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Original Research

The effects of mitochondrial DNA deletion and copy number variations on different exercise intensities in highly trained swimmers

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Abstract: It has been suggested that heavy exercise might increase oxidative stress, causing mitochondrial DNA (mtDNA) mutations as well as DNA mutations and changes in the mtDNA copy number in cells. mtDNA^{4977} deletion is one of the most common deletions seen on mitochondria. We hypothesize association between exercise induced oxidative stress and mtDNA damage in peripheral blood lymphocytes (PBLs) of highly trained swimmers. Therefore we studied the mtDNA^{4977} deletion level, mtDNA copy number and their relationship with cellular ATP and oxidative stress status in PBLs of swimmers. 8 highly trained and 8 normal trained swimmers and 8 non-athlete subjects were included in the study. The mtDNA^{4977} deletion and amount of mtDNA were measured using RT-PCR method whereas dichlorohydrofluoroscein (DCF) assay method was used to assess cellular oxidative stress and ATP levels were measured using bioluminescence method. Even though an increase in mtDNA^{4977} deletion was found in all study groups, the difference was not statistically significant (p=0.98). The mtDNA copy numbers were found to be surprisingly high in highly trained swimmers compared to normal trained swimmers and non-athlete subjects by 4.03 fold (p= 0.0002) and 5.58 fold (p=0.0003), respectively. No significant differences were found between groups by means of intracellular ATP levels (p=0.406) and oxidative stress (p=0.430). No correlation was found between mtDNA copy number and intracellular ATP content of the PBLs (p=0.703). Our results suggest that heavy training does not have a specific effect on mtDNA^{4977} deletion but it may be affecting mitochondrial copy numbers which may act as a compensatory mechanism related to ATP levels in blood.

Key words: mtDNA4977 deletion, oxidative stress, mtDNA content.

Introduction

Under normal circumstances, cells repair the damage caused by radicals and/or oxidants by normal redox state in order to protect cells, DNA, lipids and proteins. In case of an imbalance in this mechanism, free radicals may harm the cells by starting a chain reaction. Due to high muscular activity, cells produce energy and generate big amounts of Reactive Oxygen Species (ROS) and mitochondrial DNA (mtDNA) is the main target of these ROS as mitochondria are the main source of energy production by producing adenosine triphosphate (ATP) through the oxidative phosphorylation (OXPHOS) system. This system also encodes 2 rRNAs and 22 tRNAs for mitochondrial translation (1). As exposure to oxidative stress increases and repeats, the frequency of somatic mutations in genes or gene regulatory regions also increase thus affecting and harming mtDNA and making mtDNA more prone to damage caused by ROS and free radicals than nuclear DNA because the ability of repair decreases and the histones cannot be protected anymore. Due to lack of this repair system, somatic mutation rate of mtDNA is 10-20 times higher than the rate of nuclear DNA (2, 3). After the first proposal of mitochondrial aging theory by Lobachev et al. (4, 5), Miguel et al. have explained the relationship between oxidative stress and mitochondrial damage (6). In 1989, Linnane et al. showed that somatic mutations in mtDNA may cause degenerative diseases and aging (7). These first studies have been followed by numerous studies investigating the relationship of mtDNA damage in various diseases including types of cancer and oxidative stress related diseases such as aging, Parkinson's Disease, Myocardial Infarction and atherosclerosis (8-17).

Huei Wei has shown that, more than twenty different types of mtDNA deletions occur in human tissues (18) but up-to date more than 250 mutations and/or deletions have been revealed (19). One of the most studied alterations of these deletions is mtDNA^{4977} deletion. A region spanning almost a 5-kb of mtDNA between ATPase8 and ND5 (including ATPase6, ND3, ND4 and ND4L) genes has been shown to be deleted which is a 4977–base pair (bp) common deletion (mtDNA^{4977}) (nucleotides between 8.470 and 13.447 bp), a common marker of mtDNA damage (20-22). As blood leukocytes are fast replicating cells, it is also possible to detect mtDNA mutations in blood. Gatterman and von Wurmb were the first researchers reporting a deletion of mtDNA in human blood cells (23-26). Some researchers have suggested that there may be a correlation with reduced ATP production and accumulation of mtDNA^{4977} in some human tissues (27-28). It is a well-known fact that, exercise on a regular basis increases the oxidative stress and antioxidant response as a response in adults

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(29-32). As swimming is a high energy consuming activity, several studies have been made to investigate the effect of swimming on oxidative stress and it has been shown that oxidative stress increases in swimmers after training (33-36). Considering that mtDNA deletions and oxidative stress are related phenomenon, exercise may also trigger mtDNA mutations in advanced athletes but this relation has not been well documented.

As a hypothesis, we may expect to find a deleterious mutation in the mtDNA of highly trained athletes who are exposed to oxidative stress related with heavier exercise load. A vast majority of the studies have focused either on oxidative stress in blood or mtDNA^{4977} deletions in different human tissues. As far as we know, the only study connecting the bridge between exercise and mtDNA^{4977} deletions has been conducted on Wistar rats by Jafari et al. who have shown that heavily trained rats suffer from mtDNA^{4977} deletions in their soleus muscles (37). To our knowledge, this is the first study investigating the relationship between mtDNA^{4977} mutation, mitochondrial content and oxidative stress in blood of the swimmers.

Therefore, the purpose of the present study was to assess the mitochondrial common deletion in cells of different groups of highly trained swimmers with at least 4 years of swimming history who have different exercise programs and compare the effects of different exercise intensities on the mitochondrial 4977-bp deletion level, mtDNA copy number, intracellular ATP levels and degree of oxidative stress in leukocytes of swimmers.

Materials and Methods

Subjects

Eight highly trained competitive swimmers for the experiment group (HTS group) and eight normal trained competitive swimmers for the control group (NTS group) volunteered to participate in this study. Eight non-athlete normal control subjects (NAC group) were included in the study for a comparison. All subjects were males with similar anthropometric parameters (Table 1). HTS group trained two sessions a day, 6 days a week; NTS group trained one session a day and 5 days a week. The NAC group did not train on a daily basis. There were no other forms of physical activity except daily routine life. All swimmers had at least 4 years of experience in swimming. Experiment group swum 30-36 km a week; control group swum 15-20 km a week. The subjects in both groups were all competing in regional and national level competitions. They were all familiarized with short and middle distance tests (25m, 50 m, 100m) performed during training sessions. The subjects completed the general training in a 4-week period before and performed their training in an indoor pool with an average air and pool temperature of 24^{o}C. Written informed consent was obtained from all subjects and the Ethical Committee of Istanbul University Cerrahpasa Medical Faculty approved the study (No: 83045809/604/01.01/113374) that has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Experimental design

The spirometry and ergometry tests were performed on subjects at the same time, using same devices. (Forced Expiratory Volume in 1 second (FEV1), Forced Vital Capacity (FVC), Diffusing Capacity (DLCO), Total Lung Capacity (TLC) and Residual Volume (RV) tests were performed on the subjects using System Masterscreen Body Plethysmography (CareFusion, San Diego, USA) (Table 1). Pulmonary function tests were performed on the subjects using Vmax Encore PFT (CareFusion, San Diego, USA) on bicycle ergometer (Ergoline, Bitz, Germany) as there was no swim ergometer in the pool area where subjects were training. The workload intensity began at 60 W was increased systematically by 30 W/min until maximum workload was reached. Peak Oxygen Uptake (VO_{2max}), and Maximum Carbon dioxide Production (VCO_{2max}) values were determined.

The anthropometric parameters were measured in the first experimental session. Body weight was determined using a Tanita Model BC-418MA Segmental Body Analyzer Monitor (Tanita Corporation of America, Inc., USA). This device analyzes total body weight, body mass index (BMI, kg/m^2), basal metabolic rate (BMR, kj), body fat (%), fat mass (kg), free tat mass (kg). The subject's BMI was computed from the height and weight measurements (Table 1). Then the performances of 25m, 50 m, 100m swim in front crawl were determined on different days in a random order (Table 2). All tests were performed in a 25 m pool, during training sessions. Individuals performed a standard warm-up for 10 minutes before each test, and after the test

Table 1. Anthropometric parameters and respiratory function test results of experiment subjects showing the physical difference between groups.

	Highly Trained Competitive Swimmers (n=8)	Normal Trained Competitive Swimmers (n=8)	Non-Athlete Swimmers (n=8)
Age (years)	16.7±0.7	16.2±1.3	15.9±1.6
Height (cm)	173.1±3.6	165.3±6.7	161.4±5.9
Weight (kg)	58.4±6.9	70.1±9.8	60.7±5.7
Fat (%)	20.6±4.7	22.6±2.8	24.3±4.8
BMI (kg/m^2)	21.3±2.3	23.8±2.1	24.2±2.2
FEV1 (lt)	4.80±0.53*	4.42±0.43†	3.98±0.40
FVC (lt)	6.08±0.84*	4.76±0.61	4.37±0.78
FEV1/FVC (%)	88.2±3.27	84.8±3.49	82.4±2.25
TLC (lt)	7.28±1.1§	5.92± 2.02	5.15±1.05
RV (lt/sec)	1.17±0.07	1.13±0.05	1.12±0.05
DLCO (mmol/min/kPa)	30.23±5.1§	24.8±4.4	22.5±2.84
VO_{2max} (max ml/kg/min)	54.2±5.5*	45.7±2.9†	37.2±5.1

*: HTS group is significantly different from NTS and NAC groups; †: NTS group is significantly different than NAC group; §: HTS group is significantly different from NAC group ($p<0.05$; Kruskal-Wallis test and Mann-Whitney U test with Bonferroni correction. Values are means ± SD) (FEV1, Forced Expiratory Volume in 1 second; FVC, Forced Vital Capacity; TLC, Total Lung Capacity; RV, Residual Volume; DLCO, Diffusing Capacity; VO_{2max}, Peak Oxygen Uptake).

Table 2. Swimming distance and times of the swimmers.

Distance swum	Highly Trained Swimmers Total time in seconds (n=8)	Normal Trained Swimmers Total time in seconds (n=8)
25 m	13.7±0.2	14.5±0.2
50 m	29.9±0.6	31.2±1
100 m	69.9±1.3	70.9±1.7

they trained normally. Calculations were made by Casio 2000 (Casio, Japan) chronometers. Throughout the biochemical experiments, subjects were asked to maintain their usual diets, to get adequate sleep at nights, not to drink coffee or tea or undertake any resistance training before the experiments, and not to use any medication. On the day of the exercise test, the subjects ate a light, carbohydrate-rich breakfast. The exercise test was carried out 2-4 h after breakfast.

Blood sampling and preparation and isolation of leukocytes from whole blood

Peripheral blood was collected in blood collection tubes containing EDTA and leukocytes were isolated within 1 hour of collection by the density gradient centrifugation Ficoll-Hypaque (Amersham Biosciences, GE Healthcare, Chicago, IL, USA) method. The leukocytes containing fraction was collected following centrifugation at 700 g for 20 minutes. The cells were then washed twice with Phosphate Buffered Saline (PBS) and they were re-suspended in RPMI 1640 medium (Sigma, St Louis, MO, USA).

DNA extraction

DNA in leukocytes was obtained the day blood was collected using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) and stored at -80°C until used.

Analysis of amount of mtDNA and mtDNA 4977 bp deletion by real-time PCR

The levels of both 4.977 bp deletion (nucleotides between 8.470 and 13.447 bp) and mitochondrial reference fragment in DNA isolated from leukocytes were determined by fluorescence-based quantitative PCR with some modifications (38, 39). ND1 gene in undeleted region for the reference sequence of mtDNA ($mtDNA^n$) as an internal control, the remaining fragment after mtDNA 4977 bp deletion ($mtDNA^{4977}$) and human ß-globin gene for gDNA for comparison were amplified by PCR both in gDNA and mtDNA. The primers and yielded products are shown in Table 3. The DNA samples were subjected to Real-Time PCR with a Qiagen_Rotor-Gene 6000 for 40 cycles (95°C for 15 sec, 60°C for 10 s and 72°C for 30 s) by using the SYBRH Premix Ex TaqTM (TaKaRa, Japan). Real-Time PCR amplifications were performed twice in 20 µL reaction volumes containing 50 ng DNA template, 10 pmol concentration of each primer. The difference in the average threshold cycle (Ct) number values was used for the measurement of relative abundance; ΔCT ($CT=Ct_{mtDNA}^{4977}-CT_{mtDNA}^{n}$) was used to calculate the abundance of the mtDNA 4977 bp deletion; and the percentage of the mtDNA deletion was calculated as $2^{-\Delta Ct}$ x 100% (40). In addition, Ct values of the genomic DNA (gDNA) (ß-globin gene) and mtDNA were also used to determine the mtDNA content present in samples. The content of the mtDNA was calculated by using ($2^{\Delta Ct}$) method ($\Delta Ct = Ct_{mtDNA}-Ct_{gDNA}$) (41).

Cellular oxidative stress (2',7'-dichlorofluorescein (DCF assay)

Cellular oxidative stress was analyzed by determining the oxidation of 2,7-dichlorohydrofluoroscein diacetate (DCFH-DA) to the fluorescent 2′, 7′ dichlorofluorescein (DCF) (42). DCFH-DA is a ROS-sensitive probe that can be used to detect ROS production in living cells. Briefly, the cells were washed in ice-cold PBS and added to 1 ml of PBS containing 20 µM DCFH-DA. Then the cells were incubated for 30 minutes at 37°C. DCF fluorescence emission was analyzed using a Shimadzu RF-5000 spectrofluorophotometer with the excitation and emission spectra set at 480 and 530 nm (with slit widths of 3 nm), respectively. The results were expressed as arbitrary units of the fluorescence intensity. The experiments were run in triplicate and the intra-assay variation for the DCF assay was 5.7%.

Measurement of intracellular ATP levels

Frozen cell samples were thawed rapidly, cell debris was pelleted by centrifugation (10000xg, 3 min at room temperature) and the supernatant diluted in 3% perchloric acid. The samples were neutralized with 75 µl ice-cold 2 M KOH, 2 mM Na_2EDTA, 50 mM MOPS and incubated on ice for 10 min. The precipitate was pelleted by centrifugation (10000xg, 1min at room temperature) and ATP levels in the supernatant were determined using the ATP Bioluminescence Assay Kit (ATP Bioluminescence Assay Kit CLC II, ROCHE, Boehringer Mannheim, Germany) according to the manufacturer's instructions by comparison with appropriate standards. ATP content was expressed as nmoles per mg protein. The experiments were triplicated and the intra-assay coefficient of variation was found as 5.4%.

Statistical analysis

Given the small sample size, differences between independent groups and samples were compared using non-parametric Kruskal-Wallis test. If differences were

Table 3. Regions and primers amplified in Real Time PCR.

Fragments	Nucleotide Position	Product (bp)	Primers
Conserved region (ND1 gene-mtDNA)	3307-3458	152	F- 5'- AACATACCCATGGCCAAC-3' R- 5'- TCAGCGAAGGGTTGTAGTAGC -3'
$mtDNA^{4977}$ deleted region	8388-13646	281	F- 5'- TATGGCCCACCATAATTACCC-3' R- 5'- AAGCGAGGTTGACCTGTTAGG-3'
human ß-globin gene	61991-62259	268	F- 5'-GAAGAGCCAAGGACAGGTAC-3' R- 5'-CAACTTCATCCACGTTCACC-3'

found to be statistically significant, Mann-Whitney U test with Bonferroni correction would be used to analyze the differences between pairs of groups. Spearman's rho analysis was applied to analyze the relationship between mtDNA content and ATP levels in leukocytes of HTS group. Data were expressed as means ± SD and statistical significance was assumed as $p < 0.05$. All statistical analyses were performed with SPSS 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp).

Results

Anthropometric parameters and respiratory function test results

The differences between anthropometric parameters and respiratory function test results were compared with each other for similarities and differences. By means of FEV1, the HTS group was found to be significantly different from NTS and NAC groups, and the NTS group was significantly different than NAC group. Regarding FVC, HTS group was found to be significantly different from NTS and NAC groups. TLC and DLCO of the HTS group was significantly higher than the subjects in NTS and NAC groups while VO_{2max} values of the HTS group was significantly higher than NTS and NAC groups, and the NTS group was significantly higher than NAC group. ($p<0.05$; Kruskal-Wallis test and Mann-Whitney U test with Bonferroni correction. Values are means ± SD) (Table 1).

Comparison of mtDNA deletion levels between the three study groups

The percentage levels of mitochondrial deletion in leukocytes in all three study groups were compared by using Real-Time PCR method. Even though incidence of mtDNA^{4977} deletion was observed in all groups (26.93% ± 20.14 in HTS group, 21.28% ± 9.59 in NTS group and 21.05% ± 5.55 in NAC group), by using Kruskal-Wallis test, the results were not considered statistically significant when we compared the percentage of deleted region to undeleted region (p=0.98) (Figure 1).

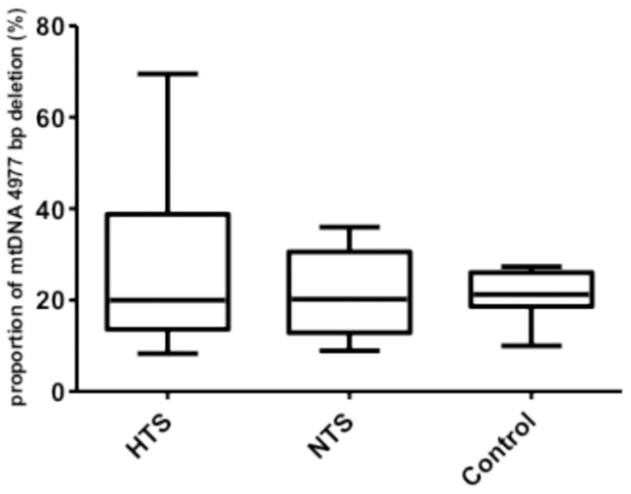

Figure 1. The relative amount of mtDNA^{4977} deletion in PBLs from HTS, NTS and NAC groups. The solid lines represent the mean values. Statistical analysis was carried out with Kruskal-Wallis test (HTS: Highly Trained Swimmers, NTS: Normal Trained Swimmers).

Comparison of mtDNA content between the three study groups

We compared the mitochondrial copy numbers of all three study groups with genomic DNA (gDNA) numbers in leukocytes using Real-Time PCR and after performing Kruskal-Wallis test a significant difference was found in copy numbers between groups (1914.14 ± 1175.73 for HTS group, 474.49 ± 79.51 for NTS group and 342.60 ± 307.47 for NAC group) (p= 0.0003). As the results were statistically significant, we compared the groups with each other using Mann-Whitney U test and found that mitochondrial copy numbers were increased in HTS group compared to NTS group by 4.03 fold (p=0.0002) and by 5.58 fold compared to NAC group (p=0.0003). On the other hand, we didn't find a significant difference between NTS and NAC groups by means of copy numbers (p=0.1) (Figure 2).

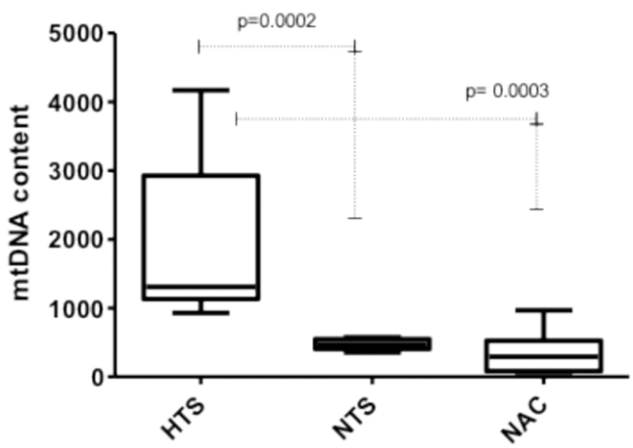

Figure 2. Box plot analysis illustrating levels of mtDNA copy number in the leukocytes from HTS, NTS and NAC. The relative mtDNA copy number was significantly different between the groups evaluated by Kruskal-Wallis test ($p<0.0003$). Horizontal lines: group medians; the box is the interquartile range, excluding outlying and extreme values (HTS: Highly Trained Swimmers, NTS: Normal Trained Swimmers, NAC: Non-Athlete Controls).

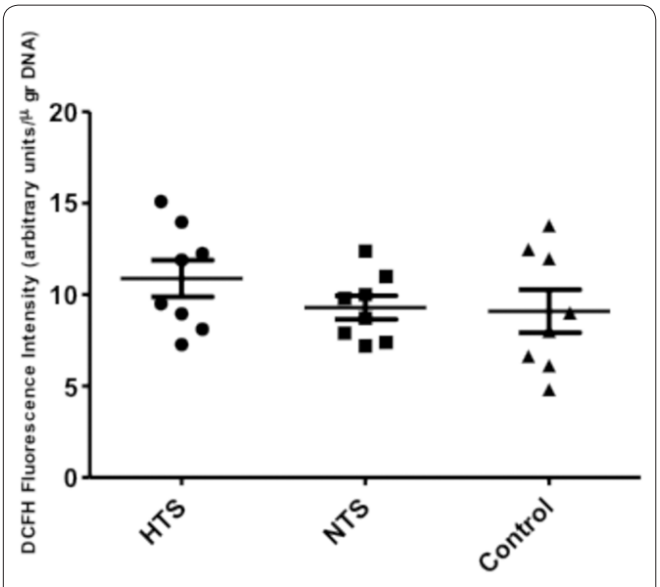

Figure 3. Cellular oxidative stress levels measured fluorometrically by the oxidation of dihydrodichlorofluorescein within leukocytes from HTS, NTS and NAC groups (HTS: Highly Trained Swimmers, NTS: Normal Trained Swimmers, Controls: Non-Athlete Controls).

Comparison of adenosine triphosphate (ATP) levels between the three study groups

To assess the effects of mtDNA^{4977} deletion on mitochondrial dysfunction, we measured levels of intracellular ATP in cells obtained from subjects. Three groups of subjects were compared; no significant differences were found in the concentration of intracellular ATP levels of leukocytes between the studied groups (p=0.406). The intracellular ATP levels in leukocytes in HTS, NTS and NAC groups were 16.00 ± 11.43, 13.26 ± 7.14 and 17.35 ± 6.88 nmol mg^{-1} protein, respectively.

Cellular oxidative stress

As it has been found that the relative content of mtDNA^{4977} deletion is correlated with increased levels of oxidative stress (43, 44), we also measured the fluorescence intensity of DCF. Although, a slight increase in DCF fluorescence levels in leukocytes from HTS subjects was present compared to those from NTS and NAC subjects, the group differences did not reach statistical significance (p=0.430) (Figure 3).

Relationship between increased mtDNA copy number in leukocytes and intracellular ATP level in highly trained swimmers

To better characterize the consequences of increased mtDNA copy number in leukocytes of HTS group, we tested for a possible relationship between this parameter and ATP levels in leukocytes of the subjects, using Spearman's correlation test. In this case, no significant correlation was found to be present in NTS subjects between the mtDNA copy number and ATP levels of leukocytes (p= 0.703) (Figure 4).

Discussion

To our knowledge, this is the first study investigating the relationship between mtDNA^{4977} deletion and oxidative stress in blood of the trained swimmers. Regarding the information given in introduction, our first expectation was that, swimmers with a heavier load of exercise program might have more mitochondrial damage and suffer more from oxidative stress than the normal trained swimmers and normal individuals because they might be producing more free radicals in the context of high density training programs. As we expected, mtDNA^{4977} deletions were observed in the leukocytes of swimmer groups but the difference between groups wasn't statistically significant.

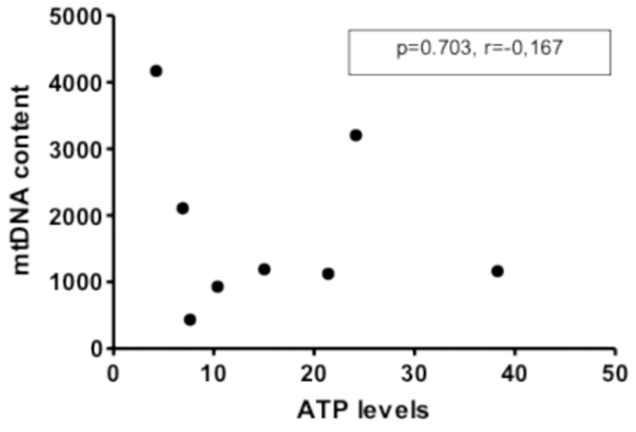

Figure 4. Correlation of mtDNA copy number levels (y-axis) and intracellular ATP content (x-axis) in leukocytes in HTS cases (n=8). Statistical analysis was carried out with Spearman's correlation test.

To ascertain whether training load in swimmers increases the fluorescent intensity of DCF, an indicator of oxidative stress in cells, we also measured the DCF fluorescence of three groups. Although DCF fluorescence levels of leukocytes were higher in the HTS group, the difference between the means of groups did not reach statistical significance, suggesting that oxidative stress was not augmented in swimmers with training load. The lack of difference observed among the groups may be due to the higher levels of defense against the causes of deletion in highly trained athletes. Although contradictory results exist, studies performed with different exercise protocols show that subjects recruited in the studies develop an adaptive response due to modifications occurring in the antioxidant system both at cellular and systemic levels (45-48). Therefore, the lack of oxidative stress change may be explained with increased antioxidant enzymes efficiency in trained subjects.

Moreover, it should also be noted that mtDNA^{4977} deletion accumulates faster in some tissues due to greater metabolic activity and minimal cell turnover compared to other tissues including blood (20). It may be expected that, a different result could be obtained if this study had been conducted in muscle cells. Therefore, further studies have to be made concerning this subject.

Meanwhile, changes in the mtDNA copy number have been observed in a variety of pathologies and in the presence of either endogenous or exogenous oxidative stress. An increased mtDNA copy number was observed as a compensatory effect of mtDNA deletions (49-51). A few risk factors such as diabetes and increased age which may contribute to an oxidative environment has been shown to induce alteration of the mtDNA copy number by increasing mtDNA defect (52, 53).

The copy number of mtDNA that reflects the abundance of mitochondria in a cell may change under different energy demands and different physiological or environmental conditions (54). Therefore, we evaluated substantive changes in mtDNA content related to training load in swimmers. Regarding that, we found a significant association in the context of mitochondrial content in swimmer groups and observed that mitochondrial copy numbers had increased in the trained swimmers group which can be the translated as a consequence of the need for high energy production to meet the oxygen demand of muscle cells. Investigations with animal models and epidemiologic studies with humans suggest that exercise increases the speed of metabolism and immune function of lymphocytes (55). Exercise induced lymphocyte proliferation has also been reported previously (56). Like all living cells, leukocytes directly or indirectly use ATP to transport organelles and to alter cellular morphology during cell locomotion and division. The ATP requirement for these processes in leukocytes is considerable. Due to this increasing ATP demand in the cells, an increase in the mitochondrial content in HTS group may be reasonable. The increased mitochondrial content may be dependent on the density of the exercise and the increased mitochondrial content may be a compensatory physiological response in order

to fulfill the demand of ATP. However, we neither observed any significant changes for ATP levels nor any correlations between the mtDNA content and the level of cellular ATP in leukocytes for each highly trained swimmer, suggesting that the increased mitochondrial content in leukocytes does not increase ATP production simultaneously. Another possible explanatory statement can be made as the production of ATP increases, consumption of ATP also increases simultaneously because ATP cannot be stored in the cell.

Another crucial point is, the subjects enrolled in the study are not world-class elite athletes. They were two different groups of swimmers with different exercise intensities. Thus, there is no doubt that further studies need to be undertaken to dissect the exact role of increased mtDNA content in highly trained swimmers. A preliminary test measuring the mtDNA copy numbers of swimmers in blood may help coaches choose more eligible candidates for a higher training load.

In conclusion, we found that mitochondrial copy numbers were interestingly high in highly trained swimmers. Despite the increased mtDNA^{4977} deletion and mitochondrial copy numbers in all groups, an association with oxidative stress and ATP levels is unlikely. To finalize, high energy consuming athletes may have developed a compensatory mechanism by producing more mitochondria to supply energy to the cells but due to very limited and specialized sample of subjects, this phenomenon must also be confirmed in muscle cells to prove a possible compensatory mechanism.

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