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Raman spectroscopy applied to identify metabolites in urine of physically active subjects

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ABSTRACT

Raman spectroscopy is a rapid and non-destructive technique suitable for biological fluids analysis. In this work, dispersive Raman spectroscopy has been employed as a rapid and nondestructive technique to detect the metabolites in urine of physically active subjects before and after vigorous 30 min pedaling or running compared to sedentary subjects. For so, urine samples from 9 subjects were obtained before and immediately after physical activities and submitted to Raman spectroscopy (830 nm excitation, 250 mW laser power, 20 s integration time) and compared to urine from 5 sedentary subjects. The Raman spectra of urine from sedentary showed peaks related to urea, creatinine, ketone bodies, phosphate and other nitrogenous compounds. These metabolic biomarkers presented peaks with different intensities in the urine of physically active individuals after exercises compared to before, measured by the intensity of selected peaks the Raman spectra, which means different concentrations after training. These peaks presented different intensity values for each subject before physical activity, also behaving differently compared to the post-training: some subjects presented increase while others decrease the intensity. Raman spectroscopy may allow the development of a rapid and non-destructive test for metabolic evaluation of the physical training in active and trained subjects using urine samples, allowing nutrition adjustment with the sport's performance.

1. Introduction

The benefits of physical exercises in improving the health of people of all ages are already well evidenced in the scientific literature. Regular exercise is capable of slowing the development of many of the organic changes that are associated with the natural and degenerative process of aging, such as chronic diseases, thus increasing longevity with quality of life [1,2]. Researchers showed that 30-min of regular walking a day can reduce the risk of mortality by 20 to 40% [1].

It has been demonstrated that it is possible to observe changes in the urine metabolic fingerprint after the practice of physical exercise in sedentary men through the analysis of urine [3]. The urine of healthy individuals consists mostly of nitrogen compounds from protein metabolism, such as ammonia and creatinine; inorganic ions and salts (sodium, chlorine, potassium); organic acids (hippuric and citric acids); urea; several water-soluble toxins; and pigmented products of hemoglobin breakdown [4–7]. Other metabolites resulting from physical exercise have been reported in the literature, such as alanine, arginine and methionine; ketone bodies; hypoxanthine; lactate; and pyruvate [3–7]. Researchers published an extensive list of metabolites and their concentrations in urine [8]. The metabolites in serum may be related to the ones found in urine; for instance the blood lactate is related to the concentration found in urine of swimmers and may be a reliable biomarker of training capability [7].

Raman spectroscopy is an optical technique based on the inelastic scattering of the light by the polarizable molecules that reveals the vibrational energy levels of the molecule's chemical bonds [9]. Thus, a rapid and non-destructive analysis of the chemical structure and the changes associated to biological processes, metabolites and infections in important bio-fluids for human health, such as the red blood cells,

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cerebrospinal fluid, plasma, serum and urine [10–17] can be done by the analysis of the Raman spectrum. These findings confirm the potential of Raman spectroscopy for the diagnosis and prognosis of diseases, proving to be a method complementary to the traditional ones.

Raman technique takes advantage over the conventional methods of biochemical testing, because it is possible to evaluate the sample quickly and non-destructively, providing a rapid and low cost technique to determine the physical condition of a particular organism without destruction, tissue removal or use of reagents, providing qualitative and quantitative information of the molecular composition in situ in real time [9,17,18] and may be used for diagnosis purposes [9–20].

Raman spectroscopy has been applied in different studies of metabolites in urine in order to detect changes in the concentrations of the compounds present. Bispo et al. [15] correlated the amount of urea, creatinine and glucose in the urine of patients with diabetes mellitus and hypertension at risk of developing renal lesions by a discrimination model based on principal components analysis applied to the Raman spectra of single (spot) urine. Saatkamp et al. [16] quantified urea and creatinine in urine of healthy subjects using Raman spectroscopy aiming to diagnose kidney disease. Researchers also applied the Raman technique to study urine metabolites in order to diagnose different diseases [17,21–23], while sports-related researches have detected the presence of illegal substances in athletes' urine [24,25]. Still there are no studies focused on the analysis of urine metabolites related to physical exercise.

Thus, the objective of this study was to identify metabolic biomarkers in the urine of physically active individuals associated to the exercises by means of Raman spectroscopy, by identifying the Raman peaks with significant differences in the intensity and position in the urine collected after a physical exercise session (30 min of vigorous pedaling or running) compared to the urine before and from sedentary (control) group, correlating these peaks with the peaks of the possible metabolites found in urine after exercises, and evaluating the intensity of these peaks after and before physical activity and compared with the sedentary group, aiming to check the ability of Raman spectroscopy to reveal the metabolic status of subjects after physical activity.

2. Materials and Methods

2.1. Urine Samples

The study was approved by the Ethics Committee of the University Santa Cecilia - UNISANTA (protocol no. 1.133.024). It was evaluated 9 physically active subjects (both gender, age 24.1 ± 7.2 years, weight 67.5 ± 9.8 kg, body weight mass 22.4 ± 2.3) doing aerobic exercises, consisted of 30 min. of vigorous pedaling (5 subjects) and 30 min. of running (4 subjects), and 5 sedentary (control group) university's students (both gender, age 31.3 ± 6.2 years, weight 68.9 ± 12.4 kg, body weight mass 24.9 ± 3.4). The participants responded an anamnesis form to obtain information on eating habits, chronic diseases, use of medicines, as these aspects may influence the response of urine metabolites.

Subjects were asked to remain 48 h without exercise. On the day of collection, a urine sample of each active subject was collected before the physical exercise session and another sample was collected immediately after the session. For the sedentary individuals, only one urine sample was collected on the same day of the assets. The urine samples were stored in 5 mL cryogen tubes and then immediately frozen and stored in freezer at − 20 °C.

2.2. Raman Spectroscopy of the Urine Samples

In order to evaluate the basal and acute effects in modulating urine metabolites (such as urea, creatinine, ketone bodies, phosphate and other nitrogenous compounds) after exercises compared to controls, Raman spectra were obtained from all the urine samples. It was used a dispersive Raman spectrometer (Dimension P-1, Lambda Solutions Inc., MA, USA) connected to a Raman probe (Vector probe, Lambda Solutions Inc., MA, USA), with excitation wavelength at 830 nm (near infrared) and a power output of 350 mW at the distal tip of the probe. The probe collects the light scattered by the sample, and a grating integrated with the spectrometer disperses the light onto a high efficiency CCD camera, in the spectral range of 400 to 1800 cm^{-1} with spectral resolution of about 2 cm^{-1}.

Before the spectral collection, the spectrometer was checked for its Raman shift calibration using the known bands of naphthalene. At the time of spectra collection, the samples were warmed up to room temperature, slightly shaked and pipetted in an aluminum sample holder with wells of about 80 μL. The spectra were obtained in triplicate for each sample, in a time of 20 s exposure for each spectrum.

Once stored, the spectra have been intensity-corrected by the spectral response of the optical components (probe, grating, spectrograph optical path, CCD camera) and preprocessed to remove the background fluorescence. Thee fluorescence background was modeled by the “mpoly” routine [26], that fits a 5th order polynomial to the baseline and subtracts from the Raman spectrum, revealing the positive, high frequency Raman bands. For better comparison of the amount of urine metabolites before and after physical activity, spectra were normalized by the area under the water band (H-O-H bending vibration) from 1600 to 1690 cm^{-1}.

2.3. Data Analysis

The mean spectrum and standard deviation of the urine from sedentary subjects was plotted and used to identify the basal compounds of urine. The mean spectra and standard deviation of the urine from the active subjects before and after physical training were plotted in order to find which Raman bands were affected by the exercises compared to sedentary controls. Then these bands were correlated with the Raman spectra from the biochemicals presented in the urine metabolome, in concentrations of the order of mg/dL [8], found in the published literature of Raman spectra, and compared to the peaks found in the urine of both active and sedentary subjects. Finally, the spectrum before and after the training for each subject was plotted and the alterations identified as changes in the Raman peaks related to urea, creatinine, phosphate, ketone bodies, and other nitrogenous compounds. Peaks may appear in some spectra, others may increase or decrease the intensity when comparing before and after training.

The area of the most relevant peaks of urine at 681, 848, 880, 983, 1006, 1079 and 1159 com^{-1} (attributed to urea, creatinine, phosphate, ketone bodies, and other nitrogenous compounds) were obtained by fitting a Lorentzian function with 5 cm^{-1} bandwidth (Microcal Origin 6.0, MA, USA, Lorentzian peak fitting function) and these peak areas were used to evaluate the changes in the biochemical compounds of urine depending of the exercises. Since the Raman bands of some other compounds may overlap with the spectra of urea and creatinine, it has been performed a data analysis based on the plot of the areas of selected peaks of these two compounds, measured in different concentrations diluted in water, aiming to identify the presence of the unique basal compound in each spectrum and an eventual contribution from another compound, which means not following a straight line. For so, spectra of urea and creatinine (urea - ref. 208,884 and creatinine - ref. C4255, Sigma Aldrich Química Brasil, SP, Brazil) were diluted in distilled water in the concentrations of 40 to 500 mg/dL for urea and 20 to 320 mg/dL for creatinine (spectra from the work of Vieira et al. [27]) and the plot of the areas of the urea peaks at 1159 and 1006 cm^{-1} and creatinine peaks at 848 and 681 cm^{-1} were obtained. These curves were plotted and the obtained straight equations were represented in the same plotting of the areas of the same peaks from the urine samples of both sedentary and active subjects. Deviation of these data points from the