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Lymphocyte antioxidant response and H_2O_2 production after a swimming session: Gender differences

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Abstract

This study evaluated the gender differences in response to intense exercise on lymphocyte hydrogen peroxide production, nitric oxide handling and mitochondrial superoxide dismutase (MnSOD) activity and gene expression. Fifteen males and nine females participated voluntarily in the study and performed a swimming session at 75–80% of the maximal capacity. In basal conditions females presented higher lymphocyte MnSOD activity compared to males ($p < 0.05$). Exercise increased MnSOD activity in males ($p < 0.05$) reaching similar values to females. MnSOD gene expression was also increased in males after exercise ($p < 0.05$) but not in females. Nitrite concentration and iNOS gene expression significantly increased only in males after swimming ($p < 0.01$). The exercise decreased UCP-3 gene expression in both genders ($p < 0.05$). Lymphocyte H_2O_2 production significantly increased in males after exercise in non-stimulated and in PMA-stimulated cells ($p < 0.01$). In conclusion, females seem to be more protected against oxidative stress induced by a swimming session. Hydrogen peroxide is mainly produced in males and this subsequently leads to increases in MnSOD gene expression and activity.

Keywords: *Oxidative stress, exercise, gender, antioxidant enzymes, iNOS*

Introduction

During exercise, the oxygen consumption is greatly increased and consequently the reactive oxygen species (ROS) formation is enhanced [1,2]. Major sources of ROS during exercise include leakage of electrons from the mitochondrial electron transport chain and enhanced purine oxidation in muscle, leukocytes activation and increased haemoglobin turnover [3–5]. ROS produced by moderate levels of exercise or regular training programme are capable to induce the expression of antioxidant defences [6,7]. The mechanism by which this regulation is carried out involves transcription factors such as nuclear factor kappa B (NFκB) and activator protein 1 (AP-1) [7,8]. Recently, a wide variety of families of proteins have been reported to be involved in the defence against oxidative stress, as is the case of uncoupling proteins (UCPs) [9]. However, ROS associated with strenuous exercise may react with lipid membranes, proteins and DNA to cause cell damage [10–12].

Inducible nitric oxide synthase (iNOS) is present in many cells involved in immunity and inflammation, which produce high-levels of sustained nitric oxide (NO) synthesis when cells are activated. NO is important as a toxic defence molecule against infectious organisms and also regulates the functional activity, growth and death of many immune cells

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[13]. When NO is generated at high concentrations it is rapidly oxidized to reactive nitrogen oxide species (RNOS) which mediate most of the immunological effects. NO production is increased during exercise [14–16]. NO has vasodilatory effects and increases blood flow, which facilitates the delivery of glucose to the capillary of skeletal muscle.

The lymphocyte antioxidant defences have shown adaptations to oxidative stress to exercise-induced oxidative stress. Increases in vitamin E contents and in antioxidant enzyme activities have been reported after different bouts of exercise [17,18]. It was reported that the expression of the antioxidant enzymes in immune cells is induced and regulated by ROS and cytokines [19]. However, the adaptive response of antioxidant defences did not prevent the oxidative damage in lymphocytes induced after intense exercise [18]. Skeletal muscle generates significant amounts of oxidants during exercise and part of these oxidants are released to extracellular fluid [20]. Repetitive contractions also result in muscle damage, inducing the release of muscle proteins and cytokines into circulation [21]. Both oxidants and cytokines could modulate the lymphocyte response to exercise.

Several studies have focused on the effect of gender on the antioxidant defences and oxidative damage. Mitochondria from females generate less amounts of hydrogen peroxide than those of males and have higher levels of mitochondrial reduced glutathione (GSH) and antioxidant enzymes [22–26]. It has been hypothesized that at least some of these differences could be attributed to the antioxidant properties of female sex hormones [27,28]. Oestrogens also bind to oestrogen receptors and increase the expression of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase [29]. The higher protection in front of oxidative stress might be related to the increased lifespan in females when compared to males [30]. Sex differences in the extent of muscle damage, in the inflammatory response and in the susceptibility to oxidation have been postulated. Generally females have been shown to be more protected than males against both basal and post-exercise oxidative stress [27,31].

In order to elucidate gender differences in response to intense exercise our aim was to study and to compare the effects of a swimming session on lymphocyte hydrogen peroxide production, nitric oxide handling and mitochondrial MnSOD gene expression and activity in males and females.

Materials and methods

Subjects and protocol

Fifteen adolescent males and nine adolescent females participated in this study. They all were swimmers belonging to amateur teams. The characteristics of subjects participating in the study are presented in Table I. Subjects and their parents were informed of the purpose of this study and the possible risks involved before both parents and adolescents gave their written and informed consent to the adolescent taking part in the study. The groups were homogeneous taking into account age, average weight and training sessions. Subjects were non-obese and non-smokers and they were free of any medication. The study protocol was in accordance with the Declaration of Helsinki and was approved by the University of the Balearic Islands bioethics committee and by the ethic committee of investigation of the Govern de les Illes Balears.

The exercise session was performed in an Olympic pool. Swimmers completed a 30 min warm-up prior to starting the exercise protocol consisting of 5 min general movement to warm the body, followed by a brief 10 min stretching session and a 15 min front-crawl swimming at low intensity. After warming the participants started with a series of intermittent 50 m swims of progressively increasing speed for 30 min, with a resting time of $\sim$10–15 s between each swim, reaching a pace corresponding to 75–80% of the maximal capacity of each participant. The correct speed was controlled by means of the time they used to complete each 50 m swim in relation to the best time they achieved in preliminary tests [32]. In these tests participants were asked to complete several bouts of 50 m at their maximum velocity. The time required to complete each 50 m swim was recorded by a trainer using a hand stopwatch and it was used to control the swim speed. These previous tests were also performed to ensure that all the swimmers would be able to complete the exercise protocol.

Experimental procedure

Peripheral venous blood samples were obtained from swimmers in suitable vacutainers with EDTA as anticoagulant before any exercise following an overnight fast and immediately after end the exercise protocol. Lymphocyte fraction was purified from whole blood and quantified in an automatic flow cytometer analyser Techicon H2 (Bayer) VCS

Table I. Anthropometric characteristics of participants.

	Male	Female
Age (years)	16.1 $\pm$ 0.5	14.7 $\pm$ 0.2#
Height (cm)	173 $\pm$ 2	168 $\pm$ 2#
Weight (Kg)	67.8 $\pm$ 3.4	61.0 $\pm$ 1.7
BMI	22.4 $\pm$ 0.9	21.7 $\pm$ 0.6
% Fat body mass	16.0 $\pm$ 1.7	23.1 $\pm$ 1.2#

The values are the mean $\pm$ s.e.m. of 15 males and eight females. # indicates significant differences between genders (Student's *t*-test unpaired data, $p < 0.05$). BMI: body mass index.

system. ROS production was determined in lymphocytes in basal conditions and after activation with PMA. MnSOD activity and the gene expression of UCP-3, MnSOD and iNOS were assessed in lymphocytes. Nitrite levels as marker of NO were also determined in lymphocytes.

Anthropometrical data

Height was determined using a mobile anthropometer (Kawe 44444, France) to the nearest mm, with the subjects head in the Frankfurt plane. Body weight was determined to the nearest 100 g using a digital scale (Tefal, sc 9210, France). Percentage of fat body mass was calculated by bioimpedance with one hand-held BIA unit (Omron®BF 300). Body Mass Index (BMI) was calculated as follows: BMI = weight in Kg/squared height in m).

Lymphocyte purification

Blood samples were processed following an adaptation of the method described by Boyum [33]. Blood was carefully introduced on Ficoll in a proportion of 1.5:1 and was then centrifuged at 900 × g, 18°C for 30 min. The lymphocyte layer was carefully removed. The precipitate containing the erythrocytes and neutrophils was then discarded. The lymphocyte slurry was then washed twice with PBS and centrifuged for 10 min at 1000 × g, 4°C. The cellular precipitate of lymphocytes was lysed with distilled water.

Enzymatic determinations

MnSOD was determined with a Shimadzu UV-2100 spectrophotometer at 37°C. The activity of SOD was estimated using a xanthine/xanthine oxidase system to generate the superoxide anion. This anion produced the reduction of cytochrome c, which was monitored at 550 nm. The superoxide dismutase of the sample removed the superoxide anion and produced an inhibition of the reduction. MnSOD was achieved after specific inhibition of Cu-ZnSOD with 5 mmol/l potassium cyanide [34].

mRNA gene expression

MnSOD, iNOS and UCP-3 gene expression were determined by real time RT-PCR with 18S ribosomal as reference gene. For this purpose, mRNA was isolated from lymphocytes by phenol-chloroform extraction. cDNA was synthesized from 1 μg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. Target cDNAs were amplified in separate tubes using the following procedure: 10 min at 95°C, followed by 40 cycles of amplification. The specific primers and the amplification conditions used for each gene are presented in Table II. The relative quantification was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$.

Nitrite levels

Nitrite levels were determined in all lymphocytes by the acidic Griess reaction using a spectrophotometric method. Lysed cells and plasma were deproteinized with acetone and kept overnight at −20°C. Samples were centrifuged for 10 min at 15000 × g at 4°C and supernatants were recovered. A 96-well plate was loaded with the samples or nitrite standard solutions (100 μl) in duplicate; 50 μl sulphanilamide (2% w/v) in 5% HCl was added to each well and 50 μl N-(1-napthyl)-ethylenediamine (0.1% w/v) in water was then added. The absorbance at 540 nm was measured following an incubation of 30 min.

Hydrogen peroxide production

H_2O_2 production in lymphocytes was measured before and after stimulation with phorbol myristate acetate (PMA) using 2,7-dichlorofluorescin-diacetate (DCFH-DA) as indicator. A stock solution of DCFH-DA (1 mg/ml) in ethanol and PMA (1 mg/ml) in DMSO were prepared and stored at −20°C

Table II. Primers and conditions used in Real Time PCRs.

Gene	Primers	Conditions
18S	Fw: **5′-ATGTGAAGTCACTGTGCCAG-3′**	95°C,10 s 60°C, 7 s
	Rv: 5′-GTGTAATCCGTCTCCACAGA-3′	72°C, 12 s
UCP-3	Fw: 5′-CGTGGTGATGTTCATAACCTATG-3′	95°C, 5 s 60°C, 7 s
	Rv: 5′-CGGTGATTCCCGTAACATCTG-3′	72°C, 10 s
MnSOD	Fw: 5′-CGTGCTCCCACACATCAATC-3′	95°C, 10 s 65°C, 5 s
	Rv: 5′-TGAACGTCACCGAGGAGAAG-3′	72°C, 7 s
iNOS	Fw: 5′-TCTGCAGACAGTGCGTTACT-3′	95°C, 10 s 60°C, 10 s
	Rv: 5′-ATGCACAGCTGAGCATTCCA-3′	72°C, 15 s

until analysis. DCFH-DA (30 μg/ml) in PBS was added to a 96-well microplate containing 50 μl lymphocytes suspension. PMA (3 μm) prepared in HBSS or HBSS alone was added to the wells and the fluorescence (Ex, 480 nm; Em 530 nm) was recorded at 37°C for 1 h in FLx800 Microplate Fluorescence Reader (Bio-tek Instruments, Inc.).

Statistical analysis

Statistical analysis was carried out using a statistical package (SPSS 14.0 for Windows). Results are expressed as mean ± s.e.m. and $p < 0.05$ was considered statistically significant. The Kolmogorov-Smirnov test was used to evaluate the fit of the data to a normal distribution. Student *t*-test for unpaired data was used to identify differences at baseline regarding the anthropometric characteristics. The statistical significance of the data was assessed by two-way analysis of variance (ANOVA). The statistical factors analysed were time swimming (S), gender (G) and the interaction of both factors. When significant effects of gender or exercise were found, a student *t*-test for unpaired data was used to determine the differences between the groups involved.

Results

Anthropometric data

The anthropometric characteristics of participants are shown in Table I. Adolescent males and females participating in the study presented similar weight and BMI. However, males were significantly older and taller and had less percentage fat body mass ($p < 0.05$).

The effect of gender and exercise on lymphocyte counts

No significant differences were observed between males and females in the lymphocyte counts in basal conditions and no changes were evidenced after exercise (data not shown).

The effect of gender and exercise on MnSOD

The enzymatic activity and the gene expression of MnSOD in lymphocytes are presented in Figure 1. The basal MnSOD activity (Figure 1A) is significantly higher in females compared to males (43%, $p < 0.05$). Exercise significantly increased the MnSOD activity in males (52% higher, $p < 0.05$) and maintained the basal values in females. The post-exercise activity in males reached to values similar to the females. The MnSOD gene expression (Figure 1B) presented similar basal values in both males and females; however, exercise significantly increased the gene expression in males (59%, $p < 0.05$) but not in females. The post-exercise MnSOD gene expression

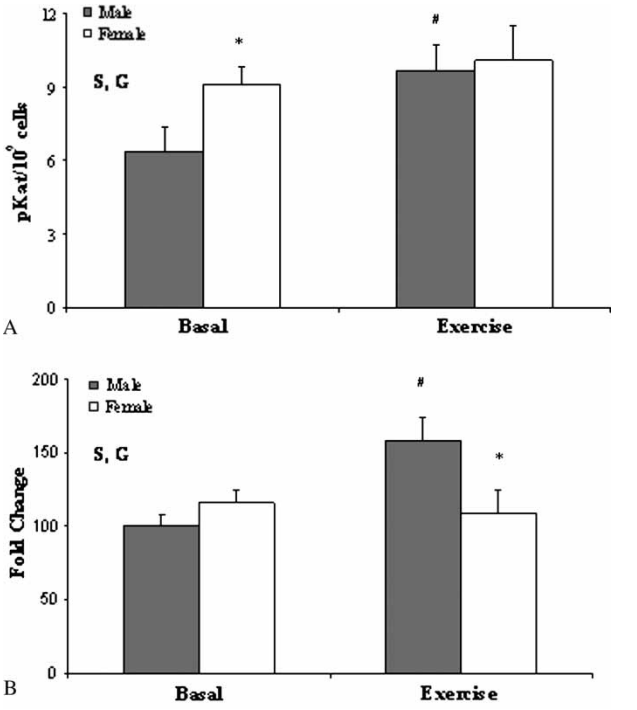

Figure 1. Effects of swimming session and gender on lymphocyte MnSOD activity (A) and MnSOD gene expression (B). The relative quantification of MnSOD expression was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 100. (S): Significant effects of swimming. (G): Significant effects of gender (Two-way ANOVA, $p < 0.05$). *Indicates significant differences between genders. #Indicates significant differences between basal an exercise values. Values are expressed as mean ± s.e.m.

is significantly higher in males when compared with females (50% higher, $p < 0.05$).

The effect of gender and exercise on iNOS

The lymphocyte nitrite levels and the gene expression of iNOS are shown in Figure 2. Both the nitrite levels and the iNOS expression showed the same pattern of change. The basal nitrite levels and iNOS gene expression presented similar values in both males and females. The swimming session significantly increased the nitrite levels and the iNOS gene expression in males (59% and 48%, respectively, $p < 0.01$), but not in females. However, the post-exercise values measured in males were not significantly different compared to the females' post-exercise values.

The effect of gender and exercise on UCP-3 gene expression

The UCP-3 gene expression is presented in Figure 3. Exercise, but not the gender, affected UCP-3 gene expression which decreased about 20% after the swimming session, in both males and females ($p < 0.05$).

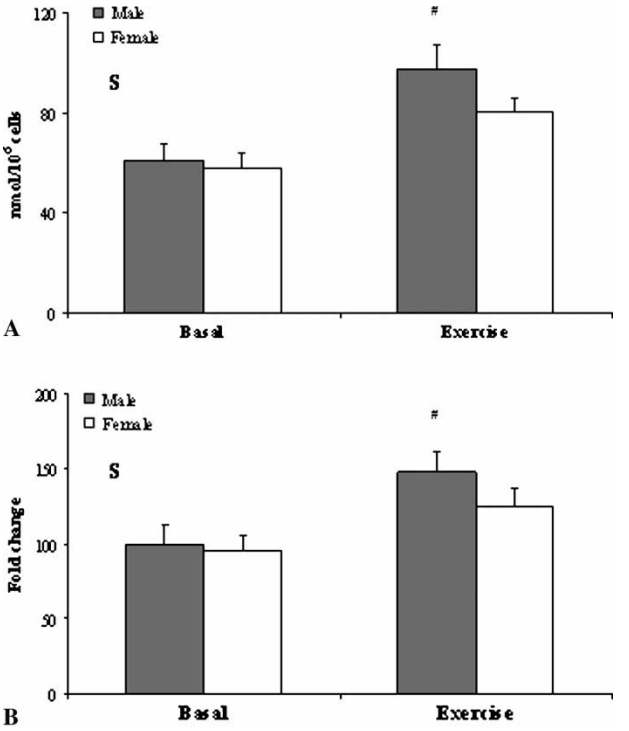

Figure 2. Effects of swimming session and gender on lymphocyte nitrite levels (A) and i NOS gene expression (B). The relative quantification of i NOS expression was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 100. (S): Significant effects of swimming (Two-way ANOVA, $p<0.05$). No significant differences were evidenced between genders. #Indicates significant differences between basal an exercise values. Values are expressed as mean ± s.e.m.

The effect of gender and exercise on H_2O_2 production

Lymphocyte H_2O_2 production without activation and after activation with PMA are presented in Figure 4. The H_2O_2 production in no-activated lymphocyte showed no significant differences between males and

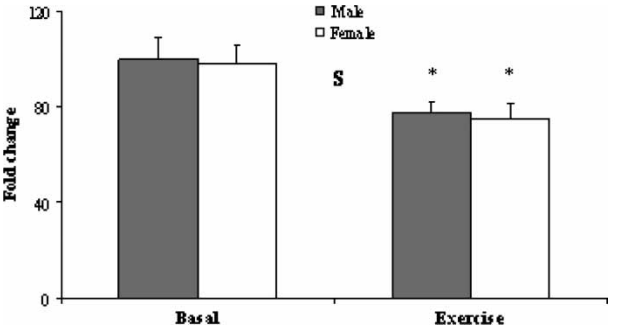

Figure 3. Effects of swimming session and gender on lymphocyte UCP3 gene expression. The relative quantification of UCP3 expression was performed by standard calculations considering $2^{(-\Delta\Delta Ct)}$. mRNA levels at the beginning of the stage were arbitrarily referred to as 100. (S): Significant effects of swimming (Two-way ANOVA, $p<0.05$). No significant differences were evidenced between genders. #Indicates significant differences between basal an exercise values. Values are expressed as mean ± s.e.m.

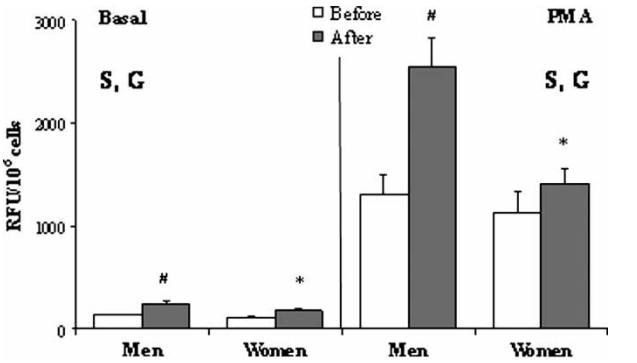

Figure 4. Effects of swimming session and gender on H_2O_2 production in no-activated lymphocytes and in PMA-activated lymphocytes. (S): Significant effects of swimming. (G): Significant effects of gender (Two-way ANOVA, $p<0.05$). *Indicates significant differences between genders. #Indicates significant differences between basal an exercise values. Values are expressed as mean ± s.e.m.

females in basal conditions. However, after exercise the H_2O_2 production significantly increased only in males (71%, $p<0.01$), being this post-exercise H_2O_2 production significantly higher when compared with the females post-exercise values (25% higher, $p<0.05$). The activation of lymphocytes with PMA increases ~10-fold the H_2O_2 production, but the pattern of response to exercise was similar to the observed in no-activated lymphocytes.

Discussion

Sex differences in muscle damage, in the inflammatory response and in the oxidative stress induced by exercise, have been evidenced by several authors [27,31]. In the present study, we evidenced that the lymphocyte oxidant and antioxidant machinery in females responds less to a swimming training session than in males, which is in agreement with previous results [35]. It has been hypothesized that some of the gender differences could be attributed to the female sex hormones [27]. Therefore, when comparing the basal parameters we only found significant differences between sexes in lymphocyte MnSOD activity, whereas the other parameters were similar in both genders. The higher MnSOD activity in females is in agreement with previous findings [26,36]. It was recently shown that the higher levels of oestrogens in females protect them against ageing by up-regulating the expression of antioxidant genes such as glutathione peroxidase (GPx) and MnSOD via activation the nuclear factor kappa β (NFκβ) [37]. In fact, inhibition of NFκβ signalling pathway prevents this gene induction [37]. Oestrogens also possess phenolic–OH groups which can act as chain-breaking antioxidants in a similar manner to that of vitamin E [5,38]. It was evidenced that the rate of oxidant production by mitochondria from female rats is

significantly lower than from males [26]. Mitochondria from female rats produce approximately half the amount of H_2O_2 generated by male mitochondria, tested in liver and brain [26]. As a result, mitochondria from females suffer less oxidative damage to critical molecules such as mitochondrial DNA or glutathione than those from males [37]. In our study, the ROS production in basal conditions was similar in both genders and this, together with the higher mitochondrial SOD activity in females, suggests that female mitochondria are better protected from oxidative damage in basal conditions.

The physiological response to exercise involves a number of changes in the oxidative balance and in the metabolism of some important biological molecules [39]. Exercise causes an increase in the generation of free radicals by cells [40]. Several authors found that these radicals cause cellular damage only when exercise is exhaustive [41]. However, free radicals not only cause damage but they also have a role in cell signalling [42–44]. The cellular damage is parallel to the increase in the activities of several antioxidant enzymes [45]. In the present study, we studied the lymphocyte capability to produce ROS before and after stimulation with PMA, an agent to activate ROS producing pathways. Lymphocytes from males significantly increased the ROS production after swimming, whereas remained unchanged in females. More than 90% of the oxygen used by cells is consumed in mitochondria being these organelles the main source of ROS [40,46]. Respiratory chain complexes I and III are the main source of mitochondria ROS generation within the cell [47] and their activities are directly correlated with their radical production [48]. Exercise, as result of increased oxygen consumption, induces an increase in mitochondrial oxidant production derived of the increased oxygen utilization. However, any change in mitochondria should be present resulting in an increased ROS production. It was evidenced that the degree of the respiratory chain reduction is inversely related to the rate of electron flow and, consequently, the ROS generation is also affected [49]. As a consequence of this increased ROS production, lymphocytes from males significantly increased the antioxidant enzyme defence to avoid possible cellular damage. The increased ROS after swimming evidenced in males could act as molecular messengers inducing the expression of MnSOD. Recent publications on the production of ROS show that a small increase in H_2O_2 is necessary in the activation of some intracellular signalling pathways, responsible for the development of an adaptive response to exercise-induced oxidative damage [7,50,51]. The ROS activated pathways result in adaptive responses to maintain cellular oxidant–antioxidant homeostasis during exercise.

NO is important for the maintenance of cardiovascular homeostasis and the basal vasodilator tone [52,53]. iNOS is present in many cells involved in immunity and inflammation, which produce high-levels of sustained NO synthesis when cells are activated. iNOS generates greater amounts of NO compared to the constitutive isoforms of NOS. In a previous study, we evidenced that iNOS levels and SOD activity dropped in neutrophils and raised in lymphocytes from male cyclists after exercise [16]. In the present study, we evidenced an increase in iNOS gene expression and nitrite levels—marker of NO—only in males, whereas females maintained basal values. The activation of NFκβ signalling cascade by ROS has been shown to activate the gene expression of iNOS [54]. This response is in accordance with the increased ROS production only evidenced in males. The intracellular accumulation of NO generated by iNOS may produce toxic levels of NO high enough to inhibit key enzymes of the oxidative phosphorylation [55]. *In vitro* experiments documented that NO can attenuate the contractile performance of the skeletal muscle [56,57]. The increased nitrite levels in males after exercise could inhibit any enzyme from the mitochondrial chain facilitating the ROS generation. Both the increased NO and ROS could interact, because the excess of NO production could promote the generation of peroxynitrite in the presence of ROS.

UCP-3 is a member of the mitochondrial uncoupling protein family mainly detected in skeletal muscle but it is not involved in thermogenesis [9]. UCP-3 has been shown to act as an antioxidant by reducing ROS production, but its primary function seems to be extracting fatty acid anions from the mitochondrial matrix, thus protecting mitochondria against the accumulation of fatty acids [58]. In our study, a swimming session induced the down-regulation of UCP-3 expression in lymphocytes from both males and females, which is in contrast with previous works that reported increases in UCP-3 expression in skeletal muscle after an acute bout of exercise [59]. The explanation of these different results could lie in the different kinds of physical activity used or, more probably, in the different cell types analysed. However, UCP-3 gene expression in lymphocytes appears unrelated to differences in radical production between genders.

In conclusion, females seem to have more antioxidant protection than males. An acute bout of exercise as a swimming session induces an activation of the antioxidant machinery only in males to prevent the negative effects of exercise-induced oxidative stress. The lower antioxidant response to exercise observed in female swimmers compared to male swimmers could be explained by the higher oestrogens concentration. These sex differences highlights the importance of studying both genders in exercise

studies rather than generalizing the studies only to males.

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