Article published online: 2021-02-10

Oxidative Stress and Abnormal Tendon Sonographic Features in Elite Soccer Players (A Pilot Study)

Estresse oxidativo e características ultrassonográficas do tendão anormal em jogadores de futebol de elite (um estudo piloto)

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Rev Bras Ortop 2021;56(4):432–437.

Abstract

Objective Sound experimental data suggest that oxidative stress plays an important role in the pathogenesis of tendinopathies. However, this hypothesis in humans remains speculative given that clinical data are lacking to confirm it. Recently, a new methodology has allowed to quantify the oxidative stress in vivo by measuring the concentration of hydroperoxides of organic compounds, which have been utilized as an oxidative stress-related marker in several pathologic and physiologic conditions. Given the reliability of this test and the lack of information in subjects with tendinopathies, the aim of the present study was to assess the oxidative stress status in elite professional soccer players with and without ultrasonographic features of tendon damage.

Methods In 73 elite players, blood metabolic parameters were evaluated and oxidative stress was measured by means of a specific test (expressed as U-Carr units). Therefore, an ultrasonographic evaluation of the Achilles and patellar tendons was performed.

Results No significant relationships were observed between metabolic parameters and oxidative stress biomarkers. The Achilles and patellar tendons showed a normal echographic pattern in 58 athletes, and sonographic abnormalities in 15. The athletes with ultrasonographic alterations, compared to those with normal US picture, showed significantly higher U-Carr levels ($p = 0.000$), body mass index (BMI) values ($p = 0.03$) and were older ($p = 0.005$). The difference in U-Carr values among the subjects remained significant also after adjustment for age and BMI.

Conclusion The results of the present study support the hypothesis that oxidative substances, also increased at systemic and not only at local level, may favor tendon damage.

Level of Evidence IV (pilot study).

Keywords
- Achilles tendon
- football
- oxidative stress
- patellar tendon
- ultrasonography

received
April 18, 2020
accepted
September 17, 2020
published online
February 10, 2021

DOI https://doi.org/10.1055/s-0040-1721364.
ISSN 0102-3616.

Thieme Revinter Publicações Ltda., Rua do Matoso 170, Rio de Janeiro, RJ, CEP 20270-135, Brazil

Resumo

Objetivo Dados experimentais ultrassonográficos sugerem que o estresse oxidativo desempenha um papel importante na patogênese das tendinopatias. No entanto, essa hipótese permanece especulativa em humanos, dado que faltam dados clínicos para comprová-la. Recentemente, uma nova metodologia permitiu quantificar o estresse oxidativo *in vivo* medindo a concentração de hidroperóxidos de compostos orgânicos, que tem sido utilizada como um marcador relacionado ao estresse oxidativo em várias condições patológicas e fisiológicas. Dada a confiabilidade desse teste e a falta de informação em sujeitos com tendinopatias, o objetivo do presente estudo foi avaliar o status de estresse oxidativo em jogadores profissionais de elite com e sem características ultrassonográficas de dano tendinoso.

Métodos Em 73 jogadores de elite foram avaliados parâmetros metabólicos e o estresse oxidativo foi medido por meio de um teste específico (expresso como unidades U-Carr). Por isso, foi realizada uma avaliação ultrassonográfica dos tendões de Aquiles e patelar.

Resultados Não foram observadas relações significativas entre parâmetros metabólicos e biomarcadores de estresse oxidativo. Os tendões de Aquiles e patelar mostraram um padrão ecográfico normal em 58 atletas, e anormalidades ultrassonográficas em 15. Os atletas com alterações, em comparação com aqueles com quadro normal, apresentaram níveis significativamente mais elevados de U-Carr ($p = 0,000$), índice de massa corporal (IMC) ($p = 0,03$) e eram mais velhos ($p = 0,005$). A diferença nos valores de U-Carr entre os sujeitos permaneceu significativa também após ajuste por idade e IMC.

Conclusão Os resultados deste estudo corroboram a hipótese de que as substâncias oxidativas, também aumentadas a nível sistêmico e não apenas a nível local, podem favorecer danos no tendão.

Nível de Evidência IV (estudo piloto).

Palavras-chave

- tendão de Aquiles
- futebol
- estresse oxidativo
- tendão patelar
- ultrassonografia

Introduction

Several experiments performed in cell cultures and animals show that oxidative stress (OS) plays a major role in the pathogenesis of tendinopathies.[1] Indeed, mechanical overload stimulates the production of reactive oxygen species (ROS), which, in combination with lifestyle and hereditary factors, influence tendon integrity and contribute to degeneration.[2]

However, despite the experimental evidence, this hypothesis in humans remains speculative given that clinical data are lacking. Recently, a simple methodology has been introduced to evaluate ROS in the blood. The Diacron - reactive oxygen metabolites (d-ROMs) test can quantify the OS by measuring the concentration of hydroperoxides of organic compounds (lipids, proteins, nucleic acids), and it has been established as an OS-related marker in the clinic.[3]

By means of this method, an OS increase has been demonstrated in several pathologic conditions: cardiac heart failure, acute myocardial infarction, hypertension, peripheral artery disease, renal insufficiency, eclampsia, rheumatoid arthritis, knee osteoarthritis, pulmonary fibrosis, chronic lymphatic leukemia, hypothyroidism, hypopituitarism, diabetes, dyslipidemia, hyperuricemia, metabolic syndrome, obesity, as well as in physiologic situations (aging, menstrual cycle, menopause, strenuous exercise).[4–19]

Given the reliability of the d-ROMs test and the lack of information about subjects with tendinopathies, the aim of the present study was to assess the OS status in elite professional soccer players with and without ultrasonographic features of tendon damage.

Methods

The present observational pilot study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments; informed written consent for participation was obtained from each patient. Institutional ethics committee approval was not required due to the nature of the study.[20]

Elite professional soccer players (Italian second division), enrolled during two consecutive agonistic seasons (2017–2018 and 2018–2019), participated in the study.

At baseline, demographic and anthropometric data were collected. The height and weight of each participant were measured, and the body mass index (BMI) was then calculated. Standard blood tests were performed, including blood glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, uric acid, and albumin. Blood samples were collected in the morning, after an overnight fast, at least 2 days after a period of avoidance from training or official games.

The d-ROMs test was conducted by the Free Radical Analytical System (Diacron, Grosseto, Italy), as reported previously.[3] Briefly, in a pipette, 25 µL of the serum sample were mixed with an acetic acid buffered solution (pH 4.8), and chromogenic substrate was added to the mixture. The mixture was then centrifuged and incubated in the thermostatic block of the system. The absorbance was recorded at 505 nm. The measurement is expressed in U Carr, and 1 U Carr corresponds to 0.08 mg/dl H_2O_2 (hydrogen peroxide).

Therefore, players underwent an ultrasound (US) and color Doppler (CD) evaluation of the Achilles and patellar tendons, using a high-resolution, multi-frequency (6-15 MHz) linear array transducer (MyLab 30 Gold, Esaote, Genova, Italy). Following a standard protocol,[21] longitudinal and transverse scans of both tendons were taken. The presence of dishomogeneous hypo- or hyperechoic thickening, diffuse or focal, of the tendon, associated with loss of the normal fibrillar pattern and/or irregularity of the tendon margins, was considered as sign of degeneration.

According to an arbitrary, but suitable for clinical purpose, classification, based on the structural abnormalities, the tendons were then stratified for severity as "mild" (one area of disorganized echotexture, i.e., focal dishomogeneous area with loss of fibrillar pattern), "moderate" (some areas of disorganized echotexture, i.e., dishomogeneous hypo- or hyperechoic tendon damage with altered fibrillar pattern), and "severe" (diffuse disorganized echotexture and hypo- or hyperechoic areas with irregularity of tendon margins and/or calcifications).[21]

The presence of neovascularization was investigated by means of Color Doppler (CD) and graded as (0), (1 +), (2 +), (3 +) according to a semi-quantitative estimate of the number of vessels. When no vessels were visible, the estimation was 0. When there were one or two small vessels mostly in the anterior part of the tendon, the estimation was (+1). When there were several irregular vessels throughout the tendon, the estimation was (+2) to (+3).[21] To avoid artifacts, sensitivity was optimized for low flow, and the gain was set just below the noise level.

Statistical Analysis

Using the Pearson correlation coefficient, the relationship between U Carr units and anthropometric and biochemical variables was assessed. Therefore, the subjects were divided in 2 groups (with and without sonographic lesions) and compared. Data are reported as mean ± standard deviation. The two-sample Student t-test was used to compare continuous variables, when the distribution of data was normal; the Wilcoxon's rank sum test was used otherwise. The significance level was determined at $p < 0.05$.

Results

Seventy-three players were included in the study. Anthropometric and laboratory data are shown in ►**Table 1**. No significant relationship was observed between these parameters and OS bio-markers. The US evaluation of Achilles and patellar tendons showed a normal echographic pattern in 58 athletes, and sonographic abnormalities in 15 (10 Achilles and 5 patellar [9 mild and 6 moderate]).

Table 1 Demographic, anthropometric, and metabolic characteristics of participants

	Mean ± SD	Correlation with U-Carr (r-value)
Age (years)	25.3 ± 5.1	0.1171
Height (cm)	182.1 ± 5.7	0.0451
Weight (kg)	75.1 ± 5.2	0.0317
BMI	22.6 ± 1.2	0.1004
Blood glucose (mg/dL)	76.6 ± 9.5	0.0291
Uric acid (mg/dL)	5.5 ± 0.7	0.0738
Cholesterol total (mg/dL)	173.1 ± 26.8	0.1178
Colesterol HDL (mg/dL)	55.7 ± 11.1	0.1295
Triglycerides (mg/dL)	83.7 ± 46.5	0.0819
Albumin (g/dL)	4.5 ± 2.3	0.0886
Oxydative stress (U-Carr)	320.1 ± 67	

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; SD, standard deviation.

Neovessels were found in 4 athletes (grade 1 in 3 players, grade 2 in 1). Out of these 15 subjects, 4 had suffered of tendinopathy in the past but had healed completely after conservative treatments.

As shown in ►**Table 2**, the athletes with sonographic lesions, compared to those with normal US picture, were older ($p = 0.005$) and showed significantly higher levels of U-Carr ($p = 0.000$) and BMI values ($p = 0.03$). The difference in U Carr remained significant also after adjustment for age and BMI.

Discussion

Tendinopathies recognize several etiopathogenetic factors. In particular, mechanical overload, by means of the increased metabolism, hyperthermia, and repetitive ischemia/riperfusion, can expose the tendons to OS. A further possibility is that the tendons are indirectly influenced by changes in ROS metabolism in other nearby tissues, such as in exercising muscles. Indeed, resting muscles generate both intra- and extra-cellular superoxide, the production of both being enhanced during contraction.[1,22] In turn, the OS, in combination with lifestyle and hereditary factors, may orchestrate tendon degeneration.[2]

Several experiments performed in animals and cell cultures support the OS hypothesis. Rats submitted to patellar window injury from weeks 3 to 5 postsurgery received 3 weekly subcutaneous injections of H_2O_2 into the tendon. On week 6, specimens were harvested for histology. In comparison to controls receiving saline, the rat treated with H_2O_2 showed

Table 2 Demographic, anthropometric and metabolic characteristics of players with and without US alterations

	US alteration	No US alterations	*p*
Number	15	58	
Age (years)	28.6 ± 5.9	24.5 ± 4.5	0.005
Height (cm)	180.2 ± 5.3	182.6 ± 5.8	0.1
Weight (kg)	74.7 ± 4.8	75.2 ± 5.4	0.7
BMI	23.2 ± 1	22.5 ± 1.2	0.03
Blood glucose (mg/dL)	80.6 ± 8.1	75.6 ± 9.6	0.06
Uric acid (mg/dL)	5.8 ± 6	5.5 ± 7.6	0.1
Total Cholesterol (mg/dL)	183.9 ± 17.9	170 ± 28.2	0.08
HDL Cholesterol (mg/dL)	56.5 ± 13.5	55.5 ± 10.5	0.7
Triglycerides (mg/dL)	95.2 ± 85.8	80.8 ± 29.7	0.2
Albumin (g/dL)	4.5 ± 2.1	4.5 ± 0.2	0.6
Oxydative stress (U-Carr)	393 ± 65.5	301.3 ± 53.5	0.000*

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; US, ultrasound.
*p = age and BMI adjusted.

impaired tendon healing and developed tendinopathic changes.[23]

Guaraldo et al.[24] submitted young and old rats to swimming exercise, and found an increased OS activity, which was more pronounced in old animals. Yuan et al.,[25] exposing cultured human rotator cuff tendon fibroblasts to H_2O_2 to create an *in vitro* OS situation, observed a pronounced apoptosis, via several pathways including the release of cytochrome-c from mitochondria to cytosol and the activation of caspase-3.

In Lee's et al.[26] experiments, tendon-derived stem cells were exposed to oxidative damage. H_2O_2 promoted apoptosis, suppressed cells viability and migration, and inhibited clonogenicity; pro-antocianine had a protective effect, mediated by an increased expression of glutamate-cysteine ligase regulatory subunit, hemoxygenase-1, and NADPH: quinone oxidoreductase via upregulating the Nrf-2 signaling pathway.[27]

An elegant study by Chen et al.[28] showed that H_2O_2 treatment of human tendon stem cells resulted in ROS accumulation and loss of self-renewal capacity, cell stemness, and differentiation capability. Starvation and the mTOR inhibitor rapamycin increased autophagic activity, reduced ROS generation, and maintained self-renewal capacity, cell stemness, and differentiation capability. Moreover, these protective effects of starvation and rapamycin were curtailed by autophagy inhibition. These results indicate that autophagy protected human tendon stem cells against OS-induced damages through suppression of ROS accumulation.

These studies support the hypothesis that OS is deleterious for cell recruitment and tendon healing, which explains why antioxidant supplementation in the early stages is beneficial.[27]

Despite the experimental evidence, applying the OS hypothesis to clinical tendinopathies remains speculative given that data in humans are lacking to confirm it. The d-ROMs test allows to quantify the OS *in vivo* and has been used successfully in a wide range of pathologic and physiologic conditions: namely, cardiac heart failure, acute myocardial infarction, hypertension, peripheral artery disease, renal insufficiency, eclampsia, rheumatoid arthritis, knee osteoarthritis, pulmonary fibrosis, chronic lymphatic leukemia, diabetes, dyslipidemia, hyperuricemia, obesity, metabolic syndrome, hypothyroidism, hypopituitarism, aging, menstrual cycle, and menopause.[4–19] Regarding the present research, it is important to remark that increased OS has been found during strenuous exercise.[13,16]

Our study is the first, to our knowledge, to address the relationship between OS and tendon disorders in humans. In athletes belonging to a professional soccer society, we found that OS was neither related to age nor to blood glucose, total and HDL cholesterol, triglycerides, uric acid, and albumin. Although the literature reports increased OS in elderly individuals[17,22] and in subjects with increased blood levels of metabolic parameters,[12,13,16] this relationship could be observed in our athletes, whose biochemical parameters, in agreement with Tamae et al.,[29] fell into a small range of juvenile values.

The main finding of the present study was the observation of higher OS levels in players with ultrasonographic tendon abnormalities. This result supports the hypothesis that oxidative substances, also increased at systemic and not only at local level, may favor tendon damage. The origin of the increased ROS blood levels remains elusive. We suppose that the athletic overuse contributed to ROS production, given that the athletes with ultrasonographic abnormalities were older in comparison with those with a normal US pattern and, therefore, had submitted their muscles and tendons to strenuous exercise for more years. In this framework, the higher BMI values could be included, because of the increased load on the lower limb tendons.[2] Alternatively, it could be hypothesized that the OS could be related to a deficit of antioxidant enzymes, such as superoxide dismutase, glutathione peroxidase and catalase.[29] Indeed, ROS are normally produced by muscles and tendons and play a physiologic role (i.e., cell signaling), but when an imbalance between the systems occurs, their level increases and they work as an aggressor.

Taking everything into account, it must be underlined that the observation of US abnormalities does not mean *per se* a clinical pathology. Indeed, these lesions do not imply a certain risk for future symptomatic tendinopathies and may remain stable or sometimes regress to normality.[30]

Given the presence of several limitations, the above speculation must be viewed with criticism and caution, and the results must be considered as preliminary. The sample size was small and the design was cross-sectional;

because only men were included, the results cannot necessarily be extrapolated to women.

The US evaluation was performed by a single researcher, and this can be a limiting factor because the component of subjectivity inherent to the US methodology. However, all the US examinations were performed by a physician with 15 years of experience in musculo-skeletal ultrasonographic methodology. In this respect, it has been demonstrated that the inter-observer's reliability in detecting tendon pathologic findings is excellent.[22]

Finally, and more importantly, the measurement of blood levels of anti-oxidants, and of OS markers other than d-ROMs, was not performed.

For these reasons, this must be considered as a pilot study, which, however, paves the way for more in-depth research. Actually, it needs confirmation from a prospective trial in a larger population, measuring additional markers, mainly those exploring the individual antioxidant status. In case our results should be confirmed, it will be possible to target a subset of individuals in whom OS plays a pathogenetic role in tendon degeneration. Moreover, it would be interesting to assess whether these subjects could benefit from an antioxidant supplementation, whose efficacy has been clearly demonstrated in different conditions and in strenuous exercise.[31]

Conclusion

The results of the present study support the hypothesis that oxidative substances, also increased at systemic and not only at local level, may favor tendon damage.

Note

The present work was developed at the Delfino Training Center (Città Sant'Angelo [Pescara]) and Centro Analisi Biochimiche dello Sport, (Lanciano [Chieti]), Italy.

Financial Support

There was no financial support from public, commercial, or non-profit sources.

Conflict of Interests

The authors have no conflict of interests to declare.

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