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INTERFACES CIENTÍFICAS

ACUTE EFFECT OF THE MAXIMAL CARDIORESPIRATORY CAPACITY TEST ON THE LEVELS OF LEUKOCYTES, NEUTROPHILS, NK, B AND T CELLS IN SOCCER PLAYERS

EFEITO AGUDO DO TESTE DE CAPACIDADE CARDIORRESPIRATÓRIA MÁXIMA SOBRE OS NÍVEIS DE LEUCÓCITOS, NEUTRÓFILOS, CÉLULAS NK, B E T EM JOGADORES DE FUTEBOL

EFECTO AGUDO DE LA PRUEBA DE CAPACIDAD CARDIORRESPIRATORIA MÁXIMA SOBRE LOS NIVELES DE LEUCOCITOS, NEUTRÓFILOS, CÉLULAS NK, B Y T EN JUGADORES DE FÚTBOL

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ABSTRACT

High-intensity exercise plays a crucial role in immune modulation, especially in the sports context, where athletes' performance may be influenced by immunological changes. Maximal cardiorespiratory capacity tests (VO_2max) are common in intermittent aerobic sports like soccer, and this strenuous test is a high-intensity exercise that can directly impact the immune response. Thus, it is necessary to understand how immunological indicators react following the VO_2max test. We analyzed the acute effect of the VO_2max test on immunological indicators in soccer athletes. This pre-experimental study involved a sample of 16 university male soccer athletes (age: 22.0±1.7). The athletes underwent the VO_2max test on a treadmill, and respiratory gases were analyzed via ergospirometry. Blood samples (20 mL) were collected pre- and post-VO_2max test. Immunological tests were performed to assess the impact of high-intensity exercise on leukocytes, lymphocytes, and neutrophils ($p=0.001$). After the VO_2max test, an increase in total leukocyte and lymphocyte counts and a decrease in neutrophil percentage were observed. There was an increase in T & B lymphocytes ($p<0.05$). These findings suggest that the VO_2max test in soccer athletes promotes significant immunological changes, with an increase in total leukocyte and lymphocyte levels and a reduction in neutrophil levels, indicating potential immune adaptation to high-intensity training.

KEYWORDS

Sport; Athlete; Immune System; Leukocytes; Lymphocytes; Neutrophils.

RESUMO

O exercício de alta intensidade desempenha um papel crucial na modulação da imunidade, especialmente no contexto esportivo, onde o desempenho dos atletas pode ser influenciado por alterações imunológicas. A realização de testes de capacidade cardiorrespiratória máxima (VO_2max) é comum em esportes aeróbicos intermitentes, como o futebol, e este teste extenuante é um exercício de alta intensidade que pode impactar diretamente a resposta imunológica. Assim, é necessário entender como os indicadores imunológicos reagem após a realização do teste de VO_2max. Analisamos o efeito agudo do teste VO_2max nos indicadores imunológicos em atletas de futebol. Ensaio pré-experimental com amostra de 16 atletas universitários de futebol (sexo masculino, idade: 22,0±1,7). Os atletas foram submetidos ao teste de VO_2max realizado em esteira mecânica; a análise dos gases respiratórios ocorreu por ergoespirometria. Amostras de sangue (20mL) foram coletadas dos atletas nos momentos pré e pós-teste de VO_2max. Os testes imunológicos foram realizados para avaliar o impacto do exercício de alta intensidade sobre leucócitos, linfócitos e neutrófilos. Após o teste de VO2max, observou-se um aumento nas contagens totais de leucócitos e linfócitos e uma diminuição na porcentagem de neutrófilos ($p=0,001$). Houve aumento nas subpopulações de linfócitos T & B ($p<0,05$). Esses achados sugerem que o teste VO_2max em atletas de futebol promove alterações imunológicas significativas, com aumento dos níveis de leucócitos e linfócitos totais e redução nos níveis de neutrófilos, implicando em potencial adaptação imunológica ao treinamento de alta intensidade.

PALAVRAS-CHAVE

Esporte, Atleta, Sistema Imunológico, Leucócitos, Linfócitos, Neutrófilos.

RESUMEN

El ejercicio de alta intensidad desempeña un papel crucial en la modulación de la inmunidad, especialmente en el contexto deportivo, donde el rendimiento de los atletas puede estar influenciado por cambios inmunológicos. Las pruebas de capacidad cardiorrespiratoria máxima (VO_2max) son comunes en deportes aeróbicos intermitentes, como el fútbol, y esta extenuante prueba es un ejercicio de alta intensidad que puede impactar directamente la respuesta inmune. Por lo tanto, es necesario entender cómo reaccionan los indicadores inmunológicos después de la prueba de VO_2max. Analizamos el efecto agudo de la prueba de VO2max en los indicadores inmunológicos de los futbolistas. Este ensayo preexperimental incluyó una muestra de 16 futbolistas universitarios varones (edad: 22,0±1,7). Los atletas realizaron la prueba de VO_2max en una cinta ergométrica y los gases respiratorios se

analizaron mediante ergoespirometría. Se recogieron muestras de sangre (20 ml) antes y después de la prueba de VO_2max. Se realizaron pruebas inmunológicas para evaluar el impacto del ejercicio de alta intensidad sobre leucocitos, linfocitos y neutrófilos. Tras la prueba de VO_2max, se observó un aumento en las concentraciones totales de leucocitos y linfocitos, así como una disminución en el porcentaje de neutrófilos (p=0,001). Hubo un aumento en las subpoblaciones de linfocitos T & B CD-20 (p<0,05). Estos hallazgos sugieren que la prueba de VO_2max en futbolistas promueve cambios inmunológicos significativos, con un aumento en los niveles de leucocitos y linfocitos totales y una reducción en los niveles de neutrófilos, lo cual indica una posible adaptación inmunológica al entrenamiento de alta intensidad.

PALABRAS CLAVE

Deporte, Deportista, Sistema Inmunológico, Leucocitos, Linfocitos, Neutrófilos.

1 INTRODUCTION

Soccer is a dynamic sport with intermittent demands that rely heavily on aerobic energy pathways, which requires high levels of physical conditioning from athletes (SLIMANI; NIKOLAIDIS, 2017). Effective training prescription is essential for these athletes to reach peak performance, particularly when considering the intense physical demands of the sport (BORGES *et al.*, 2022). To ensure adequate conditioning, it is necessary to analyze the maximum cardiorespiratory capacity (VO_2max) of players, from which training zones are established based on VO_2max or peak heart rate reached during the test (BOK; FOSTER, 2021). This VO_2max test, categorized as a strenuous form of exercise, subjects athletes to considerable physical exertion and fatigue (KOSTRZEWA-NOWAK; NOWAK, 2018).

Intense aerobic exercise, such as the VO_2max test, has been shown to impact immune responses significantly, with several immunological indicators affected in the aftermath of strenuous activity (NIEMAN, 1997, 2000). Among these immunological indicators are leukocytes and their subpopulations, including neutrophils and lymphocytes, which can be analyzed through blood count tests (ALMEIDA-NETO *et al.*, 2023a). Neutrophils are a type of leukocyte responsible for the initial recognition and defense against invading pathogens, contributing to pro-inflammatory responses (LEY *et al.*, 2018). Lymphocytes, on the other hand, are essential for antiviral responses, producing cytokines and actively eliminating infected cells. These immune cells play distinct roles, with T lymphocytes primarily involved in cellular immunity and B lymphocytes responsible for antibody production, or humoral immunity (YATIM; LAKKIS, 2015; BRODIN; DAVIS, 2017; KHAN; GHAZANFAR, 2018).

The relationship between intense aerobic exercise and immune response is particularly relevant in understanding athletes' performance and recovery. Analyzing how leukocyte subsets react to high-intensity exercise provides insight into the immune adaptations that may accompany athletic trai-

ning. Therefore, it is important to assess the behavior of immune indicators following a VO_2max test, as this information offers valuable parameters for monitoring athletes' immune status and potential responses to training (SCHLAGHECK *et al*., 2020).

Thus, the aim of the present research was to analyze the acute effect of a maximal cardiorespiratory capacity test on immunological indicators in college soccer athletes. This study has practical implications for understanding immune system adaptations in high-performance athletes. The hypothesis was that after performing a VO_2max test, soccer athletes would exhibit significant changes in total leukocytes and subpopulations (e.g., neutrophils and lymphocytes).

2 METHODS

The design of the present study was pre-experimental with a quantitative approach.

2.1 PARTICIPANTS

The sample consisted of 16 male college soccer athletes in the U-23 category. For inclusion in the study, athletes needed to be healthy, be part of the U-23 category, have weekly training frequency of four times (two hours session) and participate in university competitions at national level. We classified the sample into trained athletes as proposed by McKay *et al*. (2022) As exclusion criteria we used: being a smoker, having clinically diagnosed heart problems, making use of hormonal or anabolic therapies that could interfere with the behavior of the immune system. The characteristics of the sample are shown in table 1 in Results sections.

2.2 ETHICS

This study was approved by the Research Ethics Committee of the Onofre Lopes University Hospital of the Federal University of Rio Grande do Norte (Natal, Brazil) (#47014415.9.0000.5292). All athletes participated in the study on a voluntary basis by signing an informed consent form in accordance with resolution 466/2012 of the National Research Ethics Committee - CONEP of the National Health Council in agreement with the ethical principles expressed in the Helsinki Declaration of the World Medical Association (VAN DELDEN; VAN DER GRAAF, 2017). The protocol of the present study was registered and is publicly available on the Open Science Framework Registries platform (Doi: 10.17605/OSF.IO/XPRJ9).

2.3 BLINDING

The immunological indicators were analyzed by external collaborators who had no knowledge about the procedures of the present study; they only received the blood samples from each participant with the codes: moment I and moment II (pre and post, respectively). In addition, the statistical data processing was performed in a "blind" manner by an external collaborator.

2.4 PROCEDURES

The athletes were recruited from the U-23 soccer team of the Federal University of Rio Grande do Norte (Brazil). After recruitment, 48-h before the tests, the participants were screened through a structured anamnesis. For characterization purposes, the athletes who met the inclusion criteria were submitted to a body composition analysis using dual-energy X-ray absorptiometry (DXA); and they were instructed not to perform strenuous physical exercises 24h before the VO_2max test. All tests took place between 8h and 10h in the morning and blood samples (20mL) were collected from the athletes before and after the test.

2.5 BODY COMPOSITION ASSESSMENT

Body mass was measured with a digital scale (Micheletti®, São Paulo Brazil) with a precision of 0.01kg on a horizontal and flat surface. Height was measured using a stadiometer with an accuracy of 0.1cm (Sanny®, São Paulo, Brazil). Then, the participants had their body composition assessed in a DXA equipment (Lunar® / GE Prodigy - LNR 41.990, Washington, DC, USA) equipped with enCORE software (GE Healthcare®, version 15.0, Madison, WI, USA). The equipment was properly calibrated before the evaluations and followed the same configuration for all participants (Full Body Evaluation, Voltage (kV): 76.0, Current (mA): 0.150, Radiation dose (μGy): 0.4 (Very low, no health risk)). Subsequently, the values in Kg of bone, fat, lean and fat-free masses were acquired.

2.6 MAXIMUM CARDIORESPIRATORY CAPACITY TEST

The maximal cardiorespiratory capacity test was performed in an air-conditioned environment (24°C) on a motorized treadmill (Centuriom 300®, Brasilia, Distrito Federal, Brazil). There was a previous warm-up lasting 5-min at 4km/h and 0% inclination. After that, the speed was gradually increased according to the individual capacity estimated for each subject, based on the American College of Sports Medicine (ACSM), trying to reach the maximum oxygen consumption (VO_2max) within the period of eight to twelve minutes. We point out that the test protocol was carried out in an automated way by algorithms connected to the motorized treadmill and the ergospirometer. Details can be seen in the studies by Guazzi *et al*. (2016) and Thompson *et al*. (2009). For the analysis of respiratory gases, an ergoespirometer Model Metalyzer-3B (Micromed®, São Paulo, Brazil) was used, following the "breath by breath" method. Subsequently, with the aid of Metasoft® software, connected to a Cortex® unit calibrated by the closed-circuit method with gas calibration, we determined the maximum oxygen consumption relative to body mass (ml/kg.min-1). During the test, heart rate was measured by short-range telemetry using a Polar® strap (Model H10, Vantage NV, Finland).

2.7 RATING OF PERCEIVED EXERTION

During the incremental test we used the Rating of Perceived Exertion (RPE) scale proposed by Borg (1982). According to the procedure carried out in a previous study (ALMEIDA-NETO, *et al*.,

2023b), the scale was exposed to the participant every two minutes to assess the levels of physical exertion perceived by the participants. This scale consisted of numerical values between six and 20, where six indicates resting and 20 maximum effort. The incremental test lasted until the participant reported maximum effort on the Borg scale. We emphasize that the sample was already familiar with the RPE, which was used during conventional soccer training. In addition, we performed a previous familiarization (24h before) with the RPE.

2.8 BLOOD COLLECTIONS & IMMUNOPHENOTYPING

A nurse collected 20 mL of peripheral blood before and immediately after a high-intensity exercise using a venipuncture technique. The blood samples were gathered using a vacuum-based system called Becton - Dickinson - Vacutainer SST BD. Subsequently, 5 mL of the blood was transferred to tubes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA) for a blood count analysis conducted with a hematology analyzer known as Cell Dyn-3,000® (São Paulo, Brazil). This analysis included determining the total counts of leukocytes, lymphocytes, and neutrophils. To convert these percentages into absolute values, the percentage values were multiplied by the absolute leukocyte values and then divided by 100.

The lymphocyte subsets were assessed using a lysis technique that is part of a single-platform method. For this purpose, we utilized four different color combinations to evaluate the expression of specific antigens: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), and phycoerythrin-cyanin (PC-5). Anticoagulated whole blood (100 µL each) was divided into 12 x 75 mm polystyrene tubes (Falcon Plastics, Becton Dickinson's Biosciences), with each tube containing 20 µL of a monoclonal antibody (MoAb). These tubes were thoroughly mixed and incubated in a dark environment at room temperature for 30 minutes. Following this, 2 mL of FACS Lysing Solution (Becton Dickinson's Biosciences), which was previously prepared by diluting it with distilled water at a 1:10 ratio (v:v), was added to lyse the red blood cells. After vigorous shaking and a 10-minute incubation, the tubes were centrifuged at 600 g for 5 minutes in the dark. The supernatant was discarded, and the cell pellet was re-suspended in cold phosphate-buffered saline (PBS) with a pH of 7.2 (Sigma-Aldrich, Germany). This suspension was then centrifuged once more, with the final step being repeated for accuracy. Ultimately, the cell pellet was re-suspended in 1 mL of a 0.5% formaldehyde solution in PBS, and the cell suspension was stored in the dark at 4°C until the analysis was conducted using flow cytometry. In this manner, a total of 20,000 events were acquired per tube using the Fluorescence Activated Cell Analyzer (FACScan, San Jose, CA, USA), along with the Cell Quest software (Cell QuestTM® Software, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Examples of immunophenotyping are presented in Figure 2 in Results section.

2.9 STATISTICAL ANALYSIS

For the a priori sample calculation, we entered the effect measures in the open source software G*Power® (Version 3.1, Düsseldorf, Germany) in the configuration "T" family tests for dependent sam-

ples (T test), considering an α = 0.05 and a β = 0.8 (Details in the Results section).

The normality of the data was analyzed using the Shapiro-Wilk and Z-Score tests for skewness and kurtosis (-1.96 to 1.96). The assumption of normality was accepted. For comparisons we used paired Student's t-test (pre-test Vs. post-test). The effect size between differences was evaluated using the "d" test proposed by Cohen (1992), being interpreted by magnitude: Small<0.20; Medium>0.20 and <0.50; Large>0.50. All analyses were performed using open source software JASP® (Version 0.15.0.0; University of Amsterdam, Holland) considering p<0.05. All figures analyses were performed in GraphPad Prism software (Version 8.01 (244), California, USA).

3 RESULTS

In this section, the results are organized into topics to facilitate reading and understanding. First, we provide a descriptive overview of the sample size analysis conducted a priori in this study. The second topic presents the characterization of the 16 soccer players who participated in the study. Next, we discuss the descriptive results regarding the behavior of the Rating of Perceived Exertion (RPE) during the cardiorespiratory test. Following this, we present the descriptive results of the "gates" scheme used in the immunophenotyping analyses. Finally, the last topic addresses the comparative analyses of the immunological factors evaluated in this study.

3.1 SAMPLE SIZE

The sample size was determined a priori through a pilot study with six soccer athletes from the U-23 category (age: 21.0 ± 1.9, stature: 176.7 ± 8.3 cm, total mass: 73.1 ± 6.4 Kg, BMI: 23.2 ± 1.7 Kg/m², VO_2max: 55.3 ± 4.6 ml/kg.min-1). Thus, we acquired the effect measures of 7.9 for leukocytes, 7.1 for lymphocytes, and 0.8 for neutrophils. Next, a minimum sample size of 06 subjects for WBCs and lymphocytes and 12 subjects for neutrophils was indicated. We considered the minimum sample size of 12 subjects with an addition of four subjects (35%) thinking of possible sample losses.

3.2 CHARACTERIZATION OF PARTICIPANTS

The characteristics of the final sample of the present study (16 soccer players) are shown in table 1.

Table 1. Sample Characterization.

Variables	Values	
	Mean ± Sd	**Min; Max**
Age $_{(years)}$	22.0 ± 1.7	19.0; 24.0
Stature $_{(Cm)}$	170.0 ± 0.10	160.0; 192.0

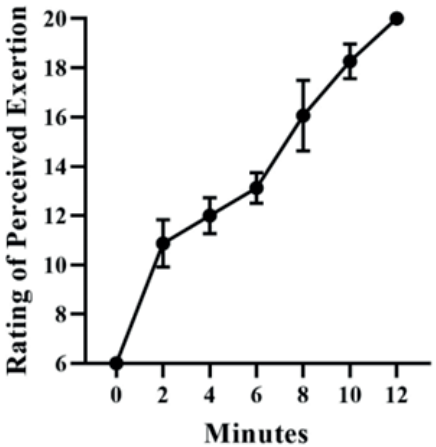
Variables	Values	
BMI $_{(Kg/cm^2)}$	24.3 ± 1.9	21.7; 28.2
Total mass $_{(Kg)}$	77.1 ± 9.1	62.0; 92.8
Bone mass $_{(Kg)}$	3.5 ± 0.4	2.7; 4.5
Fat mass $_{(Kg)}$	12.1 ± 3.1	6.9; 19.0
Lean mass $_{(Kg)}$	61.5 ± 7.1	46.4; 72.2
Fat-free mass $_{(Kg)}$	65.0 ± 7.5	49.1; 76.0
Resting heart rate $_{(bpm)}$	53.1 ± 3.2	49.0; 61.0
Peak heart rate $_{(bmp)}$	182.7 ± 28.2	126.0; 233.0
Maximum power $_{(Watts)}$	753.2 ± 276.8	46.6; 1080.6
Relative anaerobic threshold 1 $_{(ml/kg.min\text{-}1)}$	27.5 ± 5.0	18.3; 37.0
Relative anaerobic threshold 2 $_{(ml/kg.min\text{-}1)}$	49.6 ± 6.1	39.4; 57.8
VO_2 max $_{(ml/kg.min\text{-}1)}$	54.2 ± 5.1	45.2; 61.5

BMI: Body mass index. (Kg/m²): Kilograms per square meter. (Kg): Kilograms. (bpm): Beats per minute. VO_2max: Maximal oxygen uptake during maximal incremental test. (ml/kg.min^{-1}): Millimeters per body weight per minute. Sd: Standard. Min; Max: Minimum; Maximum.

3.3 Rating of Perceived Exertion

The behavior of RPE during the incremental test is displayed in Figure 1.

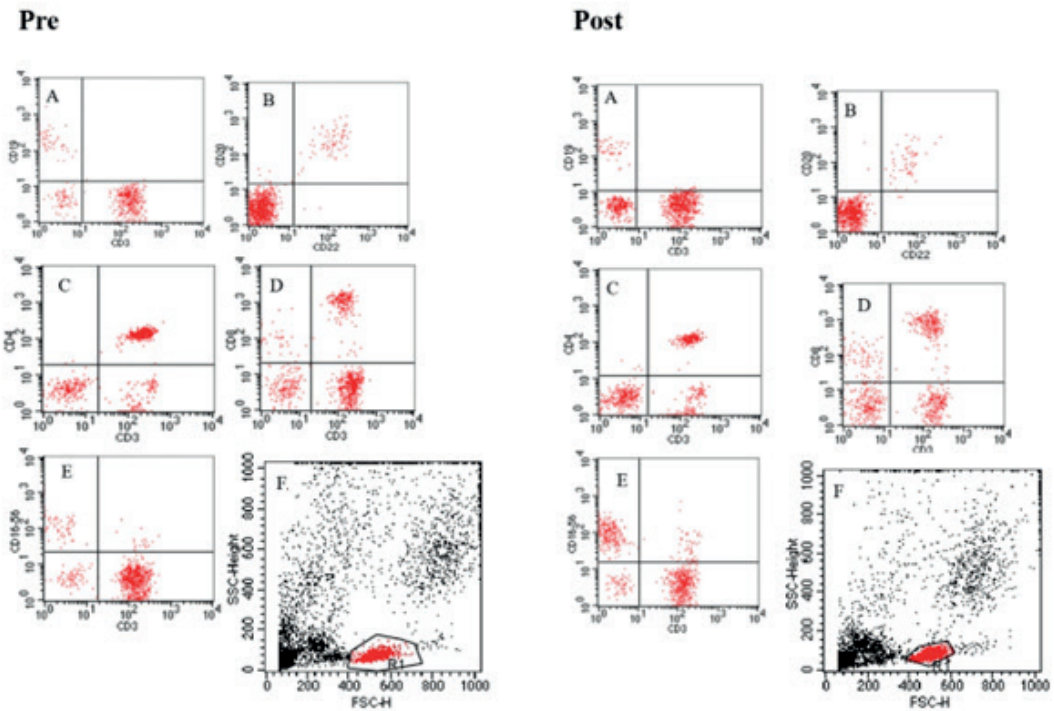
Figure 1 - Behavior of the subjective perception of effort during the maximal incremental test performed on a treadmill.



3.4 EXAMPLES OF IMMUNOPHENOTYPING

In Figure 2 we can see examples of the "gates" layout used in the immunophenotyping analysis.

Figure 2 - Dot-plot histogram obtained by flow cytometry of the lymphocyte subpopulations (A= [B-Cell CD19], B= [B-Cell CD20 / CD22], C = [T helper], D = [T cytotoxic], E = [Natural Killer: CD16-56], F = Color scheme used in flow cytometry, R1 = Total Lymphocytes), SSC: Side Scatter, FSC: Forward Scatter.



3.5 COMPARISONS IMMUNOLOGICAL FACTORS

Figure 3 reports an increase in absolute serum leukocyte levels (Figure 2-A: Effect size: 3.4, CI95%: 3.0; 5.0) and in absolute and relative lymphocyte levels (Figure 2-C: mm^3: Effect size: 3.1, CI95%: 2.6; 3.7. Figure 2-D: %: Effect size: 0.6, CI95%: 0.0; 1.1). In addition, there was a significant reduction in the relative levels of neutrophils (Figure 2-F: Effect size: 1.1, CI95%: 0.5; 1.8).

Figure 3 - Comparisons of leukocyte levels, neutrophil and lymphocyte levels at the time points of this study. Analysis performed by Student's (t) test for paired samples. (mm³): Cubic millimeters. (%): Percent.

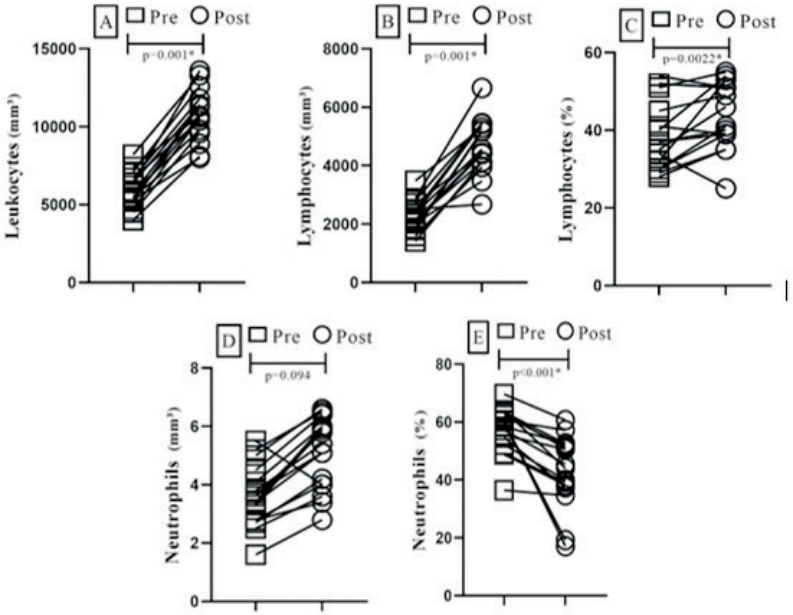


Table 2 reports the changes in T and B lymphocyte subpopulations before and after the incremental VO2max test. Increased serum values of CD16-56, CD56, CD3, CD3-CD4, CD3-CD8 and CD-20 were observed after physical exertion.

Table 2. Comparison of T and B lymphocyte subpopulation levels at the times of this study.

Variables	Moments		Effect Size	CI95%	P - Value
	Pre-test	Post-test			
CD3-CD16-56 $_{(mm^3)}$	98.6 ± 102.9	264.5 ± 323.2	0.7	0.2; 1.3	**0.009***
CD16-56 $_{(mm^3)}$	362.8 ± 251.3	1678.6 ± 692.5	2.5	1.2; 3.0	**0.001***
CD56 $_{(mm^3)}$	0.0 ± 0.0	655.0 ± 1210.5	0.8	0.5; 1.0	**0.047***
CD3 $_{(mm^3)}$	1524.2 ± 426.5	2430.9 ± 1034.1	1.2	0.5; 1.7	**0.001***
CD3-CD4 $_{(mm^3)}$	669.0 ± 210.8	1002.2 ± 252.4	1.4	0.4; 1.6	**0.001***
CD3-CD8 $_{(mm^3)}$	716.6 ± 238.3	1335.2 ± 768.1	1.1	0.4; 1.5	**0.002***
CD4/CD8 $_{(mm^3)}$	0.9 ± 0.3	0.9 ± 0.5	0.0	-0.5; 0.5	0.938

Variables	Moments		Effect Size	CI95%	P - Value
CD-19 $_{(mm^3)}$	0.5 ± 0.2	0.4 ± 0.3	0.3	-0.1; 0.8	0.2
CD-20 $_{(mm^3)}$	5.1 ± 2.7	8.0 ± 5.0	0.6	-0.4; 1.1	**0.03***
CD-22 $_{(mm^3)}$	13.2 ± 6.7	20.0 ± 13.1	-0.5	-1.0; 0.0	0.051

Analysis performed by Student's (t) test for paired samples. (mm^3): Cubic millimeters. CI95%: Confidence interval of 95% referring to the effect size. *: Statistically significant.

4 DISCUSSION

The objective of this study was to analyze the effect of a maximal cardiorespiratory capacity test on immunological indicators in soccer athletes. The initial hypothesis was that the test used would be able to promote significant changes in the immunological indicators investigated (i.e., leukocytes and subpopulations). As a result, it was found that there was an increase in leukocyte and lymphocyte levels, and a reduction in neutrophil levels soon after the end of the test, which confirmed the initial hypothesis. To optimize the prescription of physical training in soccer, it is common to perform VO_2max tests (BOK; FOSTER, 2021). However, this type of test is a strenuous physical exercise (BOK; FOSTER, 2021; BORGES *et al.*, 2022). It is known that strenuous exercise promotes modulation of the immune system (NIEMAN, 1997; REBELO *et al.*, 1998; MALM *et al.*, 2004; KOSTRZEWA-NOWAK; NOWAK, 2018). In light of this, we will discuss the acute behavior of immunological indicators in relation to strenuous exercise.

4.1 LEUKOCYTES

According to Chabot-Richards and George (2014), leukocytes are white blood cells that act as one of the primary lines of defense in the immune system, and elevated levels indicate an inflammatory response. Our findings align with prior literature that describes leukocyte increases as part of a systemic inflammatory response following strenuous exercise. Specifically, elevated leukocyte levels observed in this study corroborate evidence that exercise-induced inflammatory mediators (e.g., Interleukin-6) are released by immune cells and active muscle tissue (COOPER *et al.*, 2007). This supports an adaptive response in which leukocytes and their subpopulations are mobilized to prepare the body for post-exercise recovery and repair (BROWN *et al.*, 2015).

In a review study, Luz Scheffer and Latini (2020) addressed that inflammation is the immune system response aimed at prevention and has the objective of limiting and repairing damage caused by external agents (i.e., pathogens), by endogenous biomolecules, or by physiological stress. Exercise promotes physiological stress in the body through training variables (e.g., load, volume, and recovery interval) (GOLDBERG *et al.*, 1975; SCHOENFELD, 2013). Due to the same mechanism of biological inflammation, through the stress generated by exercise, an increase in lymphocyte levels also occurs

(KHAN; GHAZANFAR, 2018). According to Hamad and Mangla (2019) the lymphocytes are a subpopulation of leukocytes that account for 18% to 42% of circulating leukocytes.

Due to the inflammatory environment generated by physical exercise, B and T lymphocytes are stimulated to proliferate and differentiate according to local demand. According to Pennock *et al*. (2013) when the inflammatory agents and the B and T cells are eliminated, memory cells emerge that remain and proliferate to ensure a faster and more efficient immune response should a similar inflammatory event occur. After exercise, the increase in lymphocyte levels occurs in both lymphocyte subtypes (i.e., B and T), with greater emphasis on the T subtype (IBIS *et al*., 2012; BARROS *et al*., 2017).

Our study supports these findings by showing a rise in leukocyte levels, suggesting an acute inflammatory process that may play a protective role against exercise-induced damage, thereby aiding in recovery.

4.2 LYMPHOCYTES

To simplify, the T cell activation mechanism through the CD3 complex is notable due to its role in immune response modulation and repair after physical stress. The significant increase in CD3 observed in our study highlights an acute adaptive response that may enhance post-exercise recovery and performance. Specifically, the observed T cell activation suggests a mobilization that could contribute to faster recovery by mitigating any exercise-induced immune suppression (KUHNS *et al*., 2006; OWEN *et al*., 2013). This justifies the fact that the present study found a significant increase in CD3 after the VO_2max test.

According to Nieman and Nehlsen-Cannarella (1994), CD4+ T lymphocytes are more sensitive to exercise than CD8+ T cells. This heightened response in CD4+ cells may facilitate immune surveillance and response to pathogens post-exercise, contributing to overall immune preparedness in athletes. Furthermore, CD8+ cells, known for their cytotoxic role, likely provide a complementary function by targeting cells potentially damaged during exercise. The balance between CD4+ and CD8+ T cells, therefore, could play a pivotal role in immune recovery post-exercise. Kostrzewa-Nowak and Nowak (2018), identified that after U-21 soccer players performed a stress test to determine VO_2max, an increased release of CD4+ T cells into the bloodstream occurred. Stating that progressive exertion during the stress test apparently induced an anabolic effect related to the differentiation and peripheral distribution of CD4 + T cells.

The present study found changes in the levels of CD3-CD4 and CD3-CD8 lymphocytes. The answer for this behavior may lie in the function of T lymphocyte subtypes, CD4+ T cells have the function of identifying and attacking any external agents and opportunistic infections that affect the human body (RUTERBUSCH *et al*., 2020). According to Swain and Mckinstry (2012) the plasticity of effector CD4+ T cells, points to importance for multidirectional immune responses (e.g., pathogen elimination & humoral response induction). CD8+ T cells in turn are cytotoxic and act in a specific way to eliminate infectious or neoplastic cells (MAIMELA *et al*., 2019).

Another cytotoxic subpopulation of lymphocytes is the Natural Killer (NK) cells, NK cells have antiviral potential (MANDAL; VISWANATHAN, 2015). In a review study, Arachchige (2021) highlights that CD56 T lymphocytes found in the blood are a subpopulation of NK cells bound to CD-16. CD-16 is

a molecule of the immunoglobulin superfamily (IgSF) that acts as a mediator of antibody-dependent cellular cytotoxicity (ADCC) (MANDELBOIM *et al*., 1999). The present study found significant elevation of CD56 and CD16-CD56 levels. According to Llavero *et al* (2021) due to their susceptibility to catecholamines (e.g., epinephrine) NK cells are highly responsive to exercise stress.

Among the findings of the present study, we identified a significant increase in CD-20 type B lymphocytes. The compound CD-20 is a non-glycosylated transmembrane phosphoprotein, playing a crucial role in modulating the activation and proliferation processes of B lymphocytes (PAUL, 2012). Gillum *et al*. (2017), analyzed the effect of physical exercise on salivary CD-20 cells in male subjects (20.3 ± 0.8 years old), the authors did not find significant differences between the pre and post exercise moments. The fact that the present study on athletes and blood cells can be explained by the differences between the studies, apparently the behavior of the immune system can be influenced by the level of physical activity (Gonçalves *et al*., 2020) and the type of sport practiced (ISAEV *et al*., 2018).

4.3 NEUTROPHILS

Our study identified a significant reduction in neutrophil levels immediately after the test. This aligns with findings by Walsh *et al*. (2011), where an acute reduction in neutrophils was linked to the body's response to minimize muscle damage. Such reduction likely reflects a mobilization of neutrophils to muscle tissues, aiding in inflammatory responses that protect against tissue damage (KAWANISHI *et al*., 2016).

Additionally, the observed neutrophil reduction may relate to oxidative damage during exercise (FERRER *et al*., 2009). According to Chatzinikolaou *et al*. (2014) and Fatouros and Jamurtas (2016), neutrophils infiltrate muscle fibers during recovery, producing free radicals as part of the repair process, which is essential for adaptation in musculoskeletal tissue. The movement of neutrophils to muscle tissue may thus play a direct role in musculoskeletal recovery and long-term adaptation (PYNE, 1994; PIZZA *et al*., 2005; LOCKHART; BROOKS, 2008). This indicates that neutrophils have a direct relationship with exercise stimulation in musculoskeletal tissue.

Beiter *et al* (2015) address in a review study that musculoskeletal injuries generated by strenuous exercise increase local inflammation, which may cause neutrophils to organize to generate intravascular extracellular neutrophil traps (NETs). Among their functions, NETs respond to tissue injury, and may contribute to the body's improvement in maintaining immune homeostasis by promoting protection against chronic inflammation. However, the authors state that the occurrence of NETs at extensive frequencies may favor cardiovascular damage, calling attention to care for elite athletes who are often subjected to strenuous exercise.

4.4 PRACTICAL APPLICABILITY

Based on our findings, monitoring immunological indicators during training periods could offer practical insights for optimizing athlete recovery and performance. For instance, tracking leukocyte and lymphocyte levels may guide the timing and intensity of training sessions, particularly following exhaustive

cardiorespiratory tests. By incorporating immunological monitoring, coaches and physiologists can develop tailored recovery plans to help mitigate immune suppression and reduce injury risk, especially in elite athletes facing high physical demands. Studies about competitions underscore the need for athletes to peak at the right time (MORGANS *et al.*, 2014). Therefore, we suggest using immunological monitoring not only to guide training loads but also as a preventive measure to maintain immune function, reduce injury risk, and optimize performance during critical phases of the competitive season.

4.5 LIMITATIONS AND SUGGESTIONS FOR NEW STUDIES

The present study has the following limitations: (i) not having a control group; (ii) we did not analyze food or sleep history; (iii) the fact that we did not perform measurements after the incremental test; and (iv) we analyzed only male athletes.

In this sense, the methodological limitations in a scientific study can significantly impact the interpretation and validity of its findings. The absence of a control group, for example, limits the ability to compare observed effects exclusively between athletes and non-athletes, hindering the generalization of results to broader populations and affecting the understanding of baseline immune responses. Additionally, the lack of control over variables such as diet and sleep complicate the distinction between the impact of exercise and the potential modulating effect of these factors, introducing potential biases into the data collected. The absence of additional measurements at various post-exercise intervals (e.g., 1h, 3h, 24h, 48h, and 72h after the test) restricts our understanding of the duration of immunological effects and can lead to limited interpretations of the immune response to strenuous exercise over time. Without these data, there is a risk of partial assessments that could either underestimate or overestimate the impact of exercise on the immune system. Finally, focusing exclusively on male athletes limits the applicability of findings to other populations, such as female athletes, who may have distinct immunological responses to exercise. Collectively, these methodological limitations suggest that the results should be interpreted with caution, as the validity of the findings may be influenced by uncontrolled external factors within this study.

Therefore, future studies must address these gaps to strengthen the robustness and applicability of scientific results across varied contexts.

5 CONCLUSION

This study concludes that in under-23 college soccer athletes, maximal cardiorespiratory capacity testing on a treadmill acutely promotes an increase in leukocyte and lymphocyte levels, along with a decrease in neutrophil levels. These immunological alterations may play a key role in the athletes' recovery processes and performance, as elevated leukocyte and lymphocyte counts suggest an adaptive immune response that could support muscle repair and readiness for subsequent training. Conversely, the reduction in neutrophils immediately after testing may indicate a regulatory response aimed at mitigating excessive inflammation in the short term, which is essential for balancing immune activity and recovery.

These findings support the initial objectives and hypothesis of the study, highlighting that maximal exercise induces a specific adaptive response in the immune system of young athletes. This adaptive response suggests that the immune system modulates itself to handle the demands of strenuous activity while also preparing for faster recovery, potentially contributing to the athletes' resilience against infections or injuries during intensive training phases.

Future studies should explore the chronic effects of repeated maximal exercise on immune markers in athletes, particularly investigating how these acute immune responses evolve over time and during competitive seasons. Such research would offer valuable insights into the long-term impact of immune adaptations on performance and could guide optimal training and recovery strategies to prevent immune suppression or overtraining risks among elite athletes.

Data Availability: The database for this study is publicly available at: https://figshare.com, under the Doi: 10.6084/m9.figshare.21636818.

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