

## Redox status biomarkers in the fast-twitch extensor digitorum longus resulting from the hypoxic exercise

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### ABSTRACT

The fast-twitch muscle may be affected from over-produced reactive oxygen species (ROS) during hypoxia/hypoxic exercise. The study aims to investigate redox status biomarkers in the fast-twitch extensor digitorum longus (EDL) muscle after hypoxic exercise. Male Sprague Dawley rats (eight-week-old) were randomly divided into six groups of the experimental “live high train high (LHTH), live high train low (LHTL) and live low train low (LLTL)” and their respective controls. Before the EDLs’ extraction, the animals exercised for a 4-week familiarization period, then they exercised for four-weeks at different altitudes. The LHTH group had higher ratios of lipid hydroperoxides (LHPs) than the experimental groups of LHTL ( $p=0.004$ ) and LLTL ( $p=0.002$ ), while having no difference than its control ‘LH’. Similarly, a higher percentage of advanced oxidation protein products (AOPP) was determined in the LHTH than the LHTL ( $p=0.041$ ) and LLTL ( $p=0.048$ ). Furthermore, oxidation of thiol fractions was the lowest in the LHTH and LH. However, redox biomarkers and thiol fractions illustrated no significant change in the LHTL and LLTL that might ensure redox homeostasis due to higher oxygen consumption. The study shows that not hypoxic exercise/exercise, but hypoxia might itself lead to a redox imbalance in the fast-twitch EDL muscle.

Keywords: advanced oxidation protein products, fast-twitch muscle fibers, hypoxic exercise, lipid hydroperoxides, thiols

#### Abbreviations:

ROS: reactive oxygen species  
EDL: extensor digitorum longus  
LHTH: live high train high  
LHTL: live high train low  
LLTL: live low train low  
LHL: live high live low  
LH: live high  
LL: live low  
LHPs: lipid hydroperoxides  
AOPP: advanced oxidation protein products  
T-SH: total thiols  
P-SH: protein thiols  
Np-SH: non-protein thiols

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## INTRODUCTION

Hypoxic exercise like “live high train high” or “live high train low” has created interest in both scientists and athletes.<sup>1-3</sup> Hypoxia is a condition of having an inadequate or insufficient oxygen supply to the whole body.<sup>4</sup> Exposure to hypoxia itself leads to a reactive oxygen species (ROS) formation as a cellular adaptive response.<sup>5-7</sup> Furthermore, the physiological level of ROS production also takes place in muscular fibers during exercise as a process of adaptation. This event is known as hormesis.<sup>8-11</sup> In fact, ROS have played a major role in signaling cascades of muscular fibers during exercise.<sup>9,10</sup> Therefore, it is expected that hypoxic exercise will lead to ROS formation in muscle fibers. However, over-produced ROS has detrimental effects on muscular redox metabolism via the oxidation of cellular macromolecules. This undesired situation would lead to a decrease in the production of muscle power and cause fatigue.<sup>12</sup> In fact, a number of hypoxic exercise related studies indicate that oxidation biomarkers were found to be higher in both human blood and rat skeletal muscles.<sup>1-3,7,13,14</sup>

Among the redox status biomarkers, lipid hydroperoxides (LHPs) are known as the early phase-products of lipid peroxidation which are transformed into highly toxic hydroxyl radicals having the potential to induce oxidative damage to membrane lipids, cellular proteins and thiol (-SH) groups of proteins which also have a free radical buffering capacity.<sup>15</sup> Proteins may be able to buffer nearly 50–75% of the total ROS production due to being mainly found in the macromolecules of cells.<sup>16</sup> Both protein thiols (P-SH) and non-protein thiol groups (Np-SH) form total thiol (T-SH) fraction.<sup>17,18</sup> In addition, Np-SH fraction mainly consists of a reduced form of glutathione (GSH).<sup>17</sup> Advanced oxidation protein products (AOPP), also known as another redox status biomarker includes a group of non-enzymatic oxidation products such as dityrosine, pentosidine, and protein carbonyl groups.<sup>15,19,20</sup> Conversely, there are a limited number of studies related to all these redox status biomarkers in skeletal muscle at hypoxic exercise.<sup>3,14</sup>

Li and Zhang (2011) studied the lipid peroxidation formation rate of the gastrocnemius muscle at two different hypoxic exercise models: the LHTH and the LHTL.<sup>14</sup> The soleus muscles of the rats for the cited redox status biomarkers have been recently studied in both the LHTH and the LHTL hypoxic exercise groups.<sup>3</sup> While the soleus muscle consists of mainly slow twitch fibers, which are rich in mitochondria, the gastrocnemius muscle is composed of both slow and fast twitch fibers.<sup>21,22</sup> Both gastrocnemius and soleus muscles are more prone to ROS attacks than fast-twitch muscles, since these muscles have a higher ratio of mitochondria and a higher activity of NADPH oxidases.<sup>23</sup> On the other hand, it was known that the mitochondria from fast-twitch muscle fibers have unique characteristics that stimulate a higher rate of ROS production compared to the mitochondria from slow-twitch muscle fibers.<sup>24</sup> Moreover, it was reported that the fast twitch type of the plantaris muscle has had an indication of over-produced ROS after 10-days of endurance exercise.<sup>25</sup>

The endurance type of exercise depends on aerobic energy metabolism, in which mitochondria rich oxidative muscle fibers are mainly active. However, it was reported that the ROS formation occurs via NADPH enzymes' oxidization without the contribution of mitochondria during exercise.<sup>23</sup> The NADPH oxidases are mainly localized within the sarcoplasmic reticulum, transverse tubules, and the sarcolemma of skeletal muscles fibers.<sup>26</sup> NADPH oxidases may have played physiological roles during exercise in order to optimize muscular contraction.<sup>26</sup> In addition, the increased activity of NADPH oxidases may lead to the atrophy of glycolytic muscles due to their high level of potential ROS formation.<sup>27</sup> On the other hand, mitochondria were reported to

produce ROS at normoxia.<sup>26</sup> In fact, they turn out to be the focal point of ROS generation in hypoxia due to electron leakage from the inner mitochondrial membrane complexes I and III.<sup>28,29</sup>

Even though fast twitch fibers have few mitochondria and are poor in NADPH oxidases, we believe that fast twitch fibers may also be affected from excessive ROS formation during hypoxic exercise. As mitochondria produce ROS due to electron leakage from complexes I and III in hypoxia, the mitochondria of fast twitch muscles may also produce more ROS. It was also reported that NADPH oxidases are more active during muscular construction as ROS diffuses from one cell to another in muscular tissue.<sup>30</sup> Therefore, hypoxic exercise may lead to ROS attacks to fast twitch skeletal muscle fibers as well. The fast-twitch extensor digitorum longus (EDL) muscle of rats was chosen in the context of the present study. The EDL has higher ratio of fast twitch fibers with deep EDL having about 10% type I, 26.7% type IIa, 63.3% Type IIb, the superficial EDL 11.2% type IIa and 88.8 type IIb.<sup>31</sup> The type I fiber is oxidative, type IIa is both oxidative and glycolytic and type IIb is mainly glycolytic.<sup>23,32,33</sup> Both type IIa and type I have mitochondria and NADPH oxidases exist for every type of fiber. All these mean ROS may have some detrimental effects on EDL during hypoxic exercise. In addition, to the best of our knowledge, the current study is the first experimental research assessing the “AOPP, LHPs, T-SH and P-SH” of the EDL during different hypoxic exercises. As a result, the aim of the current study is to determine the possible effects of three different types of altitude exercises “the LHTL, the LHTL and the LLTL” on the muscular redox status biomarkers “AOPP, LHPs, T-SH and P-SH” of the EDL.

## MATERIALS AND METHODS

### *Chemicals*

Analytical grade reagents were used for the assessment of redox biomarkers in the redox status assays. All the reagents were prepared with deionized water and stored at + 4°C. The assay reagents were equilibrated at an ambient temperature for 30 min prior to the redox biomarker analysis.

### *Apparatus*

An environment of a normobaric hypoxic condition was established with the oxygen generator device (8850 SUBMIT 3 in 1, Altitude Tech. Co., Canada) while the oxygen detector device (IBRID MX6, Industrial Scientific Co., USA) continuously monitored the oxygen level inside the hypoxic tent. Also, the Testo 435 device (Testo AG, Germany) was run regularly to monitor the ratio of CO<sub>2</sub>, the percentage of humidity, and the temperature in degrees. To keep the ratio of CO<sub>2</sub> at an optimum level inside the tent, a CO<sub>2</sub> cleaning device (Altitude Tech. Co., Canada) was also run. All the rats exercised on a motorized treadmill (MAY-TME 9805, Commat, Turkey). The muscular tissue was weighted with a CP225D Analytical Balance Scale (Sartorius, Germany). The X250D homogenizator (CAD, Germany) device was used to homogenize the EDL samples. For all centrifugation procedures a Z 323 K cooling centrifuge (Hermle, Germany) was employed and the Power Wave XS spectrophotometer (BioTek., USA) measured the absorbance values of the redox status biomarkers “T-SH, Np-SH, AOPP and LHPs”.

### *Experimental animals and procedures*

**Animal subjects.** Eight-week-old male Sprague-Dawley rats were supplied from the Experimental Animal Production and Research Center, at Pamukkale University in Denizli. According to the study of Andreollo et al (2012), the rats in our study were in their period of prepubescent.<sup>34</sup>

In order to provide a parametric statistical analysis, a minimum number of rats were considered for this research in each group. The total number of rats was 36 rats with 6 animals per group.

All the rats were run on a motorized treadmill between 0.3 km/h – 0.5 km/h, at a 0% grade, for 10 min/day, 4 days a week in order to test their running ability.<sup>35</sup> Then the rats having the ability to run were selected and randomly divided into six groups: “three experimental groups (LHTL, LHTH, LLTL) and their respective control groups (the live high group (LH), the live high and low group (LHL), the live low group (LL))” (Figure 1). All the rats in the groups had a similar body weight prior to the familiarization exercise. A standard diet of laboratory chow (20% protein, 55% carbohydrate, 3% fat) was given to all the groups of rats. The rats were housed in conventional wire-mesh cages (two rats per cage) and allowed free access food and tap water. Additionally, the animals were kept in 12-h light–dark cycles, at a 21-23 °C regulated room temperature with 45–55% humidity throughout the housing period and the course of the experiments. We did not lose any animals throughout the experimental periods. All the procedures performed in the study involving experimental animals were carried out in accordance with the ethical standards of Pamukkale University’s Ethics Committee of Animal Care and Usage (PAUHDEK-2009/014).

**Exercise protocol.** All the experimental animals in the established training groups “LHTL, LHTH and LLTL” were given an exercise familiarization routine to ensure that all the rats are trained at the same altitude. The exercise familiarization period lasted for 4 weeks, 4 days a week (2 days running, 1 day off rotation, 2 days running), for 15–30 minutes per day in the laboratory’s stated conditions, at an altitude of about 350 m above sea level (Denizli/Turkey). Then, the training groups of rats all exercised for 30 minutes in order to be able to run at a speed of 1.5 km/h at the end of the familiarization period. All the training groups of animals were rested for a two-days at the end of the training period.

Following the resting period, a maximal aerobic velocity test was run for all the rat groups to evaluate their running capability and to supervise the training intensities with precision for the main training period of the subsequent 4 weeks. The maximal aerobic velocity test was obtained at both normoxia and hypoxia using a treadmill during both continuous and progressive maximal exercise tests. During the test at normobaric hypoxia (~3,000 m, 15 % O<sub>2</sub>) for the LHTH group, the speed of the treadmill at the beginning was at 0.3 km/h with a 0% grade. Then, it was increased by 0.3 km/h every 3 minutes until attaining the maximal exercise intensity for each rat. No animals could maintain their running positions upon reaching the maximal exercise intensity. The maximal aerobic velocity test at normoxia (~350 m, 20.9% O<sub>2</sub>) for the LLTL, the LHTL group was run with the same protocol, but at a starting speed of 0.6 km/h.<sup>35,36</sup> In order to establish relatively similar exercise intensities in hypoxic training and normoxic training groups, starting speeds were set at 0.3 km/h and 0.6 km/h respectively.<sup>35,36</sup>

After the maximal aerobic velocity test, the rats with running abilities were selected from the group and the exercise period was started. The exercise session was four days per week in the first week and five days per week in the following weeks. The running speed was set to 60% of the maximal aerobic velocity test for 20 min in the first week, 65% of the maximal aerobic velocity test for 25 min in the second week, 70% of the maximal aerobic velocity test for 30 min in the third week, and 70% of the maximal aerobic velocity test for 35 min in the fourth week. All the exercise programs of the familiarization (2 days exercise + 1 day off + 2 days exercise) and hypoxic training (2 days exercise + 1 day off + 3 days of exercise) were implemented in the mornings between 06:00 – 09:00 a.m. All the control groups “LH, LHL and LL” were kept in the same altitude as in the exercised animals. The details of the study’s design are given in Figure 1.

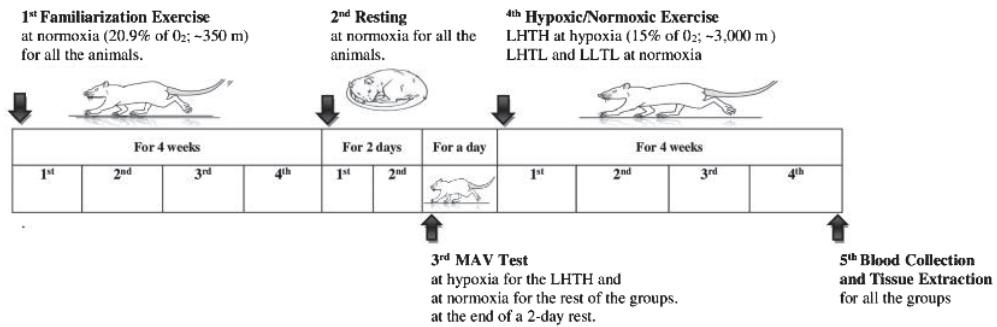


Fig. 1 Experimental design of the research

A hypoxic tent and oxygen generator were used to create the normobaric hypoxic condition (15% O<sub>2</sub>, altitudes of about 3,000 m) which was previously designed and used by Erken.<sup>37</sup> The levels of O<sub>2</sub> and CO<sub>2</sub> inside the hypoxic tent were continuously monitored throughout the experiment and the excessive CO<sub>2</sub> inside the tent was eliminated by the CO<sub>2</sub> cleaner. The ambient CO<sub>2</sub> level was kept under 0.03% during the experimental period while 21–23 °C temperature and 50–55% humidity were ensured throughout the experiment inside the hypoxic tent. The research laboratory, situated at an altitude of about 350 m above sea level (=20.9% O<sub>2</sub> of normoxia) provided a normoxic condition inside the laboratory. The LHTH group and its control group LH were exposed to a hypoxic condition for 24 hours, the LHTL group and its control group LHL were exposed to a 12-hour hypoxia /normoxia period per day, while the LLTL group and its control group LL were exposed to a 24-hour normoxic condition during the four-week training period.

After the exercise period all the groups of rats were euthanized at normoxia by an intraperitoneal injection of ketamine hydrochloride (Ketasol 10%, Richter-Pharma, Wels, Austria) and xylazine (Alfazyne 2%, Alfasan, Woerden, The Netherlands). Then, the blood samples were drawn from the abdominal aorta of all the animals and anticoagulated with heparin (15 IU/ml). The drawn samples of whole blood were used within two hours to determine the erythrocyte count.

**Preparation of tissue samples.** Afterwards, the EDL muscles of both legs of all the rats were extracted from their tendons' attachments. The obtained tissues were then immediately immersed in ice-cold saline. After that, all the excessive connective and fat tissues of the EDLs were trimmed away. The cleaned muscles were weighted and directly frozen in liquid nitrogen. The EDL muscle samples were kept at –80°C until the day of biochemical analysis. The muscular tissue samples were homogenized with a dilution ratio of 1:20 (w/v) in an ice-cold homogenizing buffer (KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub>, 100 mM, pH 7.4). Homogenate suspension was centrifuged at + 4°C 2800xg for 10 min with a cooling centrifuge. The supernatant fraction was then assayed for the assessment of redox biomarkers levels.<sup>3</sup>

*Analytical methods*

**Assessment of lipid hydroperoxides.** The LHP groups oxidize ferrous ions (Fe<sup>2+</sup>) in an acidic environment. The Xylenol orange dye binds ferric ions (Fe<sup>3+</sup>) and forms a blue-purple chromogen complex. A Ferrous Oxidation with Xylenol orange, version 2 (FOX2) reagent was pipetted into the assay tubes containing the supernatant samples. After a 30 min incubation time in the dark at room temperature, the final test samples were centrifuged at 3,000xg for 10 min

and the resulting absorbance values of the final samples were recorded at 560 nm wavelength.<sup>38</sup>

**Assessment of total thiol fractions.** The T-SH, Np-SH, and P-SH concentrations in the EDL homogenates were assayed according to the colorimetric methods of Sedlak and Lindsay (1968) and revised with the volumetric modifications of Yanar et al (2019).<sup>39,40</sup> The concentration of T-SH was analyzed by using Ellman's chromogenic reagent 5,5'-dithiobis (2-nitrobenzoic acid (DTNB). The Ellman's reagent was stoichiometrically reduced by free thiol groups and form mixed disulphide and 5-thio-2-nitrobenzoic acid (TNB). The absorbance value of the resulting chromophoric TNB was recorded as a 412 nm wavelength against a reagent blank. For the Np-SH sample assessment, aliquots of 20 µL of the supernatants were mixed in 400 µl of 50 % trichloroacetic acid (TCA). The assay tubes were shaken intermittently for 10 minutes and centrifuged at 3,000xg for 15 minutes. The final samples were assayed as performed for the T-SH groups. The absorbance values were read at 412-nm against a reagent blank. The molar extinction coefficient for thiol groups at wavelength 412 nm was  $e = 13,100 \text{ L mol}^{-1} \text{ cm}^{-1}$ . The P-SH groups were calculated by subtracting the Np-SH from T-H.

**Assessment of advanced protein oxidation products.** The supernatant samples and chloramine-T standards (0 to 100 µM) were mixed with citric acid (20mmol/L) and potassium iodide (1.16 M) reagents, respectively. The spectrophotometric readings were run in duplicate to increase their precision and performed within two minutes after the addition of potassium iodide. The absorbance value of the final reaction mixture was recorded at 340 nm wavelength against a reagent blank. The AOPP' concentration was expressed as micromoles per liter of chloramine-T equivalents.<sup>41</sup>

**Hematological parameters.** The erythrocyte count of the whole blood samples was determined by using a hematology autoanalyzer.

**Determination of total protein concentrations.** The Lowry's method (1951) was used to assess the total protein concentration in the supernatant fractions of the EDL samples.<sup>42</sup>

#### *Statistical analysis*

The descriptive statistics were expressed as both mean and standard error (SE). The distribution of the data was determined with Kolmogorov Smirnov's normality test and the homogeneity of variances was evaluated by Levene's test. One-Way Analysis of Variance (ANOVA) and Post Hoc Least Significant Difference (LSD) tests were run to analyze the differences between the experimental groups. The Statistical Package for the Social Sciences (SPSS) V.22 (IBM, Chicago, IL, USA) was used in processing the data. When not specified,  $p < 0.05$  was considered significant.

## RESULTS

All the groups of animals survived throughout both the familiarization exercise and the hypoxic/normoxic experimental protocols. No experimental animals passed away until euthanizing them. Only two of the rats had a resistance to exercise, and they were excluded from each of the hypoxic and normoxic exercise groups (one from each) as the exclusion criteria for our study was excessive weight loss due to hypoxia and an unwillingness to exercise.

The rats' food consumption during the hypoxic/normoxic exercise sessions were found to be similar except for the control group LL as they consumed more food than the LHTH ( $p=0.001$ )

and LH ( $p=0.004$ ) groups. The body weights of each of the rats were similar between pre- and post-exercise sessions and among all the groups (Table I). The total count of erythrocytes in the LHTH group was higher than the LLTL ( $p=0.047$ ) and LL ( $p=0.002$ ) groups. Furthermore, the control group LL had a lower level of blood erythrocytes than the LHTL ( $p=0.040$ ), LH ( $p=0.004$ ) and LHL groups ( $p=0.015$ ) (Table 1).

**Table 1** General physiological parameters of the experimental and control groups

Groups	FC (g/day)	Initial BW (g)	Final BW (g)	EC ( $10^6/\mu\text{l}$ )	TP (mg pr./g wt)
<b>LHTH</b>	20.01 $\pm$ 1.25 <sup>a3*</sup>	233.57 $\pm$ 4.63	232.36 $\pm$ 5.02	10.15 $\pm$ 0.19 <sup>a(1,3)*</sup>	90.45 $\pm$ 4.83 <sup>a2*</sup>
<b>LHTL</b>	22.54 $\pm$ 0.84	228.42 $\pm$ 3.18	235.28 $\pm$ 4.22	9.60 $\pm$ 0.31 <sup>b3*</sup>	97.19 $\pm$ 3.53 <sup>b(1,2)*</sup>
<b>LLTL</b>	22.46 $\pm$ 0.58	233.33 $\pm$ 3.72	244.16 $\pm$ 5.86	9.16 $\pm$ 0.30	96.97 $\pm$ 2.22 <sup>c*</sup>
<b>LH</b>	20.74 $\pm$ 1.01 <sup>d*</sup>	238.16 $\pm$ 2.88	241.00 $\pm$ 4.87	10.01 $\pm$ 0.23 <sup>d*</sup>	75.03 $\pm$ 5.28 <sup>d*</sup>
<b>LHL</b>	22.05 $\pm$ 0.73 <sup>e*</sup>	237.50 $\pm$ 4.38	243.50 $\pm$ 8.69	9.82 $\pm$ 0.58 <sup>e*</sup>	78.31 $\pm$ 4.34 <sup>e*</sup>
<b>LL</b>	24.85 $\pm$ 0.87	241.33 $\pm$ 1.99	253.02 $\pm$ 3.80	8.54 $\pm$ 0.38	93.75 $\pm$ 6.69

Values are given as means ( $\pm$  SEM).

\*  $p<0.05$

Experimental Groups:

LHTH: live high train high

LHTL: live high train low

LLTL: live low train low

Control Groups:

LH: live high

LHL: live high live low

LL: live low

FC: food consumption for each animal

BW: body weights

EC: erythrocyte count

TP: total protein concentration

g wt: gram wet tissue

pr: proteins

<sup>a1</sup>LHTH versus LLTL

<sup>a2</sup>LHTH versus LH

<sup>a3</sup>LHTH versus LL

<sup>b1</sup>LHTL versus LH

<sup>b2</sup>LHTL versus LHL

<sup>b3</sup>LHTL versus LL

<sup>c</sup>LLTL versus LHL

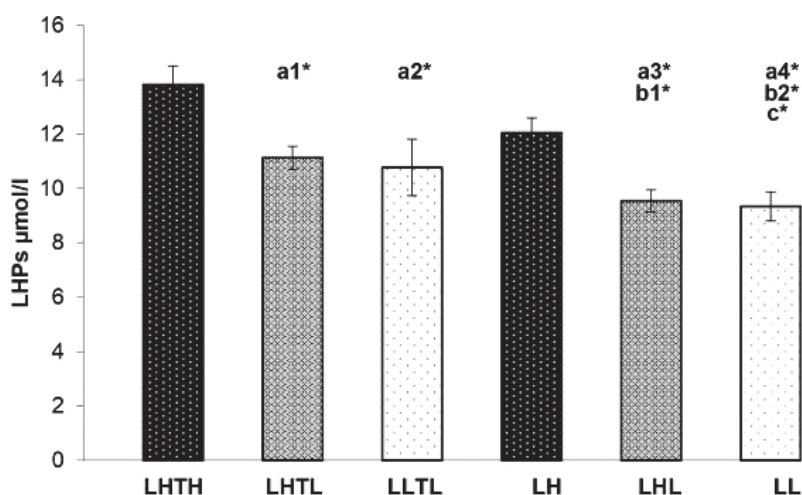
<sup>d</sup>LH versus LL

<sup>e</sup>LHL versus LL

The concentration of protein in the EDL was found to be higher in the LHTH than LH ( $p=0.027$ ). The LHTL and LLTL also had an increased protein concentration than the LHL ( $p=0.011$ ;  $p=0.011$  respectively). In addition, the LHTL had a higher protein concentration than LL ( $p=0.002$ ). Among the control groups, the protein concentration in the LL was found to be higher than LH ( $p=0.008$ ) and LHL ( $p=0.033$ ) (Table 1). The general physiological features of

the experimental animals are given in Table 1.

The LHTH group had higher LHPs than the LHTL ( $p=0.004$ ), LLTL ( $p=0.002$ ), LHL ( $p=0.000$ ), and LL ( $p=0.000$ ). However, the concentration of LHPs in the LHTH group had no difference from LHs (Figure 2). The LHTL group had a similar ratio of LHPs to all the experimental and control groups except for the LHTH and LL groups. The LHPs' level was lower in the LL group ( $p=0.049$ ). Moreover, the control group LH had a higher level of LHPs than the LHL ( $p=0.009$ ) and LL ( $p=0.005$ ) (Figure 2). The muscular concentrations of LHPs in the hypoxic/normoxic experimental groups and the control groups are given in Figure 2.



**Fig. 2** The ratios of LHPs in the experimental and control groups

\*  $p < 0.05$  (exact  $p$  values are given in the result section)

$n=6$  for each group

LHTH: live high train high

LHTL: live high train low

LLTL: live low train low

LH: live high

LHL: live high live low

LL: live low

<sup>a1</sup>LHTH versus LHTL

<sup>a2</sup>LHTH versus LLTL

<sup>a3</sup>LHTH versus LHL

<sup>a4</sup>LHTH versus LL

<sup>b1</sup>LH versus LHL

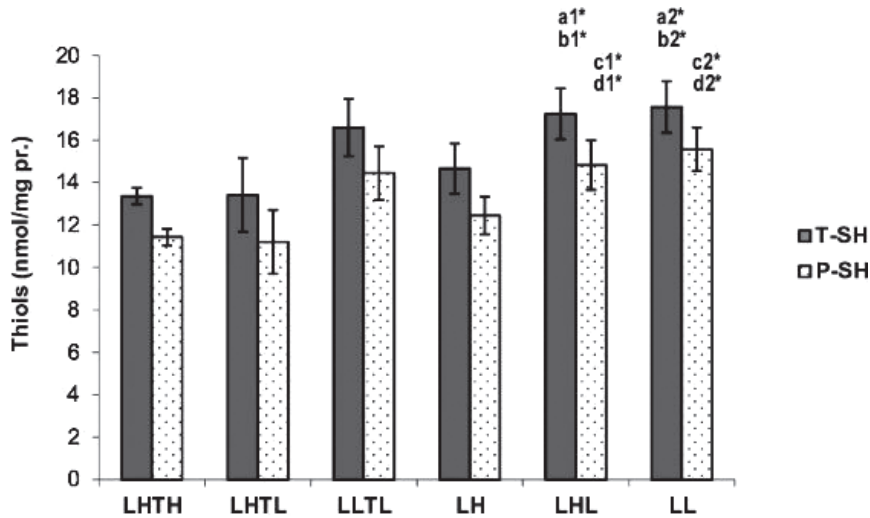
<sup>b2</sup>LH versus LL

<sup>c</sup>LHTL versus LL

There were no significant differences in the T-SH and P-SH rates among all the experimental groups (LHTH, LHTL, LLTL) including the live-high control group (LH). The T-SH fractions were assayed to be lower in the LHTH than the LHL ( $p=0.041$ ) and LL ( $p=0.035$ ) control groups. Similarly, a lower level of P-SH was also found in the LHTH than the LHL ( $p=0.043$ ) and LL ( $p=0.020$ ) groups. The T-SH and P-SH fractions were also found to be lower in the LHTL groups than in the LHL ( $p=0.035$ ;  $p=0.025$  respectively) and LL ( $p=0.030$ ;  $p=0.011$



respectively). Moreover, there were no significant differences found in the Np-SH fractions of rats' EDL muscles among all the groups (Figure 3). The T-SH, Np-SH and P-SH fractions are given in Figure 3.



**Fig. 3** The ratios of T-SH and P-SH in the experimental and the control groups

\*  $p < 0.05$  (exact p values are given in the result section)

n=6 for each group

LHTH: live high train high

LHTL: live high train low

LLTL: live low train low

LH: live high

LHL: live high live low

LL: live low

pr: protein

<sup>a1</sup>LHTH versus LHL

<sup>a2</sup>LHTH versus LL

<sup>b1</sup>LHTL versus LHL

<sup>b2</sup>LHTL versus LL

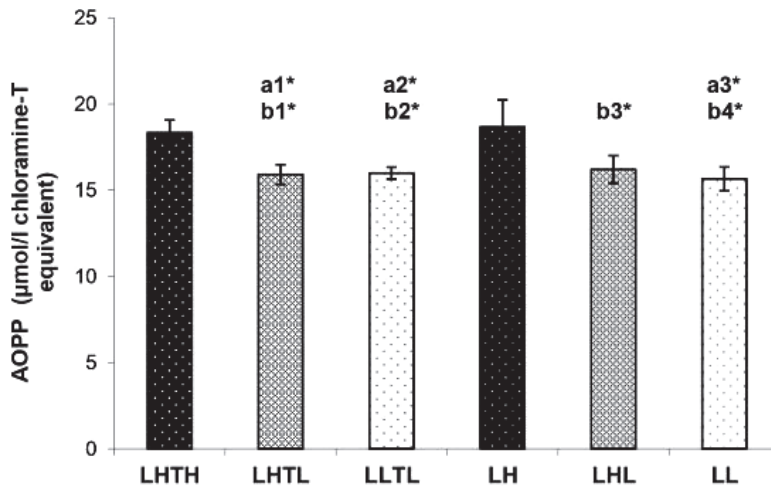
<sup>c1</sup>LHTH versus LHL

<sup>c2</sup>LHTH versus LL

<sup>d1</sup>LHTL versus LHL

<sup>d2</sup>LHTL versus LL

The higher percentage of advanced oxidation protein products formation was observed in the LHTH than the LHTL ( $p=0.041$ ) and LLTL ( $p=0.048$ ), and the control groups LHL ( $p=0.071$ ) and LL ( $p=0.031$ ). Both the LHTH and the control group LH had similar AOPP levels. Moreover, the AOPP percentage in the LHTL group had no difference from the LLTL group. The AOPP concentration was found to be lower in both the LHTL ( $p=0.027$ ) and LLTL ( $p=0.031$ ) than the hypoxic control group LH. Furthermore, among the control groups, the LH had a higher AOPP percentage than the LHL ( $p=0.046$ ) and LL ( $p=0.020$ ) (Figure 4). The AOPP levels in the EDL muscle of the experimental and control groups are given in Figure 4.



**Fig. 4** The ratios of AOPP in the experimental and the control groups

\*  $p < 0.05$  (exact  $p$  values are given in the result section)

$n=6$  for each group

LHTH: live high train high

LHTL: live high train low

LLTL: live low train low

LH: live high

LHL: live high live low

LL: live low

<sup>a1</sup>LHTH versus LHTL

<sup>a2</sup>LHTH versus LLTL

<sup>a3</sup>LHTH versus LL

<sup>b1</sup>LH versus LHTL

<sup>b2</sup>LH versus LLTL

<sup>b3</sup>LH versus LHL

<sup>b4</sup>LH versus LL

## DISCUSSION

Normally, the duration and severity of hypoxic exercise affect food consumption and body weight.<sup>43,44</sup> In our research, only the normoxic control LL group consumed more food than the hypoxic exercised group LHTH and the control group LH. However, there was no difference in the body weights of the rats between the pre- and post-training sessions and among the groups. The altitude level of 3,000 m (15% of  $O_2$ ) in the present study may not be considered as leading to a retardation in growth. On the other hand, in our study it was observed that all the hypoxic groups including the controls had higher erythrocyte counts than the normoxic control group LL which were observed as resulting from the physiological effects of hypoxia. In addition, the hypoxic control group LHL and LH had the lowest muscular protein concentrations compared to the normoxic control LL and the hypoxic/normoxic exercised groups. We assume that our hypoxic control group may normally exhibit a higher mitochondrial electron leakage, and a lower ATP production rate. Therefore, there would be an inadequate ATP supply for anabolic reactions, a higher ratio of ROS generation, and an increased rate of oxidatively modified protein degradation.

Lipid hydroperoxides have a significant potential for muscular malfunction. In our study, the highest instance of LHPs was observed in the hypoxic exercised group of LHTH, but not in

the normoxic exercised group of LHTL. In addition, the LHTH had similar LHP levels with the hypoxic control group LH. These outcomes suggest a hypoxia-induced imbalance in the redox homeostasis of the EDL rather than it being exercise-induced. Ağaşcıoğlu et al (2019) assayed the higher rate of LHPs in the left ventricles of young and aged rats in severe hypobaric hypoxia.<sup>45</sup> Furthermore, our experimental findings also show that the ratio of LHPs in the LHTL group was similar to the LLTL. All these findings also indicate that after hypoxic exposure, normoxic exercise or normoxia may hinder the catastrophic effects of ROS attacks. It is assumed that, since there is more oxygenation during normoxic exercise or normoxia, it might induce the higher ratio of aerobic ATP formation and anabolic reactions. There are a limited number of reports in the current literature related to the redox status of skeletal muscle during hypoxic exercise. Yet, in these studies, the lipid peroxidation rate in the soleus muscle and the gastrocnemius muscle were found to be lower in the LHTL group than the LHTH group.<sup>3,14</sup>

The redox homeostasis is primarily ensured by muscular thiol fractions, since cellular thiol groups are very sensitive to ROS attacks.<sup>46,47</sup> The redox buffering feature of muscular thiol fractions makes them an important part of non-enzymatic antioxidant defense systems. A limited number of studies are related to different muscular thiol fractions (T-SH, P-SH and Np-SH) and hypoxic exercise approaches. It was reported that there was no difference in the mitochondrial T-SH status in rats' myocardia after 6,000 m altitude exercise which was implemented five days a week during a five-week period.<sup>7</sup> The findings of this study are supported by our recent study which found no significant difference in the T-SH, P-SH and Np-SH fractions among the experimental groups of the LHTH, LHTL and LLTL in the soleus muscle.<sup>3</sup> We also observed no significant differences in the levels of the T-SH and P-SH fractions among the hypoxic/normoxic exercised groups. All these outcomes emphasize that the effect on muscular thiol fractions of T-SH and P-SH were caused by hypoxia, but not the exercise itself since the hypoxic control had lower levels of T-SH and P-SH than the normoxic controls. Furthermore, the T-SH and P-SH ratios of the normoxic exercised group LLTL were similar to all the normoxic-control groups LHL and LL, while the LHTH and LHTL had lower T-SH and P-SH than the normoxic control groups. Our recent findings also indicate the hypoxic effect itself on the ROS-induced depletion of T-SH and P-SH for the EDL. In the current literature, a decreased level of T-SH in the soleus, gastrocnemius, quadriceps and tibialis anterior muscles and P-SH in the heart muscle were reported after acute hypobaric hypoxia.<sup>11,12,45</sup> Moreover, the Np-SH exhibits no variation among all the groups of the experimental and control animals in our research. Similarly, Çolak et al (2020) reported no change in the levels of the Np-SH in the slow-twitch soleus muscle.<sup>3</sup> A great percentage of Np-SH is represented by a reduced form of glutathione (GSH), which was oxidized by mitochondrial ROS at hypoxia.<sup>17</sup> It would seem possible that GSH is pre-emptively replenished right away. Since Np-SH is represented as a small percentage of the T-SH and an analytic estimation of Np-SH might not be feasible in each routine assay.

The fairly new biomarker of advanced oxidation protein products consists of miscellaneous non-enzymatic oxidative products like dityrosine, pentosidine, and the protein carbonyl groups.<sup>20</sup> As far as we are concerned, there is no available report existing for AOPP formation in the fast-twitch muscle available in the literature related to hypoxic exercise. Both the hypoxic control group LH and the hypoxic exercised group LHTH had the highest ratio of AOPP in EDL. However, the normoxic exercised group LHTL and LLTL, the normoxic control group LL and the hypoxic-normoxic control group LHL demonstrated lower rates of AOPP formation. Similarly, the highest level of AOPP was found in the slow-twitch soleus muscle of the LHTH group.<sup>3</sup> According to our current experiment's findings, hypoxia itself has a more detrimental effect on the fast-twitch EDL muscle than the exercise itself. The reason for this was determined to be an increased rate of AOPP formation in the myocardia of both young and aged rats at severe

hypobaric hypoxia.<sup>45</sup>

In our study, ROS-mediated muscular protein, and lipid damages were observed in both the hypoxic-lived and exercised group LHTH and the hypoxic control group LH in their fast-twitch EDL muscles. In the same way, Çolak et al (2020) reported the highest levels of redox biomarkers in the soleus muscles of their LHTH group.<sup>3</sup> The ratios of redox status biomarkers in the soleus muscle might be higher than the EDL since the soleus is more prone to ROS attacks due to its higher mitochondrial content and NADPH oxidases activity. In spite of this, the EDL seems to be more vulnerable to ROS attacks according to our redox status related findings. As mentioned in the previous research of Anderson and Neuffer (2006), mitochondria in the fast twitch muscle fibers have unique redox properties and have higher ROS formation potential than those in the slow twitch muscles.<sup>24</sup> Therefore, it might be possible that the mitochondrial electron transfer chain in the EDL is more prone to form ROS during hypoxia.<sup>28</sup> Furthermore, cellular NADPH oxidases in the fast twitch muscle fibers overproduce ROS not only during the adaptation process of exercise,<sup>26</sup> but also might play a role in the adaptation process to hypoxia. Since atrophy in the glycolytic muscle was determined in relation to the overactivity of NADPH oxidase during the myocardial failure of rats.<sup>27</sup> The overproduction of the superoxide radical anion by NADPH oxidase and/or mitochondria at hypoxia may transform into hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). H<sub>2</sub>O<sub>2</sub> is more stable and can easily diffuse from the formation site to the other cellular compartments or the surrounding tissues.<sup>30</sup> The excessive rate of H<sub>2</sub>O<sub>2</sub> formation may lead to an insufficient muscular redox buffering capacity and cause a ROS induced oxidative macromolecular damage. However, H<sub>2</sub>O<sub>2</sub> signals at the physiological concentration also fine-tune responses to growth factors and cytokines to control cell division, differentiation, and migration.<sup>48</sup> Therefore, an impaired redox status in muscular tissue at a high altitude may also be considered as logical reason for the increased fast twitch myofibrillar protein oxidation and impaired muscular activity. Previously, myofibrillar protein loss in the gastrocnemius muscle was reported during hypoxic exposure.<sup>49</sup> It should be noted that the gastrocnemius is composed of both fast and slow-twitch muscle fibers.<sup>21,22</sup> However, the EDL mainly consists of fast twitch fibers.

According to our current findings, not hypoxic exercise but hypoxia itself may cause a muscular redox imbalance in the fast-twitch EDL muscle. The experimental results presented in the context of this paper will hopefully clarify the reasons for the impaired redox status-related fast-twitch skeletal muscle insufficiency at hypoxia to some extent. Furthermore, our recent results may put forward some reliable explanations for the skeletal muscle mass loss and strength due to hypoxic exposure since skeletal muscle may possibly have an overall limited oxygenation. Therefore, endogenous antioxidants activities may be diminished due to this limitation.

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## CONFLICT OF INTEREST

All authors declare that there is no competing financial interests.

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