Biochemical biomarkers are not dependent on physical exercise in patients with spinal cord injury

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ABSTRACT

Aims: This work presents an evaluation of the impact of physical exercise of the upper limbs in patients with muscular atrophy in the lower limbs by analysis of specific biomarkers.

Methodology: It is a cross-sectional study. Patients were recruited using convenience sampling: control group (C: n = 12) and two groups of wheelchair users: non-athletes (NAth: n = 12) and athletes (Ath: n = 13, professional basketball players). Plasmatic biomarkers analyzed: fibrinogen, TBARS and NO. Comparisons were assessed by one-way ANOVA and Newman–Keuls Multiple Comparison post-hoc.

Results: Plasma fibrinogen values were not different between Ath (3.67 ± 0.44 g/L) and NAth (3.44 ± 0.38 g/L) groups. It was observed difference between fibrinogen levels from both wheelchair user groups (Ath and NAth) when comparing to control group (C: 2.27 ± 0.08 g/L) and standard values of fibrinogen (1.8 g/dL–3.1 g/dL). The TBARS values were not different between the wheelchair users Ath (3.21 ± 0.24 nmol/mL) and NAth (3.66 ± 0.27 nmol/mL). Independently of practicing physical activity, the TBARS values from both wheelchair users, Ath and NAth, were different when compared to the TBARS values from control group (C: 24.11 ± 1.75 nmol/mL). The plasma levels of NO were not different among the groups.

Conclusion: Under SCI conditions, the upper body exercise practicing did not alter plasma levels of NO and ROS production neither rheological changes in viscosity indicated by blood clotting studies (fibrinogen levels).

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1. Introduction

Important clinical conditions in spinal cord injury (SCI) are related to damages resulted from loss of functions such as mobility and sensitivity. Frequent causes of SCI are trauma (car accident, gunshot, falls, etc.) or disease (polio, spina bifida, Friedrich’s Ataxia, etc.) [1]. While the atrophy is established after a SCI, there are intrinsic skeletal muscle changes, such as in mitochondrial oxidative capacity, which is the main contributor to the metabolic abnormalities [2]. Independently of the type of SCI injury, complete or incomplete [3], it is observed removal of the supply trophic substances from the nerve to muscle and also decreased muscle electrical/contractile activity, leading to a sharp drop in the rate of synthesis of muscle proteins and increased rate of degradation [4].

Classical studies about SCI demonstrated muscle and vascular changes below the level of injury [5–13]. These peripheral circulatory and skeletal muscle adaptations contribute to the increased risk of cardiovascular disease in SCI patients [14]. In fact, cardiovascular disorders are the substantial causes of morbidity and mortality in both acute and chronic stages of SCI [15–17]. The muscle atrophy and extensive physical deconditioning combined with reduced cardiac output impair the demand of oxygen to the muscle, leading to vascular atrophy [18].

Following SCI, blood flow to inferior limbs diminishes about 50–67% mainly due to the loss of the autonomic nervous system control and to the reduction of the local blood flow [19]. Changes in the sympathetic activity especially into large vascular beds, as in skeletal muscle vessels, the abolished compensatory vasoconstriction associated with reduced venous blood return, contribute to low blood pressure. In fact, venous thromboembolism has been detected in acute spinal cord injury patients [20].

Regarding the vascular events in the chronic phase of SCI, among other issues, there is reduced blood volume, decreased muscle or tissue pressures in the extremities, or functional alterations in the sympathetic nervous system [21]. There are well-reported imminent risks for developing deep vein thrombosis, which is lower in 8–12 weeks, but highest in 7–10 days after the injury and during the early phases of recovery and rehabilitation [19]. Causal factors related to deep venous thrombosis are venous stasis in inferior limbs after muscle paralysis and lack of muscle pump activity. In addition, there is hypercoagulability as consequence of

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reduction of fibrinolytic activity and raised activity of factor VIII from blood coagulation cascade [22].

An important mechanism that may contribute to the vascular injuries associated to hemodynamic alterations in inactive individuals with SCI is the reduced NO availability, due to endothelial dysfunction and increasing humoral or local vasoconstrictors, such as reactive oxygen species (ROS) [23]. On the other hand, exercise is an important stimulus for regulating blood flow, which is partially due to enhanced metabolic rate and NO production, affecting vascular relaxation and inhibition in platelet aggregation [24]. While regular physical activity increases the bioavailability of NO [25,26], the cardiovascular decondition is associated with the altered nitric oxide (NO) metabolism under SCI [27,28]. According to Buck and Chojkier [29], physical activity practice increases endothelial oxidative stress and stimulates the release of NO, which leads to vasodilatation.

Mitochondrial signaling contributes to disuse muscle atrophy due to oxidative stress [30]. In fact, in muscle tissue the elevated metabolic rate associated with physical exercise increases mitochondrial O2 consumption and energy production during cellular metabolism. Oxygen- and nitrogen-derived free radicals are then generated and are involved in oxidative damage to cell components.

Oxidative stress, produced by mitochondrial activity, can be evaluated in plasma through reaction of lipid peroxidation products with Thiobarbituric Acid Reactive Substances (TBARS) [31]. Djordjevic et al., [32] demonstrated athletes with higher VO2max, compared to athletes with poorer aerobic power, had higher levels of TBARS as an accepted index of lipid peroxidation. The authors conclude that TBARS supported the positive correlation found between muscle percentage and TBARS as a consequence of the higher working capacity and consequently increased oxidative stress in working muscleculature of these athletes.

It is already known reactive hyperemia [33] and arterial blood flow are extensively used to determine hemodynamic parameters in SCI patients, mainly male aged 20 and 40 years [5]. In such condition, resting metabolic demands are so low that resting blood flow might be of little diagnostic value. Blood flow during exercise is influenced by cardiac output, making changes in peripheral vascular function [5,34].

Thus, analysis of factors that inform about blood clotting, intrinsic vascular function and the cellular metabolic damages might be useful as additional tools to understand cardiovascular aspects in SCI, mainly when the interest is to know the impact of physical conditioning in such condition.

In face of the data stated above there is a potential positive association among mitochondrial metabolism, NO production and blood clotting after SCI. Considering the disuse of the lower limbs muscles leads to decrease of the local muscle mass and consequent metabolic alterations in installed blood flow reduction, we hypothesized in such condition there is lack of hemodynamic homeostasis which should be detected by biochemical biomarkers. To explore the mentioned potential, the aim of this study was to evaluate, as biomarkers, the blood levels of TBARS, NO and fibrinogen of SCI patients, who are professional basketball players, in comparison with SCI patients that did not practice physical exercise after the injury.

It was included subjects aged between 20 and 60 years old. The sample was assembled in the following groups: wheelchair users who regularly practiced physical exercises (athletes: professional basketball players); wheelchair users who did not practice physical exercises (non-athletes); able bodied (control group).

Description of the groups:

Group 1: Ten wheelchair users who are professional basketball players since the 2nd year after the SCI. The members of this group were called athletes (Ath). Along the last 7 years before the present research, all of them have regularly played basketball 3 times a week, on the Municipal Sports Center located at 627, Camilo Matos Street, Ribeirão Preto, São Paulo, Brazil (zip code: 14090-210).

Group 2: Ten wheelchair users who did not practice physical activities regularly. The members of this group were called non-athletes (NAth). Along the last 7 years before the present research all of the patients were being clinically followed up in the University Physiotherapy Clinic — University of Ribeirão Preto, (UNAERP), which is located at 2201, Costabile Romano Avenue, Ribeirão Preto, São Paulo, Brazil (zip code: 14096-030).

Evidence of the muscle atrophy for groups 1 and 2: All included patients suffered damage to the spinal cord due to trauma that resulted in a loss of functions of mobility and sensitivity of lower limbs. In all wheelchair users, the type of injury was complete. In the studied cases, the injuries were to the five Lumbar vertebra (the vertebra in the lower back between the thoracic vertebra, where the ribs attach, and the pelvis; L-1 thru L-5) or also to the fifth Sacral Vertebra (from the Pelvis to the end of the spinal column S-1 thru S-5). The patients’ injury caused loss of functioning in the hips and legs. The wheelchair users included as volunteers had had the complete SCI at least seven years before the development of the present work. The medical records of patients since the trauma had occurred included detailed physical and medical history, sensor-motor neurological examination to confirm the level, completeness of the lesion and was useful to get the time-course from the trauma at the moment of the present research. The evidence for the skeletal muscle atrophy was certified by previous diagnosis of the time of SCI, respective levels of injury and also on literature data [1].

Group 3: Twelve participants able-bodied. This was the control group (C). None of them were athletes.

Exclusion criteria: Acute coronary syndromes, coronary artery bypass grafting or percutaneous intervention during the first three months of these events were excluded as well as those with renal insufficiency (serum creatinine > 2.0 mg/dL), hepatic insufficiency and uncontrolled hypothyroidism. Those who were chronic users of vitamin C, vitamin E and beta-carotene supplementation were also excluded.

2. Materials and methods

2.1. Subjects/groups, inclusion and exclusion criteria

At the first contact with the potential subjects, they received an explanation concerning the objective of the study and, then, they were invited to be part of the research. Thirty-two volunteers attended to the study.

In any type of procedure, volunteers were held solely and exclusively after approval and agreement, by means of signing the Informed Consent Form (ICF) on a voluntary basis. The study was previously approved by the Ethics Committee of The National Ministry of Health/University of Ribeirão Preto (CAAE: 18388513.7.0000.5498/protocol: 462.531/2013).

2.2. Study procedures

2.2.1. Protocol

Subjects were submitted to blood collection for the analysis of biochemical markers: fibrinogen, TBARS and nitric oxide. All the blood collection took place between September 2014 and October 2014.

2.2.2. Fibrinogen

Blood was collected by venipuncture, using vacuum collection disposable tubes containing 2.7 mL of sodium citrate, immediately centrifuged at 2000 rpm for 15 min. After the centrifugation process, the supernatant plasma was collected for storage at −20 °C. The Multifibrin® commercial kit was used to measure the serum fibrinogen
concentration. The reference values must be between 1.8–3.1 g/L (the standard reference values).

The principle of the fibrinogen measurement was based on Clauss [35]. In brief, thrombin has clotted the previously diluted plasma (usually 1:10) in order to minimize the effect of inhibitory substances, such as heparin. To clot the diluted plasma, thrombin was used in high concentration (100 U/mL), to ensure that the clotting times are independent of thrombin concentration over a wide range of fibrinogen levels [35].

As reference to construct the calibration curve standard plasma was used in a series of dilutions (1:5–1:40). This plasma was prepared in buffer to give a range of fibrinogen concentrations. The reference plasma must present a known level of fibrinogen calibrated against an international standard also known. The clotting time of each plasma dilutions was established in duplicate samples. The results were expressed as clotting time(s)/fibrinogen concentration (g/L) [log–log]. To normalize, 1:10 concentration was considered 100%. According to Clauss [35], a precise curve must present linear correlation between clotting times in the region of 10–50 s.

2.2.3. TBARS (thiobarbituric acid reactive substances)

The OXltek® TBARS Assay commercial kit is designed to provide standardized and reproducible assay with consistent results. Each lot of reagents is quality controlled, which includes a malondialdehyde (MDA) standard [36]. MDA is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA, its level is commonly known as a marker of oxidative stress. Due to extremely short half-lives, Reactive oxygen species (ROS) are difficult to measure directly. Instead, what can be measured are several products of the damage produced by oxidative stress, such as TBARS [37].

Assay of TBARS measures MDA present in the sample, as well as malondialdehyde generated from lipid hydroperoxides by the hydrolytic conditions of the reaction [38].

Samples were prepared according to Yagi [36], where 20 μL of plasma was diluted in 4.0 mL of H2SO4 (0.04 mol/L). 0.5 mL of 10% phosphogluconate was added in 4.0 mL of H2SO4 (0.04 mL) following the addition of 0.3 mL of 10% phosphogluconate solution. The results were expressed as nmol/mL plasma.

2.2.4. NO (Nitric Oxide): measurement of plasma NOx (nitrate + nitrite) concentration

To determine the NO production, NO2⁻ (nitrite) + NO3⁻ (nitrate) plasma concentrations were measured by the Griess–nitrate reductase method [39]. The blood was collected in heparinized vacuum collection tubes (in triplicate) and promptly mixed with a nitrite preservation solution: diethylene triamine pentaacetic acid (DTPA; 0.1 mmol·L⁻¹) and N-ethylmaleimide (NEM; 8 mmol·L⁻¹) [40,41] and centrifuged at 3500 rpm for 5 min at 5 °C. After the centrifugation process, the supernatant plasma was collected for storage at −70 °C. DTPA + NEM block SH-groups and inhibiting transition metal-catalyzed transnitrosation reactions, preventing artificial nitrosation, as well as thiolate and ascorbate mediated degradation of endogenous S-nitrosothiols (RSNOs) and nitrite [42–44].

Numerous factors possibly affect the plasma levels of nitrate, such as medications, nutrition status/diet, gender differences, ethnicity, clinical conditions, smoking and environmental chemicals [45], thus limiting the clinical utility of measuring nitrate to assess endogenous NO production. On the other hand, measuring plasma nitrite level reflects NO synthase activity under normal or pathophysiologic conditions [46–48].

The plasma nitrite + nitrate (NOx) concentration were determined in duplicate by using the adapted Griess reaction from [40,41]. Briefly, 40 μL of plasma was incubated with the same volume of nitrate reductase buffer (0.1 mol·L⁻¹ potassium phosphate, pH 7.5, containing 1 mmol·L⁻¹ β-nicotinamide adenine dinucleotide phosphate and 2 U of nitrate reductase·mL⁻¹) in individual wells of a 96-well plate. Samples were allowed to incubate overnight at 37 °C in the dark. Nitrite was first treated with a diazotizing reagent (sulfanilamide, SA), in acidic media to form a transient diazonium salt. Ten minutes later, this intermediate was allowed to react with a coupling reagent, N-naphthyl-ethylenediamine (NED), to form a stable azo compound; briefly, 80 μL of freshly prepared Griess reagent (1% sulfanilamide, 1% naphthylethenediamine dihydrochloride in 5% phosphoric acid) was added to each well and the plate was incubated for an additional 10 min at room temperature.

A standard nitrate curve was obtained by incubating sodium nitrate (0.2 mmol·L⁻¹ to 200 mmol·L⁻¹) with the same reductase buffer. The intense purple color of the product allows nitrite assay with high sensitivity and can be used to measure nitrite concentration as low as −0.5 μmol/L level. The absorbance of this adduct at 540 nm is linearly proportional to the nitrite concentration in the sample [39].

2.3. Statistical analysis

The results are shown as the means (M) ± standard error mean (SEM). Numerical variables comparisons between the groups were performed using the one-way ANOVA following by Newman–Keuls Multiple Comparison post-hoc test. P value less than 0.05 (IC95) was considered statistically significant.

All statistically calculations were performed using Graph Prism 5.03 (San Diego, CA-USA) software package.

2.4. Materials

Multifibrin®U: bovine thrombin (50 IU/mL), fibrin-aggregation retardung peptide (gly-proarg-pro-ala-amide, 0.15 g/L), calcium chloride (1.5 g/L), hexadimethrine bromide (15 mg/L), polyethylene glycol 6000 (0.8 g/L), sodium chloride (6.4 g/L), Tris (50 mmol·L⁻¹), bovine albumin (10 g/L); Preservative: sodium azide (<1 g/L), were purchased from Dade Behring Inc. Newark, U.S.A.

Thiobarbituric Acid, TBARS diluents 1 and 2, SDS solution (sodium dodecyl sulfate in distilled), MDA (malonaldehyde) standards and diluents were purchase from Enzo life sciences (Farmingdale, NY – USA).

3. Results

3.1. Fibrinogen

According to Fig. 1, there was no difference between wheel chairs user Ath (3.67 ± 0.44 g/L) and N Ath (3.44 ± 0.38 g/L). On the other hand, it was observed difference between both wheelchair users Ath and N Ath, when compared to C (P < 0.01) (2.27 ± 0.08 g/L) and also the standard reference values (ref: 1.8–3.1 g/L; P < 0.05).

3.2. TBARS

Data from Fig. 2 demonstrated that there is no difference between the N Ath (3.21 ± 0.24 nmol/mL) and Ath groups (3.66 ± 0.27 nmol/mL). Conversely, it was observed in both wheelchair users groups decreased TBARS serum level when compared to C group (24.11 ± 1.75 nmol/mL; P < 0.001).
3.3. NO (nitric oxide)

As presented in the Fig. 3, there was no difference in plasma levels of NO products among the C (30.98 ± 0.92 μM/L), the NAth (32.45 ± 3.85 μM/L) and Ath (40.77 ± 3.12 μM/L) group.

4. Discussion

This is the first study analyzing the impact of physical activity to consider a triangulation among blood clotting, oxidative stress and nitric oxide, as biochemical markers, in paired groups of patients with disuse muscle atrophy of lower limbs because of complete SCI (spinal cord injury). In this study, it has been demonstrated hyperfibrinogenemia in individuals with SCI, despite of upper limbs physical activity (Ath and NAth). Similarly, the plasma levels of TBARS were lower in both groups of wheelchair users, Ath and NAth, than in the able-bodied control group. Conversely, the able-bodied control volunteers and all wheelchair users did not present different levels of blood NO. The physical exercise practice using the upper body (Ath) was an independent variable for all biochemical markers evaluated.

Several works have demonstrated the time after the SCI and tissue/organ injuries [49–59]. Taking in account the time-course (more than 7 years) of the injury of the patients included in the present research, it is evident to assume the disuse muscle atrophy of the Ath and NAth wheelchair users was definitely installed as well-evidenced in the literature. According to numerous articles, the body composition degenerates markedly during the first 6 months after SCI [49–59]. Therefore, it can be considered that a series of tissue injuries, including vascular damages, were substantially established after this elapsed time.

Hyperfibrinogenemia is a well-known risk factor for both arterial and venous thrombosis [60,61] and often is a characteristic of vascular endothelium injury [62,63]. High plasma levels of fibrinogen in muscle atrophy condition leads to injury of the vascular endothelium [64]. Notwithstanding of increased level of fibrinogen in the plasma of both SCI individuals, Ath and NAth, we do not have enough results to speculate whether muscle atrophy precedes vascular atrophy (oxygen demand–oxygen delivery) [64]. Taking into consideration their hyperfibrinogenemia, without changes in the NO plasma levels (as an indicator of endothelial integrity), the occurrence of a compensatory mechanism is a possible explanation.

We have observed greater production of TBARS in the able-bodied subjects than in wheelchair users (Ath and NAth). As shown by Zago and Zanesco [24] and Pattwell and Jackson [31], the chronic physical exercise increases the NO and TBARS production in the able-bodied. Levels of TBARS are often investigated as an index of oxygen radical-induced lipid peroxidation [32]. Acute exercise can induce oxidative stress and tissue damage in both animals and humans [65–68]. At the same time that exercise increases the ROS production, mainly through mitochondrion as source [69–72], adaptive mechanisms [3,73–75] seem to decrease oxidative stress, by means of increased antioxidant defenses, reducing basal production of oxidants, and also the reduction of radical leak during oxidative phosphorylation [76]. These statements support our hypothesis that wheelchair users may have additional compensatory mechanisms, which may improve the NO levels balance closer to physiological levels.
Regarding to vascular adaptive mechanisms, Hopman et al. [5], compared male individuals with paraplegia with able-bodied control subjects aged between 20 and 40 years to examine properties of the venous vascular system in SCI condition to prove the hypothesis of venous atrophy as an adaptation to inactivity and muscle atrophy rather than the effect of venous blood pooling caused by sympathetic denervation or muscle pump inactivity. It is widely recognized that vascular reactivity increases after exercise training [77] facilitating venous blood flow return to the heart [78–80]; while cardiovascular and venous return are impaired in deconditioned [81,82], immobilized patients [83] and in incomplete SCI individuals [84].

Cardiovascular comorbidities are implied in clinical conditions related to skeletal muscle atrophy as cause or consequence for metabolic abnormalities [2,85]. In terms of metabolic impairments, the spinal cord does not have to be severed for occurring a loss of functioning [86,87]. Muscle atrophy by denervation or disuse leads to increased mitochondrial ROS production and oxidative stress signaling clinical conditions of vascular damages [30].

Since the 1960s [88–90] important data have established a causal link between the increased mitochondrial network and denervation-induced muscle atrophy [91] to explain the origin of significant protection against muscle wasting [30,91–93]. As described by De Groot et al. [18], decreased vascular diameter and blood flow reduction was observed in adults (male and female) individuals with longstanding SCI. The authors explained this result in function of compensatory mechanisms by an adjustment due to the lower metabolic necessity under muscle atrophy condition. The explanations for correlation between mechanisms of muscular and vascular adaptations under SCI conditions are poorly studied in humans but studies in animal models are in line with the scarce clinical data available in the literature. Langille and O’Donnell [94] have shown that reductions in blood flow and arterial diameter produced by chronic decreases are endothelium dependent in rabbits. Arterial remodeling in response to chronic changes in blood flow occurs in an endothelium-and NO-dependent manner [94–96], and an increased muscle blood flow with elevated shear stress can trigger NO production. Shear stress and altered blood flow are important sources of ROS that plays a key role in the signaling mechanisms and affect vascular homoeostasis [97–99] of factors such as NO. In fact, under shear stress NO production is increased in endothelial cells modulating various cellular processes that are essential for endothelial integrity [100].

NO levels, through its endogenous products nitrate + nitrite, were similar both in able-bodied individuals and in wheelchair users. In this sense we are likely to confirm physiological levels of NO as an adaptive process to protect the vascular function from oxidative stress. Comparable condition involving muscle atrophy and reduced oxidative capacity were observed by Zizola and Schulze [101]. Our present results allow us to rationalize the new hypothesis that the skeletal muscle atrophy, as cause or consequence, is benefited by compensatory mechanisms able to maintain the NO production closer to physiological levels. Further, the reduced levels of TBARS in both SCI groups, despite of upper limbs physical activity, is also due to compensatory and protective mechanisms of mitochondrial metabolic activity reduction.

5. Conclusions

The results of the present study demonstrated that, similarly in athlete (Ath) and non-athlete (NAth) wheelchair users, the lower limb muscle disuse potentially increases blood clotting, but it does not change nitric oxide production and oxidative stress. The proposed explanation is a metabolic protective mechanism triggered in SCI condition involving vascular factors, independently of upper limb exercises.

Since data of this study did not find difference in the studied biomarker levels between both in Ath and NAth wheelchair users, the upper limb physical stimulation does not alter the rheological changes in viscosity, the red cell aggregation (indicated by fibrinogen levels), the vasodilatation (indicated by NO measure) and ROS production (indicated by TBARS levels). Nevertheless, these biomarkers are not the only ones that could be evaluated in these conditions. Further studies should be conducted to further clarify the effects of upper body exercise in wheelchair users.

6. Study limitation

1- Groups with small number of subjects.
2- Procedures limitations:
   - Hemolysis, icteric or grossly lipemic plasma samples are not suitable for using in TBARS analysis.
   - Non-lipid TBARS may be present in the sample. It is recommended that a sample with elevated TBARS levels be tested by a more specific test for lipid peroxidation such as HPLC.
   - Normal tissues contain very low levels of free malondialdehyde (MDA). Overall, TBARS method is convenient, simple, and low cost, but the inherent problems of specificity and variability of data make it difficult to be a reliable quantitative biomarker of lipid peroxidation in vivo, although it may be used as a screening tool when measured with tightly controlled consistent protocol and/or control groups for comparisons.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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