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ORIGINAL ARTICLE

Effects of moderate aerobic exercise, low-level laser therapy, or their combination on muscles pathology, oxidative stress and irisin levels in the mdx mouse model of Duchenne muscular dystrophy

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Abstract

This study aimed to investigate how the combined use of low-level laser therapy (LLLT) and exercise, to reduce the possible side effects and/or increase the benefits of exercise, would affect oxidative stress, utrophin, irisin peptide, and skeletal, diaphragmatic, and cardiac muscle pathologies. In our study, 20 mdx mice were divided into four groups. Groups; sedentary and placebo LLLT (SC), sedentary and LLLT (SL), 30-min swimming exercise (Ex), and 30-min swimming exercise and LLLT (ExL). After 8 weeks of swimming exercise, muscle tests, biochemically; oxidative stress index (OSI), utrophin and irisin levels were measured. Skeletal, diaphragmatic and cardiac muscle histopathological scores, skeletal and cardiac muscle myocyte diameters were determined under the light and electron microscope. While only irisin levels were increased in group SL compared to SC, it was determined that OSI, heart muscle histopathological scores decreased and irisin levels increased in both exercise groups ($p < 0.05$). In addition, in the ExL group, an increase in rotarod and utrophin levels, and a decrease in muscle and diaphragm muscle histopathological scores were observed ($p < 0.05$). It was determined that the application of swimming exercise in the mdx mouse model increased the irisin level in the skeletal muscle, while reducing the OSI, degeneration in the heart muscle, inflammation and cardiopathy. When LLLT was applied in addition to exercise, muscle strength, skeletal muscle utrophin levels increased, and skeletal and diaphragmatic muscle degeneration and inflammation decreased. In addition, it was determined that only LLLT application increased the level of skeletal muscle irisin.

Keywords Duchenne · Mdx mice · Exercise · Low-level laser therapy · Irisin · Muscle · Heart

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Introduction

Duchenne muscular dystrophy (DMD) has been caused by genetic mutations resulting in the loss of the dystrophin protein [1]. The incidence of DMD has been reported to be 1/3000–1/5000 (mean 1/4000) live male births. The estimated number of patients with DMD in Turkey is approximately 15,000 (aged 1–60 years), with 140–150 new cases detected annually [2]. With the use of transgenic animal models, such as the mdx mouse, great strides have been made in the discovery of potential therapeutic strategies. The mdx mouse is the most common DMD mouse model. The mdx mouse has a point mutation in the DMD gene that changes the amino acid coding for glutamine to a STOP codon. This change causes the muscle cells to produce a small, nonfunctional dystrophin protein. When compared with patients having DMD, the mdx mice have less progressive muscle damage and less muscle tissue conversion to connective and adipose tissues [3].

Exercise plays an important role in the treatment of children with DMD. Although exercise is known to be beneficial in healthy individuals, there is ongoing investigation on whether it has a therapeutic effect or whether it damages muscle tissue in individuals with muscular dystrophy [4]. It has been reported in the literature that low-to-moderate intensity aerobic exercises in particular can be beneficial [1, 3].

Low-level laser therapy (LLLT) is the application of light for therapeutic purposes using a class 3B laser device, usually with an average output range of 10–500 mW [5]. There is strong evidence that LLLT promotes tissue regeneration, reduces inflammation, and relieves pain [6]. The use of LLLT is a new area of research to manage skeletal muscle fatigue and facilitate skeletal muscle recovery. Studies have shown that LLLT applications in the mdx mouse model reduce inflammatory markers and creatine kinase (CK) levels and may help in the regeneration cycle [7–9]. In a study by Silva et al. in 2015 [9], LLLT was administered into the gastrocnemius muscle of the mdx mice for 3 days, followed by a session of high-intensity exercise. It was observed that the mdx mice treated with LLLT worked longer on the treadmill during high-intensity exercise and the redox status improved, that is, muscle fatigue was delayed in these animals [9]. This result suggests that LLLT could be used as a new and promising therapeutic agent in the treatment of DMD symptoms and may be effective against the possible side effects of exercise. However, there is no study in the literature on the combined effect of regular exercise and LLLT treatment in DMD.

Irisin was first identified in 2012 as a hormone secreted by the muscle cells. In humans, serum irisin levels have been shown to be positively correlated with biceps muscle circumference measurement and insulin growth factor-1 [10]. Thus, it has been predicted that irisin may also be involved in increasing the muscle mass. In a study by Reza et al. in 2017, intraperitoneal application of irisin to mdx mice was shown to increase grip strength and muscle weight in dystrophic muscles, decrease the percentage of fibrotic and necrotic tissues, and preserve sarcolemmal stability [11]. There is no information in the literature on how irisin levels change in the mdx mice subjected to exercise or LLLT. Therefore, in the light of the above information, it aimed to investigate how the combined use of LLLT therapy and exercise to reduce the possible side effects and/or increase the benefits of exercise would affect oxidative stress, utrophin, irisin peptide, and skeletal, diaphragmatic, and cardiac muscle pathologies.

Materials and methods

Study design and animals

This study was approved by the Local Ethics Committee, and 6–8-week-old male transgenic (20 g) mdx mice (n = 20) were obtained from Acıbadem University Experimental Animal Center (ACU-DEHAM). The mice were kept in cages maintained at 20–22 °C in groups of five in a way that they could move freely and with 12-h light/12-h dark cycle in the laboratory. The animals were cared for from the beginning until the end of the experiment in the XXX-DEHAM laboratory by paying attention to ventilation and other hygiene rules and in accordance with the laboratory animal care guidelines (1985 revised NIH publication number 85–23). (United States, Department of Health and Human Services, NIH publication, 1985 revised). The animals were given water and feed (standard laboratory feed) ad libidum. The protocol of the experiment is shown in Fig. 1A.

The sample size was computed using the G Power 3.1 program (University of Kiel, Kiel, Germany) in a manner to have minimum 5 mice in each group and 20 in total. Furthermore, 95% power ratio was maintained with 95% confidence limit and 0.05 margin of error by considering the mean muscle strength increase and standard error in the study by Zelikovich et al. [1]. The animals were divided into four groups as follows:

1. Sedentary mdx mice + placebo LLLT group (SC): n = 5
2. Sedentary mdx mice + LLLT group (SL): n = 5
3. mdx mice doing 30-min swimming exercise (Ex): n = 5
4. mdx mice doing 30-min swimming exercise + LLLT group (ExL): n = 5

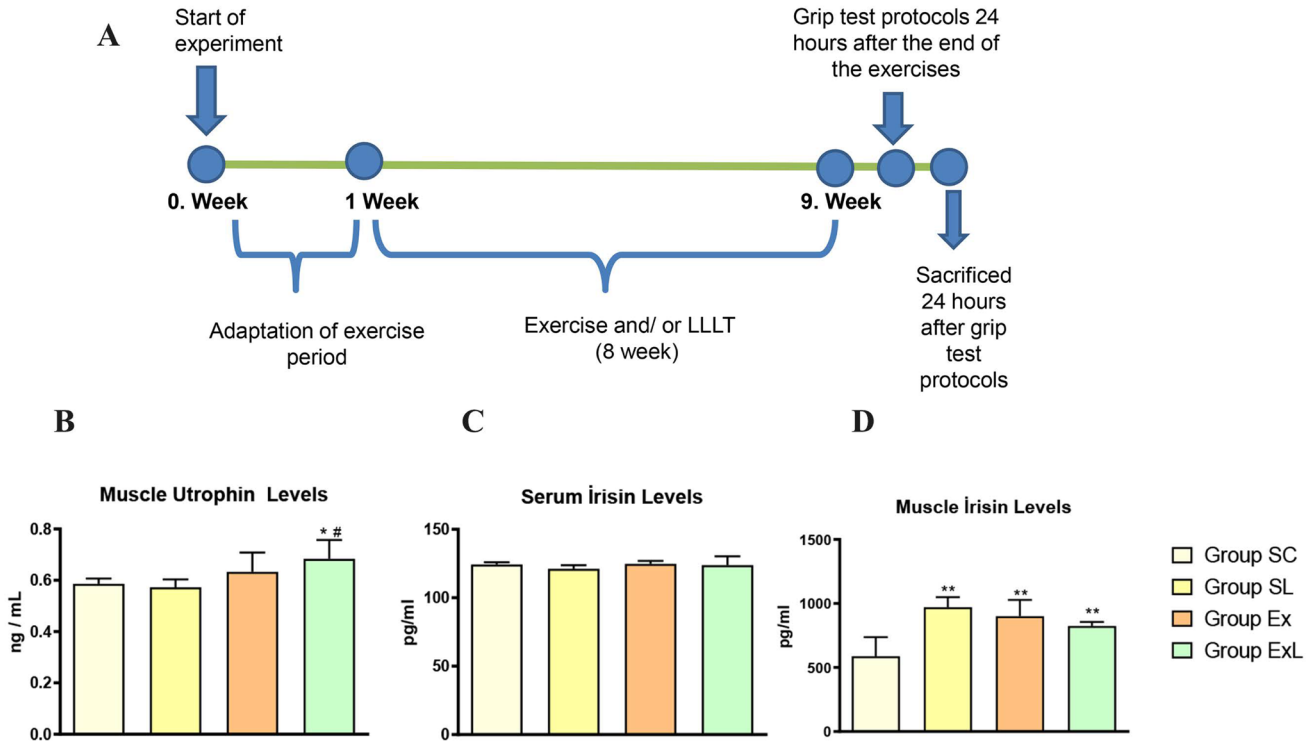

Fig. 1 Schematic showing the experimental timeline and muscle utrophin, serum and muscle irisin levels. **p < 0.05, ** p < 0.001* significance with respect to group SC. *# p < 0.05,* significance with respect to group SC. **A**: Schematic showing the experimental timeline; **B**: Muscle utrophin levels; **C**: Serum irisin levels; **D**: Muscle irisin levels; Group SC: Sedentary mdx mice + placebo LLLT group; Group SL: Sedentary mdx mice + LLLT group; Group Ex: mdx mice doing 30-min swimming exercise; Group ExL: mdx mice doing 30-min swimming exercise + LLLT group; One-way ANOVA; Tukey–Kramer post-hoc test

Exercise training

The animals were set on a swimming exercise protocol (30 min of swimming exercise 3 days a week for 8 weeks), which is a moderate aerobic physical activity [12] (except sedentary groups). The training protocol was divided into two phases consisting of adaptation and exercise. On the first day of the adaptation phase, the animals were set on swimming in a round plastic tank [60 cm × 150 cm × 45 cm; water temperature 30 ± 1 °C] for 10 min. During the adaptation period, the animals were adapted to swimming by gradually increasing the 10-min exercise on a daily basis until they were able to exercise for 30 min without interruption. After a 1-week adaptation phase, the exercise phase was started [13].

LLLT administration

SC group animals received placebo LLLT (Polar 2 Astar laser®, BielSCo-Biata, Poland) on the gastrocnemius three times a week for 8 weeks. SL and ExL group animals received point LLLT (Polaris Probe Puntale IR, 400 mW/808 nm, see Table 1) on the gastrocnemius three times a week for 90 s. ExL group received LLLT after exercise. The dose of the LLLT was chosen according to the manufacturer's instructions, with an appropriate power acting on the muscle tissue.

Table 1 Low-laser therapy parameters

Manufacturer	Astar Co, Biala, Poland
Model identifier	Polaris 2
Year produced	2016
Types of laser applicators	Point Probe Puntale IR 808 Nm/400 Mw
Wavelength and bandwidth (Nm)	Infra-Red: 808 Nm
Maximum output power (Mw) of the laser	400 Mw
Type of polarization	Linear
Power adjustment	75%
Operation mode	Continuous
Beam spot size at target [Cm^2]	1 Cm^2
Mode of laser exposure	Non-contact
Exposure duration [s]	60 s -IR
Area İrradiated [Cm^2]	1 Cm^2
Room temperature (°C) during irradiation	23 ± 2
Room humidity (%) during irradiation	70

Muscle strength tests

Once the animals completed 8 weeks of exercise (24 h after last exercise session), they were subjected to the following test protocols to test the muscle strength, as described by Aartsma-Rus et al. [3]. Muscle strength was evaluated using hanging tests (two-limb and four-limb) and Rotarod test [3].

Collecting blood and tissue samples

Twenty-four hours after the muscle strength testing (Ketamine/Xylazine 200/10 mg/kg), the animals were anesthetized intraperitoneally and were sacrificed by drawing blood from their hearts. Blood samples taken for serum preparation were immediately centrifuged at 6000 rpm for 10 min without allowing them to coagulate and stored at − 80 °C. Gastrocnemius, diaphragm muscles, and heart tissue were removed. Right gastrocnemius, diaphragm muscles, and heart tissue were placed in 10% buffered formaldehyde fixative for histopathological analysis. The left gastrocnemius was stored at − 80 °C for enzyme-linked immuno sorbent assay (ELISA).

Creatine kinase, irisin, and utrophin measurements

CK in serum samples and irisin and utrophin levels in serum and muscle samples were computed using a commercially available ELISA kit (ZellBio GmbH, Germany) according to the manufacturer's instructions (Reader device: Biotek ELx800, Washer device: Biotek Elx50, Germany).

Total oxidant capacity (TOS) and total antioxidant capacity (TAS) measurements

Oxidative stress index was determined by measuring serum TAS and TOS. The TAS and TOS levels were measured using commercially available kits (Relassay, Istanbul, Turkey) as described by Harma et al. [14]. In addition, the TOS/TAS ratio was accepted as the oxidative stress index (OSI) [14]. The following formula was used for the calculation:

$$\text{OSI} = \text{TOS}\ (\mu\text{mol}\ H_2O_2\ \text{equivalent/L})/\text{TAS}\ (\mu\text{mol Troloks equivalent/L})$$

Histological analyses

Gastrocnemius muscle, diaphragm, and heart tissue samples taken after dissection were fixed with 10% neutral buffered formalin (Cat #245–684; Fisher Scientific, Waltham, MA, USA) for 72 h. The tissues were then embedded in paraffin. Sections of 5-µm thickness obtained from tissue blocks were placed on slides for histological staining. The sections were stained with hematoxylin and eosin (Catalog #12013B, 1070 C; Newcomer Supply, Middleton, WI, USA), Masson trichrome (HT15; Sigma-Aldrich, Milan, Italy), and picrosirius dyes (Catalog #P6744–1GA; Sigma–Aldrich; St. Louis, MO, USA) to reveal the histopathological changes in the gastrocnemius muscle, diaphragm, and heart tissue. For histopathological evaluation of the tissue healing/damage degree in the obtained preparations, each preparation was shifted clockwise in the healing and damage areas. Five similar areas that were randomly selected were evaluated with 400 × magnification to obtain histopathological scores (maximum score value 9) by using histopathological parameters based on muscle degeneration, leukocyte infiltration, and vasocongestion. A scoring system ranging from 0 to 3 was used for each criterion (0: none; 1: mild; 2: moderate, and 3: severe) [15]. The diameters of the cardiomyocyte and gastrocnemius muscle were calculated by measuring the mean diameter of 100 myofibers. Fibrosis was measured using k-means segmentation in ImageJ software for the treatment groups. Sections with picrosirius staining were used for measuring fibrosis cross-sectional areas of gastrocnemius and diaphragm tissues, and Mason staining sections were used for heart tissue cross-fibrosis cross-section measurement [16, 17].

Transmission electron microscopic examination

Skeletal and cardiac muscle tissue samples were fixed in 2.5% 0.1 M phosphate buffered saline (pH 7.2) glutaraldehyde fixative at 4 °C for 4 h, and after washing in buffer, were postfixed for 1 h with 1% osmium tetroxide. The samples were dehydrated by passing through a series of ascending alcohol (70%, 90%, 96%, and 100%) and then passed through propylene oxide and buried in Epon 812 in an oven at 60 °C. Semi-thin Sects. (1 µm) obtained using an ultramicrotome were stained with toluidine blue dye. After localization of the semi-thin sections, thin sections of approximately 60 nm on copper grids were contrasted with uranyl acetate and lead citrate. Post-contrast sections were viewed using the transmission electron microscope (Thermo scientific Quattro, Czech Republic Vlastimila Pecha 1282/12) for examination of skeletal and cardiac muscles.

Statistical analyses

The GraphPad software was used for statistical analysis (Prism 9.0; GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± standard deviation. The Shapiro–Wilk test was used to determine the normality distribution of the data. The statistical significance was determined by one-way analysis of variance (ANOVA), followed by Tukey–Kramer multiple comparison post hoc

Table 2 Muscle test results of the groups

Muscle strength tests	Group SC *Mean ± SD*	Group SL *Mean ± SD*	Group Ex *Mean ± SD*	Group ExL *Mean ± SD*
Two-limb hanging tests (s)	45.00 ± 7.80	69.00 ± 4.77	108.3 ± 57.73	91.00 ± 78.58
Four-limb hanging tests (s)	56.25 ± 6.59	121.5 ± 69.02	77.25 ± 12.09	116.5 ± 55.43
Rotarod test (s)	75.5 ± 62.04	157.5 ± 54.71	177.5 ± 78.27	190.3 ± 57.05*

*$p < 0.05$. significance with respect to group SC

Group SC: Sedentary mdx mice + placebo LLLT group; Group SL: Sedentary mdx mice + LLLT group; Group Ex: mdx mice doing 30-min swimming exercise; Group ExL: mdx mice doing 30-min swimming exercise + LLLT group; Mean ± SD: mean ± standard deviation. One-way ANOVA; Tukey–Kramer post-hoc test

tests. For all studies, two-sided $P < 0.05$ was considered significant.

Results

The results of the two-limb and four-limb hanging and rotarod tests by groups are shown in Table 2. Biochemical measurements, including the mean and standard deviations of serum CK and serum and muscle TAS, TOS, and OSI, are shown in Table 3.

Comparison of the groups for muscle utrophin levels showed statistical significance (F (3, 14) = 3.478, $P = 0.040$, Fig. 1B). Utrophin was significantly increased in group ExL when compared with group SC ($P = 0.040$, Fig. 1B) and group SL ($P = 0.014$, Fig. 1B). No statistical significance was found in the comparison of serum irisin levels between the groups (F (3.14) = 0.665, $P = 0.584$, Fig. 1C). On the other hand, statistical significance was found in the comparison of the groups for muscle irisin values (F (3.14) = 10.180, $P = 0.008$, Fig. 1D). When compared with group SC, muscle irisin levels were significantly increased in group SL ($P = 0.001$), group Ex ($P = 0.017$), and group ExL ($P = 0.001$) (Fig. 1D).

The histopathological score results combining the degeneration of gastrocnemius muscle tissue, leukocyte infiltration indicating inflammation, and vasoconjection scores were statistically significant between the experimental groups (F (3, 16) = 1.846, $P = 0.170$, Fig. 2 and 3A). The histopathological score for group ExL was statistically significantly lower than that of group SC ($P = 0.004$) and group SL ($P = 0.017$) (Fig. 2 and 3A). No significant difference was found in the gastrocnemius muscle diameter measurements (F (3, 194) = 1.923, $P = 0.124$, Fig. 2 and 3D).

The histopathological score results for the diaphragm muscle tissue were statistically significant between the experimental groups (F (3, 16) = 4.489, $P = 0.011$, Fig. 3B and Supplementary Fig. 1). The scores based on histopathological parameters were statistically significantly lower in the samples of the ExL group when compared with those of the SC group ($P = 0.022$, Fig. 3B and Supplementary Fig. 1).

A statistically significant difference was found between the experimental groups for the histopathological score results of cardiac muscle tissue (F (3, 16) = 9.846, $P = 0.001$, Fig. 2 and 3C). In the experimental groups, a statistically significant score reduction was observed for histopathological

Table 3 Biochemical analysis results of the groups

Biochemical analysis	*Group SC* *Mean ± SD*	*Group SL* *Mean ± SD*	*Group Ex* *Mean ± SD*	*Group ExL* *Mean ± SD*
Serum CK (U/L)	6731 ± 1935	7318 ± 1133	6993 ± 819.6	6623 ± 1522
Serum TAS (mmol/L)	1.890 ± 0.33	1.740 ± 0.13	1.952 ± 0.28	1.746 ± 0.17
Serum TOS (μmol/L)	14.86 ± 7.86	12.77 ± 8.29	6.360 ± 1.21*	5.322 ± 1.58*
Serum Oxidative Index	0.96 ± 0.58	0.77 ± 0.52	0.32 ± 0.04*	0.30 ± 0.08*
Muscle TAS (mmol/L)	0.93 ± 0.22	1.136 ± 0.22	1.204 ± 0.18	1.080 ± 0.10
Muscle TOS (μmol/L)	19.68 ± 3.33	20.07 ± 2.50	13.65 ± 4.90*#	13.75 ± 2.00*#
Muscle Oxidative Index	2.179 ± 0.47	1.621 ± 0.15	1.109 ± 0.27*#	1.279 ± 0.19*#

*$p < 0.05$, significance with respect to group SC

# $p < 0.05$, significance with respect to group SC

CK, creatine kinase; *TOS*, total oxidant capacity; *TAS*, total antioxidant capacity; Group SC: Sedentary mdx mice + placebo LLLT group; Group SL: Sedentary mdx mice + LLLT group; Group Ex: mdx mice doing 30-min swimming exercise; Group ExL: mdx mice doing 30-min swimming exercise + LLLT group; Mean ± SD: mean ± standard deviation. One-way ANOVA; Tukey–Kramer post-hoc test

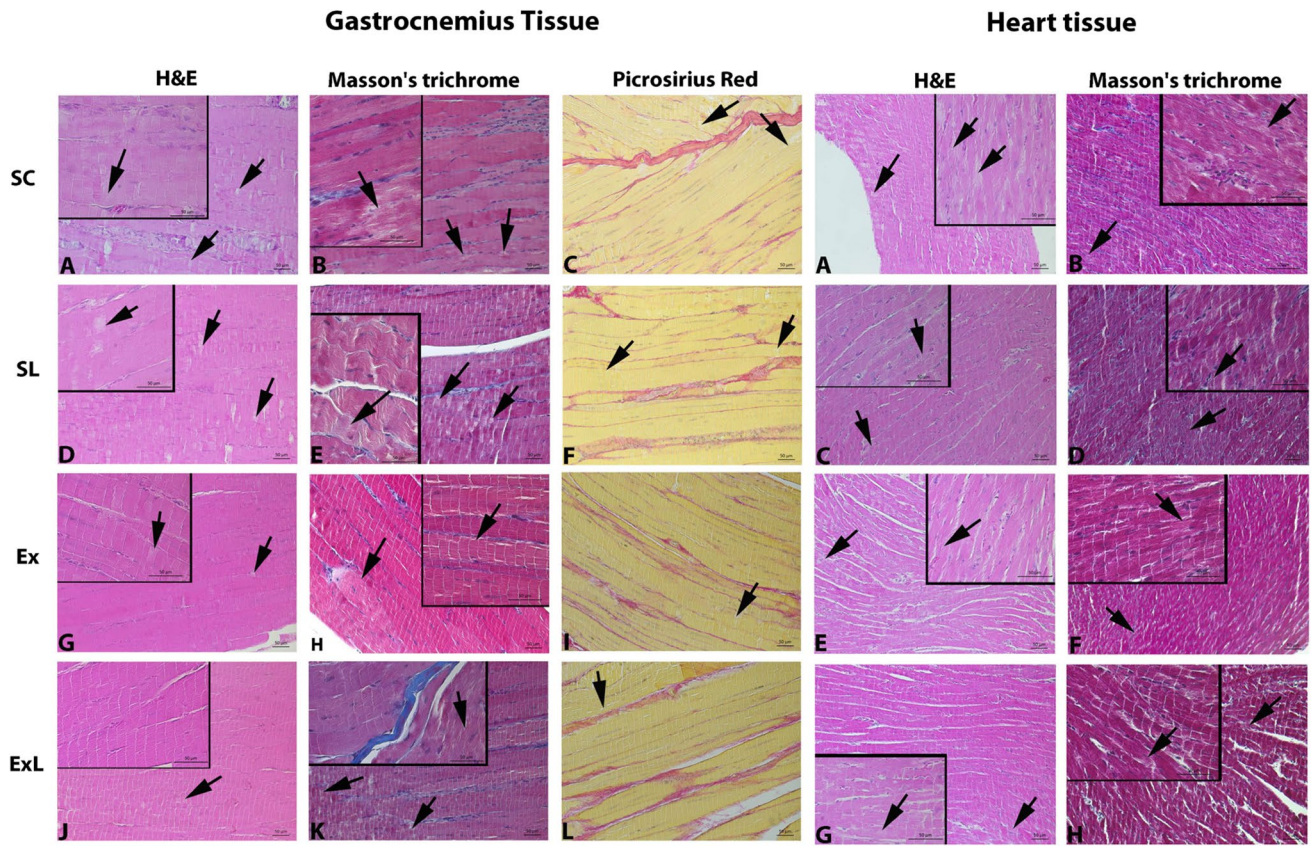

Fig. 2 Presentation of stains used for histopathological examination of the gastrocnemius and heart muscle tissue. **Gastrocnemius tissue: A–C**: Sedentary mdx mice + placebo LLLT group; **D–F**: Sedentary mdx mice + LLLT group; **G–I**: mdx mice doing 30-min swimming exercise; **J–L**: mdx mice doing 30-min swimming exercise + LLLT group; Arrow: degenerated muscle fiber (**A–J**: Hematoxylin and Eosin, **B–K**: Masson trichrome, **C–L**: Picrosirius, Paraffin section). **Heart tissue: A**, **B**: Sedentary mdx mice + placebo LLLT group; **C**, **D**: Sedentary mdx mice + LLLT group; **E**, **F**: mdx mice doing 30-min swimming exercise; **G**, **H**: mdx mice doing 30-min swimming exercise + LLLT group; Arrow: degenerated muscle fiber (**A–G**: Hematoxylin and Eosin, **B–K**: Masson trichrome, **B–H**: Picrosirius, Paraffin section). Bar = 50 µm

parameters in the samples belonging to the Ex ($P = 0.018$) and ExL ($P = 0.018$) groups when compared with the SC group. (Fig. 2 and 3C). Cardiomyocyte diameters differed significantly between the groups (F (3, 203) = 12,051, $P < 0.001$, Fig. 3E). Among the experimental groups, there was a significant decrease in cardiomyocyte diameter measurements in the Ex ($P < 0.001$) and ExL ($P = 0.037$) groups when compared with the SC group (Fig. 2 and 3E). In addition, a statistically significant decrease was observed in the cardiomyocyte diameter measurements of the Ex ($P < 0.001$) and ExL ($P = 0.041$) groups when compared with those of the SL group (Fig. 2 and 3E). No statistically significant results were found in the fibrotic cross-sectional area results for muscle (Fig. 2 and Supplementary Fig. 2A), diaphragm (Fig. 2 and Supplementary Fig. 2B), and heart (Fig. 2 and Supplementary Fig. 2C) muscle ($P > 0.05$).

The electron micrograph results for the gastrocnemius and cardiac muscle tissue by groups are shown in Fig. 4 and Fig. 5, respectively.

Discussion

The current study showed that in the sedentary group with LLLT, only irisin levels were increased when compared with the SC. However, in both exercise groups, improved heart muscle histopathology, TOS (hence OSI), cardiomyocyte diameters decreased and irisin levels increased when compared with the SC group. Moreover, the group with combined exercise and LLLT had increased rotarod duration and utrophin levels and improved histopathology of skeletal and diaphragmatic muscles.

The use of the LLLT device to manage skeletal muscle fatigue and facilitate skeletal muscle recovery is a new area of research. In the literature, it is reported that LLLT acts in a manner akin to cellular energy metabolism by stimulating photochemical and photophysical events in the cell mitochondria. Also, LLLT increases the mitochondrial membrane potential in the muscle and the enzyme activity of the respiratory chain [18, 19]. Structural changes include

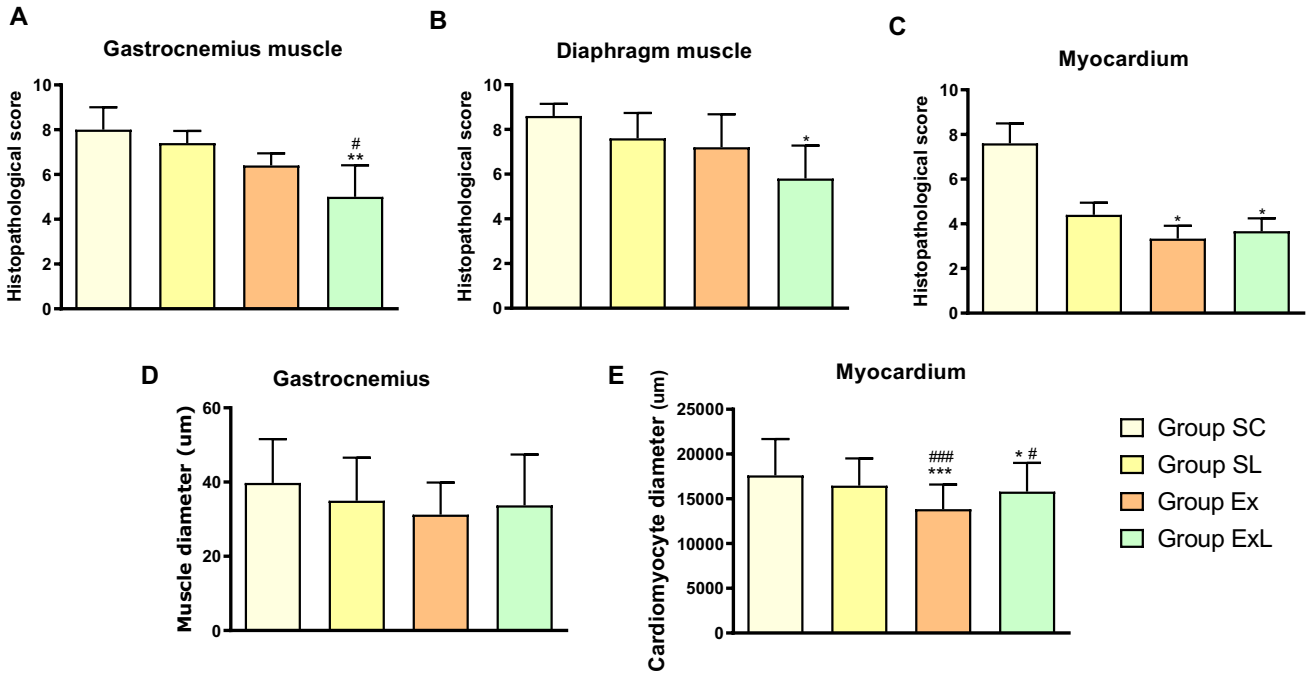

Fig. 3 Muscle, diaphragm, and heart muscle histological analysis results. *$p<0.05$, ** $p<0.01$ significance with respect to group SC. # $p<0.05$, ### $p<0,001$ significance with respect to group SC. **A**: Histopathological score of gastrocnemius muscle, **B**: Histopathological score of diaphragm muscle, **C**: Histopathological score of cardiac muscle, **D**: Gastrocnemius muscle diameter, **E**: Cardiomyocyte diameter; Group SC: Sedentary mdx mice + placebo LLLT group; Group SL: Sedentary mdx mice + LLLT group; Group Ex: mdx fare ve LLLT. Group Ex: mdx mice doing 30-min swimming exercise; Group ExL: mdx mice doing 30-min swimming exercise + LLLT group; One-way ANOVA; Tukey–Kramer post-hoc test

the formation of giant mitochondria via the fusion of the membranes of smaller and neighboring mitochondria [20]. These changes allow the mitochondria to provide higher levels of respiration and ATP to the cells [21–23]. Furthermore, the ability of LLLT to stimulate stem cells and progenitor cells signifies that muscle satellite cells may respond well to LLLT and aid in muscle repair [24]. In a study by Macedo et al. [8], primary cultures of mdx skeletal muscle cells were irradiated only once with LLLT at a flow of 5 J/cm^2 at a wavelength of 830 nm and analyzed after 24–48 h. Their results showed a decrease in markers of inflammation and oxidative stress and an increase in the level of regeneration. Leal-Junior et al. [5] have stated that 6-week-old mdx mice were treated with LLLT (904 nm, 15 mW, 700 Hz, 1 J) or placebo LLLT to a point above the tibialis anterior muscle five times a week for 14 weeks. The LLLT was found to reduce the mRNA gene expression of various inflammatory markers and CK activity than in the placebo-control group. The significant difference seen in these studies was not replicated in the LLLT-only sedentary group in our study. We believe that this difference may be due to the fact that the power of the LLLT used in our study was slightly lower than that used in other studies. Nevertheless, we used the power declared by the manufacturer for it to be effective on the muscle. This aspect constitutes a limitation of our study.

Wineinger et al. [25] set 4-week-old mdx mice on a swimming exercise for 30 min a day for 56 weeks and concluded that muscle fatigue decreased. In a study by Hayes et al. [26] in 1993, 5-week-old mdx mice were set on a swimming exercise in which the swimming time was increased by 5 min every day for 15 weeks so that they performed a total of 2 h of swimming. In the present study, although there was an increase in the hanging tests indicating the grip strength in the exercise groups, no statistically significant increase was detected. The rotarod test, which requires balance and coordination and also measures muscle strength, was statistically higher only in the group with combined LLLT and exercise when compared with the animals in the SC group. In addition, the present study found no change in skeletal muscle diameter, which is similar to the observation of Zelikovich et al. [1]. This finding may explain why some of the muscle strength tests were not significant and could be explained by a shorter (8 weeks) exercise period when compared with the other studies [25–27]. Another limitation of our study was the shorter exercise time than that used in other studies. We recommend that the LLLT power and exercise duration should be increased in future studies.

In a study by Hayes et al. [27] in 1998, it was shown that moderate exercise training, hydrogen peroxide production by mitochondria isolated from the quadriceps muscle

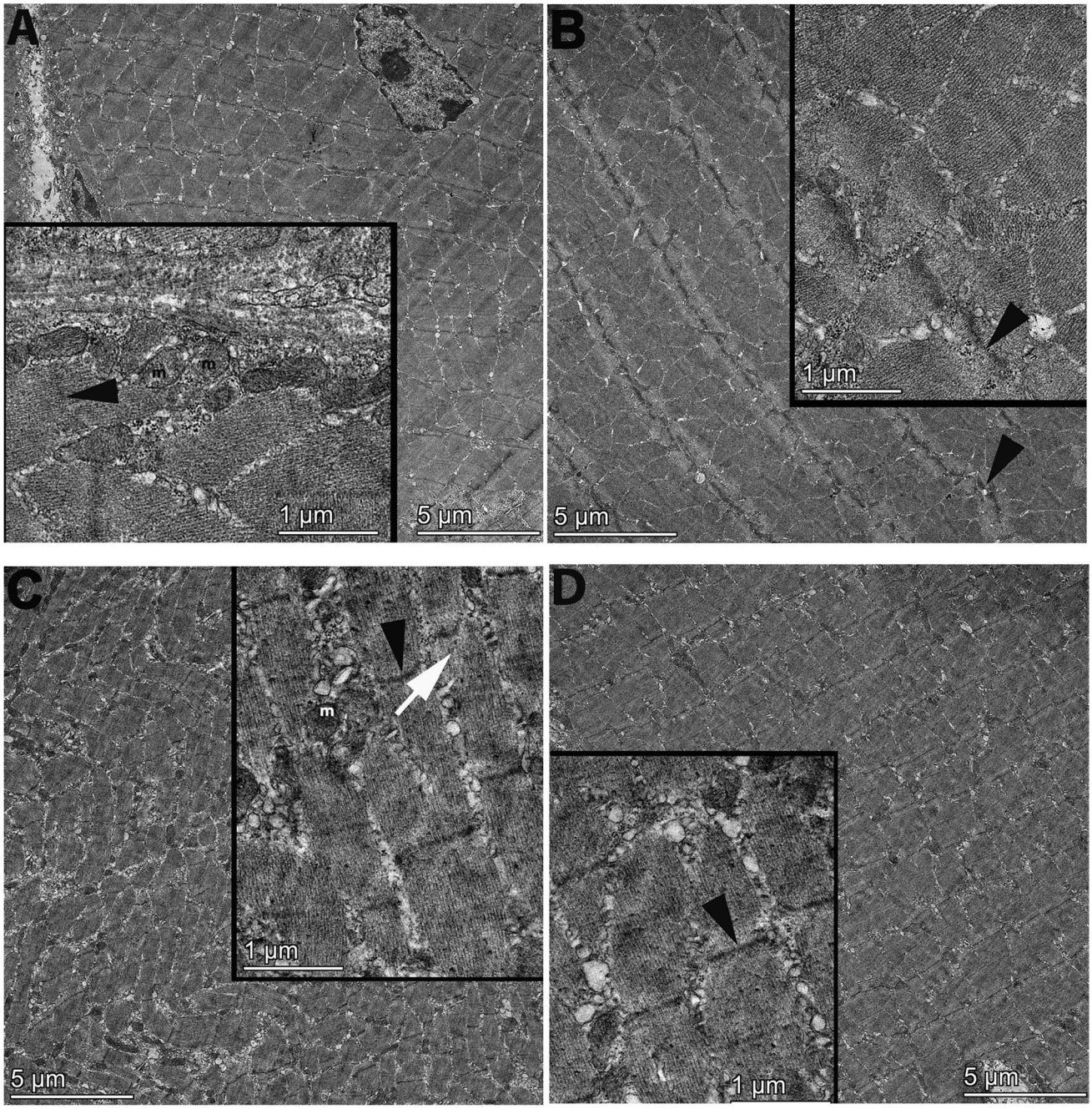

Fig. 4 Electron micrograph of the gastrocnemius muscle in the experimental groups. Mitochondrial degenerations (m) and disorganized Z-line (arrowhead) ultrastructure were observed in SC group (**A**), (TEMX2600, inset: × 11000). Regular sarcomere arrangement and some minor degeneration of Z-line (arrowhead) were observed in the SL group (**B**), (TEMX2600, inset: × 11000). Mild disorganization in sarcomere, mitochondria with erased cristae (m), discontinuous Z-line (arrowhead), and disturbed myofilament organization (white arrow) were observed in the Ex group (**C**), (TEMX2600, inset: × 11000). Regular sarcomere arrangement and some disorganized Z-line (arrowhead) ULTRASTRUCTURE were seen in ExL group (**D**). Group SC: Sedentary mdx mice + placebo LLLT group; Group SL: Sedentary mdx mice + LLLT group; Group Ex: mdx mice doing 30-min swimming exercise; Group ExL: mdx mice doing 30-min swimming exercise + LLLT group

is lower in mdx mice that are subjected to the exercise when compared with the sedentary mdx mice. This result may explain why exercise reduced TOS in our study. In addition, aerobic exercise also compensates for the decrease in the PGC1α pathway [28], which is the main transcriptional coactivator involved in the regulation of mitochondrial biogenesis and metabolism during aging, where oxidative stress is also involved. In our study, OSI was found to be low in

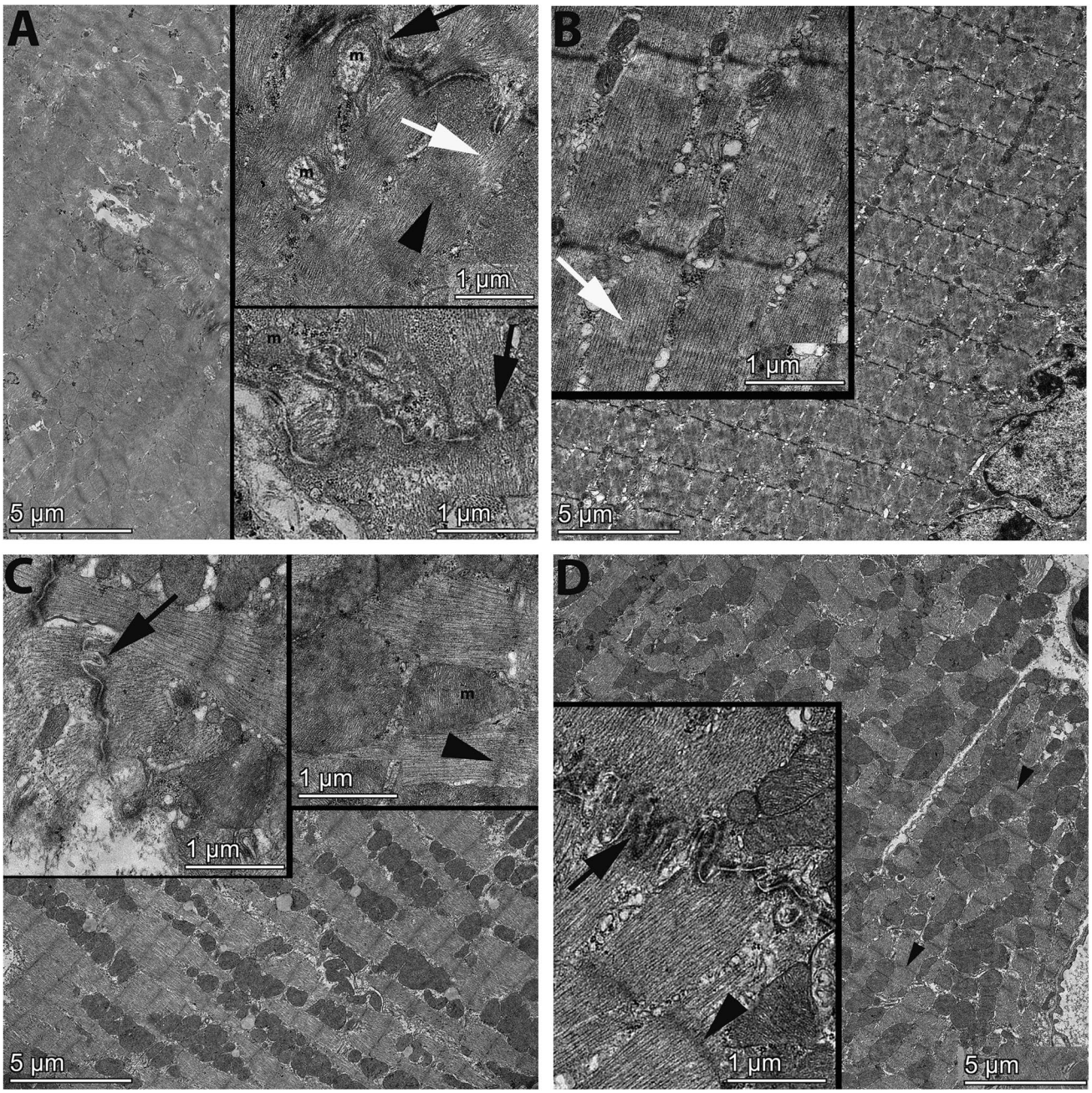

Fig. 5 Electron micrograph of the cardiac muscles in the experimental groups. Disrupted integrity of intercalated disk and Z-line (arrowhead) mitochondrial degeneration with erased cristae (m), depicted prominent sarcomere degeneratıon in SC group (**A**), (TEMX2600, upper inset × 8500 and lower inset: × 11000). Regular sarcomere with few minor myfilamentous degenerations (white arrow) and intact mitochondrial ultrastructure along were observed in SL group (**B**), (TEMX2600, inset: × 11000). Loss of integrity in intercalated disk with segments exhibiting disarrangements of filaments (arrow), mildly degenerated mitochondria (m), disorganized Z-line (arrowhead) were seen in the Ex group (**C**), (TEMX2600, left inset: × 11000, right inset: × 8500, × 11000). Regular intercalated disk and Z-line (arrowhead) ultrastructure presented intact sarcomere organızation in ExL group (**D**), (TEMX2600, inset: × 11000). Group SC: Sedentary mdx mice + placebo LLLT group; Group SL: Sedentary mdx mice + LLLT group; Group Ex: mdx mice doing 30-min swimming exercise; Group ExL: mdx mice doing 30-min swimming exercise + LLLT group

the muscle and serum, which is consistent with the literature findings. However, this decrease was caused only by the decrease in TOS. Lower markers of free radical damage following low-intensity exercise training in the mdx mice without a significant increase in antioxidant enzyme activities and protein content suggest that reactive oxygen production is lower in the mdx mice after such exercise intensity [27]. In LLLT studies, it is also stated that LLLT

reduces oxidative stress [8, 9]. In our study, we could not see similar results in the LLLT-only groups. However, the OSI decreased in the exercise groups when compared with the sedentary groups. This decrease seems to be related to exercise training.

The use of LLLT after physical exercise improves mitochondrial and metabolic dysfunction. LLLT is added to the positive effects of aerobic exercise on mitochondria, repair the micro lesions and reactive oxygen/nitrogen metabolites can be modulated [23]. Giant and more functional mitochondria (higher enzyme activity) can provide higher levels of cellular respiration and ATP synthesis [19, 29, 30] during these exercises, thereby increasing the oxygen consumption [28] and reducing the muscle fatigue [22]. LLLT inhibits LDH activity even when oxygen supply is slow and ATP synthesis is insufficient in the mitochondria during physical exercise [24, 29, 31–33]. In addition, LLLT uses cytochrome c oxidase as the primary photoreceptor. The main effects of this interaction are increased ATP synthesis and increased mitochondrial function [12, 18, 19, 27]. In this way, it improves mitochondrial function during exercise, delaying muscle fatigue and increasing muscle performance. In the current study, these changes caused by LLLT delivered after exercise may constitute the underlying mechanism of how it increased the rotarod test, increased the utrophin expression (dystrophin homolog) in muscle tissue, decreased the histopathology in the heart, diaphragm, and gastrocnemius muscle, and decreased the oxidative index in the serum and muscle.

DMD results from the loss of dystrophin, a structural protein in the skeletal, diaphragm and cardiac muscle fibers [34, 35]. More than 90% of patients with DMD develop cardiomyopathy, and approximately 10–20% die from heart failure [36, 37]. Physical exercise has previously been shown to activate calcineurin signaling [37] or mdx heart muscle to increase cardiac fibrosis [33] or impair heart function and lead to cardiomyopathy [38]. Chronic, moderate-intensity exercise has been described to have cardio-protective properties in normal-type mice [37–40]. When Barbin et al. [35] set the mdx mice on a swimming exercise for 1 h, 6 days a week, for 8 weeks, they observed an increase in diaphragm and cardiac muscle fibrosis and thought that exercise might be harmful for these muscles. In a study by Zelikovich et al. [1], it was shown that in mdx mice, low and moderate aerobic exercise 3 days a week led to improvement in skeletal muscle and respiratory function, delaying the progression of cardiomyopathy without adversely affecting the electrical function of the dystrophic heart. In addition, they stated that it improved dystrophic heart, diaphragm, and muscle function without significantly increasing fibrosis, central core fibers, or serum CK. In the present study, similar to the observations of Zelikovich et al. [1], the heart histopathological score decreased significantly in both the exercise groups without increasing fibrosis in the heart and without causing cardiopathy. In addition, in the group with combined LLLT and exercise, there was an improvement in the histopathological score without an increase in the fibrotic areas of the diaphragm and skeletal muscle.

In a study by Reza et al. [11], intraperitoneal administration of irisin in mdx mice was found to increase the grip strength and muscle weight, decrease the percentage of fibrotic and necrotic tissue, and preserve sarcolemmal stability in dystrophic muscle. It was suggested that irisin caused these improvements without increasing the utrophin level. The current study is the first to show that irisin is increased after moderate aerobic exercise in the mdx mouse model. However, interestingly, in the LLLT-only group, the irisin level of the gastrocnemius muscle was found to be increased when compared with the SC group.

The peroxisome proliferator-activated receptor involved in the molecular pathway leading to irisin release leads to increased PGC1α, which results in the expression of the FNDC5-containing fibronectin type III domain. FNDC5 is a type of transmembrane protein encoded by the Fndc5 gene and is known as the irisin precursor [10, 11, 41]. In a previous study, it was shown that exposure to near-infrared light increased the expression of PGC1α in mouse muscle cells by approximately 20% [42]. Therefore, the increase in the irisin with LLLT could be attributed to this reason. However, this may not be the only reason as the irisin level in our study improved almost twofold when only LLLT was applied to the irisin muscle. Therefore, the increase in the level of the muscle irisin makes us think that the LLLT applied might have had a stimulating effect on the irisin peptide, like cold [43] and hot [44]. Therefore, more research is needed, especially on the effects of exercise and LLLT on the irisin and the mechanisms of these effects on recovery parameters in the mdx mouse model. We measured the irisin level in the muscle using only the ELISA method in our study, and we could not use other molecular methods to measure mRNA or protein expression. This constitutes the most important limitation of our study.

As a result, we determined that swimming exercise performed for 30 min 3 days a week was beneficial in oxidative stress, irisin level, and cardiac muscle pathology without increasing the fibrosis and cardiopathy in the mdx mouse model. When exercise was combined with LLLT, it additionally had beneficial effects on utrophin levels and diaphragm and skeletal muscle pathology. Therefore, we predict that LLLT delivered after exercise may be beneficial. However, more research is needed on this subject.

It was determined that the application of swimming exercise in the mdx mouse model increased the irisin level in the skeletal muscle, while reducing the OSI, degeneration in the heart muscle, inflammation and cardiopathy. When LLLT was applied in addition to exercise, muscle strength, skeletal

muscle utrophin levels increased, and skeletal and diaphragmatic muscle degeneration and inflammation decreased. In addition, it was determined that only LLLT application increased the level of skeletal muscle irisin.

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Declarations

Ethics approval and consent to participate This study was approved by Acıbadem University Animal Experiments Local Ethics Committee (Ref No. HADEK-2020–21).

Conflict of interest The authors declare no competing interests.

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