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Keywords:	Raman Spectroscopy, Placenta, hypoxia, pattern recognition

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SHORT COMMUNICATION

RAMAN SPECTROSCOPY AS A NOVEL METHOD IN PLACENTAL RESEARCH: RECOGNIZING THE PATTERN OF PLACENTAL HYPOXIA

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ABSTRACT

Raman spectroscopy (RS) is a non-elastic photon scattering technique. We evaluated whether a specific RS pattern exists in fetal venous perfusates obtained at 30-60 min intervals from the *ex vivo* human dual placental perfusion model under hypoxic (n=6) and normoxic (n=5) conditions. A stratified Principle Component and a linear support vector machine analyses showed that the separation between two conditions was readily feasible at 60-120 min of perfusion; after this any apparent differences were most likely the result of artifact over-fitting. This report is the first attempt to identify the RS fingerprint associated with placental hypoxia.

Keywords: Raman spectroscopy, placenta, hypoxia, pattern recognition.

INTRODUCTION

In the 21st century diagnostic methods expanded beyond the identification of single molecular species [1] [2]. Raman Spectroscopy (RS), a method based on inelastic light scattering when excitation photons interfere with specific molecular bonds [3], represents a unique tool currently being applied to the diagnosis of cancer, Alzheimer and cardio-vascular diseases[4] [5]. Resonance RS and Raman microspectroscopy have been applied for detection of hemo-and myo-globin oxygenation for monitoring of tissue oxygenation[6] [6-7] and for molecular fingeprints of pre-eclamptic placentas[8]. Orbital Raster Scanning (ORS) is a novel RS sampling technique based on rastering laser beam, which repetitively sweeps the sample volume providing the opportunity to represent thousands of chemical compounds simultaneously within a spectral "fingerprint". The rastering approach allows scan the larger area, e.g. while the focused laser beam, has a diameter of 50µm, the raster spot diameter is approximately 1 mm. This gives an ORS sampling area of approximately 0.076 mm^2 versus an instantaneous sampling area of 0.0078 mm^2. [9] The application of mathematical models of pattern recognition enables an analysis between experimental study groups. Here we demonstrate, for the first time, the application of RS-ORS to detect fetal hypoxia in physiological perfusates following the adaptation of the *ex vivo* dual perfused human placenta to different oxygenation conditions.

MATERIALS AND METHODS

We analyzed 500 µL of fetal perfusate, obtained using the human *ex vivo* dual placental perfusion technique, which had been modified to normoxic (N; n=5) and hypoxic (H; n=6) conditions, with mean soluble oxygen tension within the intervillous space (IVS) of 5-7% and < 3%, respectively [10]. These adaptations have been proven to have specific changes in

inflammatory mediation within the perfused tissue, demonstrated as an altered biochemical release of cytokines [10a]. The perfusate (modified Earle' bicarbonate buffer) composition was as follows 2.4 mM CaCl2; 0.4mM MgSO4; 117 mM NaCl; 5.4mM KCl; 0.41mM NaH2PO4; 26mM NaHCO3; 5.6mM D-glucose; 5,000 IU heparin, sodium; 0.04mM L-arginine; 0.1% (w/v) bovine serum albumin (fraction IV); 3.5% (w/v) dextran (clinical grade; 70KDa)[10b]. Fetal venous perfusate samples were collected at 60, 120, 150, 210, 240, 300, and 330 minutes (Supplementary material, Fig. 1S). The RS of the samples were collected using Mira Cal software (Mira M-1, Metrohm, USA), which was connected to the hand-held RS device (Mira M-1, Metrohm, USA). In MIRA M1 device, exposure time is automated be the analyzer, the exposure time was 5 second with spectral resolution: 14 to 16 cm^{-1}.

Insert Fig. 1 here

For the Principal component analyses the spectral analysis began by constructing a tuple of information from the twelve fingerprint regions, identified by picking maximum peak heights for peaks that were greater than the mean value of all spectra values (Fig 1). For each of these regions, a feature vector was constructed based on three peak attributes: the peak's value (x), the derivative for the peak (x'), and the second derivative for the peak (x''). Thus for a given time (min) and class (i.e., H vs. N), a 36 dimensional feature vector was constructed (i.e. 12 peaks times, 3 attributes per peak).

A stratified Principle Component Analysis (PCA) [11] was performed independently on the feature vector data aggregated at each time point. For each time point, a set of distinct principle components were identified where each subsequent set of components describes an increasing amount of variance in the feature vector data: e.g., 50%, 60%, …, 100%. A linear support vector

machine (SVM) [12] was then used to compute the accuracy of each component set. In this context, the analysis contrasts how increasing information content (as represented by different principle components) impacts classification performance.

RESULTS AND DISCUSSION

Twelve Raman peaks were detected at 415 cm^{-1}, 440 cm^{-1}, 541 cm^{-1},785 cm^{-1}, 851 cm^{-1}, 920 cm^{-1}, 1020 cm^{-1}, 1086 cm^{-1}, 1129 cm^{-1}, 1341 cm^{-1}, 1457 cm^{-1}, 1632 cm^{-1} (Fig.1S). The hypoxic treatment of placenta *ex vivo* in our study represents the models, mimicking pre-eclampsia (PE) [10a], interestingly, these spectra are similar to the Raman shift, reported for human normal and pre-eclamptic placentas [8] and blood samples from women with pre-eclampsia[13]. The Raman peak of 1129cm^{-1} was quite close to 1127 cm^{-1} hemoglobin-derived hemoporfirin shift reported in exposed to hypoxia erythrocytes and myocardium[6, 14] and the same as found in placentas from women with pre-eclampsia [8]. 1128 cm^{-1} Raman shift was identified for myristic acid, which has also chain vibration expansion shift of 414 cm^{-1}[15] – close to detected in our study 415 cm^{-1}. Band at the shift of 1341 cm^{-1} had decreased intensity in samples with hypoxia in our study and is similar to reported 1342 cm^{-1}shift with decreased intensity in serum of women with PE, which has been identified as CH bending of amino acids[13]. The 1457 cm^{-1} shift is located within 1420-1500 cm^{-1} region of described deformities for δ (CH_2) and δ (CH_3) region of L-arginine[15]. The peak of 440 cm^{-1} corresponds to β-D-glucose and peak at 541 cm^{-1} -to D (+) dextrose [15] – components of the perfusion media. At the 60 min time point the separation between N and H (Figure 2A) is easily obtained with 100% accuracy using principle components that capture 80%

of the variability in the feature vector data. Even when using fewer features where 50% of the variability is captured, separation is feasible with 80% accuracy.

Insert Fig. 2 here

At 120 minutes (Figure 2B), a slight degradation in accuracy is observed. Specifically, we now need almost 88% of the variance captured by the principle components to provide 100% accuracy in separating two conditions (as opposed to 80% seen in 60 minutes). Additionally, classifying based on PCA that captures 50% of the variance only provides about 75% accuracy.

Beyond 120 minutes (Figure 2C and D), significant degradation of the differential signal is observed. Though 100% classification accuracy is feasible (i.e. using principle components that capture *all* variance in data), removing even a little of the information in the data (i.e. from 95% to 100%) dramatically reduces accuracy. Such behavior strongly indicates that beyond 120 minutes of perfusion, separation between two conditions is an artifact of overfitting or could mirror the phenomenon of slow progression of normoxia toward hypoxia in the artificial *ex vivo* perfusion system [10b]. To more confidently address this artifact and confirm the ability (or inability) to separate data beyond 120 minutes, an additional number of samples is required. Commensurate with a reduction in classification accuracy after the 120 minute time point between normoxic and hypoxic groups, the fetal-side release profile of endogenous placental substances appears to be strongly governed by intervillous space soluble oxygenation within this timeframe, since the difference in this variable is highest within the first two hours and diminishes thereafter [10b]. Raman spectra are produced by the portion of photons, scattered by electron density of specific molecular bonds [5a] and depend on vibrational activity of the bonds. Therefore, it is also possible that the intervillous space hypoxia results in the differences in

vibrational activity of the bonds, prior to the release of any active substances in the fetal circulation and therefore could be a sensitive method of diagnosis of fetal hypoxia.

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FIGURE LEGENDS

Figure 1. Example of representative peaks used for the vector construction.

Figure 2. The accuracy of a linear support vector machine to separate hypoxia from normoxia at 60, 120, 150 and 210 min of the placental perfusion *ex vivo*.

SUPPLEMENTARY MATERIAL

Figure 1S. Mean Raman Spectroscopy patterns in the fetal perfusates obtained at 30-60 min intervals in *ex vivo* placental perfusion: hypoxic (n=6) and normoxic (n=5) conditions [10b].

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