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Time and Time-frequency Analysis of Near-Infrared Signals for the Assessment of Ozone Autohemotherapy Long-Term Effects in Multiple Sclerosis*

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Abstract— **Ozone autohemotherapy is an emerging therapeutic technique that is gaining increasing importance in treating neurological disorders. A validated and standard methodology to assess the effect of such therapy on brain metabolism and circulation is however still lacking. We used a near-infrared spectroscopy system (NIRS) to monitor the cerebral oxygenation of 9 subjects: 4 remitting-relapsing multiple sclerosis (MS) sufferers and 5 controls. Subjects were tested before, during, and after ozone autohemotherapy. We monitored the concentration changes in the level of oxygenated and deoxygenated haemoglobin, and in the level of the Cytochrome-c-oxidase (CYT-c). From the time and time-frequency analysis of the NIRS signals we extracted 128 variables, which were used to characterize the metabolic brain pattern during the therapy. We showed that by using only 7 NIRS variables out of 128 it is possible to characterize the metabolic brain pattern of the two groups of subjects. The MS subjects showed a marked increase of the CYT-c activity and concentration about 40 minutes after the end of the autohemotherapy, possibly revealing a reduction of the chronic oxidative stress level typical of MS sufferers. From a technical point of view, this preliminary study showed that NIRS could be useful to show the effects of ozone autohemotherapy at cerebral level, in a long term monitoring. The clinical result of this study is the quantitative measurement of the CYT-c level changes in MS induced by ozone autohemotherapy.**

Keywords—**ozone autohemotherapy, near-infrared spectroscopy, cerebrovascular reactivity, time-frequency analysis.**

I. Introduction

Recent studies showed that ozone autohemotheraphy could be very useful to treat vascular diseases [1], wounds [2], and to prevent limb ischemia in dialysed subjects [3]. The above referenced studies demonstrated the ozone capabilities of boosting the overall metabolism and, particularly, of enhancing peripheral tissue oxygenation. Particular attention has been given to the possibility of utilizing ozone in neurology, in order to enhance brain oxygenation [1]. Reduced oxygen supply to brain tissues and cerebral chronic venous insufficiency have, in fact, been related to different neurodegenerative disorders [4]. Besides increasing the oxygenation level of the tissues, ozone is known to induce an increase in the metabolic rate and to lower the overall inflammation state [5]. This anti-inflammatory effect could be particularly useful in treating autoimmune chronic pathologies, such as multiple sclerosis (MS).

Previous studies tried to quantify the effect of ozone autohemotherpy by considering mainly biochemical reactions [5] and vascular changes [3], but an *in-vivo* uniformed and standardized evaluation protocol of such effects on brain tissue is still missing.

Near-infrared spectroscopy (NIRS) is a non-invasive technique to monitor the changes in the brain concentrations of oxygenated (O_2Hb) and reduced (HHb) haemoglobin in real-time. Additionally, NIRS can measure changes in Cytochrome-c-oxidase (CYT-c) concentration, an enzyme involved in the chain of cellular respiration and therefore representative of cellular energy consumption, particularly at mitochondrial level.

In this study, we used NIRS to monitor the long-term effects of ozone autohemotherapy in neurological subjects. The overall duration of the monitoring was about 2.5 hours. We wanted to focus on the long-term effect to avoid the obvious increase of blood oxygen saturation that is caused by ozone autohemotherapy. We studied patients affected by Multiple Sclerosis (MS) and controls. Since the spectral content of the NIRS signals contains information about cerebral vasomotor reactivity, we studied the signals in the time-frequency domain. Traditional time analysis was carried out as well. PCA, two way ANOVA and MANOVA were used to study the differences of the ozone authoemotherapy effects between patients and controls, and particularly to show the effects on brain metabolism of ozone. To the very best of our knowledge, this study is the first one investigating the long-term effect of ozone therapy in neurological patients.

II. Methods

A. Experimental setup

The ozone therapy protocol started with the drawning of 240 grams of blood from the subjects' antecubital vein. The blood was then mixed with 180 ml of O_2/O_3, composed by O_2 at 50%, with an O_3 concentration equal to 40 μg/ml (M95, Multioxygen, Gorle (BG), Italy). The ozonized blood was then slowly reinjected.

Hence, the overall protocol consisted of *a*) baseline initial recording (average duration 258±58 s), *b*) blood

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drawing and ozonization (326±154 s), *c*) reinjection (1520±804 s), *d*) post-injection monitoring (1.5 hours).

The NIRS recordings were made using a commercially available oximeter (NIRO300, Hamamatsu Photonics K.K., Japan) and the sampling rate was set to 2 Hz. The NIRS probe, consisting of a photo-detector and four infrared LED sources (wavelengths equal to 775, 810, 830 and 910 nm) was placed on the subject's forehead 2 cm away from midline and 1 cm above the supraorbital ridge. During all the test, the subjects were asked to rest in supine position, in a quiet room and with eyes closed.

TABLE I. SUBJECTS DEMOGRAPHICS

	Controls	MS subjects
Number of subjects (# females)	5 (4)	4 (1)
Age (years)	68.2 ± 3.3	38.0 ± 3.4
BMI (kg/m^2)	25.57 ± 2.71	24.23 ± 3.49

After having been instructed about the overall procedures and having signed a written informed consent, 9 subjects (5 controls and 4 MS sufferers) underwent ozone therapy. We enrolled in the study patients suffering from relapsing-remitting MS. Table I summarizes the patients' demographics.

B. *NIRS signal processing*

NIRS monitoring was carried out during the entire therapy protocol and lasted for about 1.5 hours after blood reinjection. We investigated the acquired signals in 8 different time intervals, lasting 256 s each. These 8 analysis windows were centred on *I)* baseline recording, *II)* blood drawing, *III)* middle of reinjection period, *IV)* end (last 256 s) of reinjection, *V)* 20 minutes after reinjection, *VI)* 40 minutes after reinjection, *VII)* 1 hour after reinjection and *VIII)* 1.5 hours after the reinjection. We selected these windows because we wanted to monitor each specific phase of the ozone autohemotherapy. For each window, on each patient we performed a time domain analysis and a time-frequency domain (TF) analysis. In fact, it was already shown that NIRS cerebral signals are characterized by a marked nonstationarity [5]. We analyzed the following 4 signals: O_2Hb, HHb, CYT-c, and Tissue Oxygen Index (TOI). The TOI is defined as the ratio between O_2Hb and the total haemoglobin (*i.e.* O_2Hb + HHb).

The time analysis was made by averaging the signal amplitude in each of the observation windows, in order to analyze the changes in the haemoglobin concentrations in the brain tissue. Therefore, thirty-two variables were generated by time domain analysis (*i.e.* four signals in eight observation epochs).

The time-frequency analysis was made by means of the Choi-Williams distribution (CW) of the Cohen's class (with $\sigma = 0.5$). From the CWs, we measured the signals' power in two frequency bands: very low frequency (VLF: 20mHz - 60mHz) and low frequency (LF: 60mHz - 140mHz). Also, we measured the total signal power (P_{TOT}) for the 4 signals. Fig.1 shows the CYT-c signal during the VI observation window in both time and time-frequency domains. The VLF band is related to the long-term vasomotor reactivity, whereas the LF band is correlated to the activity of the sympathetic system and, hence, to the cerebral autoregulation [6]. We then computed the relative percentage powers P_{VLF}/ P_{TOT} and P_{LF}/ P_{TOT} for the O_2Hb, HHb, CYT-c and TOI concentrations, in the 8 analysis windows and for all the sample population. Thus, we computed 96 variables using the time-frequency analysis. Considering the 32 variables computed from the time domain analysis, the total number of variables was 128.

C. *Variable reduction and supervised/unsupervised analysis*

The aim of our analysis was to find the most important variables characterizing the NIRS pattern of controls and MS subjects during the entire experiment. Therefore, as first

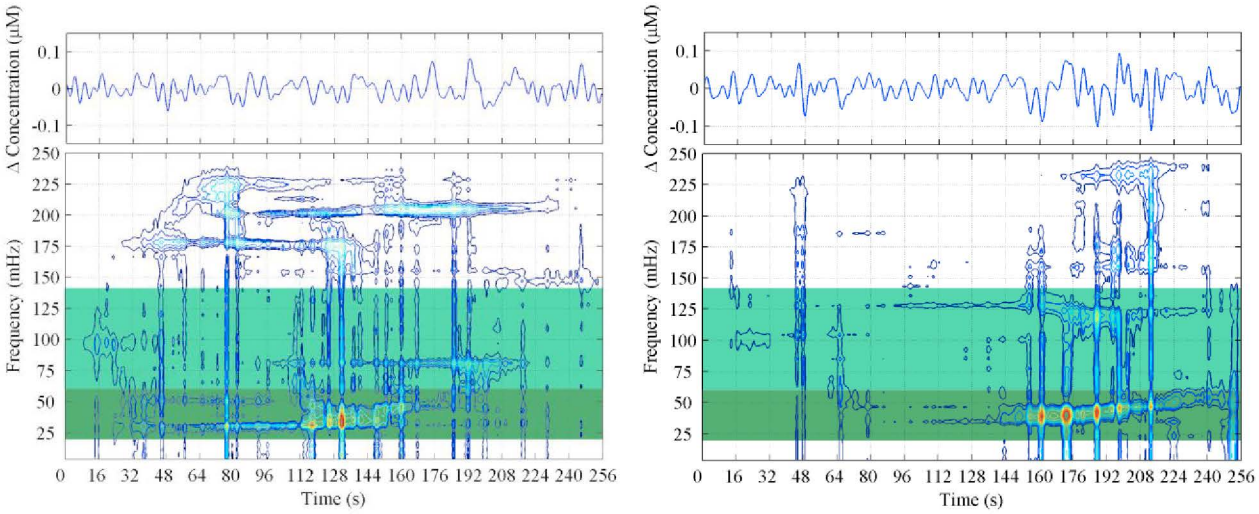

Figure 1. The CYT-c signal is showed in the time and time-frequency domain during the observation window number VI. *a)* is referred to a control subject while *b)* has been obtained from an MS sufferer. VLF and LF frequency bands have been superimposed for easiness of reading. The TF analysis highlights a pronounced difference between the two subjects.

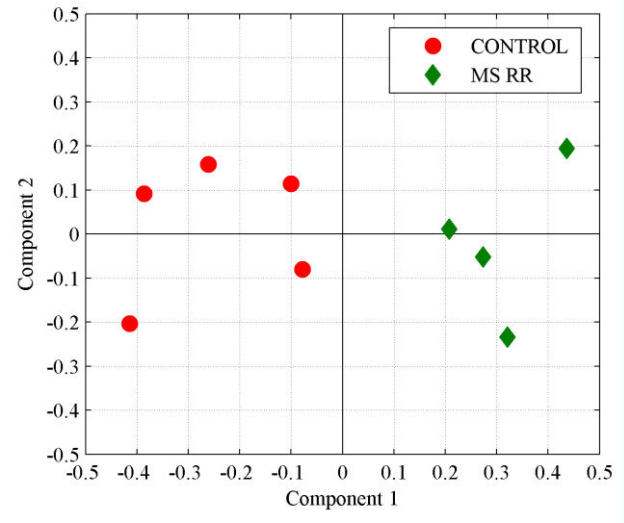

Figure 2. PCA, the figure represents the patients in the space of the first two PCs. The subjects can be divided in two groups accounting on the value of the first PC.

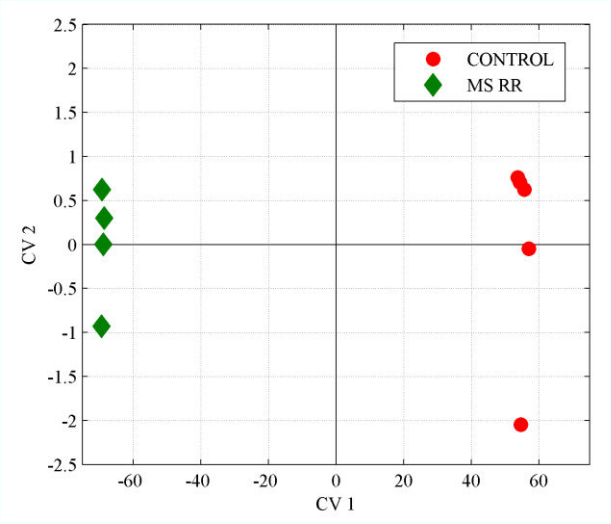

Figure 3. MANOVA, the figure represents the subjects in the space of the first two canonical variables. The subjects can be divided in two groups accounting on the value of CV 1.

step, we removed the collinear variables. The number of variables was reduced by means of a balanced one-way ANOVA between each of the dependent variables and the subject's pathology (considered as independent variable). We considered significant those variables that reported a p-value equal or smaller than 0.01. The following part of the work has been carried on the remaining 17 variables as reported by Table II.

TABLE III. VARIABLES AFTER ANOVA

TIME DOMAIN	
VARIABLE	OBSERVATION WINDOWS
Mean O_2Hb	II, IV, V, VI, VII, VIII
Mean CYT-c	II, III, VI, VII, VIII
TIME-FREQUENCY DOMAIN	
P_{VLF} CYT-c	I, VI
P_{LF} CYT-c	I, VI
P_{TOT} CYT-c	I, VI

In order to discover any difference in the therapy response of the two groups, we applied a Principal Component Analysis (PCA) to the normalized variable set. Fig. 2 shows how the first two PCs (accounting for almost the 80% of the total variance) can easily differentiate the response of MS patients from the controls' one. The five higher loadings for PC1 were assigned to the Mean O_2Hb in windows VI, IV, V, to the Mean CYT-c III and to the P_{TOT} CYT-c VI. We completed our analysis investigating the source of variance in the data set by means of a two way ANOVA. We focused the ANOVA on both the variance generated by the different patients and the variance generated by the different observation windows. The p-values obtained were smaller than 0.01 and therefore we rejected the three different null hypothesis (*i.e.* all patients belong to the same population, all the observation windows belong to the same population and there is no relation between patient group and window).

To discriminate those variables responsible for the biggest part of the total variance we performed a *Wilks' lambda test* on the reduced variables set as in equation (1):

$$\Lambda = \frac{|W|}{|B|+|W|}. \quad (1)$$

Here $|\boldsymbol{W}|$ is the determinant of the *within* group variance matrix, while $|\boldsymbol{B}|$ is the determinant of the *between* groups variance matrix. As a consequence the variables with the smaller Λ have been chosen as the more suitable for subjects classification in the two different groups.

In order to group the subjects using a single parameter we performed a linear combination of the available variables by means of a MANOVA. To avoid singularity problems when working with variance and covariance matrices, we used the first 7 variables showing the smaller Λ (Table III).

This analysis reported that the dimension of the space containing the group means is equal to one, i.e. it was possible to differentiate the subjects with a single canonical variable as showed in Fig. 3.

III. RESULTS AND DISCUSSION

We used NIRS long-term monitoring to detect possible differences in the cerebrovascular metabolic pattern of MS sufferers compared to healthy volunteers. We coupled a time analysis to time-frequency analysis to investigate both the volumetric and metabolic effect of the ozone

TABLE II. VARIABLES AFTER ANOVA

TIME DOMAIN	
VARIABLE	OBSERVATION WINDOWS
Mean O_2Hb	II
Mean CYT-c	II, VIII
TIME-FREQUENCY DOMAIN	
P_{VLF} CYT-c	I, VI
P_{LF} CYT-c	VI
P_{TOT} CYT-c	VI

autohemotherapy (time domain) and its effect on the cerebral vasomotor reactivity (frequency domain). Time-frequency analysis was needed to cope with the overt signals' nonstationary nature. Of the 7 variables that resulted as discriminant, three were computed in the time domain and four in the time-frequency domain (Table III).

Our results showed that by using 7 variables computed on the NIRS signals, it is possible to differentiate the metabolic pattern of the two groups. The three discriminant variables computed in the time domain were the O_2Hb and CYT-c concentrations during blood drawing (phase II of the protocol) and the CYT-c concentration at the end of the monitoring (phase VIII), after 1.5 hours from blood reinfusion. The remaining four discriminant variables were all computed in the time-frequency domain and were all relative to the CYT-c signal. Three of these were the power of the CYT-c signal in the VLF and LF bands, and the total power of the signal recorded during phase VI of the protocol, which was 40 minutes after reinjection. All variables were statistically different between the two groups (Student's *t-test*, $p < 0.05$). Table IV summarizes the mean values of the variables in the two groups.

TABLE IV. MEAN VALUES OF THE 7 DISCRIMINANT VARIABLES. ALL VARIABLES RESULTED STATISTICALLY DIFFERENT BETWEEN THE TWO GROUPS (STUDENT'S T-TEST, $P < 0.05$).

VARIABLE	Mean ± σ CONTROLS	Mean ± σ MS
Mean O_2Hb II	-0.66 ± 0.51	1.99 ± 1.22
Mean CYT-c II	0.08 ± 0.05	-0.19 ± 0.12
Mean CYT-c VIII	0.00 ± 0.20	-0.41 ± 0.13
P_{VLF} CYT-c I	0.02 ± 0.00	0.05 ± 0.01
P_{VLF} CYT-c VI	0.02 ± 0.00	0.05 ± 0.01
P_{LF} CYT-c VI	0.06 ± 0.00	0.11 ± 0.02
P_{TOT} CYT-c VI	0.14 ± 0.01	0.23 ± 0.04

Our results demonstrate that the ozone autohemotherapy has a clear effect in triggering the CYT-c signal, particularly in MS patients. The CYT-c levels in MS are lower than in controls due to mitochondrial damage that is characteristic of the MS disease and our *in-vivo* data confirm this finding [7]. A previous study hypothesized the presence of oxidative damage to DNA in association with inflammation in chronic active plaques, which are the typical white matter damages associated to MS [8]. The same study showed how the oxidative damage developed in association with inflammation in the central nervous system, and might contribute to a decline of energy metabolism in affected cells. Consequently, there could be impairment in the CYT-c production in MS patients due to inflammation induced oxidative stress. In a recent study, Sagai *et al.* showed that ozone induced a mild oxidative stress [5]. Such mild action triggered the production of free antioxidants and anti-oxidative enzymes, which not only protected cells from oxidation and inflammation, but also reversed the chronic oxidative stress. Our findings about the increased activity (higher power in time-frequency representations of the CYT-c signal) seem to confirm that ozone promotes the reduction of chronic oxidative stress and, consequently, enhances the mitochondrial functionality of neural cells. This effect is particularly visible on the MS patients because of their starting conditions of hyper-oxidative stress and lower CYT-c levels.

We coupled unsupervised (PCA) and supervised (MANOVA) analysis strategies in order to better understand if the independent and non-collinear variables were actually descriptive of our sample population. The two approaches gave substantially the same result in terms of subjects' clustering, therefore indicating that the selected variables were discriminant between the two groups. Therefore, we believe that the NIRS technique could be suitable for the long-term monitoring of the effects on brain metabolism and vasomotor reactivity induced by ozone therapy.

In conclusion, we propose the joint approach of NIRS recordings, time and time-frequency analysis, and supervised/unsupervised clustering techniques as suitable in physiological and neuroscience experimental protocols [9,10].

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