MALE ADULT RAT SPERM PARAMETERS AFTER SKELETAL MUSCLE INJURY

PARÂMETROS ESPERMÁTICOS DE RATOS ADULTOS APÓS LESÃO MÚSCULO-ESQUELÉTICA

PARÁMETROS ESPERMÁTICOS DE RATAS ADULTAS DESPUÉS DE LESIÓN MÚSCULO-ESQUELÉTICA

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ABSTRACT

Introduction: Skeletal muscle injuries stimulate a systemic inflammatory response which may interfere in species reproduction. Objective: To evaluate the effects caused by skeletal muscle injuries on the inflammatory response and sperm parameters of male adult rats. Methods: The sample group was composed of 30 Wistar rats distributed evenly across control and injury groups. Muscle injury was induced by bruising, caused by the release of a 200 g weight from a height of 30 cm onto the gastrocnemius muscle. Blood (CBC and damage/muscle inflammation markers), muscle (oxidative stress) and gonad (sperm parameters) samples were collected 72h after the injury. Results: The muscle injury increased monocytes, creatine kinase, C-reactive protein, reactive oxygen species (ROS) concentration and lipid peroxidation. In contrast, the injury reduced antioxidant capacity against peroxyl radicals (ACAP), membrane integrity (36%) and sperm acrosome (33%). Membrane integrity and acrosome ($p<0.05$) correlate directly with ACAP ($\rho=0.602$; $\rho=0.513$ respectively) and inversely with monocytes ($\rho=-0.703$; $\rho=-0.635$, respectively), creatine kinase ($\rho=-0.450$; $\rho=-0.603$), C-reactive protein ($\rho=-0.511$; $\rho=-0.703$) and parameters of oxidative stress (ROS $\rho=-0.703$; $\rho=-0.635$; lipid peroxidation $\rho=-0.494$; $\rho=-0.559$). Conclusion: The acute systemic inflammatory response arising from skeletal muscle injury interferes in the male reproductive cell organelles (membrane and acrosome). ***Level of Evidence V; Experimental study.***

Keywords: Reproduction; Spermatozoa; Muscle, skeletal; Inflammation; Oxidative Stress.

RESUMO

Introdução: As lesões músculo-esqueléticas estimulam uma resposta inflamatória sistêmica que pode interferir na reprodução das espécies. Objetivo: Avaliar os efeitos causados pelas lesões músculo-esqueléticas sobre a resposta inflamatória e os parâmetros espermáticos de ratos machos adultos. Métodos: O grupo da amostra foi composto por 30 ratos Wistar uniformemente distribuídos nos grupos controle e grupo lesionado. A lesão muscular foi induzida por meio de contusão, causada ao se soltar um peso de 200 g de uma altura de 30 cm sobre o músculo gastrocnêmio. Foram coletadas amostras de sangue (hemograma completo e marcadores de danos e inflamação muscular), músculo (estresse oxidativo) e gônadas (parâmetros espermáticos) 72 horas após a lesão. Resultados: A lesão muscular aumentou os monócitos, creatina quinase, proteína C-reativa, concentração de espécies reativas de oxigênio (ROS) e lipoperoxidação. Por outro lado, a lesão reduziu a capacidade antioxidante contra os radicais peroxil (ACAP), a integridade da membrana (36%) e o acrossoma espermático (33%). A integridade da membrana e o acrossoma ($p<0,05$) se correlacionaram diretamente com ACAP ($\rho=0,602$; $\rho=0,513$ respectivamente) e inversamente com os monócitos ($\rho=-0,703$; $\rho=-0,635$, respectivamente), creatina quinase ($\rho=-0,450$; $\rho=-0,603$), proteína C-reativa ($\rho=-0,511$; $\rho=-0,703$) e parâmetros de estresse oxidativo (ROS $\rho=-0,703$; $\rho=-0,635$; lipoperoxidação $\rho=-0,494$; $\rho=-0,559$). Conclusão: A resposta inflamatória sistêmica aguda decorrente da lesão músculo-esquelética interfere nas organelas das células reprodutivas masculinas (membrana e acrossoma). ***Nível de evidência V; Estudo experimental.***

Descritores: *Reprodução; Espermatozoides; Músculo esquelético; Inflamação; Estresse oxidativo.*

RESUMEN

Introducción: Las lesiones músculo-esqueléticas estimulan una respuesta inflamatoria sistémica que puede interferir en la reproducción de las especies. Objetivo: Evaluar los efectos causados por las lesiones músculo-esqueléticas sobre la respuesta inflamatoria y los parámetros espermáticos de ratas macho adultas. Métodos: El grupo de la muestra fue compuesto por 30 ratas Wistar distribuidas uniformemente en los grupos control y grupo lesionado. La lesión muscular fue inducida por medio de contusión, causada al soltarse un peso de 200 g desde una altura de 30 cm sobre el músculo gastrocnemio. Fueron colectadas muestras de sangre (hemograma completo y marcadores de daños e inflamación muscular), músculo (estrés oxidativo) y gónadas (parámetros espermáticos) 72 horas después de la lesión. Resultados: La lesión muscular aumentó los monocitos, creatina quinasa, proteína C reactiva, concentración de especies reactivas de oxígeno (ROS) y lipoperoxidación. Por otro lado, la lesión redujo la capacidad antioxidante contra los radicales peroxilo (ACAP), la integridad de la membrana (36%) y el acrosoma espermático (33%). La integridad de la membrana y el acrosoma ($p<0,05$) se correlacionan directamente con ACAP ($\rho=0,602$; $\rho=0,513$ respectivamente) e inversamente con los monocitos ($\rho=-0,703$; $\rho=-0,635$, respectivamente), creatina quinasa ($\rho=-0,450$, $\rho=-0,603$), proteína C reactiva ($\rho=-0,511$; $\rho=-0,703$) y parámetros de estrés oxidativo (ROS $\rho=-0,703$; $\rho=-0,635$; lipoperoxidación $\rho=-0,494$; $\rho=-0,559$). Conclusión: La respuesta inflamatoria sistémica aguda proveniente de la lesión músculo-esquelética interfiere en los orgánulos de las células reproductivas masculinas (membrana y acrosoma). ***Nivel de evidencia V; Estudio experimental.***

Descriptores: *Reproducción; Espermatozoides; Músculo esquelético; Inflamación; Estrés oxidativo.*

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INTRODUCTION

The testosterone signaling present over spermatogonia in the seminiferous tubules in sync with the paracrine activity of different substances promotes proliferation and differentiation of sperm cells.[1] The spermatogenesis differs from the development of other cells by the occurrence of meiosis, chromatin remodeling and formation of specialized structures such as the acrosome and the flagellum.[2,3] In epididymis, these cells mature and are subsequently propelled by the vas deferens wherein they are bathed by the seminal plasma, which is characterized by intense antioxidant activity. However, this process may undergo changes in its mechanisms as a result of an inflammatory process, resulting in an imbalance in oxidative stress (OS) parameters of this region and consequent dysfunction of fertilizing capacity.[4-9]

The inflammatory phase of musculoskeletal injuries usually occurs in the first three days, triggering a series of physical and chemical events that promote tissue repair.[10] This process activates circulating leukocytes,[11] vascular permeability rise, chemotaxis and diapedesis of leukocytes into the damaged tissue,[10,12] with an ensuing increase in the concentration of reactive oxygen species (ROS) and lipoperoxidation (LPO). Muscle damage is usually observed systemically by increases in plasma concentrations of creatine kinase (CK), lactate dehydrogenase (LDH)[13,14] and acute phase proteins such as C-reactive protein (CRP).[15]

Changes in fertilizer capacity may occur as a result of systemic effects, where the resulting OS imbalance is appointed as a factor in infertility scenario[4-9]. Excessive concentrations of ROS may cause sperm deficiency during spermatogenesis and/or through the vas deferens. The inflammatory phase of musculoskeletal injuries may cause systemic effects, as a result of a state of OS, caused by the release of inflammatory mediators and leukocytosis,[10,12,13,16] which may interfere with sperm structure and function. The aim of this study was to evaluate the effects of skeletal muscle injury on the inflammatory response and sperm parameters in adult male rats.

METHODS

Animals and experimental groups

The study used 30-week-old adult male Wistar rats that weighed between 300 and 400g. The animals were kept in cages with three animals in light/dark cycles of 12h and temperature between 21ºC and 24ºC. Food and water were given *ad libitum* during the entire experimental protocol. For sample calculation, a sample size of 30 animals in the study was estimated.[16] The calculation used had a 90% detection rate for distinguishing 100% difference between means of CK and results with 110% standard deviation for α=0.05. After anesthesia, the animals were randomized into Control Group (CG, n=15) and Lesion Group (LG, n=15).

Injury protocol

The right gastrocnemius muscle was injured by mechanical crushing[16]. Before the procedure, all animals were shaved for better muscle exposition and anaesthetized with intraperitoneal injections of ketamine hydrochloride (80mg/kg) and xylazine (15mg/kg). Then, the animals were placed at the base of the equipment in the prone position for maximum knee and ankle extension. The lesion was induced by a metal mass (200g) released from a height of 30cm[16] Rats from the CG were anesthetized and handled in the same way, but without injury induction. No bone fracture was verified at the moment of injury or after dissection.

Tissue sampling

Animals were euthanized by decapitation 72h after injury protocol and blood, muscle, gonads and epididymis were collected. Blood was collected in heparinized vacutainers and centrifuged at 3000×g for 15min at 4°C in order to separate blood cells from plasma, which were transferred to micro tubes stored with the muscles in Ultrafreezer -80 until analysis. For assessment of semen parameters, epididymis and a portion of vas deferens were collected. To facilitate the departure of sperm, the tissue was immersed in 1mL of half M2 and disrupted with the aid of a needle (18G) in 24 sterile culture plates. The evaluation of sperm quality was carried out after incubation of samples for 10min at 37°C.[17]

Evaluation of Hemograms, markers of inflammation, muscle damage and parameter of oxidative stress

Erythrocyte and total leukocyte counts were performed in a veterinarian automatic cell counter (Sysmex®, model pocH-100iV Diff). Leukocyte differential was obtained from the fabrication of fresh and stained blood smears with subsequent microscopic analysis.[11] Plasma activities of CK and LDH as well as the plasma concentration of CRP were spectrophotometrically determined.[16]

The total concentration of ROS and antioxidant capacity against peroxyl radicals (ACAP) were determined using a reaction media containing (30mM HEPES, 200mM KCl, and 1mM $MgCl_2$ adjusted to pH 7.2). For ROS analysis 2'.7'-dichlorofluorescein diacetate (H_2DCF-DA; Invitrogen TM) was used at a final concentration of 40μM. Total fluorescence production was calculated by integrating the fluorescence units (FU) over time and results were expressed as ROS=relative area. ACAP analysis used 2.2'-azobis (2-methylpropionamidine) dihydrochloride (20mM ABAP; Aldrich TM) as a ROS generator after thermal decomposition at 37ºC using 488ηm (absorption) into 60min, and results were expressed as 1/relative area with/without ABAP.[18]

The Fox assay (ferrous oxidation/xylenol orange method) was performed to measure lipoperoxidation (LPO).[16] Frozen tissues were homogenized in methanol (5:1) and centrifuged at 1000xg for 10min at 4°C. Lipoperoxidation was determined using a reaction media containing 1mM $FeSO_4$, 0.25M H_2SO_4 and 1mM xylenol orange. The absorbance was measured at 550ηm in a microplate reader after 4h of incubation at 37°C. Cumenehydroperoxide (1.75nM) was employed as a standard. LPO values were expressed in ηmoles CHP/g of wet tissue.

Analysis of sperm parameters

The total sperm motility was assessed in a 10μL aliquot of semen from a 200x magnification in a bright-field heated optical microscope (Olympus BX41-PH-III, SP, Brazil). The motility was determined by the percentage of motile cells identified in three separate fields of the microscope with scale from 0 to 100%.[17]

Membrane integrity was assessed by fluorescent probes of carboxyfluorescein diacetate and Propidium Iodide (IP).[19] Evaluation was performed with an epifluorescence microscope with a magnification of 400x (Olympus BX51, SP, Brazil). After 200 sperm count per slide, sperm cells presenting green fluorescence were considered intact.

Mitochondrial functionality was assessed using Rhodamine 123 along with IP probes. Two hundred cells were analyzed with an epifluorescence microscopeata magnification of 400x (Olympus BX51, SP, Brazil). Cells that presented the intermediate piece with an intense green fluorescence were considered with functional mitochondria.[20]

The assessment of acrosome integrity was based on a technique described with subsequent modifications.[21] Initially, 20μL of semen was added and a smear slide was made. After drying, an aliquot IP 20μL was added to the swab and then dried. Slides were submerged in absolute ethyl alcohol 95.55% (Sigma Chemical Company, MO, USA) for 5min and then washed in PBS. In a dark room, 20μL de Lectin from Arachishypogaea FITC Conjugate (20mg/mL) (Sigma Chemical Company, MO, USA) was added to the samples for 10min. Subsequently, the slides were washed in deionized water and drained. The slides were examined with an immersion epifluorescence microscope with a magnification of 1000x (Olympus

BX51, SP, Brazil). After 200 sperm count per slide, cells that had green fluorescence in the acrosome were considered with integrity acrosomes.

The evaluation of sperm morphology consisted of verifying all changes of sperm acrosome, head, midpiece and tail. For this purpose, a double staining was carried out by eosin and nigrosin, adding a drop of the coloring solution of 10μL in a drop of semen of 10μL. After 1min, some smear was made and when dried, a count of 200 cells was performed in optical microscope with phase contrast in immersion (Olympus BX41-PH-III SP, Brazil) [22]

DNA integrity was verified by acridine orange probe, with assessments being performed by an epifluorescence microscope with a magnification of 400x. Two hundred sperms were assessed per sample. Cells that showed green fluorescence were considered with normal DNA (double-stranded), whereas red or orange cells were considered with denatured DNA (monocatenary).[23]

This study was approved by the Ethics Committee on Animal Use from the Federal University of Rio Grande (CEUA/FURG #P034/2012 addendum 002/2013).

Statistical analysis

Continuous variables are reported as means (X) ± standard deviation (SD). All continuous variables were tested for normal distribution with the Kolmogorov-Smirnov test. Normal distribution data were compared by "t" and nonparametric distribution by Mann Whitney tests. The Pearson (*r*) and Spearman (ρ) correlations were applied when appropriate. p<0.05 was considered to be statistically significant.

RESULTS

Results of haematological parameters in control and injury groups 72h after application of the Protocol are shown in Table 1. Hematocrit, erythrocytes, hemoglobin, platelets and total plasma proteins were similar among groups. The animals submitted to the injury protocol increased by 17% the total leukocytes (p=0.013). This result was due to an increase of neutrophils (p=0.02) and monocytes (p<0.001), which showed a relative increase of 50% and 160%, respectively. Eosinophils, Lymphocytes and basophils (<1%, data not presented) did not differ between groups.

Results of the OS parameters and plasma concentrations of inflammatory muscle markers are shown in Figure 1. The LG showed an increase in the concentration of ROS (p<0.001, Figure 1A), in LPO (p<0.001, Figure 1B) and a reduction in the ACAP (p<0.001, Figure 1C) in relation to the CG. Musculoskeletal injuries increased by 45% the plasma levels of CK (p<0.001, Figure 1D) and 82% in relation to the LDH (p<0.001, Figure 1E) in relation to the CG. The CRP raised more than twice the LG (p<0.001, Figure 1F) in relation to the CG.

Variables of the fertilizing capacity 72h after experimental protocol are shown in Table 2. Motility parameters, sperm morphology, mitochondria function and DNA integrity were similar between groups. The LG showed a reduction in membrane integrity (p=0.002) and in acrosome (p<0.001) when compared to the CG.

Table 1. Hematological variables at the end of the experimental protocol.

Hematologic variable	Unit	Control Group (n=15) X ± SD	Lesion Group (n=15) X ± SD	p
Hematocrit	%	46.1 ± 1.6	46.1 ± 1.5	0.764
Erythrocytes	$x10^5/mm^3$	8.82 ± 0.39	8.84 ± 0.35	0.878
Hemoglobin	g/dL	15.7 ± 0.6	15.8 ± 0.5	0.766
Platelets	$x10^3/mm^3$	661 ± 109	611 ± 125	0.301
TPP	g/dL	6.62 ± 0.31	6.64 ± 0.26	0.846
Leukocytes Total	$x10^3/mm^3$	5710 ± 1042	6667 ± 961	0.013
Neutrophils	$x10^3/mm^3$	1254 ± 649	1876 ± 735	0.021
Monocytes	$x10^3/mm^3$	77 ± 44	202 ± 99	<0.001
Eosinophils	$x10^3/mm^3$	134 ± 94	141 ± 103	0.842
Lymphocytes	$x10^3/mm^3$	4245 ± 936	4451 ± 865	0.536

Data are reported as means (X) ± standard deviation (SD). TPP: Total Plasma Proteins.

The weight of animals was similar between groups at the end of the study (p=0.208, CG: 363 ± 23g vs. LG: 353 ± 24g). The concentration of ROS correlated negatively with the membrane integrity (Figure 2A) and with the acrosome (Figure 2B). LPO also presented this behavior (Figure 2C and 2D) in relation to the fertilizing capacity of the variables. ACAP was positively correlated with changes in membrane integrity (Figure 2E) and with the acrosome (Figure 2F). CK (Figure 2G and Figure 2H), PCR (Figure 2I and Figure 2J) and monocytes (Figure 2K and Figure 2L) inversely correlated with the integrity of the membrane and acrosome.

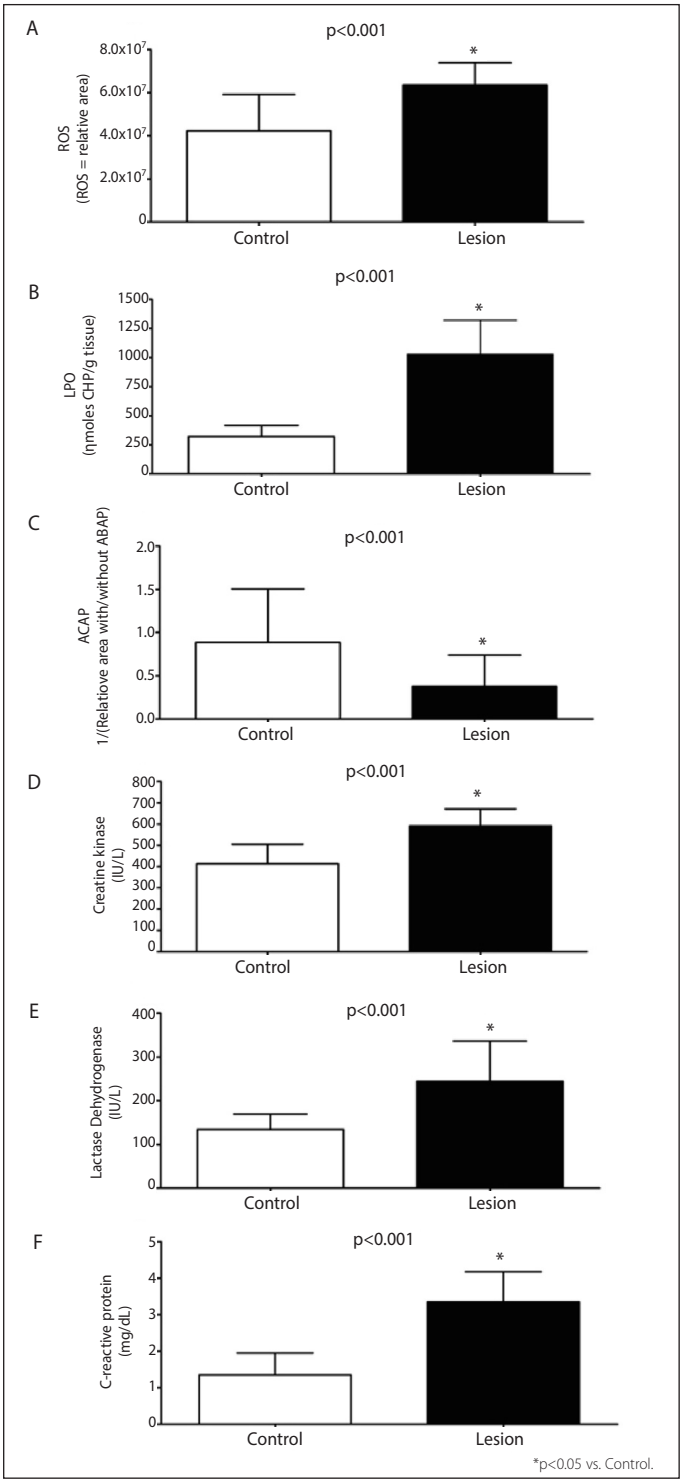

Figure 1. Parameters of oxidative stress and markers of damage and muscle inflammation. Parameters of oxidative stress, muscle damage and inflammation. Data are reported as means ± standard deviation (SD). ROS: reactive oxygen species; LPO: lipid peroxidation; ACAP: antioxidant capacity against peroxyl radicals.

Table 2. Sperm parameters.

Variable	Unit	Control Group (n=15) X ± SD	Lesion Group (n=15) X ± SD	p
Motility (%)	%	85.6 ± 9.6	85 ± 7.3	0.838
Membrane (%)	%	66.8 ± 27.4	31.2 ± 29.4	0.002
Mitochondria (%)	%	84.1 ± 9.2	79.9 ± 22.2	0.491
Acrosome (%)	%	83.9 ± 17.7	51.2 ± 18.5	0.001
Morphology (%)	%	95.7 ± 4.1	97.6 ± 1.5	0.091
DNA (%)	%	98.2 ± 4.3	99.1 ± 3.5	0.562

Data are reported as means (X) ± standard deviation (SD). DNA: Deoxyribonucleic acid.

DISCUSSION

The main discovery of the study is that musculoskeletal injuries interferes sperm parameters (reduction of membrane integrity and acrosome). Changes in hematological concentrations of white blood cells and plasma markers of muscle damage 72h after injury protocol are results already demonstrated in previous studies.[11,16] These changes occur because of the rapid necrosis of myofibrils and the production of proinflammatory cytokines that activate the innate immune response.[24] Neutrophilia occurs immediately after injury with these cells migrating to the damaged tissue,[25] playing an essential role in the repair of the production and release of ROS specific proteases and cytokines that facilitate the phagocytosis and the recruitment of circulating monocytes.[26] Monocytes, in turn, also migrate to the damaged tissue, where they differentiate into macrophages (increasing the concentration of these cells already resident in the tissue) and perform phagocytosis of damaged myofibrils,[25,26] phagocytosing, eventually, viable damaged cells possibly causing secondary damage muscle.[26]

CK and LDH are muscle damage biomarkers which increase as a result of the primary lesion (compromised muscular structure directly by trauma) and effects of OS, demonstrated by the excessive increase in the generation of ROS[14,16,27], ACAP reduction and increase of LPO,[16] with resultant damage to macromolecular structures. The interaction of these mechanisms results in lipoperoxidation or myocyte membrane damage, causing leakage of the mitochondrial metabolic enzymes and sarcoplasm to blood.[14] CRP is an inflammatory marker of acute phase and nonspecific; in hepatocytes[28] its production is stimulated by IL-6. In the adipose tissue and mononuclear blood cells its production is stimulated by TNF-α and IL-1β.[15] These mechanisms explain in part the correlation of these markers with changes in sperm acrosome and membrane found.

Systemic inflammation exerts inhibitory effects on spermatogenesis due to the action of inflammatory cytokines and ROS on the hypothalamic-pituitary-Leydig axis, circular axes and seminiferous epithelium interfering with the fertilizing capacity.[4] These effects may be more pronounced in the seminiferous epithelium during VIII-IX phases by the specific sensitivity of this stage and by the action of inflammatory mediators in the activity of Sertoli cells by inhibiting the expression of essential proteins to the structure of the sperm.[5] The systemic inflammation increases IL-6 expression, which interacts with TNF-α and decreases the secretion of testosterone by Leydig cells and transferrin Sertoli cells with effects on the signaling spermatogenesis.[6] The inflammatory cytokine phase also modulates the expression of cyclooxygenase-2 and prostaglandin by Leydig and Sertoli cells, interfering with the production of androgens and glucose uptake by these cells, affecting signaling and energetic metabolism of the spermatogenic process[7]. Thereby, dysfunctions in this phase may be attributed to the high production of genes related to inflammation of the systemic circulation and therefore, in the testis and epididymis, exerting adverse effects on developing and maturing sperm.[8] In this study, systemic inflammation resulted from the interaction of different evaluated mechanisms thereby changing the membrane and acrosome sperm quality.

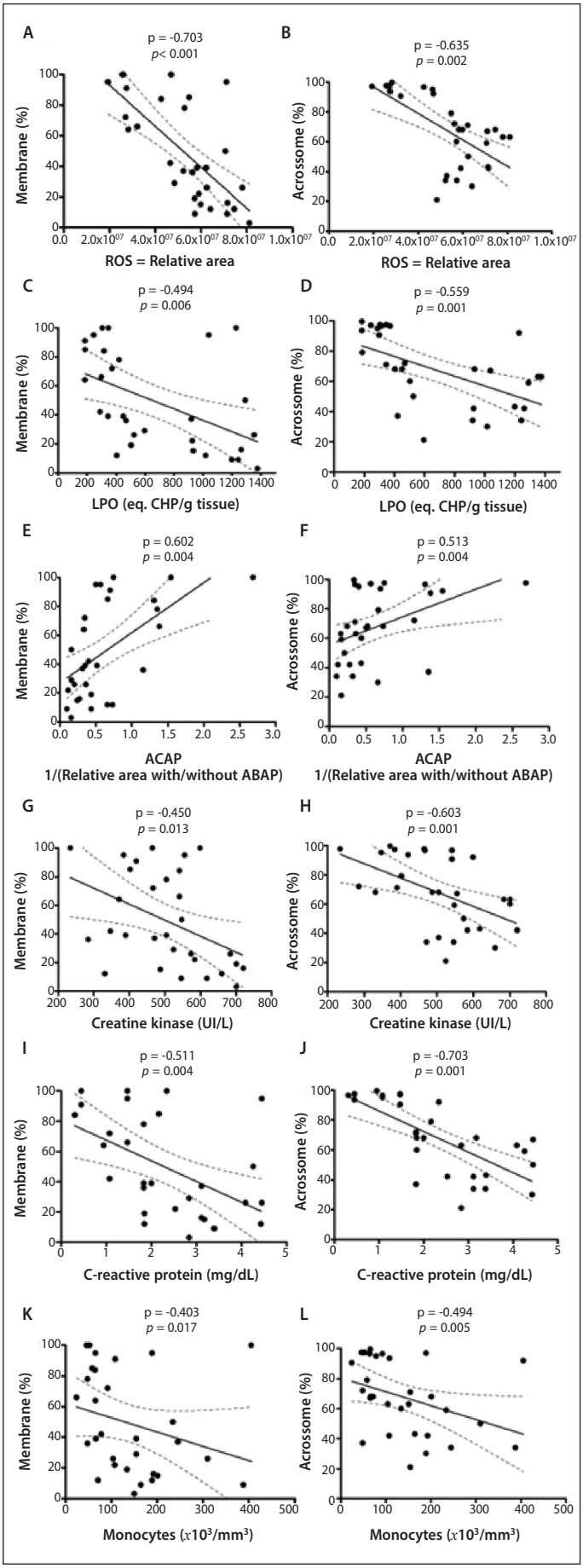

Figure 2. Correlations between sperm parameters, oxidative stress and inflammatory markers and leukocytosis. Correlation between parameters of oxidative stress, muscle damage and inflammation with changes in fertilizer capacity. ROS: Reactive Oxygen Species; LPO: Lipid Peroxidation; ACAP: Antioxidant Capacity Against Peroxyl Radicals.

Modifications of these sperm parameters and the interaction with the evaluated molecules also occur due to the high degree of conformation of these markers in their binding sites with these molecules having highly reactive cysteines when in the presence of OS.[15] This state is potentially present in the reproductive organs, where leukocytes and sperm are considered the main producers of ROS in this location.[2,3] In contrast to the ROS effects, seminal plasma has antioxidant systems that maintains the functionality of the spermatozoa.[9] In the present study, this balance was possibly affected by the rise of ROS concentrations in the epididymis, resulting from the interaction of systemic inflammation at the site, which may cause damage to sperm membrane via lipoperoxidation. Nevertheless, these cells are unable to restore their membrane after damage.[29] OS also activates protein kinase C signaling, resulting in a dysfunction in the activity of Ca^{2+} channels, and generating premature acrosomal and exocytosis, leading to dysfunction and consequent reduction of fertilization capacity.[30] In this context, the systemic action of inflammatory markers and optimization of ROS concentration in tissue may lead to an imbalance between the oxidant/antioxidant systems affecting sperm structure with consequent impairment of its fertilizing capacity.

The absence of measures of the central and peripheral temperatures, as well as evaluations of cortisol, testosterone and plasma interleukin (IL-6 and TNF-α) levels are limitations of this study. The gonadal OS measures also contribute to a better understanding of the results found.

CONCLUSIONS

The present study demonstrates that the inflammatory phase of musculoskeletal injuries by bruise causes a state of muscle oxidative stress and leukocytosis, increases markers of systemic inflammation and muscle damage as well as reduces sperm variables. The integrity of the membrane and the sperm acrosome are decreased, and these measures are related to oxidative stress parameters and plasma markers of muscle damage and systemic inflammation.

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