Analysis of serum cortisol levels by Fourier Transform Infrared Spectroscopy for diagnosis of stress in athletes

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Abstract **Introduction** Fourier-transform infrared (FT-IR) spectroscopy is a technique with great potential for body fluids analyses. The aim of this study was to examine the impact of session training on cortisol concentrations in rugby players by means of infrared analysis of serum. **Methods:** Blood collections were performed pre, post and 24 hours after of rugby training sessions. Serum cortisol was analyzed by FT-IR spectroscopy and chemiluminescent immunoassay. **Results:** There was a significant difference between the integrated area, in the region of 1180-1102 cm^{-1}, of the spectra for pre, post and post 24 h serums. The cortisol concentration obtained by chemiluminescent immunoassay showed no significant difference between pre, post and post 24 h. Positive correlations were obtained between the techniques (r = 0.75), post (r = 0.83) and post 24 h (r = 0.73). **Conclusion:** The results showed no increase in cortisol levels of the players after the training sessions, as well as positive correlations indicating that FT-IR spectroscopy have produced promising results for the analysis of serum for diagnosis of stress.

Keywords: Infrared spectroscopy, Blood, Cortisol, Sports medicine.

Introduction

The goal in training of elite athletes is to provide training loads that are effective in improving performance during competitions. A successful process requires the interaction between the training load and recovery time, in order to avoid stress, which can lead to decreased performance and increased risk of injury (Ferrari et al., 2013; Meeusen et al., 2013).

This imbalance between the overall strain experienced during exercise training and the athlete's tolerance to such effort may induce overreaching or overtraining syndrome, which are characterized by diminished sport-specific physical performance, accelerated fatiguability and subjective symptoms of stress (Urhausen et al., 1995).

Exercise produces stress response activating the sympathoadrenal medullary system and the hypothalamic pituitary adrenal axis (HPA). Thus, researchers have examined the human stress response to exercise by means of cortisol (a stress biomarker) in various situations including pre-exercise, during exercise, and post-exercise (Backes et al., 2015; Cunniffe et al., 2010; Dickerson and Kemeny, 2004; Hill et al., 2008; Moreira et al., 2013).

Traditional clinical trials used for analysis of cortisol are relatively expensive and laborious, making it difficult for many teams to monitor athletes without substantial financial support. In order to minimize

such difficulties, new laboratory tools have been proposed to characterize biological samples as an alternative technique for health monitoring of athletes (Ellis and Goodacre, 2006; Khaskheli et al., 2013; Petibois et al., 2000).

FT-IR spectroscopy is a technique with great potential for the analysis of body fluids (Petibois et al., 2000; Yoshida et al., 2013), which has some advantages compare to standard laboratory analysis, for example, it can provide real-time information without the use of reagents and requires a small sample volume (Franck et al., 1998; Khaustova et al., 2010; Petibois et al., 2000). FT-IR spectroscopy allows measuring the frequency and intensity at which a given sample absorbs infrared radiation, providing the identification of functional groups like carboxyl, amine, hydroxyl, carbonyl, etc. The quantification by FT-IR spectroscopy is done using the absorption bands of the vibrational modes of molecular radicals in spectral range of 4000-700 cm^{-1} (Khaustova et al., 2010; Petibois et al., 2000; Stuart, 1997).

Some studies have shown the determination of glucose (Petibois et al., 1999) and immunoglobulins (Sankari et al., 2010) in serum samples, as well as lactate, glycerol, triglycerides, fatty acyl, total amino acids plasma concentrations (Petibois et al., 2002) using FT-IR spectroscopy. However, there are few

studies using FT-IR spectroscopy for the diagnosis of stress in athletes.

Thus, the aim of this study was to examine the impact of training sessions on the concentration of serum cortisol in rugby players, to help coaches prescribe effective training loads during periods of training and competition. In addition, we studied the use the FT-IR spectroscopy as a tool to identify serum cortisol levels in rugby players, in order to facilitate the diagnosis of stress.

Methods

Participants

The sample was composed of 10 male rugby players (Age: 22 ± 3 years; Height: 1.79 ± 6 cm; Body mass: 87 ± 10 Kg). All players had experience with this sport for approximately five years and train twice a day (90-120 minutes per session), four days per week.

This study was approved by the Ethics Research Committee of the University of Vale of Paraiba (No. 255.474). All volunteers provided written informed consent before participation.

Training session

The training session was performed at 21:30 h, which was started with a 20 min warm-up (stretching and running) and sport-specific exercises (technical drills and tactical drills). After this stage the players performed a simulated rugby match. In addition, all official rules were maintained and no player was substituted during the match.

The intensity of the training session was assessed by training load, which was calculated by multiplying the exercise duration (min) by the 'session rating of perceived exertion' scale (Session-RPE), as defined by the Borg CR-10 scale (Foster, 1998). The RPE of each player was recorded 30 min after the training session.

Blood samples and analyses

Blood collection was performed approximately 30 min before (~21:00 h - pre-session), after (~23:30 h - post-session) and 24 h after the training session. The blood sample was collected from a superficial forearm vein with subjects in the seated position using standard venipuncture technique. Eight milliliters of blood was collected into sterile tube, gel-barrier vacuum (BD Vacutainer SST Advance Tube, 5 mL, Gold). After collection, the serum was separated by centrifugation at 3000 rpm for 10 minutes and stored in sterilized tubes of 2 ml at -20°C until the analysis.

Cortisol was assessed using chemiluminescent immunoassay method.

FT-IR spectroscopy analysis of blood serum

The blood samples were defrosted and 15 µl of serum was deposited on a calcium fluoride (CaF_2) window and dried for 60 min using an incubator (Quimis, Q317B-53, Brazil). The spectra of the samples were recorded in the range of 4000-750 cm^{-1}, with 32 scans and a spectral resolution of 4 cm^{-1}, using a Spectrum 400 spectrophotometer coupled to a microscope (Perkin-Elmer, Spotlight 400, USA), controlled by a computer using Spotlight 400 Software. For each sample, eight random points were performed along the thin film.

Statistical analyses

Data were reported as means and standard deviation (SD). After checking the normality of distribution (Kolmogorov-Smirnov test), the differences between the data obtained by FT-IR spectroscopy and chemiluminescent immunoassay methods (pre, post and post 24h session training) were determined using a one-way ANOVA with a post hoc test (Tukey). Statistical significance was set at $p < 0.05$. The relationships between the integrated area of spectra of serum and cortisol concentrations were analyzed using Pearson's coefficient.

The FT-IR spectra of serum sample were standardized by baseline correction and normalization (0-1). The area was calculated by integrating the spectral region within 1180-1102 cm^{-1}. All data were analyzed using Excel 2007, Origin V8.5, and OPUS V4.2.

Results

The study included 10 players, which ranked sessions as follows: 7 players = intense; 3 players = somewhat intense. Training load was rated 480 ± 98 (Means $\pm$ SD), indicating an intense training session.

The average FT-IR spectra of the serum samples collected pre-session training (Pre), post-session training (Post) and 24 h after session training (Post 24 h) are shown in Figure 1A. In order to improve the visualization of differences between the absorption bands, the second derivative of FT-IR spectra was used, as shown in Figure 1B. The results showed differences between the samples in the spectral region 1590-1004 cm^{-1}. The region 1180-1102 cm^{-1} (marked) is highlighted because it is predominantly attributed to cortisol (Caetano et al., 2016; Khaustova et al., 2010).

Figure 2 shows the results of the integration of the serum spectra areas. This statistics was used to show the spectral differences of serum samples
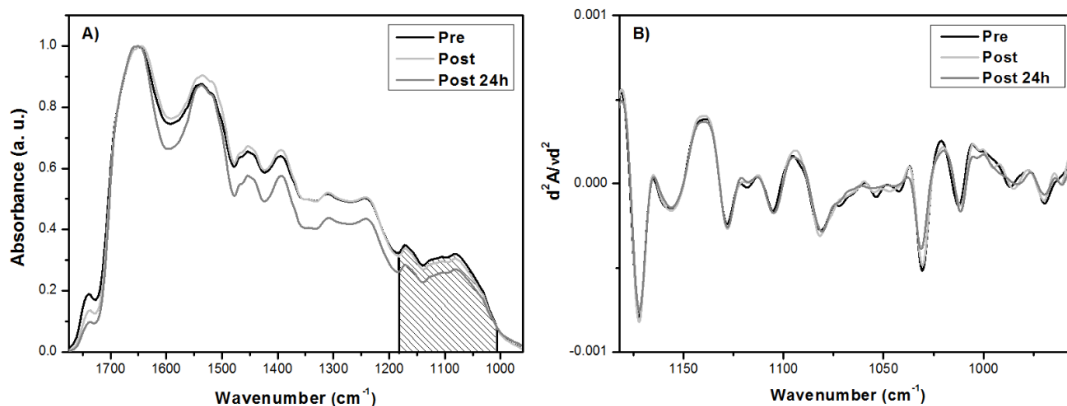
Figure 1. (A) Average FT-IR spectra of the serum samples recorded in the 1800-900 cm^{-1} region for samples collected pre, post and post 24 h training session. (B) Second derivative of serum spectra in the region of 1180 to 1102 cm^{-1}.
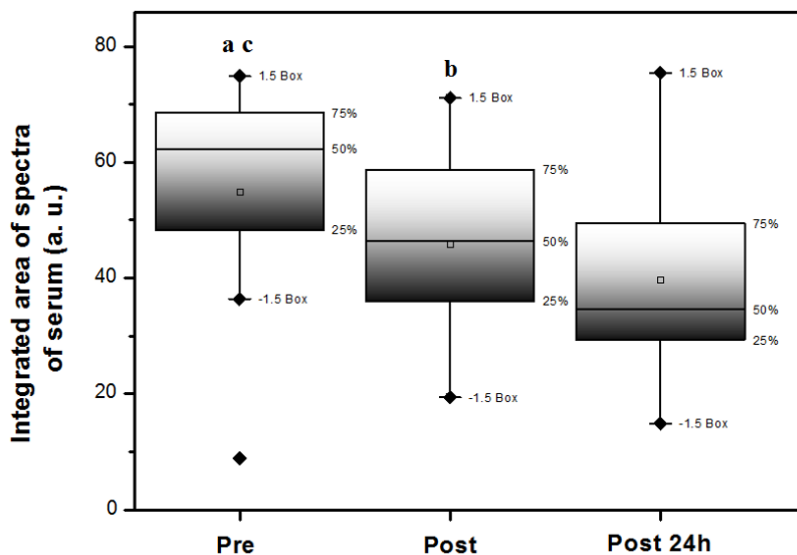

Figure 2. Mean (and SD) of integrated area of spectra of serum sample collected before, after the training session, and after 24 h of recovery for spectral region 1180-1102 cm^{-1} (a = significant difference between pre and post; b = post and post 24h; c = pre and post 24h ($p<0.05$)).

collected pre and post-session training and post 24 h of recovery for the spectral region 1180-1102 cm^{-1}, predominantly attributed to cortisol. There was a significant difference between the integrated area of spectra of serum pre, post and post 24h.

The cortisol concentrations obtained by chemiluminescent immunoassay method are shown in Figure 3. There were no significant differences between the cortisol concentrations in pre, post and post 24h session training. These results are in accordance with the FT-IR results, showing that the training session did not cause relevant changes in cortisol concentrations.

The correlation between the integrated area of the spectra of the serum samples and cortisol concentrations

obtained by chemiluminescent immunoassay method are shown in Figure 4. Positive correlations were observed between the integrated area of the spectra regions predominantly associated with cortisol and cortisol concentrations, with the values obtained before (r=0.75) (Figure 4A), after (r=0.83) (Figure 4B) and after 24 h (r=0.73) (Figure 4C) the rugby training session.

Discussion

The aim of this study was to examine the impact of session training on the serum cortisol concentrations in rugby players, using FT-IR spectroscopy and chemiluminescent immunoassay method.
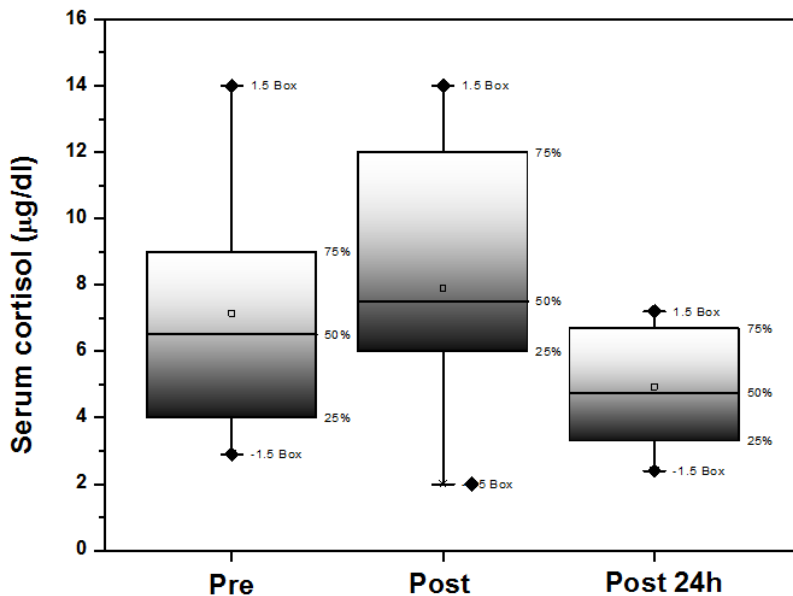
Figure 3. Concentration of serum cortisol pre, post and post 24 h training session, obtained by the chemiluminescent immunoassay method. No significant difference between the serum samples (p<0.05).

The training load was used as an indicator of exercise intensity since it has been widely used as a useful tool for prescribing exercise intensity, due to its relationship with physiological indicators of exercise stress (Irving et al., 2006; Nakamura et al., 2010). This method showed that the training session performed by the volunteers was intense.

Infrared analysis was focused on the spectral region of 1180 to 1102 cm⁻¹, which is attributed predominantly to cortisol. In order to amplify the spectral variations between the average FT-IR spectra of the serum samples collected pre, post and post 24 h, the second derivative of the spectra was used and showed that the main differences occur in the following spectral regions: 1104, 1117, 1128, 1156 and 1172 cm⁻¹. These results indicate that biochemical changes do exist in serum after a training session, as shown in other studies (Déléris and Petibois, 2003; Galliera et al., 2014).

The results of the integrated area were significantly different for three sampling times (pre, post and post 24h). There was a decrease in the spectral band for serum collected after physical effort, which can be explained by factors such as no increase in cortisol concentrations and the complexity of the serum spectra due to the absorption of proteins, hormones, lipids, carbohydrates, etc. (Petibois et al., 2000).

The spectral region of 1180 to 1102 cm⁻¹ corresponds to group C-O and C-O-C, which is assigned to cortisol, because this hormone is formed by $C_{21}H_{30}O_5$ (Khaustova et al., 2010). However, other components present in serum, such as lactate and glycerides, may also have absorption bands in this region (Petibois et al., 2000).

The cortisol response was confirmed by chemiluminescent immunoassay method, which is used to determine the concentration of samples according to the intensity of the luminescence that the chemical reaction emits. The immunoreaction indicates the interaction between antigen and antibody. This method has high sensitivity and good specificity, but requires some specific reagents and always needs a long incubation period, which, in turn, leads to several hours to complete the analysis (Wang et al., 2012).

The results obtained by chemiluminescent immunoassay showed that the cortisol concentration was not significantly different for pre, post and 24h session. Thus, the stress imposed by the training session was not enough to increase cortisol concentrations. These results show that the FT-IR spectroscopy has the necessary sensitivity for this type of analysis, even in the case were no increase in the absorption bands assigned to cortisol was observed.

Some authors report that high-intensity running and impacts commonly associated with rugby have caused significant changes in markers of muscle damage, psychophysiological stress and immune system function (Cunniffe et al., 2010; Smart et al., 2008; Takarada, 2003; Thompson et al., 1999). However, rugby is an intermittent sport governed by high force and high frequency impacts (Lindsay et al., 2015a;
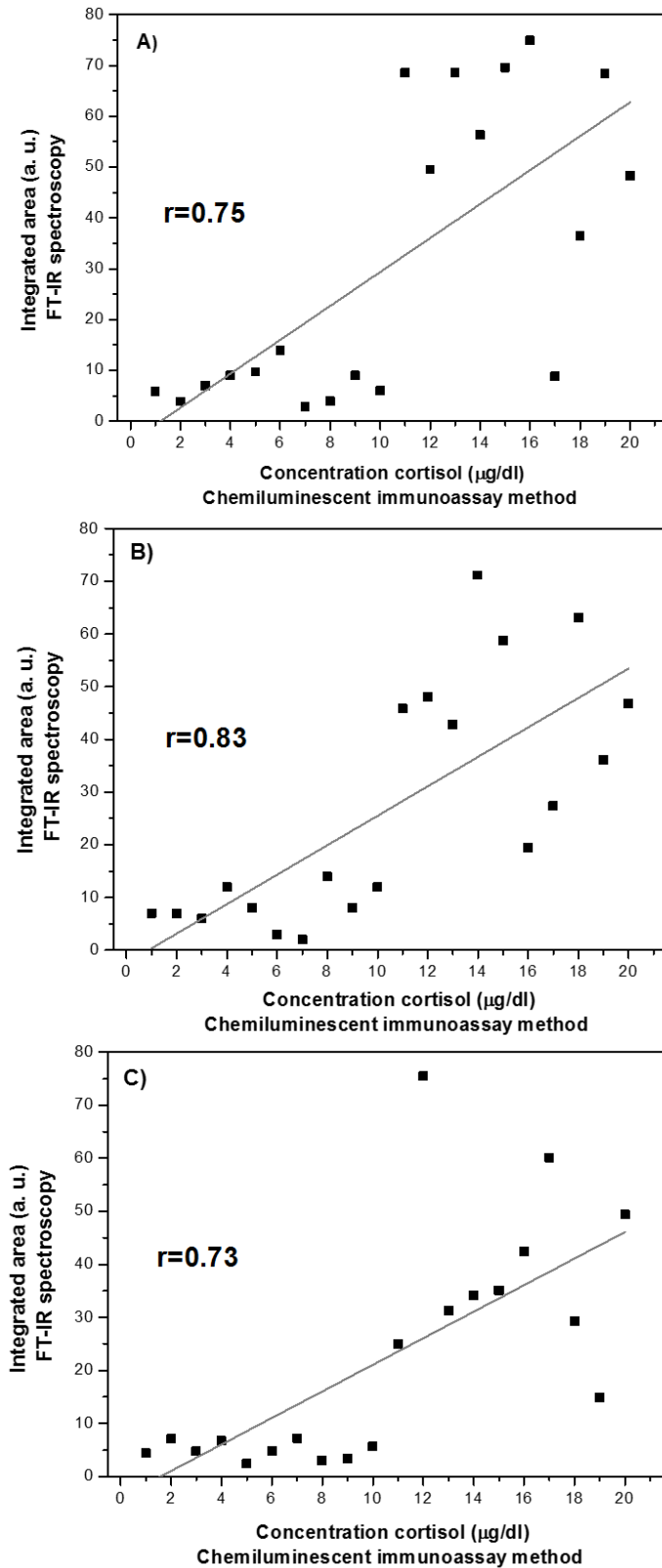
Figure 4. Correlation (R) between the integrated area of the serum spectra and cortisol concentrations obtained by chemiluminescent immunoassay, for values obtained pre (A), post (B) and post 24 h (C).

Quarrie and Hopkins, 2007), with recovery periods during the match. Thus, factors such as recovery periods and physiological adaptations resulting from proper physical training can avoid high stress levels during rugby matches and training sessions (Hejazi and Hosseini, 2012).

Cortisol is a glucocorticoid produced in the adrenal glands that are controlled by the hypothalamus-pituitary-adrenal axis in response to stress, which could also be related to emotional, physiological, and behavioral dimensions (Sapolsky et al., 2000). If it is so, the psychological factor, which is considered an additional stressor in sports, helps to explain our results, since blood collection was performed around a training session. In other words, a representative increase in cortisol would be found if our experiments were performed around an official match, as shown by Lindsay et al. (2015b), who found a significant increase in cortisol after an official rugby game. These results area corroborated by other studies that showed that levels of cortisol after an official match are considerably higher compared to a simulated match (Haneishi et al., 2007; Moreira et al., 2013).

In addition, cortisol levels show a circadian rhythm in which levels increase dramatically on awakening and gradually decrease throughout the day, reaching the lowest levels late in the evening (Dickerson and Kemeny, 2004). Thus, this could also explain part of our results, since the experiment was performed at night (~21:30h).

Correlation coefficient was used to establish the statistical associations between the parameters obtained by FT-IR spectroscopy and chemiluminescent immunoassay method. There were positive correlations between the integrated area of the serum spectra and cortisol concentrations for pre (r = 0.75), post (r = 0.83) and post 24 h (r = 0.73).

In addition to checking the relationship between the techniques, the results of this study showed that the training load could be increased during the training sessions of the volunteers, since no relevant changes were found in the levels of cortisol pre, post, and post 24 h of recovery after the training session. Unlike the training load, that showed an intense session, the results of FT-IR spectroscopy and chemiluminescent immunoassay showed that the game was somewhat intense. Thus it is important that the training load is associated with another test to properly rate the intensity of training sessions. This is important because a successful training not only must involve overloads but must also avoid the combination of excessive overload and inadequate recovery. When athletes do not sufficiently respect the balance between training and recovery, overtraining

syndrome can occur. This syndrome is characterized by an accumulation of training and/or nontraining stress resulting in long-term decrease in performance capacity with or without related physiological and psychological signs and symptoms of maladaptation (Meeusen et al., 2013). The diagnosis of overtraining syndrome is very difficult and depends on the clinical outcome of other blood biomarkers such as, immunoglobulin, testosterone, creatine kinase, as well as exclusion diagnosis (Ferrari et al., 2013; Meeusen et al., 2013; Mujika et al., 1996). In this sense, FT-IR spectroscopy can be used for this purpose and/or used as a complementary technique, especially for monitoring players that belong to teams without proper financial support.

In conclusion, the results showed that no increase in cortisol levels were found in players after the training session, as well as positive correlations were obtained between infrared analysis and chemiluminescent immunoassay. FT-IR spectroscopy has produced promising results for serum analyses involving the detection of stress without the use of any additional reagent.

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