ORIGINAL

TNFA expression level changes observed in response to the Wingate Anaerobic Test in non-trained and trained individuals

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
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abstract

Background: Literature reports indicate changes in the expression of genes encoding proinflammatory factors are observed as a result of physical exercise. The stress changes caused by high-intensity loads and adaptive changes induced by the planned long-term training are less studied. The aim of this study was to determine the impact of intense anaerobic effort in people adapted to regular trainings and in non-trained persons on the expression levels of the *TNFA* gene.

Material and methods: 50 experienced soccer players and 50 non-trained participants were recruited for the study. Anaerobic capacity was measured by means of the Wingate Anaerobic Test. To evaluate the expression of *TNFA* gene, a QRT-PCR was applied.

Results: A comparison of the *TNFA* expression profiles between well-trained athletes and non-trained controls revealed that transcript levels were higher in non-trained participants when compared with soccer players in all the studied time points, with the exception of the second post-test; however, this difference was the only one that was statistically insignificant.

Conclusions: The *TNFA* mRNA expression characteristic described in our study indicated a significant downregulation of the *TNFA* expression observed in the course of time in experienced athletes reflecting molecular adaptation to physical effort caused by long-term training regime.

Key words: *TNFA*, *TNFα,* tumor necrosis factor alpha, gene expression changes, Wingate Anaerobic Test.

INTRODUCTION

Regular physical activity is one of the most important factors for the prevention of lifestyle diseases, also known as civilization diseases. However, the detailed molecular mechanism of changes caused by physical effort in the human organism has not been found yet. There are many factors influencing the body's response to physical effort, including, among others, individual predispositions, types of physical effort, types of training, the degree of training, age, gender, and others. Additionally, this response applies not only to the muscle tissue, but also to other organs, especially blood cells in which changes in the expression show the systemic response and play a significant role in the immune response [1].

So far, genes becoming overexpressed in working muscles have been identified. Literature shows that these are genes associated with the cellular response to stress, which manifests itself, among others, in the production of anti-apoptotic, proliferous pro- and anti-inflammatory factors [2–5]. The stress changes caused by high-intensity loads and adaptive changes induced by the planned long-term training (sports and recreational) are less studied. Many literature reports indicate that changes in the immune system, including changes in the expression of genes encoding interleukins and other proinflammatory factors, are observed as a result of regular physical exercise [6–8]. On the other hand, there are also publications showing that physical activity reduces the inflammatory response in favor of the anti-inflammatory one [9–11].

Despite the growing availability of methods of molecular genetics, relatively few academic papers have been associated with research on gene expression changes in response to a different type of physical activity. In view of the above, the purpose of the presented study was to determine the impact of intense anaerobic effort in people adapted in the course of long-term sports training and in non-training persons on the expression levels of selected genes encoding factors associated with the inflammatory response of cells. For this purpose, the *TNFA* gene has been selected. *TNFA* encodes tumor necrosis factor alpha, that is a factor associated with an inflammatory response of cells, playing a central role in inflammation, especially in the acute phase reaction and regulation of immune cells as well as is engaged in immune system development and apoptosis [12–14]. In many reports it has also been shown that impairments of the *TNFA* gene expression are associated with different human diseases, such as Alzheimer's disease [15], cancer [15], depression [16], psoriasis [12] and inflammatory bowel disease [5].

The direct aim of the study was to describe the changes in the *TNFA* gene expression levels under the influence of supramaximal anaerobic effort in well-trained elite athletes adapted to long-term sports training and in non-trained participants.

MATERIAL AND METHODS

An experiment based on an ex post facto research plan was used in the study, due to the lack of manipulation of the independent variable (grouping variable). In the whole experiment, the group ("Trained", "Non-trained") was the independent variable, while the expression levels of the studied genes measured in pre-test and post-tests were the dependent variables. The recruitment for the "Trained" (study group) and the "Non-trained" (control group) was based on the purposive choice approach.

PARTICIPANTS

The procedures followed in the study were conducted ethically according to the principles of the World Medical Association Declaration of Helsinki and ethical standards in sport and exercise science research. The whole experimental protocol was approved by the Ethics Committee of the Regional Medical Chamber in Gdansk (approval number: KB-35/17). All participants were given a consent form and a written information sheet concerning the study, providing all pertinent information (purpose, procedures, risks, benefits of participation). All participants had time to read the information sheet and the consent form. After ensuring that the participant had understood the information, every participant gave written informed consent (a signed consent form) to participate in the project on the understanding that it was anonymous and that the obtained results would be confidential. The experimental procedures were planned in accordance with the set of guiding principles for reporting the results of genetic association studies defined by the STrengthening the REporting of Genetic Association studies (STREGA) Statement [17].

Participants for the study group (designated as "Trained") were recruited by direct contacts with coaching staff, targeting national teams and information provided to athletes attending training camps, sports tryouts as well as based on public announcements or voluntary letters of intent. The criteria for inclusion in the "Trained" group were:
- gender (only males),
- all competitors participating in the study group were qualified on the lists of Polish Sports Associations of their discipline, which means that they had license to compete,
- all participants had documented history of systematic sport training and participation in a given sports discipline for over 10 years,
- all participants underwent a regular medical examination allowing them to exercise and participate in sports competitions,
- good current health condition (no coexisting injuries) and a negative medical interview for disorders of the cardiovascular system, the autonomic nervous system, mental disorders, head injuries and other diseases which might directly affect the experimental procedures,
- not taking any supplements or medication during the study which might directly influence the obtained results.

In order to achieve the objectives of the study, 50 experienced soccer players (mean age 21.9 ±5.9 years) were recruited for the "Trained" group.

Non-trained males were recruited for the control group (designated as "Non-trained") based on a public announcement or voluntary letters of intent. The criteria for inclusion in the "Non-trained" group were:
- gender (only males),
- control participants were matched to the participants from the study group by age and characteristics of general morphology indicators,
- a low level of physical activity self-reported by each participant with use of Global Physical Activity Questionnaire (according to the World Health Organization in the Polish adaptation),
- a good current health condition (no coexisting injuries) and a negative medical interview for disorders of the cardiovascular system, the autonomic nervous system, mental disorders, head injuries and other diseases which might directly affect the experimental procedures,

• not taking any supplements or medication during the study which might directly influence the obtained results.

In order to achieve the objectives of the study, 50 participants (mean age 20.6 ±0.9 years) were recruited for the "Non-trained" group.

Measurement of anaerobic capacity by the Wingate Anaerobic Test

To assess the impact of the supramaximal anaerobic effort on the changes of the expression levels of the genes encoding factors associated with inflammatory response, the measurements of anaerobic capacity were conducted through the Wingate Anaerobic Test (WAnT) in the 30-second version with full resistance of the flywheel from the beginning of the effort. All participants performed the test on their lower limbs on a bicycle ergometer Ergomedic E818 (Monark, Sweden), with the flywheel resistance selected individually (0.075 kg per kg of body weight). To calculate the mechanical parameters in the above test the computer program MCE v5.1 was used. The following parameters were calculated: the amount of total work (Wtot), the maximum anaerobic power (PPWAnT), the time to obtain the maximum power (TUZ), the time to sustain the maximum power (TUT) accurate to the nearest 0.01s and the power decline rate (WSM) in percent values.

Biological material samples collection

Venous blood samples (volume 500 µl) were taken to the RNAprotect® Animal Blood Tubes (Qiagen, Germany) form "Trained" and "Non-trained" participants before WAnT (pre-test) and immediately after WAnT (0h) as well as 30 min (0.5h), 6 hours (6h) and 24 hours (24h) after WAnT (post-tests).

Isolation of the RNA and cDNA synthesis

The total RNA was extracted from the blood samples using a RNeasy® Protect Animal Blood Kit (Qiagen, Germany) according to the manufacturer's protocol. All of the extracts were treated with DNAse in order to avoid genomic DNA contamination. The obtained isolates were spectrophotometrically controlled for RNA quantity and quality with use of the Spectrophotometer apparatus (Eppendorf, Germany). 100 ng of each RNA sample was subjected to the reverse transcription with use of iScript cDNA Synthesis Kit (Biorad, USA), and the resulting cDNA samples were stored at -20°C until further analysis.

Application of Quantitative Real-Time PCR

To evaluate the expression of the *TNFA* gene, a Quantitative Real-Time PCR (QRT-PCR) was applied in duplicate for each sample using a StepOne Real-Time Polymerase Chain Reaction instrument (Applied Biosystems, USA). QRT-PCR fluorescence detection was performed in 96-well low-skirted plates (Applied Biosystems, USA) with a PrimePCR™ SYBR® Green Assay (Biorad, USA) and SsoAdvanced Universal SYBR Green SuperMix Reagents Kit (Biorad, USA) according to the manufacturer's protocol under the following conditions: 95°C for 30s for initial denaturation, followed by 40 cycles of 10s at 95°C for denaturation and 30s at 60°C for annealing, extension and plate read. A quantified transcript of gene encoding human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene, and no significant differences were observed in the GAPDH mRNA copy number between the analyzed groups.

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Statistical analysis

The preliminary statistical analyses encompassed the calculation of means and standard deviations of the gene expression parameters and analysis of the expression levels of the *TNFA* gene obtained from the QRT-PCR reaction with use of the $\Delta\Delta C_T$ method [18]. The distribution of variables was evaluated using the Shapiro-Wilk test. Because most data were not normally distributed, the post hoc nonparametric Mann-Whitney U-test was used in order to evaluate significant differences between the *TNFA* expression levels observed in the "Non-trained" and "Trained" group in post-tests time points (0h, 0.5h, 6h, 24h). The results were considered statistically significant for $p < 0.05$. All calculations were conducted with the use of Microsoft Excel 2017 (Microsoft, USA) and PQStat software (PQStat Software, Poland).

Results

The results of the *TNFA* expression fold changes calculated by $\Delta\Delta C_T$ method made between the "Non-trained" and "Trained" group in four time points of post-tests after WAnT are illustrated in Figure 1.
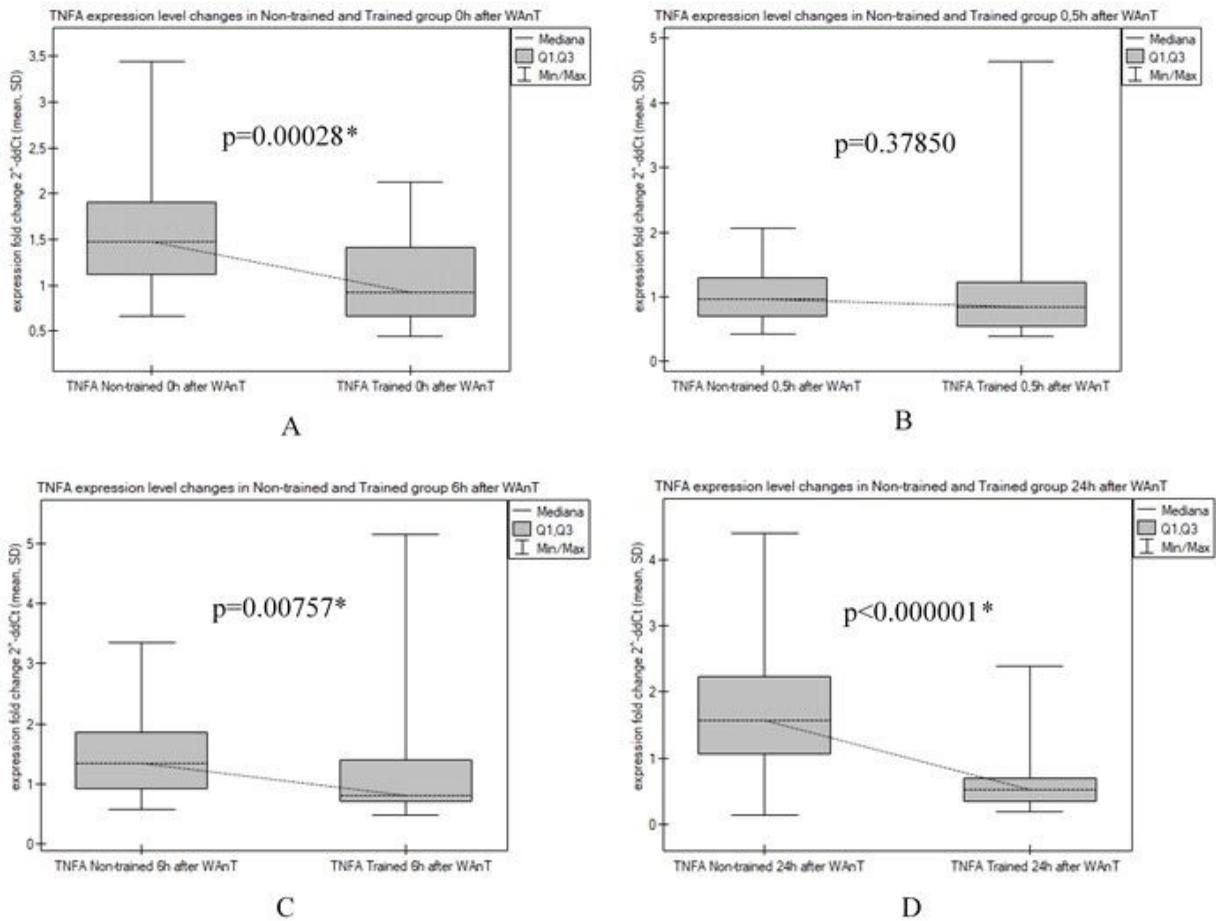
In the "Trained" group, the *TNFA* mRNA expression showed a relatively low level when measured immediately after WAnT, followed by a continuous increase during the course of time with a maximum in 6th hour after WAnT, while after 24 hours the *TNFA* transcript level reached a minimum level. The trend of *TNFA* expression level changes observed for non-trained participants was different: the number of *TNFA* transcripts was relatively high in the first post-test after WAnT (time point 0h), while in the next time point (0.5h after WAnT) the *TNFA* expression level was lower with a continuous increase during the course of time with a maximum observed 24 hours after WAnT (Fig. 1).

A direct comparison of the *TNFA* expression levels measurements between the "Non-trained" and "Trained" group in four post-tests after WAnT revealed that *TNFA* transcript levels measured immediately after WAnT (time point 0h) as well as 6h and 24h after WAnT were higher in non-trained participants ("Non-trained" group) when compared with trained soccer players (the "Trained" group). Only in the second post-test after WAnT (time point 0.5h) the expression of *TNFA* was slightly higher in the "Trained" group (Fig. 1).

Fig. 1. *TNFA* expression level changes in "Non-trained" and "Trained" group in four post-tests after WAnT

The statistical comparisons between the *TNFA* expression levels measured in the "Non-trained" and "Trained" group during subsequent post-tests (Fig. 2) revealed that the observed differences were statistically different in time points 0h, 6h and 24h after WAnT (p = 0.00028, p = 0.00757, p < 0.000001, respectively).

p values are Mann-Whitney U-test asymptotic p values corrected for ties, *statistically significant differences
Fig. 2. A comparison between the *TNFA* expression levels measured in the "Non-trained" and the "Trained" group in four post-tests: (A) 0h after WAnT, (B) 0.5h after WAnT, (C) 6h after WAnT, (D) 24h after WAnT

DISCUSSION

Changes in expression levels of selected genes have been implicated in the pathogenesis of severe diseases and health problems in humans, not only with those associated with impairment of the immune system function (e.g. cutaneous T cell lymphoma [19]) or autoimmunological reaction (e.g. asthma, rheumatoid arthritis [20], chronic granulomatous disease [21]), but also with lifestyle diseases (e.g. arterial hypertension [22], coronary disease [23], coronary artery disease [24], myocardial infarction [25], arteriosclerosis [26], diabetes mellitus [27], obesity [28]).

Nowadays, the application of molecular biology techniques in the practice of physical education and sport sciences seems to be essential for further dynamic development of this field of science. Research defining the molecular characteristics of the response to physical effort gives a new insight into the underlying mechanisms, among others, adaptation to effort, and thus it enables a better understanding and explaining of the nature of an athlete's response to specific effort. Widely recognized scientific literature provides numerous

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reports describing the molecular genetics techniques used mainly for detection of DNA diversity in athletes representing various sports disciplines in the context of the genetic markers application in talent search programs [29–33] or using them for anticipation of a post-training physiological response in carriers of specific alleles or genotypes [34, 35].

On the other hand, research projects focused on the expression of genes analyzed in professional athletes or even in physically active amateurs are not frequent, neither in Poland nor in the world. Despite the fact that there are numerous reports defining the profile of an inflammatory reaction as a consequence of performed physical activity in different forms and time of duration [36, 37], still very few papers raise the issues related to the adaptation of the human body in the form of activation and/or braking the pro- and anti-inflammatory cascades as a consequence of performing a supramaximal physical effort in a group of athletes at a sports championship level.

Several cytokines have already been identified to play a pivotal role in post-exertion immunological changes and have therefore been chosen for the present investigation. The research approach proposed in the presented study includes the precise descriptions of changes in expression levels of the *TNFA* gene encoding tumor necrosis factor alpha (*TNF*-α) associated with the inflammatory response observed during or directly after the supramaximal anaerobic effort, especially in reference to elite athletes representing the highest level in championship standings, characterized by a long-term training experience. *TNF*-α is a multifunctional cytokine involved in the advancement of inflammatory responses and is critical in the pathogenesis of inflammatory, autoimmune and malignant diseases [38]. *TNF*-α also has a high potential regarding the primary systemic response after physical effort – it is well known that *TNF*-α produced by Th1 cells is responsible for stimulation of Tc cell-associated immune response [39].

Via detecting very early mRNA expression changes of the *TNFA* gene, we could demonstrate a characteristic gene expression kinetic following exertion. To our knowledge, this is the first study evaluating very early functional changes of the human immune system made by investigating mRNA expression profiles of the *TNFA* gene directly following supramaximal physical effort in a group of well-trained athletes and non-trained controls. Thereby, we could show that *TNFA* expression levels compared between the "Non-trained" and "Trained" group were different. In well-trained athletes, the *TNFA* mRNA levels were relatively low immediately after exertion, followed by a continuous increase during the course of time with a maximum in the 6th hour after WAnT, to finally show a significant decrease over the next 24 hours – while in non-trained controls the number of *TNFA* transcripts was relatively high directly after supramaximal physical effort, with a significant decrease 0.5h after WAnT, followed by a continuous increase during the course of time with a maximum observed 24 hours after the test. The comparison of the *TNFA* expression profiles between athletes adapted to physical effort and controls without long-term training experience revealed that *TNFA* transcript levels were higher in non-trained participants when compared with trained soccer players in all the studied time points, with the exception of the second post-test (time point 0.5h after WAnT); however, the Mann-Whitney U-test showed that this difference was the only one that was statistically insignificant. In summary, the *TNFA* mRNA expression characteristic described in our study indicated a significant downregulation of *TNFA* expression observed in the course of time in experienced athletes reflecting molecular adaptation to physical effort caused by a long-term training regime.

CONCLUSIONS

In contrast to numerous studies that investigate cytokine changes in serum or plasma after exertion, a major strength of this work is the evaluation of mRNA expression changes. However, this missing protein analysis and a relatively small sample size might be seen as a limitation of our work, as mRNA levels do not necessarily correlate with protein levels. But it should be taken into account that one of the main advantages in studying gene expression profiles is the detection of very early transcriptome changes. This is based on the idea that changes in the genome level can improve the understanding of the underlying immune functionality. By evaluating molecular changes starting at the moment when physical effort occurs, we could demonstrate that these fundamental functional immunological changes already start immediately after the exertion.

To our knowledge, there are solely some experimental studies available that evaluate mRNA expression levels after supramaximal physical effort in trained and non-trained participants. In view of the fact that there have been only few scientific studies at least partly engaged in this subject matter, the proposed research could be relevant and fill the existing gaps in the knowledge of sport sciences. Moreover, there were no or very few studies aiming to determine the molecular adaptation profile manifested by the range and direction of changes in the expression levels of genes engaged in the immune reaction under the influence of long-time physical training of a specific character (anaerobic, mixed) causing such adaptations. Such genetic expression profile generates very valuable information about the systemic response induced by physical effort and long-term training. Such data may be used as an innovative characteristic that could be applied to the capabilities assessment of athletes at the highest level of sports proficiency. In the future, the analyses of the athlete's genetic expression profile may be applied in practice, enabling determination of the appropriate level of cellular activity that favors the functional improvement of one's effort abilities and, at the same time, may protect against the development of overtraining or injuries.

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