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A new oxidative stress indicator: Effect of 5-hydroxytryptophan on thiol-disulfide homeostasis in exercise

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ABSTRACT

Objectives: The aim of the present study was to evaluate the relationship between exercise and both 5-hydroxytryptophan and oxidative stress using thiol-disulfide homeostasis via what is likely a novel biomarker.
Methods: Male albino Wistar rats ($n = 32$) were randomly divided into four groups as follows: control, exercise group, 5-hydroxytryptophan group (5H), and 5-HTP + exercise group (5Hex). Exercise and 5-HTP administration (25 mg/kg per d) were performed 5 d/wk for 10 wk. After completion of the experimental protocol, to determine oxidative stress parameters, serum total thiol and native thiol concentrations were measured. Dynamic disulfide status, reduced thiol, oxidized thiol (OT), and thiol oxidation reduction percentage ratios were compared between the groups. The methods used in the present study to measure dynamic thiol-disulfide homeostasis as calorimetric and duplex quantities were developed in 2014. These new methods are simple, reliable, and sensitive, with both high linearity and repeatability.
Results: Compared with the control group, serum dynamic disulfide levels were significantly lower in the 5H group and highest in the control group. The lowest OT and the highest reduced thiol rates were determined to be in the 5H group. The highest OT value was found in the 5Hex group. Thiol oxidation reduction values were found to be highest in the 5H group and lowest in the 5Hex group.
Conclusions: Both 5-HTP and moderate exercise seem to be significantly effective in inhibiting oxidative damage. In addition, the new oxidative stress measurement method used in this study is a promising practical and useful method to evaluate and improve the performance of athletes.

Introduction

Free radicals are atoms, molecules or ions with unpaired electrons, which are highly active to chemical reactions with other molecules. Biologically, free radicals are often derived from oxygen, nitrogen, and sulfur molecules. These free radicals are parts of groups of molecules called reactive oxygen species (ROS), reactive nitrogen species, and reactive sulfur species [1]. ROS are produced during cellular metabolism and functional activities and have important roles in cell signaling, apoptosis, gene expression, and ion transportation [2]. However, excessive amounts of ROS can have deleterious effects on many molecules, including protein, lipid, RNA, and DNA because they are very small and highly reactive [3].

Heavy exercise brings heavy oxygen consumption, especially in skeletal muscles [4,5]. Oxygen also increases because of the increase in metabolic activity. As a result, ROS are released [5]. ROS increases that are greater than the physiological level cause oxidation between two electron or redox modification of radical-based cysteine residues. In this redox reaction, a sulfur atom that is found in the cysteine side chain gets oxidized and transforms into disulfide [6]. Thus dynamic thiol-disulfide homeostasis moves toward disulfide form, and the first stage of the oxidative damage is attached to oxidant radicals when the cellular level begins [7]. Many studies have confirmed that long-term or high-intensity exercise can cause excessive amounts of oxidative stress, damaging macromolecules in both blood and skeleton [8]. Although the tissues responsible for ROS production during exercise remain a subject of debate, strong evidence suggests that muscle activity increases oxidant production in skeletal fibers [5].

The interest in using free radical scavengers, called antioxidants, to inhibit oxidative stress and its harmful effects arising from exercise and to develop effective strategies in this regard increases with each passing day. Oxidative stress occurs because of the imbalance between antioxidant molecules [7,9]. This chemical has been associated with stress, exercise, and use of amino acids. The use of amino acids has been recommended to reduce the

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effects of this biological stress caused by exercise [10], and one of these amino acids is tryptophan. This amino acid has been reported to be a potent free radical scavenger [11] and the main antioxidant in human placental extracts that can suppress lipid peroxidation [12]. This property has been associated with the metabolites it contains; 5-hydroxytryptophan (5-HTP), one of these metabolites, is a naturally occurring amino acid and is the precursor of the neurotransmitter serotonin [13]. The serotonin system in living beings is very influential, especially in neuronal conduction and neuromodulation. It is also very important for mood, aggression, sexual function, nutrition, thermoregulation, and sleep/wakefulness [14]. Exercise is a promising adjuvant therapy for depressive behavior, sleep/wakefulness abnormalities, and cognition and motor dysfunction. However, sleep deprivation negatively affects emotional, cognitive, and functional performance. The serotonin pathway begins with the production of 5-HTP by tryptophan hydroxylase from tetrahydrobiopterin-dependent family. The 5-HTP is converted to serotonin by aromatic amino acid decarboxylase and pyridoxal phosphate. Finally, it is metabolized by 5-hydroxyindoleacetic acid–producing monoamine oxidase and aldehyde dehydrogenase [15]. Studies have found that 5-HTP can prevent protein oxidation and reduce lipid peroxidation in rats [16], and one reasonable explanation for the protective effect of 5-HTP against oxidative stress is its ability to neutralize free radicals in the membranes [11].

Oxidative stress resulting from exercise can be triggered by oxidative agents such as reactive oxygen species, reactive nitrogen species, and free radicals. Therefore the study of chemical reactions between these species, and of chemical compounds that can neutralize them, represents an important and fundamental area of research in exercise physiology.

To the best of our knowledge, there is no automated colorimetric measurement method for plasma/serum dynamic disulfide (μmol/L) levels [17]. In a few recent studies, the disulfide and thiol levels of low-molecular-weight disulfide compounds of plasma have been determined using high-performance liquid chromatography [18,19], fluorescence capillary electrophoresis [20], and bioluminescent systems [21]. In these sophisticated systems, separation processes such as the removal of the remaining reductants, which are NaBH4, Tris(2-carboxyethyl) phosphine, and tributyl phosphine, as well as precipitation of proteins, are also needed [17]. These pretreatment applications and measurement procedures are time consuming, labor intensive, and costly and require complicated techniques.

The methods that measure dynamic thiol-disulfide homeostasis as calorimetric and duplex quantities were not developed until 2014. A fully automated colorimetric method was developed by Erel and Neselioglu [22]. These new methods used in the present study are simple, reliable, and sensitive, with both high linearity and repeatability [22].

In this context, this study aims to investigate the effect of 5-HTP on a new oxidative stress marker, thiol-disulfide balance, in rats that perform treadmill exercise.

Material and methods

Animals

The experimental protocol was approved by the Animal Ethics Committee (No. 2016/36). A total of 32 male albino Wistar rats, weighing 180 to 200 g and 12 wk old, were kept under standard environmental conditions for temperature (21.5 ± 2°C) and humidity (60 ± 1%) and on a 12:12 h light/dark cycle. The rats were kept in a well-ventilated room and allowed free access to a standard pellet diet along with water ad libitum. The rats were randomly divided into four groups: control (C), exercise (EX), 5-hydroxytryptophan (5H), and 5-HTP + exercise (5Hex).

5-HTP dose adjustment

The 5-HTP was dissolved in sterile physiological saline (0.9%; Sigma-Aldrich, St. Louis, MO, USA). A gavage tube was used to deliver the substance orally, which is the clinically approved method of delivery. The volume of administrated dosage was kept at 2 mL per animal (25 mg/kg per d in 2 mL saline; 5H and 5Hex group) or saline alone (C group). This dose was a dose determined for rats [23]. In literature, there are different doses in human studies. For example, the effect of 5-HTP given daily to the performance of the Grooved Pegboard Test (150 mg) was investigated, and it was found that 150 mg oral dose did not affect fine motor functions [24]. In a different study, 50 patients with primary fibromyalgia syndrome were given 100 mg 3 times a day for a month [25]. However, one study reported that in humans the administered dose of 5-HTP should not exceed the daily recommended dose, which is approximately 4 mg/kg body weight (e.g., 280 mg for an adult weighting 70 kg [26]). In addition, the serum levels of 5-HTP in humans are reported to be in the range of 100 to 200 ng/mL (0.45–1 μM) [11,27].

Exercise protocol

Exercise and 5-HTP administration was performed 5 d/wk for 10 wk. The Conformité Européene (CE)-certified four-lane animal treadmill (May Time 0804, Animal Treadmill) with adjustable settings for rate, distance, running time, speed, and inclination and a built-in memory to store data was used for exercise experiments. To avoid any stress that may have arisen during physical exercise, all rats were preliminarily subjected to a conditioning exercise series at the lowest speed in 5-min sessions for 10 d. After the treadmill adaptation period, control group rats were put in cages with the standard conditions until surgery, whereas the exercised groups continued to be trained according to the treadmill exercise protocol. The exercise workload consisted of running at the speed of 2 m/min for the first 5 min, 5 m/min for the next 5 min, and then 8 m/min for the last 20 min with a 0° angle inclination [5] (Fig. 1).

Biochemical parameters

At the end of the experiment, rats in all groups were starved overnight for 12 h and sacrificed under ketamine hydrochloride (10 mg/kg intraperitoneally) anesthesia. After entering abdominal and thoracic cavities, blood from the heart was collected into biochemistry tubes using 10 mL syringes (5 mL). The samples were centrifuged (1500 g for 10 min) after waiting 30 min. Subsequently, the separated serums were stored in tubes with Eppendorf (Isolab centrifuge tubes 2.0 mL, flat cap without skirt) with −80° cap. The samples were transferred to the laboratory

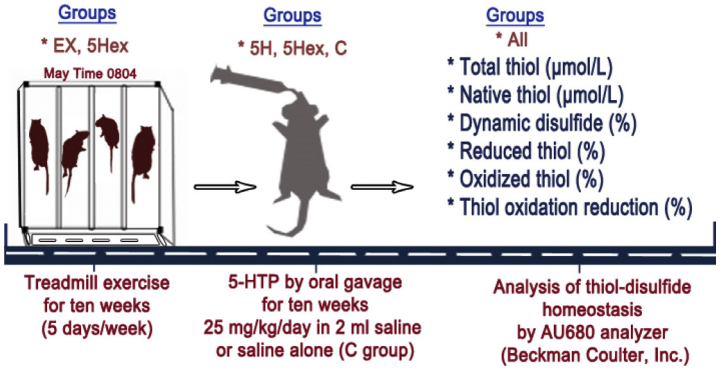

Fig. 1. Experimental application process carried out in the present study. C, control; 5H, 5-hydroxytryptophan group; 5Hex, 5-hydroxytryptophan + exercise group.

with a dry ice system at 24 h before the working day. The incoming samples were again microcentrifuged, and the study test parameters were studied in the Rel Assay Diagnostics kits. Biochemical analysis of this study was also done in the Research Hospital Clinical Biochemistry Laboratory (fully automated chemistry analyzer AU680, Beckman Coulter, Tokyo, Japan). In this study, dynamic thiol-disulfide homeostasis in the serum samples of rats was identified by using an automated method newly developed by Erel and Neselioglu [22].

Properties of the new method

Precision

Three levels of a plasma pool were tested to determine the precision of the new test. A plasma pool that had high disulfide levels was obtained from the samples of patients with diabetes mellitus. The plasma pool with medium disulfide levels was obtained from the samples of healthy persons. The plasma pool with low disulfide levels was obtained from the samples of patients with urinary bladder cancer. Percentage of coefficient variation (%CV) was 4 ($\bar{X}$ = 29.12 and σ_X = 1.2) for high levels, 5 ($\bar{X}$ = 16.03 and σ_X = 0.79) for medium levels, and 13 ($\bar{X}$ = 7.15 and σ_X = 0.98) for low levels.

Total thiol (−SH + −S−S−) and native thiol (−SH) concentrations in the samples were measured by using Ellmann's and modified Ellmann's reagent. Native thiol content was subtracted from total thiol content, and half of this difference gave the amount of dynamic disulfide bonds (−S−S−). In addition, the (−S−S−) × 100/(−SH), (−S−S−) × 100 / (−SH + −S−S−), and −SH × 100 / (−SH + −S−S−) ratios were calculated using these parameters.

Analytical recovery

The percentage recovery of the novel method was determined via the addition of 200 μM oxidized glutathione to plasma samples. The mean percentage recovery was 98% to 101%.

Linearity

The linearity of the native thiol measurement was the same with that of Ellman's reagent assay. Serial dilutions of the glutathione solution were generated. The upper limit of the linearity for the native thiol measurement was 4000 μM. Linearity of the total thiol measurement was also dependent on the amounts of NaBH4 and formaldehyde concentrations. Serial dilutions of the oxidized glutathione solution were also generated. The upper limit of the linearity for the disulfide measurement was 2000 μM. Dilution of plasma samples did not affect the novel assay.

Lower detection limit

The detection limit of the assay was determined by evaluating the zero calibrator 10 times. The detection limit, defined as the mean value of zero calibrator + 3 SD, was 2.8 μM.

Analytical sensitivity

As the slope of the calibration line, analytical sensitivity was found to be 7.9 × 10 − 4 Absorbance / Amount (A × [μM] − 1).

Interference

It was found that hemoglobin, EDTA, citrate, and oxalate did not interfere with the assay developed, but bilirubin did negatively interfere with the assay. Lipemic and uremic plasma samples did not interfere with the assay. Plasma and serum samples can be used as samples.

Storage

Storage at 4°C for 1 d led to a 7% decrease in the native thiol amount and 170% increase in the disulfide amount (total thiol, native thiol, and disulfide levels of fresh and stored plasma samples were 391 μmol/L, 357 μmol/L, and 17 μmol/L and 391 μmol/L, 333 μmol/L, and 29 μmol/L, respectively). Plasma native thiol, total thiol, and disulfide concentrations were not affected by storage at −80°C for 3 mo [22].

Statistical analyses

All results are presented as the means ± SD. Statistical analyses were performed by SPSS version 21.0 (IBM Corp., Armonk, NY, USA). The normality of the data was tested before analyses. One-way analysis of variance and Tukey honest significant difference post hoc tests were performed for normal distribution data (Table 1). For the data with no normal distribution, the Kruskal-Wallis and Tamhane T2 multiple comparison tests were performed to determine the significance of the differences between the groups (Tables 2 and 3). For all statistical tests, $P < 0.05$ was considered statistically significant.

Table 1
One-way ANOVA analysis of RT, OT, and TOR parameters used in thiol disulfide homeostasis

ANOVA Tukey HSD				
Parameters	Groups	Mean	SD	*P*
RT (%)	EX	17.656	4.926	0.687
	5H	32.095	18.931	
	5Hex	17.094	8.158	
	C	19.862	17.251	
OT (%)	EX	164.691	9.563	0.596
	5H	135.835	37.018	
	5Hex	165.826	16.726	
	C	160.293	34.022	
TOR (%)	EX	10.896	3.614	0.668
	5H	27.936	18.853	
	5Hex	10.849	6.615	
	C	15.231	16.107	

ANOVA, analysis of variance; C, control; EX, exercise group; HSD, honest significant difference; OT, oxidized thiol; RT, reduced thiol; TOR, thiol oxidation reduction; 5H, 5-hydroxytryptophan group; 5Hex, 5-hydroxytryptophan + exercise group.
There was no statistically significant difference among the percentages of reduced thiol, oxidized thiol, and thiol oxidation reduction.

Results

Serum total thiol (μmol/L), native thiol (μmol/L), and disulfide concentrations were determined using a novel automated measurement method. In addition, dynamic disulfide status (DD; %), reduced thiol (RT; %), oxidized thiol (OT; %), and thiol oxidation reduction (TOR; %) percentage ratios were compared among the groups.

According to the normality test, native and total thiol and dynamic disulfide values indicated a non-homogeneous distribution.

Analysis of variance was used for parametric tests for RT, OT, and TOR values, which were accepted as normal in the groups (Table 1).

The highest OT value was found in the 5Hex group (165.82 ± 5.95%). TOR values were found to be the highest in the 5H group (27.93 ± 6.68%) and lowest in the 5Hex group (10.84 ± 2.25%). The lowest OT (135.83 ± 13.23%) and the highest RT (32.09 ± 6.61%) rates were determined to be in the 5H group (Table 1). Compared with the C group, serum DD levels were significantly lower in the 5H group (70.67 ± 39.772%) and highest in the C group (225 ± 114.56 %) (Table 3; Fig. 2). No significant difference was found in the groups' native thiol levels (μmol/L). Total thiol levels (μmol/L) were significantly lower in the 5H group (Fig. 3A, B).

Discussion

The effect of 5-HTP and exercise on oxidative stress was investigated from the perspective of thiol-disulfide homeostasis in the present study. When statistically significant parameters were examined, the lowest DD value was found in the 5H group, followed by the 5Hex and EX groups (Table 3; Fig. 2). The use of

Table 2
Kruskal-Wallis test of DD, NT, and TT parameters used in thiol disulfide homeostasis

Test	DD (%)	NT (μmol/L)	TT (μmol/L)
χ^2	9.718	1.748	8.393
Asymptotic significance	0.021*	0.626	0.039*

DD, dynamic disulfide status; NT, native thiol; TT, total thiol.
*The statistically significant difference was found in DD and TT values from the measured parameters. The level of significance among the groups was determined by the Tamhane's T2 multiple comparison.

Table 3
Tamhane's T2 multiple comparisons of NT, TT, and DD parameters used in thiol disulfide homeostasis

Parameters	Groups	Mean	SD	*P*
NT (μmol/L)	EX	14.670	6.945	0.765
	5H	21.333	17.44	
	5Hex	15.67	23.066	
	C	104.166	145.545	
TT (μmol/L)	EX	78.666 ab	22.384	<0.05
	5H	56.566 b	36.236	
	5Hex	70.001 ab	68.014	
	C	327.166 a	299.510	
DD (%)	EX	128.00 b	31.343	<0.05
	5H	70.667 b	39.772	
	5Hex	108.666 b	90.656	
	C	446 a	324.029	

C, control; DD, dynamic disulfide status; EX, exercise group; NT, native thiol; TT, total thiol; 5H, 5-hydroxytryptophan group; 5Hex, 5-hydroxytryptophan + exercise group
The letters a and b indicate the differences among the groups in the same column
There was a statistically significant difference among the groups in terms of total thiol and dynamic disulfide parameters. The lowest dynamic disulfide level in the groups was found to be statistically significantly lower in the 5H group.

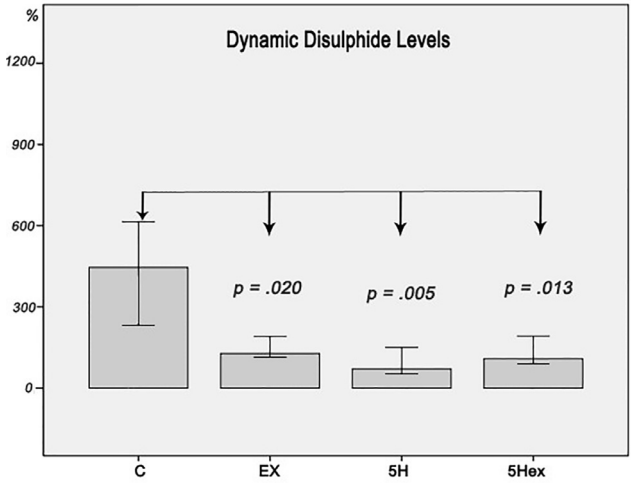

Fig. 2. Groups' dynamic disulfide levels (%). The figure shows that serum DD levels were significantly lower in 5H group (70.667 ± 39.772) and highest in the C group (446 ± 324.029). C, control; DD, dynamic disulfide; EX, exercise group; 5H, 5-hydroxytryptophan group; 5Hex, 5-hydroxytryptophan + exercise group.

5-HTP alone appeared to be most effective in reducing the oxidative stress level.

Although antioxidants in general do not eliminate all free radicals, an increase in antioxidant consumption can affect a variety of pathways, including cellular signaling pathways, which are important for exercise adaptation [28]. Serotonin (5-hydroxytryptamine) is a monoamine neurotransmitter synthesized by aromatic amino acid decarboxylase using 5-HTP as a substrate. Studies have found that serotonin and its precursor have strong antioxidant properties [29,30]. Accordingly, the potential role of tryptophan as a protector against oxidative stress has been attributed to its metabolites. The hydroxylated metabolites of tryptophan have been suggested to be responsible for the peroxyl radical scavenging activity of tryptophan. One of the hydroxylated metabolites of tryptophan is 3-hydroxyanthranilic acid (2-amino-3-hydroxybenzoic acid). It is an aminophenol that is naturally produced in the nicotinamide pathway of tryptophan catabolism [31]. It has been proposed that 3-hydroxyanthranilic acid plays anti-inflammatory and neuroprotective roles during inflammation processes [32,33]. This is in line with other findings suggesting that tryptophan catabolism is triggered in response to inflammation and oxidative stress. Researchers also found 3-hydroxyanthranilic acid to be more efficient than tryptophan and its other catabolites for deactivating reactive oxygen and chlorine species [31]. This finding led to the hypothesis that tryptophan metabolism might be a self-regulatory mechanism to limit the tissue damage caused by the free radicals produced by macrophages. In addition, 3-hydroxyanthranilic acid has been found to efficiently inhibit the oxidation of low-density lipoproteins and to have a synergistic effect [34].

Muñoz-Castañeda et al. [35] found that in rats the antioxidant properties of serotonin protected against basal oxidative stress in the brain. Keithahn and Lerchl [11] reported that 5-HTP was a stronger in vitro hydroxyl radical scavenger than were melatonin or vitamin C. Similarly, Yoo et al. [36] stated that N-acetyl serotonin produced from serotonin in rats activated antioxidant system pathways and enzymes, indicating its antioxidant and antiapoptotic properties. Phenolic compounds and some of their species are very effective in preventing auto-oxidation. Serotonin and its compounds are likely to be effective as a phenolic component in antioxidant processes. In fact, because of its phenolic group characteristics, serotonin has been reported to have higher radical scavenging and copper ion–lowering activities than those of

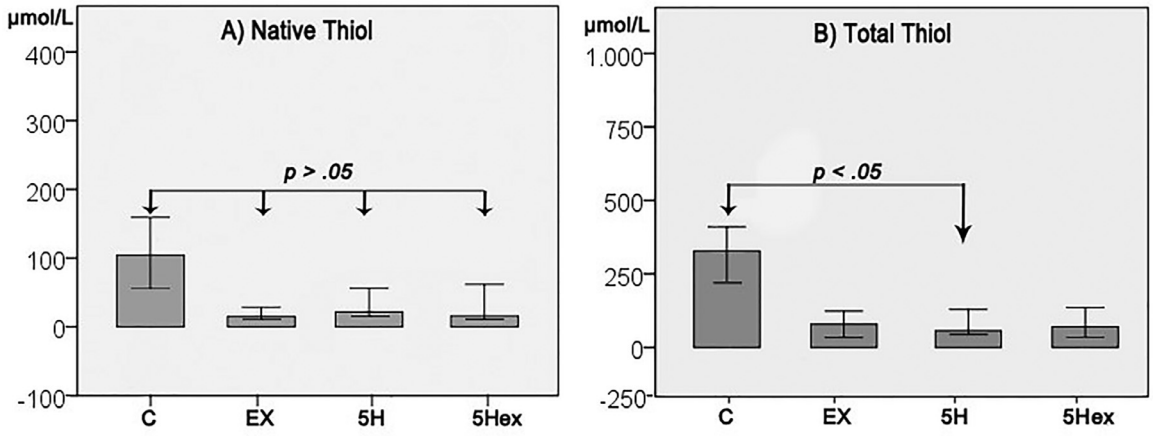

Fig. 3. Groups' (A) native and (B) total thiol levels (μmol/L). Total thiol (functional thiol groups [–SH] + dynamic disulfide bonds [–S–S–]) and native thiol (–SH) concentrations in the samples were measured by using Ellmann's and modified Ellmann's reagent. No significant difference was found in the groups' native thiol levels. Total thiol levels were significantly lower in the 5H group. C, control; EX, exercise group; 5H, 5-hydroxytryptophan group; 5Hex, 5-hydroxytryptophan + exercise group.

melatonin, which is known to have antioxidant properties and with which these compounds function synchronously [30].

In this study the lowest disulfide levels (the earliest manifestation of radical-mediated protein oxidation) were found in the 5H and 5Hex groups. Studies of humans and mice have found that nourishment with antioxidant vitamins has positive effects on postexercise lipid peroxidation [37,38]. In this context the present study results are consistent with the literature, and the effect of 5-HTP alone and in combination with exercise in reducing oxidative stress has been clearly established.

In the present study, DD level was significantly lower in the exercise group. This result indicates that moderate exercise alone was successful in reduction of oxidative stress.

Oxidative stress can damage important biomolecules such as DNA, lipids, and proteins. Although there are many studies examining the relationship between oxidative stress and exercise, it has been found that increased reactive oxygen species production resulting from exercise may be associated with the pathogenesis of many diseases [39]. Many studies also suggest that physical exercise has a positive effect on brain functions [40–42]. It has been reported that mood regulation is directly related to the serotonergic system. A decrease of depression risk can be achieved by increasing the synthesis of serotonin, which can be induced by increased physical activity [43]. However, concentrations of extracellular serotonin, which show an excretion-related increase in various regions of the brain, cause fatigue during prolonged exercise [44–46]. This suggests that the serotonin system plays an important role in the development of exercise-dependent chronic fatigue. Exercise can be very beneficial for health, but it can also produce dangerous compounds. However, physical activity may lead to the formation of more free radicals by increasing metabolic processes and oxygen consumption in proportion to its severity and duration, and intensive exercise can cause an increase in the production of oxidants in skeletal muscles [5,47,48]. Therefore, a moderate exercise protocol was preferred in the present study. Although it has been reported that athletes may be exposed to exercise-induced free radical flow risk, which is particularly proportional to increased exercise intensity and duration [49–51], different studies have reported that individuals doing regular exercise and athletes adapt to such a program over time and are more resistant to oxidative damage [52,53]. As a general principle, the activity of antioxidant enzymes increases considerably in humans and rats doing exercise. The increase in antioxidant activity also responds by preventing lipid peroxidation, which is caused by increased oxidative stress resulting from exercise [54]. In addition, individuals exercising regularly have an advantage over sedentary individuals because exercise supports activities of many large antioxidant enzymes and development of overall level of antioxidants. This confirms the present study results of the EX and 5Hex groups.

Studies have found that, as a general principle, antioxidant vitamin supplements can be offered to individuals doing regular heavy exercise. Although there are still some debated points, the question of whether antioxidant vitamins and enzymes play a protective role in exercise-induced muscle damage can be answered positively.

Conclusions

This is the first study that examined the effect of 5-HTP use together with exercise on oxidative stress over the thiol-disulfide homeostasis perspective using a new measurement method. As a result, 5-HTP and moderate exercise were found to be significantly effective in inhibiting oxidative damage. It is known that athletes doing regular exercises use supplements intensively to improve performance or accelerate recovery. In this context it is anticipated that serotonin and precursors may help in preventing cellular damage. In addition, the new oxidative stress measurement method used in this study is recommended as a practical and useful method with a prognostic value to assess athletes' performances and detect early damage at the tissue level.

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