increments of 30 g were added till the load did not let the rats climb the whole length of the ladder. It is noteworthy that the given process was iterated, so that the animals were not voluntarily capable of climbing the entire length of the ladder on three subsequent efforts. Finally, the MVCC was viewed as the successfully greatest load over the whole length of the ladder [47].

In resistance training protocol, the rats became familiarized with the apparatus by climbing it without the weights attached to their tails. Moreover, the weights were then fastened to the animals' tails. In crucial circumstances, a physical stimulus with finger pinching on the rats' tails was further employed as a stimulus for initiating the climbing movements. Upon the arrival of the animals in a housing chamber, they were let to rest for two min. Then, the procedure was iterated till the animals could voluntarily climb for three consecutive attempts [48]. The resistance training group also performed the ladder resistance training at 60% of the MVCC, 14-20 climbs in each session, with a one-min rest between each two trials, five days a week [49]. Finally, the group with diabetes was restricted to do any physical exercise training beyond the normal cage activities.

Within the eight-week endurance training, the animals were exposed to endurance training programs. Then, the training intensity was calculated by vVO$_{2max}$. At the beginning of the endurance training, the animals were trained at 40-50% of vVO$_{2max}$ for five min and 0% incline for warm-up. The endurance training protocol consisted of repeated bouts of high- and low-intensity training, two min of running with 60% vVO$_{2max}$ in the course of the first week, 65% vVO$_{2max}$ during the second week, 70 % vVO$_{2max}$ in the course of the third week, and finally 75% vVO$_{2max}$ during the fourth week to the completion of the training time. Moreover, low-intensity bouts involved two-min running with 40% vVO$_{2max}$ from the first week to the end of the third week and 30% vVO$_{2max}$ from the onset of the fourth week to the completion of the eighth week.

At the end, the number of the high-intensity interval bouts increased from two to eight reps from the first to the end of the eighth week [50].

Blood sample and collection of Aortic tissue

The rats were weighed and anesthetized through intraperitoneal administration of a mixture of 90 mg/kg ketamine and 10 mg/kg xylazine. The animals were then sacrificed 48 h following the final

resistance/endurance training session. The thoracic aortas were then gently dissected out and immediately immersed in liquid nitrogen.

Western Blotting

According to the research design, Western blotting was run on the homogenates of the aortic tissues. Briefly, a pestle was employed to powder about 50 mg of the aortic tissue piece in liquid nitrogen and then lysed with a 1 mL of phosphate buffered saline (PBS). Moreover, this buffer was complemented with a protease inhibitor cocktail consisting of pepstatin, leupeptin, aprotinin, antipain, as well as chymostatin (5 µg/ml each) and rotated for twenty min at 4°C. In addition, they were centrifuged at 12,000×g at a temperature of 4°C for ten min. Hence, this supernatant was gathered and kept at -80°C. The total protein content of the tissue extract was correspondingly determined by the Bradford method (Bio-Rad Laboratories, CA, USA) and spectrophotometric measurements (Jenway™ 6305 UV/Visible Spectrophotometer, Bibby Scientific Ltd., UK). The proteins were then isolated through the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique (10 µg protein loaded in the wells) and transported electrophoretically over the polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membranes. It should be noted that the non-specific binding was further obstructed by a two-h incubation of the membrane in 5% (w/v) non-fat dry milk in the Tris-buffered saline (TBS) at a power of hydrogen (pH) of 7.5. After that, the incubation of the blots was done for two h at the room temperature or over-night at a temperature of 4°C with the primary antibodies; that is, anti-eNOS, SIRT1, as well as β-actin (Santa Cruz, USA) 1:500, diluted in an antibody buffer consisting of 1% (w/v) non-fat dry-milk in TBS and polysorbate 20 (Tween 20) (0.05% (v/v) in the TBS). Afterwards, they were washed three times with TBS-T and consequently incubation was fulfilled for one h with a secondary antibody, that is, goat anti-rabbit (IgG) (Santa Cruz, USA) 1:5000, in the antibody buffer. Next, the blots were developed to visualize by means of the enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL, USA). Finally, antiβ-actin was employed as one of the loading controls. The band intensity on immunoblots was further quantified via densitometry using the Image Studio Lite software.

Histological Analysis

The aortic tissues were fixed in 10% formalin solution in the continuous dehydration in the growing grades of ethanol (Merck: Germany), then cleared, and put in xylol and paraffin. Afterwards, 5 µm sections were taken and stained with Hematoxylin & Eosin (H & E). In the next step, a pathologist examined them under a light microscope (Olympus BH-2; Tokyo: Japan) in a blinded manner. In addition, a minimum of five fields for all aorta slides were investigated and allocated to determine the severity of the tissue modifications (n= 8 for each group).

Plasma Glucose, Insulin, and Lipid measurement

In this step, fasting blood glucose (FBG) in the tail vein blood sample was measured at the beginning of the experiment through a blood glucose meter to ensure that the animals had euglycemia. Following the HFD/STZ-induced T2D, blood glucose level was measured to assess the initiation of hyperglycemia (FBG

200 mg/dl). Moreover, the enzyme-linked immunosorbent assay (ELISA) (Mecodia, Winston-Salem Inc., NC, USA) was utilized to measure the plasma insulin levels in the rats that had been fasted for four h. In addition, total cholesterol, fasting plasma TG, LDL-c, as well as HDL-c were measured with the use of a commercial diagnostic kit (Randox, UK), based on the company's directions.

Antioxidant activity

To evaluate NO, SOD, MDA and TAC ZellBio GmbH Nitric Oxide (NO) assay kit (CAT No. ZB-NO-96A), ZellBio GmbH Superoxide Dismutase (SOD) assay kit (CAT No. ZB-SOD-96A), and Biocore (ZellBio) Malondialdehyde (MDA) Assay kit (ZB-MDA96) and TAC assay kit (cat. number NX2332) (Randox Laboratories, Crumlin, UK) were used, respectively according to the company's guidelines.

Data analysis and statistics

According to the research design, the statistical analyses were completed using the SPSS Statistics software (ver. 21). Then, two-way analysis of variance (ANOVA) with training conditions in three levels (namely, resistance, endurance, and no exercise training) and supplementation in two levels (UA & no supplementation) were applied to reflect on the impact of the training as well as supplementation on modifications in the serum and gene expression outcomes. As well, the two-way ANOVA with group status in six levels (viz. D, DE, DR, DU, DEU, and DRU) and time with three to eight levels (that is, three levels for vVo_2 and MVCC [i.e., weeks one, four, and eight], five levels for blood glucose [namely, weeks one, two, four, six, and eight], and eight levels for body weight [viz. weeks one, two, three, four, five, six, seven, and eight]) was employed to determine the impact of group and time status on the alterations in descriptive outcomes. It should be noted that the sham group data were not included in the statistical analysis. As well, the Partial Eta squared (ηp^2) was utilized to indicate the effect size with the main effect as well as the interaction effect from the two-way ANOVA. Furthermore, Tukey's test was employed for each analysis, which required a post-hoc test. To examine the correlation between the parameters, the Pearson correlation coefficient was consequently practiced. The level of statistical significance was estimated to be $p < 0.05$.

Results

Blood Glucose and Weight Changes in Rats during the Study in Each Group

The two-way ANOVA results did not show any remarkable group main effect ($p=0.151$, $\eta p^2=0.041$), time main effect ($p=0.091$, $\eta p^2=0.061$), as well as group by time interaction effect ($p=0.998$, $\eta p^2=0.001$) on the body weight following the eight weeks of interventions.

No significant group main effect ($p=0.071$, $\eta p^2=0.080$) and group by time interaction effect ($p=0.307$, $\eta p^2=0.061$) were also observed, but a considerable time main effect ($p=0.017$, $\eta p^2=0.095$) was reported on the glucose levels after the completion of the eight-week interventions. The post-hoc comparisons for

the time main effect correspondingly showed that the glucose levels had significantly decreased in the fourth (p=0.045) and sixth weeks (p=0.042) compared with the first one.

MVCC and vVO$_{2max}$ Changes in Study Groups

A significant group main effect (p=0.001, ηp^2=0.361) and a time main effect (p=0.001, ηp^2=0.206), but no group by time interaction effect (p=0.178, ηp^2=0.171) was observed for the MVCC. The post-hoc comparisons for the group main effect also revealed that the MVCC had remarkably enhanced in the DR (p=0.010) and the DRU (p=0.001) groups compared with that in the D one, in the DRU (p=0.001) as well as the DR groups (p=0.015) in comparison with the UA one, as well as in the DRU group (p=0.002) as compared with the DE one. Moreover, the post-hoc comparisons for the time main effect demonstrated that the MVCC had been significantly boosted in the post-test in comparison with that in the mid-test (p=0.001) and the pre-test (p=0.004).

As well, the vVo$_{2max}$ showed a significant main group effect (p=0.001, ηp^2=0.657), time main effect (p=0.001, ηp^2=0.201), as well as group by time interaction effect (p=0.001, ηp^2=0.464). The post-hoc comparisons for the group main effect also revealed that the vVo$_{2max}$ had significantly increased in the DEU, DE, DRU, and DU groups compared with the D one. This was similarly evident in the DEU, DE, and DRU groups compared with the DR one; as well as in the DEU and DE groups in comparison with the DRU and DU ones (p<0.05).

The post-hoc comparisons for the time main effect correspondingly revealed that the vVo$_{2max}$ had remarkably elevated in the mid-test (p=0.019) and the post-test (p=0.001) as compared with that in the pre-test. The post-hoc comparisons for the group by time interaction effect additionally established that the vVo$_{2max}$ had significantly increased in the DEU and DE groups compared with that in the D, DR, DRU and DU ones; as well, in the DRU group in comparison with the D and DR ones in the mid-test. Moreover, a significant growth in the vVo2$_{max}$ in the post-test was observed in the DEU and DE groups compared with that in the D, DR, DRU, and DU ones, and in the DRU group compared with that in the D one in the mid-test (p<0.05) (**Figure 2**).

Serum Glucose, Insulin, and Lipid Changes in Study Groups

The two-way ANOVA results demonstrated no remarkable training main effect (p=0.465, ηp^2=0.067), supplement main effect (p=0.976, ηp^2=0.001), as well as training by supplement interaction effect (p=0.534, ηp^2=0.055) on the glucose levels. Similarly, there were no significant training main effect (p=0.163, ηp^2=0.152), supplement main effect (p=0.138, ηp^2=0.097), and training by supplement interaction effect (p=0.327, ηp^2=0.097) on the insulin.

Likewise, the HDL-c, TG, and cholesterol did not show any significant training main effect (p=0.403, ηp^2=0.079; p=0.229, ηp^2=0.125; p=0.693, ηp^2=0.033, respectively), supplement main effect (p=0.568, ηp^2=0.015; p=0.733, ηp^2=0.005; p=0.708, ηp^2=0.007, respectively), and training by supplement interaction

effect (p=0.960, ηp^2=0.004; p=0.582, ηp^2=0.048; p=0.107, ηp^2=0.184, respectively). Furthermore, the LDL-c also did not establish a considerable training main effect (p=0.906, ηp^2=0.009) as well as a supplement main effect (p=0.557, ηp^2=0.016), but it revealed a significant training by supplement interaction effect (p=0.042, ηp^2=0.251). The post-hoc comparisons also found that the LDL-c significantly decreased in the DE (p=0.040) and DU groups (p=0.025) compared with that in the DEU one (**Figure 3**).

Expression of SIRT1-eNOS Axis Changes in Study Groups

In this step, the two-way ANOVA results illustrated a considerable supplement main effect (p=0.001, ηp^2=0.420) but no training main effect (p=0.125, ηp^2=0.159), as well as training by supplement interaction effect (p=0.481, ηp^2=0.059) on the SIRT1/β-actin. The post-hoc comparisons also showed that the UA supplementation had significantly increased the SIRT1/β-actin compared with no supplementation condition (p=0.001). As well, there was a significant training main effect (p=0.089, ηp^2=0.183) as well as supplement main effect (p=0.011, ηp^2=0.241) but no training by supplement interaction effect (p=0.418, ηp^2=0.070) on the eNOS/β-actin. The post-hoc comparisons for the supplement main effect also revealed that the UA supplementation had significantly boosted the eNOS/β-actin compared with none (p=0.011) (**Figure 4**).

Expression of MDA, Superoxide Dismutase (SOD), NO, and TAC Changes in Study Groups

The two-way ANOVA results indicated a considerable training main effect (p=0.010, ηp^2=0.321) and training by supplement interaction effect (p=0.001, ηp^2=0.471), but no supplement main effect (p=0.247, ηp^2=0.055) on the levels of MDA. The post-hoc comparisons for the training main effect also showed that the MDA levels had significantly declined in the endurance training compared with the resistance condition (p=0.040) and no training (p=0.025). As well, the post-hoc comparisons for the interaction effect revealed that the levels of MDA had significantly diminished in the DE group in comparison with that in the DEU (p=0.047), DR (p=0.001), and DU ones (p=0.001).

Moreover, a significant training by supplement interaction effect (p=0.009; ηp^2=0.218) but no training main effect (p=0.307, ηp^2=0.094) as well as supplement main effect (p=0.645, ηp^2=0.009) was found on the NO. The post-hoc comparisons showed that the NO had significantly decreased in the DR (p=0.007) and DEU (p=0.013) groups in comparison with the CD one. Similarly, the NO had significantly reduced in the DEU group compared with that in the DRU one (p=0.037). Additionally, a considerable decrease was found in the DR group in this respect in comparison with the DRU one (p= 0.019). Similarly, a significant training by supplement interaction effect (p=0.016, ηp^2=0.293) but no training main effect (p=0.992, ηp^2=0.001) and supplement main effect (p=0.645, ηp^2=0.009) were observed on the TAC. The post-hoc comparisons also revealed that the TAC had significantly augmented in the DE group compared with that in the DR (p=0.041) and DEU ones (p=0.028). In addition, the TAC had considerably elevated in the DRU group in comparison with that in the DEU (p=0.033) and DR (p=0.019) ones.

There was also a significant supplement main effect (p=0.013, ηp^2=0.232), but no training main effect (p=0.318, ηp^2=0.091) as well as training by supplement interaction effect (p=0.122, ηp^2=0.161) on the SOD. The post-hoc comparisons for the supplement main effect additionally revealed that the SOD had significantly increased in the UA supplementation condition compared with that in no supplement (p=0.013) (**Figure 5**).

General Microscopic Evaluation of Rat Aorta in Experimental Groups

As illustrated in Figure 6, the H&E-stained cross-section specimens of the aortas from the control group revealed the typical and normal appearance of all tunicae. In this respect, the tunica intima had a normal arrangement of ECs with the tunica media showing the regular organization of the elastic lamellae as well as the horizontally-oriented spindle-shaped nuclei of the smooth muscle cells. In addition, the tunica adventitia had a normal appearance in most sections. In contrast, the sections obtained from the group with diabetes showed altered morphology, including partial degeneration of the smooth muscle-cell nuclei in the tunica media and partial disarrangement and/or discontinuity of the elastic lamellae in some areas. Such pathologic alterations also existed to some extent in all diabetic groups treated. However, these inappropriate changes were less noted in the DEU group.

Discussion

Understanding how endothelial-vascular cells sense and respond to distinct training modalities plus UA supplementation has been thus far highly researched in aged diabetic animals. In the present study, the effects of chronic oral UA supplementaion, alone and in combination with endurance/resistance training, on vascular aging signaling proteins such as the SIRT1-eNOS axis and the OS indices were evaluated in old male wistar rats with HFD/STZ-induced T2D.

Overall, there are some trials evaluating the impact of different training modalities and/or anti-aging/diabetes supplements such as UA on major hallmarks of EC senescence, SIRT1-eNOS axis, and OS indices in aortic ECs in aged male Wistar rats with T2D. Due to the higher life-threatening risks in aged patients with T2D, pharmacological treatmetns and physical activity rehabilitation options have been always discussed for vascular aging interventions. Training as a safe modality may thus offset age-associated vascular dysfunctions such as impaired endothelial function, aortic stiffness, plaque formation, and impaired angiogenic responses [51, 52]. Furthermore, it has been shown that UA may become a vascular-protective, anti-inflammatory, anti-oxidative, and anti-diabetes supplement for the treatment of vascular aging-associated dysfunctions [53–55]. As well, UA can mediate hyperglycemia through stimulating glucose uptake and reducing blood glucose levels and glycogen synthesis [56, 57], but the mechanisms by which UA improves glycemia remain to be completely elucidated [56].

All study data showed that the daily food intake did not differ between the groups. These results indicated that UA with/without training protocols did not alter feeding behaviors of the experimental

Wistar rats. The animals in the supplement groups also took 500 mg of UA/kg of high-fat diet/day, resulting in a daily UA intake of approximately 250 mg UA per kg of body weigh rat/day.

The findings in the present study revealed that a combination of UA and training did not improve vascular aging biomarkers such as serum glucose, insulin, HDL-c, TG concentration, and aortic SIRT-eNOS content following eight weeks of intervention, which were not consistent with the results reproted by Jang et al., wherein the UA consumption had improved lipid profiles, glucose utilization, and glycogen storage in the liver in STZ-induced diabetic mice [58]. In another study, Jayaprakasam et al. had found that eight weeks of UA plus anthocyanin supplementation (500 mg/kg of the high-fat diet) had ameliorated glucose tolerance in the C57BL/6 mice, receiving high-fat diets.

The discrepancy between the results of the present study and the ones reproted by Jayaprakasam et al. may be attributable to the difference in the compounds other than UA. The study by Jayaprakasam et al. had accordingly shown that improving glucose status might be associated with UA ability to enhance insulin levels, that was not established in the present study with a combination of UA plus training although it did not change the body weight compared with the D group. It seems that old animals having HFD/STZ-induced T2D might have become insulin-resistant, and UA plus training intervention has failed to decrease the blood glucose levels.

Therefore, HFD/STZ-induced T2D aged animals are susceptible to atherosclerosis. In this study, a combination of training and UA summplementiaon was not a potent inhibitor of vascular aging in the HFD/STZ induced T2D old animals. One possible reason was that UA could reduce in the flux of the reactive oxygen species (ROS) in monocytes that would be essential for oxidation, mitogen-activated protein kinase phosphatase 1 (MKP-1) inactivation, as well as S-glutathionylation of protetin, but, UA chemical structure illustrated that it would not be readily oxidized and hence there would be a poorer scavenger of ROS [57].

It was established in this study that oral 0.5% UA supplementation did not prevent vascular aging biomarkers in a HFD/STZ-induced T2D model. In addition, the UA supplement possibly did not enjoy the benefits of protecting the endothelium against aging-dependent dysfunctions [55, 59]. Hence, in this study, lack of protection of vascular against oxidative stress induced dysfunctions involved in ineffective anti-vascular aging properties of UA. Another possibility was that the given dose of UA did not modulate the expression of cytokines related to T-helper 1–2 cells, that had possibly influenced the pro-atherogenic T-cell responses [60].

However, UA could not reduce food intake and weight gain. It seems that UA may not enhance the metabolic rate of this HFD/STZ-induced T2D model. In the present model, UA did not affect the lipid profile or the blood glucose levels. Nonetheless, physiologically relevant doses of UA were injected.

Furthermore, a decrease in the SIRT1-eNOS axis and the OS indices related to vascular aging were not mediated by the protective impact of UA on the metabolic stress-induced monocyte-priming and dysfunctions that declined the recruitment of the monocyte-derived macrophages into the aortic tissues.

On the other hand, UA supplementaion could enhance the SIRT1 expression in the vein ECs. Jiang et al. had also shown that 10 weeks of UA supplementation following high-fat diet had improved serum lipid profile, serum antioxidant status, and morphology of the aorta in the human umbilical vein ECs (HUVECs) [61]. Contrary to the results of the present study, Chang et al. had shown that a long-term 14 week aerobic training (60 min per day) for five days/per week had improved atherosclerosis status via activating the SIRT1 and inhibiting the OS in the C57BL mice [37]. Recently, Donniacuoa et al. had established that long-term moderate training had diminished the apoptosis of cardiac myocytes and had consequently promoted SIRT1 nuclear localization [16].

One possibility was that there was a strong correlation between obesity, body mass index (BMI), and SIRT1 gene polymorphisms. As well, SIRT1 expression was nutrient-sensitive [62]. Therefore, the HFD/STZ-induced T2D rat models in the present study had no significant responses to combined UA supplementation and training considering the SIRT1-eNOS axis. Contrary to these results, Lin et al. had illustrated that resveratrol treatment could increase the beneficial impact of training in aged rat heart models.

It has been shown that training combined with the use of natural antioxidants improves antioxidant system [63]. UA can further increase the expression of endothelial NOS, reduce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase expression, enjoy potency of reversing eNOS un-coupling, and prevent damage to ECs [64, 65]. It seems that UA plus different types of training modalities possess strong antioxidant properties and this bioactive free radical scavenger compound has potentials to remove free radicals, which is helpful in maintaining the ROS levels [66, 67]. SOD is also considered as an enzyme that regulates the production of oxygen (O_2) and prevents oxidative damage in the cells.

Another prominent downstream mechanism that has been regarded for the survival signalling pathway of ECs via UA [28, 68, 69] plus training [70–72] was that UA may accelerate the expression and phosphorylation of the eNOS, with subsequently increased NO production.

Furthermore, the consumption of UA plus training did not improve the lipid profile as a vascular aging biomarker in the HFD/STZ-induced T2D model. The lipid profile in the UA plus training-treated HFD/STZ induced-T2D rats were similar to those of DC. It had been established that UA could moderately decline in the liver lipid deposition [73]. Thus, the consumption of UA plus training might not modulate the lipid metabolism via influencing the oxidation and lipogenesis of the hepatic lipid in the HFD/STZ-induced T2D model. As UA did not protect the islet architecture, it augmented insulin levels in the HFD/STZ-induced T2D model. The observations in the present study also illustrated that UA supplementation plus training may not be influencing bio-synthesis, secretion of insulin, or insulin clearance. It had been also found that HFD/STZ-induced T2D model had expanded the islets with diffuse staining as well as irregular structures [45]. It seems that UA intake plus training protocols may not protect islets from the metabolic insults linked to the HFD/STZ-induced T2D and enhanced insulin secretion, as there is a correlation between blood insulin levels and islet size [45].

Another possibility discussing that the consumption of UA plus training did not improve vascular aging was that the side effects of UA of a supra-physiological dose were capable to make platelets more susceptible to aggregation [74], that was not for anti-vascular aging in ECs. Indeed, anti-platelet aggregation of UA supplementation was not enough to exert a significant effect. In addition, it had been shown that the UA solubility was too low to be applied to platelets. Instead, it has been supposed that UA supplementation might make platelets sensitive to stimuli [74]. Furthermore, circulating UA concentrations were not measured in the present study, but an oral dose of 250 mg UA per kg of body weight rat/day was used, which could be lower than the concentration enough for physiological effects in vascular aging biomarkers.

As expected, this study confirmed that the increased SOD, NO, and TAC content and the decreased MDA levels were significant following the use of a combination of UA and training interventions, indicating that UA supplement plus training could play a protective role against oxidative damage in vascular aging in old diabetic rats. These results showed that UA, especially 250 mg UA per kg of body weight rat/day plus resistance/endurance training could ameliorate antioxidant balance parameters of MDA, NO, TAC, and SOD in aortic tissues. In other words, UA consumption plus training elevated power and capacity of the antioxidant system. Indeed, the greatest reductions in the MDA and the largest increases in the SOD, NO, and TAC levels were identified in the rats that had been fed with UA supplement and had received the training programs (Fig. 4), suggesting a synergistic effect between training and supplementation with UA on antioxidant capacity. The oral administration of UA, during eight weeks, combined with training in HFD/STZ-induced T2D model also improved vascular antioxidant capacity.

The UA combined with training modalities similarly increased eNOS expression in the aorta. It seems that a combination of UA and training may reduce the NADPH oxidase 4 (NOX4) contents, inhibit the eNOS un-coupling, which augmented NO, and thus decline the levels of ROS in the aorta following the UA supplementation and training interventions [75]. This study was not consistent with the outputs presented by Steinkamp-Fenske et al., wherein UA had increased eNOS in ECs [75].

Considering MDA as an endogenous product of both enzymatic and oxygen radical-induced lipid peroxidation [76], Somova et al. (2003) had found that UA had increased glutathione peroxidase and SOD levels in insulin-resistant rats [77]. In another study, it had been established that UA had boosted SOD activity and had diminished MDA formation in PC12 cell line [78], so that decreased levels of MDA implicated that the UA could inhibit lipid peroxidation. Altogether, this study showed that the use of UA plus training in combination could increase vascular antioxidant capacity in HFD/STZ-induced T2D old Wistar rats while it had no effect on vascular aging biomarkers, lipid profile, and plasma glucose levels.

Conclusion

It was concluded that UA supplementation plus resistance/endurance training did not affect vascular aging biomarkers, so there is a need to establish practical nutritional strategies involving UA. Further studies are also required to clarify how chronic consumption of UA with/without training protocols

reverses vascular aging process. As expected, this study confirmed the improved antioxidant capacity by a combination of UA supplementation and two different training modalities, indicating that UA plus training could play a protective role against oxidative damage in vascular aging in HFD/STZ-induced T2D old male Wistar rats.

Abbreviations

UA: Ursolic acid, NO: nitric oxide, eNOS: endothelial nitric oxide synthase, HFD/STZ-induced T2D: high-fat diet and low-dose streptozotocin-induced type 2 diabetes, SIRT1: sirtuin 1, OS: oxidative stress, MDA: malondialdehyde, TAC: total antioxidant capacity.

Declarations

Acknowledgements

Data Availability Statement

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

Authorship contribution statement

M.K, E.B, M.R, and R.H devised the research. M.K and E.B were the supervisors of the training protocols. M.K, E.B, M.R, and R.H were the supervisors of laboratory exams as well as data collection. M.K, E.B, M.R, and R.H addressed data analysis and interpretation. M.K, E.B, M.R, and R.H compiled the first draft of this manuscript. E.B and M.R were responsible for editing the manuscript. Each author contributed to the manuscript writing, they also read and confirmed the final draft.

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Authors' contributions

Masoumeh Kazemi, Ebrahim Banitalebi, Mehrdad Rowghani, and Roohullah Hemmati devised the research. Masoumeh Kazemi and Ebrahim Banitalebi were the supervisors of the training protocols. Masoumeh Kazemi, Ebrahim Banitalebi, Mehrdad Roghani, and Roohullah Hemmati were the supervisors of laboratory exams as well as data collection. Masoumeh Kazemi, Ebrahim Banitalebi, Mehrdad Roghani, and Roohullah Hemmati addressed data analysis and interpretation. Masoumeh Kazemi, Ebrahim Banitalebi, Mehrdad Roghani, and Roohullah Hemmati compiled the first draft of this

manuscript. Ebrahim Banitalebi and Mehrdad Rowghani were responsible for editing the manuscript. Each author contributed to the manuscript writing, they also read and confirmed the final draft.

Availability of data and materials

The datasets used and/or analysed in the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

The local Ethics Committee for Laboratory Animals of Shahrekord University (Shahrekord, Iran) approved the study. All methods were performed in accordance with the relevant guidelines and regulations. The experiment was conducted in accordance with the ARRIVE guidelines (Animal Research) for the care and use of research animals.

Consent for publication

Not applicable.

Competing interests

There was no competing interest.

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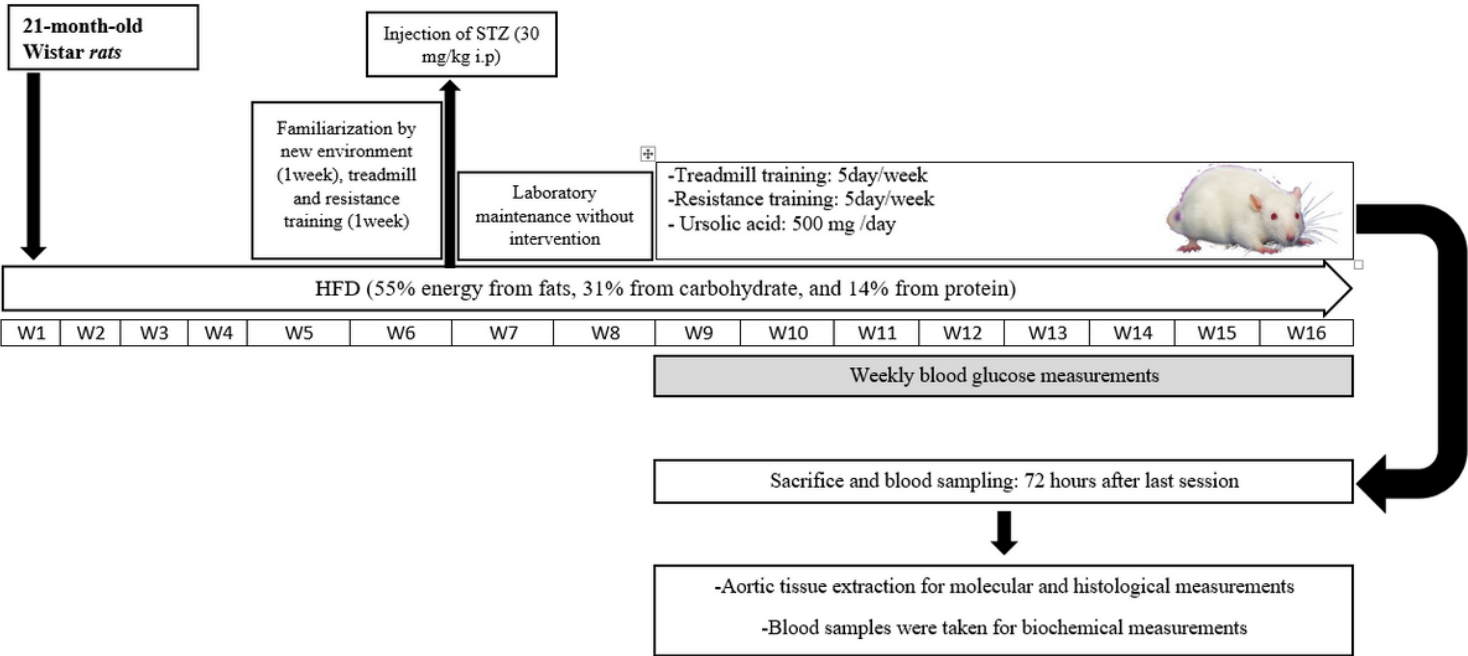
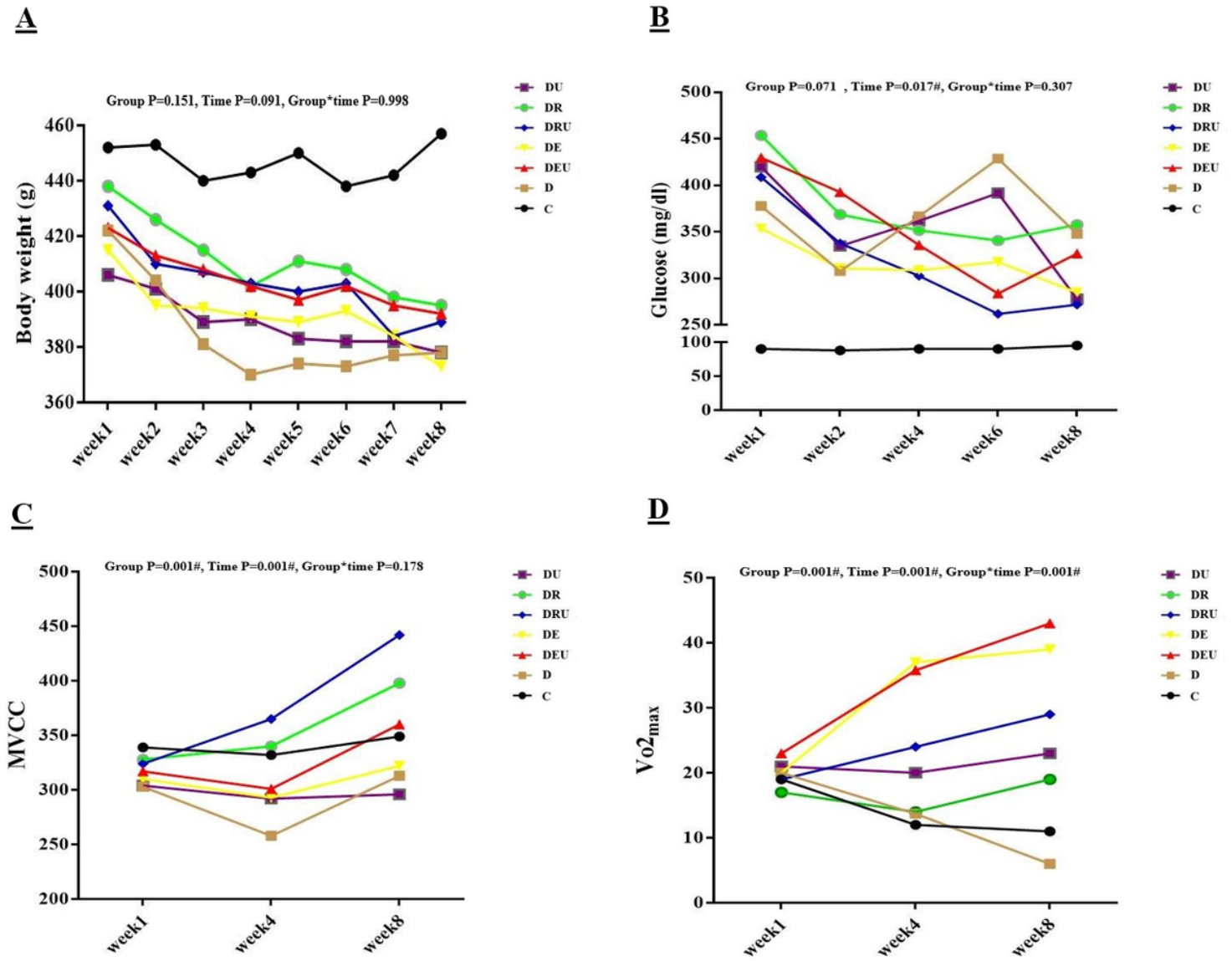# Figures

Figure 1

Study design

Figure 2

Body weight (A), glucose (B), Maximal voluntary carrying capacity (MVCC) (C) and Velocity at VO2max (vVO2max) (D) changes following 8-weeks exercise training and ursolic acid. DU: Diabetic+Ursolic Acid; DR: Diabetic+Resistance Training; DRU: Diabetic+Resistance Training + Ursolic Acid; DE: Diabetic+Endurance Training; DEU: Diabetic+Endurance Training + Ursolic Acid; D: Diabetic; C: Control.
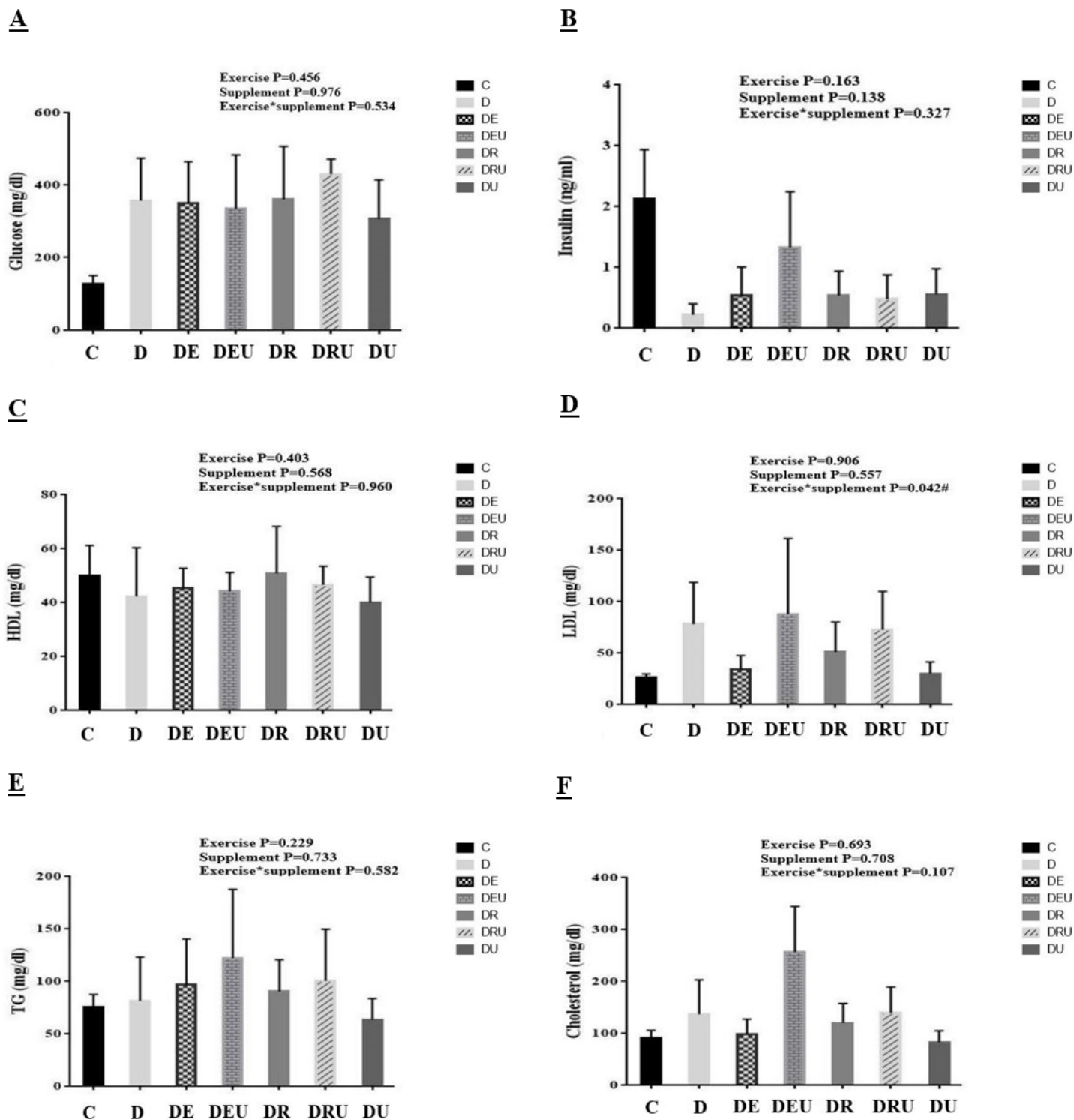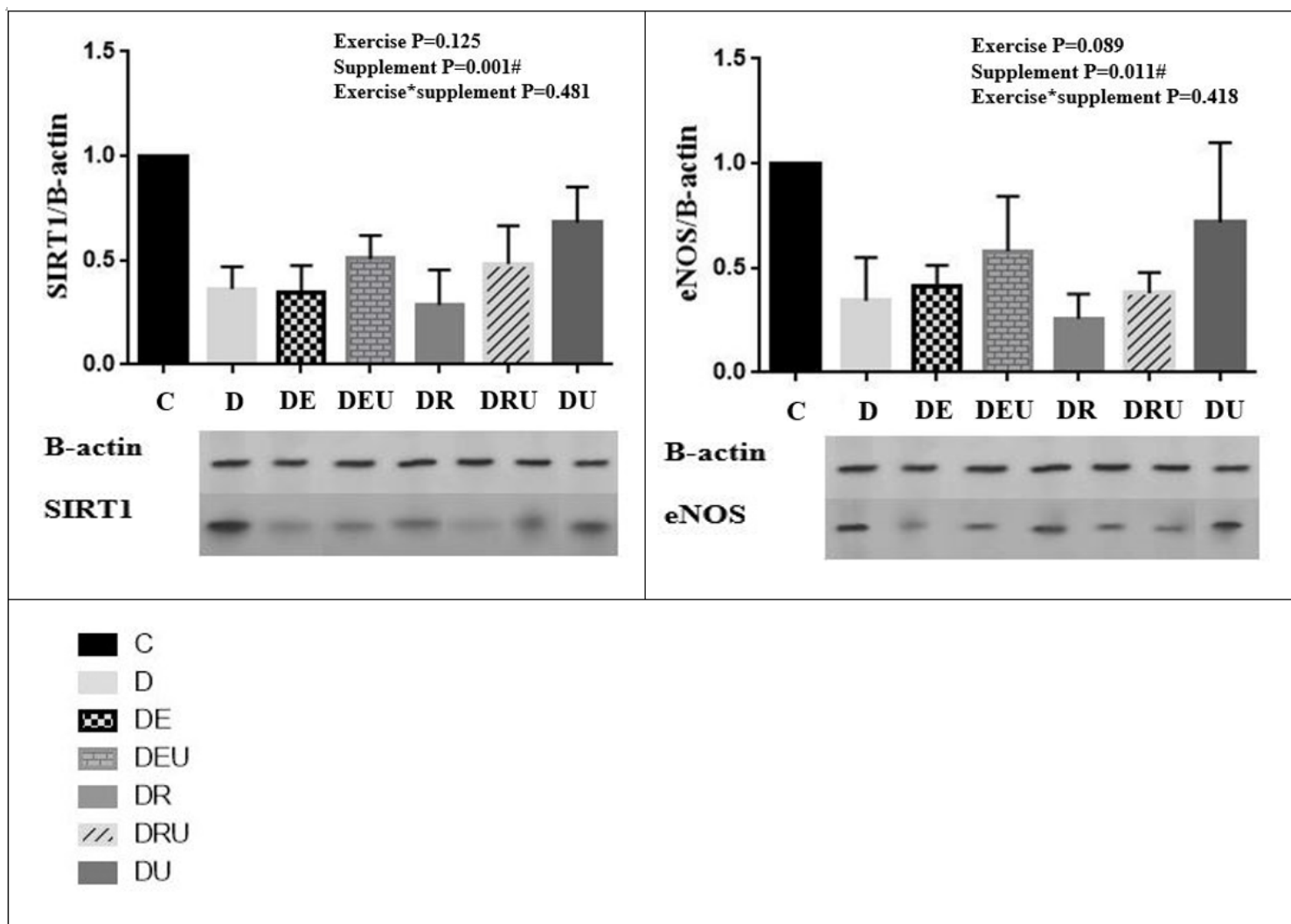
Figure 3

Serum insulin (A), glucose (B), High-density lipoprotein (HDL) (C), low-density lipoprotein (LDL) (D), triglyceride (TG) (E) and Cholesterol (F) changes following 8-weeks exercise training and ursolic acid. DU: Diabetic+Ursolic Acid; DR: Diabetic+Resistance Training; DRU: Diabetic+Resistance Training + Ursolic Acid; DE: Diabetic+Endurance Training; DEU: Diabetic+Endurance Training + Ursolic Acid; D: Diabetic; C: Control.

Figure 4

SIRT1 and eNOS gene expression changes following 8-weeks exercise training and ursolic acid. DU: Diabetic+Ursolic Acid; DR: Diabetic+Resistance Training; DRU: Diabetic+Resistance Training + Ursolic Acid; DE: Diabetic+Endurance Training; DEU: Diabetic+Endurance Training + Ursolic Acid; D: Diabetic; C: Control.
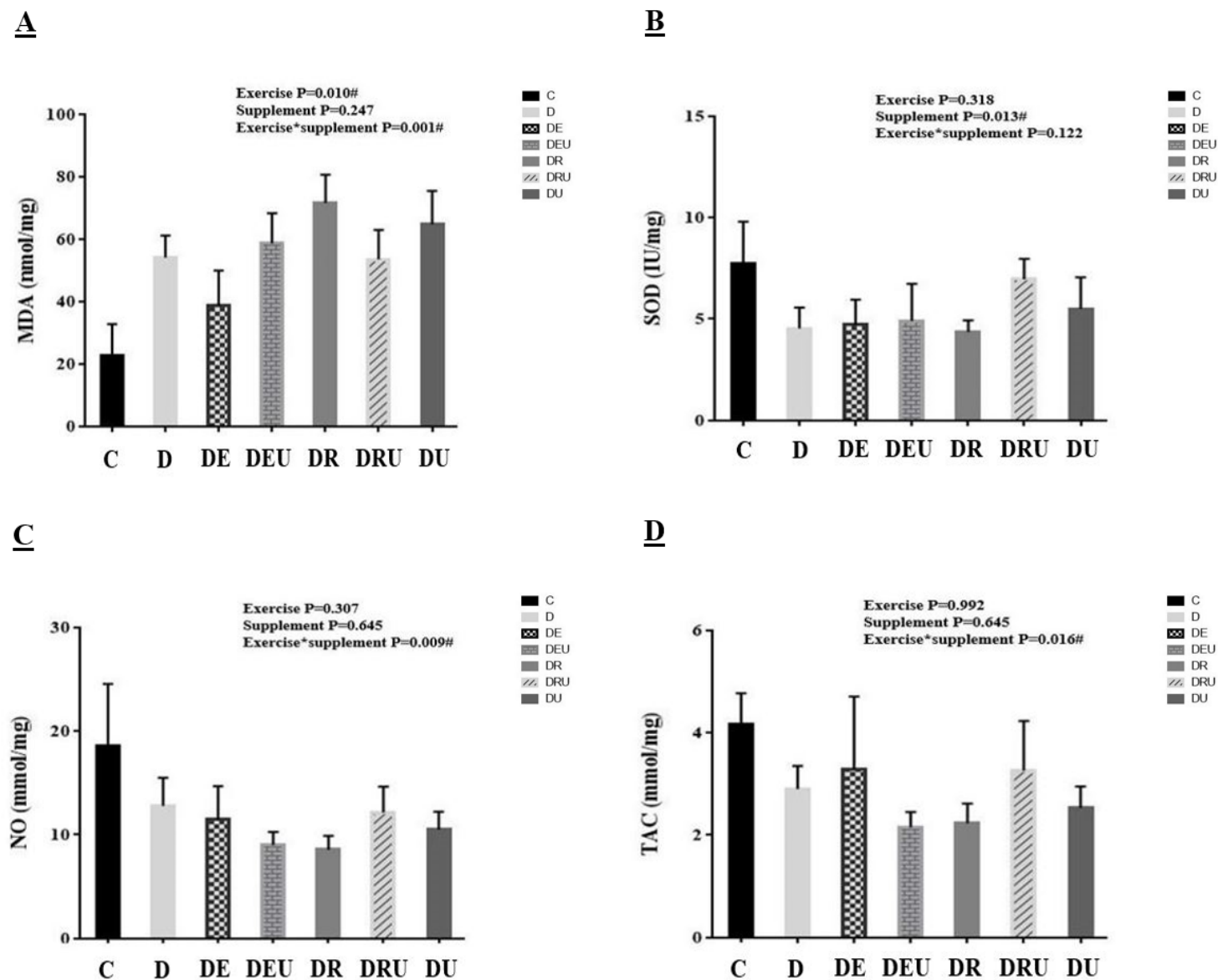
Figure 5

Malondialdehyde (MDA) (A), Superoxide dismutase (SOD) (B), Nitric oxide (NO) (C) and Total antioxidant capacity (TAC) (D) changes following 8-weeks exercise training and ursolic acid. DU: Diabetic+Ursolic Acid; DR: Diabetic+Resistance Training; DRU: Diabetic+Resistance Training + Ursolic Acid; DE: Diabetic+Endurance Training; DEU: Diabetic+Endurance Training + Ursolic Acid; D: Diabetic; C: Control.
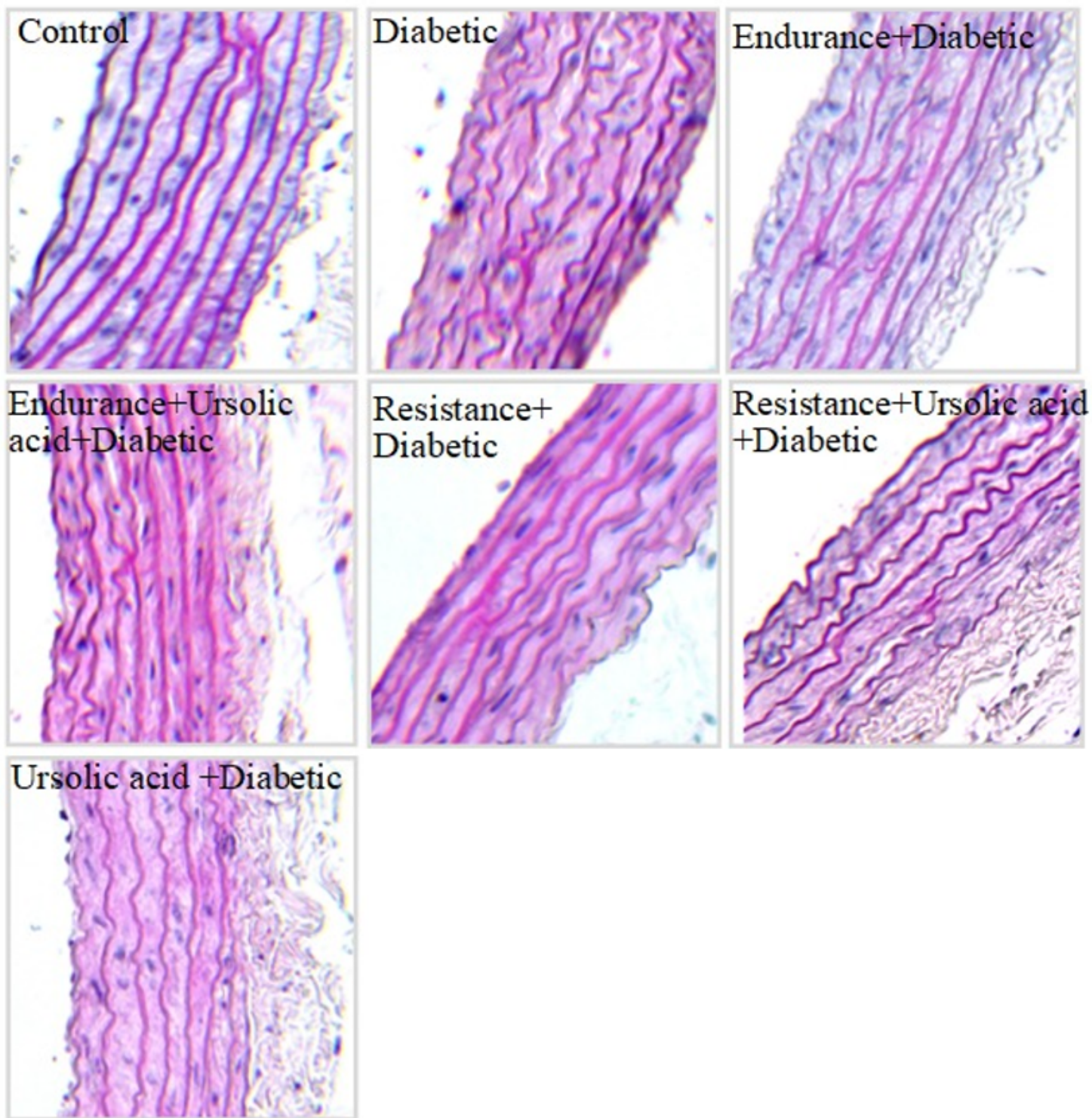
Figure 6

General microscopic evaluation of rat aorta following 8-weeks exercise training and ursolic acid. DU: Diabetic+Ursolic Acid; DR: Diabetic+Resistance Training; DRU: Diabetic+Resistance Training + Ursolic Acid; DE: Diabetic+Endurance Training; DEU: Diabetic+Endurance Training + Ursolic Acid; D: Diabetic; C: Control.