INTERNATIONAL JOURNAL OF IMMUNOPATHOLOGY AND PHARMACOLOGY Vol. 27, no. 3, 379-89 (2014)

OZONE AUTOHEMOTHERAPY INDUCES LONG-TERM CEREBRAL METABOLIC CHANGES IN MULTIPLE SCLEROSIS PATIENTS

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Received March 26, 2014 – Accepted June 30, 2014

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 (NIRS) system to monitor the cerebral metabolism and a transcranial Doppler (TCD) to monitor the blood flow velocity in the middle cerebral arteries. Fifty-four subjects (32 neurological patients and 22 controls) 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). As a primary endpoint of the work, we showed the changes in the brain metabolism and circulation of the entire population. The concentration of oxygenated hemoglobin increased after the reinjection of the ozoned blood and remained higher than the beginning for another 1.5 hours. The concentration of the deoxygenated haemoglobin decreased during the therapy and the CYT-c concentration markedly increased about 1 hour after the reinjection. No significant changes were observed on the blood flow velocity. As secondary endpoint, we compared the NIRS metabolic pattern of 20 remitting-relapsing multiple sclerosis (MS) patients against 20 controls. We showed that by using only 7 NIRS variables it was 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.

Recent studies showed the effect of ozone autohemotheraphy in the therapy of vascular diseases (1), wounds (2), macular degeneration (3) and in the prevention of limb ischemia in dialysed subjects (4). The above-referenced studies demonstrated the ozone capabilities of enhancing peripheral tissue oxygenation. In 2013, Percorelli et al. (5) demonstrated a mechanism by which ozonated

Key words: ozone autohemotherapy, near-infrared spectroscopy, transcranial Doppler sonography, cerebrovascular reactivity, time-frequency analysis, MANOVA, cytochrome-c-oxidase

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0394-6320 (2014)

DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

serum was able to up-regulate the heme oxygenase-1 expression in endothelial cells; thus providing a potential therapeutical application in pathologies aggravated by a chronic oxidative stress.

Particular attention has been given to the possibility of utilizing ozone in neurology, in order to enhance brain oxygenation (1) and to reduce the chronic oxidative stress (6). Reduced oxygen supply to brain tissues coupled to the cerebral chronic venous insufficiency have been related to severe and degenerative neurological disorders (7). Even though in studies related to multiple sclerosis (MS) the presence of a chronic cerebral venous insufficiency has been questioned (8), experimental evidence showed that the improvement of the venous brain circulation (via percutaneous transluminal angioplasty) led to a decreased relapsing in MS subjects and to an overall improvement of the clinical conditions (7). Therefore, overall, the cerebral circulation and the oxygen level seem to play a role in the progression and symptomatology of MS.

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 (6). This anti-inflammatory effect could be particularly useful in treating autoimmune chronic pathologies, such as MS. It was proposed that the white matter lesions, typical finding in the magnetic resonance images of MS subjects, could be related to a chronic increased oxidative stress (9). At the same time, the chronic increased level of reactive oxygen species could be related to a reduced metabolic activity in the cells mitochondria. Hence, ozone autohemotherapy could be useful to treat MS patients, particularly because of its boosting effect on mitochondria activation.

Previous studies tried to quantify the effect of ozone autohemotherapy by considering mainly biochemical reactions (6) and vascular changes (4), 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 that enables the real-time monitoring of the changes in the brain concentrations of oxygenated (O_2Hb) and reduced (HHb) haemoglobin. Moreover, NIRS measures change in the Cytochrome-c-oxidase (CYT-c) concentration. CYT-c is an enzyme involved in the chain of cellular respiration and therefore representative of cellular energy consumption, particularly at mitochondrial level (10).

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. Our primary endpoint was to focus on the long-term effects of ozone autohemotherapy, because we wanted to avoid the obvious and expected immediate increase of blood oxygen saturation that is caused by ozone autohemotherapy during treatment. In our secondary endpoint, we studied patients affected by MS and compared them to healthy controls. Since the spectral content of the NIRS signals contains information about cerebral vasomotor reactivity (11-13), we studied the signals in the time-frequency domain. Two-way ANOVA and multivariate ANOVA (MANOVA) were used to study the differences of the ozone autohemotherapy effects between patients and controls, and particularly to show the effects on brain metabolism of ozone. To the best of our knowledge, this study is the first one investigating the long-term metabolic cortical effects of ozone therapy in neurological patients.

MATERIALS AND METHODS

Experimental setup

The ozone therapy protocol consisted of the drowning of 240 gr of whole blood from each subject's 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 re-infused into the same vein. The overall signal recording protocol, therefore, consisted of:

a) baseline initial recording (average duration 258±58 s);

b) blood drowning and ozonization (326±154 s);

c) reinjection (1520±804 s);

d) post-injection monitoring (1.5 hours).

The NIRS recordings were made by a commercially available device (NIRO200, Hamamatsu Photonics K.K., Japan), with the sampling rate set to 2 Hz. The wearable NIRS probe consisted of a photo-detector and four infrared LED sources (wavelengths equal

to 775, 810, 830 and 910 nm) and it was placed on the subject's forehead 2 cm away from midline and 1 cm above the supraorbital ridge (11). During all the tests, the subjects were asked to rest in a supine position, breathing normally in a quiet room and with eyes closed, in order to avoid as much as possible any external vaso-active stimulation.

During the therapy, we also measured the cerebral blood flow velocity in the middle cerebral arteries of the subjects by means of a dual-probe Doppler Transcranial (TCD) device (Delica EMS-9UA, Shenzhen Delicate Electronics Co., Ltd - China) equipped by 2 MHz probes. The probes were positioned in order to insonate the M1 tract. The TCD provides information about the blood flow velocity in the major cerebral arteries, which is related to systemic blood pressure changes and the subsequent vasomotor reactivity (14). However, the penetration of the ultrasounds into the skull is highly dependent on the individual bone window (14). Thus, in about 25% of the subjects the TCD signal is either too weak or too noisy to be suitably used to assess the vasomotor changes. In previous studies, we adopted a joint monitoring approach in which we simultaneously used NIRS and TCD. The advantage in using both the methodologies is that the TCD is more accurate in detecting small changes in the cerebral hemodynamic variables (15), whereas the NIRS provides metabolic and functional information (11, 16).

The systemic oxygen saturation and the arterial blood pressure were also monitored.

Patient demographics

After having been instructed about the overall procedures and having signed a written informed consent, 54 subjects underwent ozone autohemotherapy. The tests were conducted between May 2012 and February 2013.

The average age of the subjects was 64.7±3.5 years (range 26 – 72 years). Twenty subjects were female. The sample population consisted of 22 subjects suffering from remitting-relapsing MS, 10 by other neurological diseases (2 Parkinsonian patients, 6 migraineurs, and 2 hydrocephalic subjects), and 22 healthy volunteers.

To test the secondary endpoint (i.e. to compare the effects of ozone autohemotherapy between controls and MS subjects), we selected a subgroup of 20 subjects (10 controls and 10 MS sufferers). We excluded all the subjects that had insufficient quality of the acquired signals (either NIRS or TCD), those who interrupted the recording and did not reach the end of the monitoring, and those who showed an excessive increase of the arterial blood pressure during the reinfusion. Table I summarizes the patient demographics for this subgroup.

Signal processing for the primary endpoint

We investigated the acquired signals in 8 different time intervals, lasting 256 s each. These 8 analysis windows were taken in correspondence of:

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;
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 and on each patient, we performed a time domain analysis of the signals, by averaging the signal values in each of the observation windows. This led us to compute the average blood flow velocities (from the TCD signals) during all the protocol and the changes in

Table I. *Patient demographics.*

	Controls	MS subjects
Number of subjects (# females)	10 (6)	10 (4)
Age (years)	65.4±3.7	38.0±3.8
BMI (kg/m^2)	25.6±2.7	24.2±3.5

the haemoglobin concentrations in the brain tissue (from the NIRS signals). We analyzed the following 4 NIRS 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). Also, we analysed the left and right blood flow velocity in the middle cerebral arteries. Overall, we had 48 variables from the time domain analysis for each subject (*i.e.* six signals in eight observation windows).

Signal processing for the secondary endpoint

The NIRS signals were further analysed by time-frequency (TF) approach. In fact, it has already been shown that NIRS cerebral signals are characterized by a marked nonstationarity (12, 17). The time-frequency analysis was made by means of the Choi-Williams distribution (CW) of the Cohen's class (with $\sigma = 0.5$) (18). From the CWs, we measured the signals' power in two frequency bands: very low frequency (VLF: 20 mHz – 60 mHz) and low frequency (LF: 60 mHz – 140 mHz). Also, we measured the total signal power (P_{TOT}) for the 4 signals. As an example, 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 (12, 19, 20). From the power of the VLF and LF bands, we then calculated the relative percentage powers as P_{VLF}/ P_{TOT} and P_{LF}/ P_{TOT} for all the signals (*i.e.* O_2Hb, HHb, CYT-c and TOI), 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 of the NIRS signals (*i.e.* four signals in eight windows), the total number of variables was 128 per subject.

Variable reduction and supervised/unsupervised analysis for the secondary endpoint

The aim of our secondary endpoint was to find the most important variables characterizing the NIRS pattern of controls and MS subjects during the entire experiment. The process of feature selection and extraction is essential when dealing with systems characterized by a high amount of data and of possible redundancy (21, 22). Therefore, as first step, we removed the collinear variables, as suggested by previous studies (23). The number of variables was reduced by means of a balanced

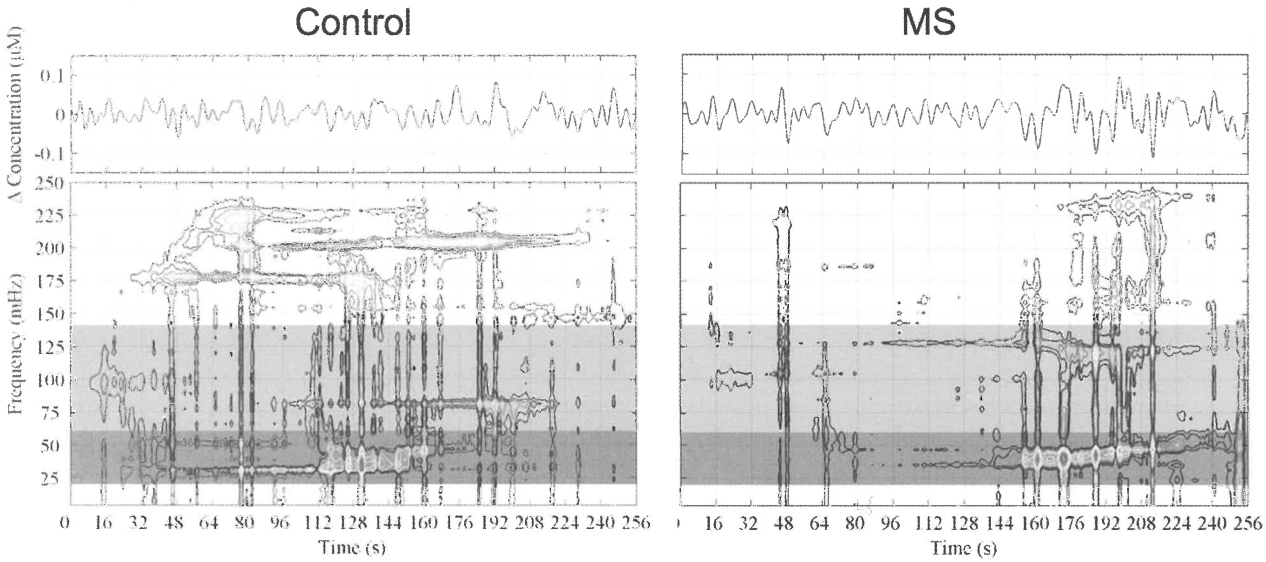

Fig. 1. *Time course (upper panels) and time-frequency representation of the cytochrome-c-oxidase signal for a healthy subject (left panels) and for an MS patient (right panels). The dark gray region depicts the VLF band (20-60 mHz), the light gray region the LF band (60 – 140 mHz).*

Table II. *Significant variables after ANOVA analysis.*

Variable	Observation Window
Mean O_2Hb	II, IV, V, VI, VII, VIII
Mean CYT-c	II, III, VI, VII, VIII
P_{VLF} CYT-c	I, VI
P_{LF} CYT-c	I, VI
P_{TOT} CYT-c	I, VI

The first two rows report variables relative to the time domain. The last three rows report variables relative to the time-frequency domain.

Table III. *The seven most significant variables for the MANOVA.*

TIME DOMAIN

Variable	Observation window
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

By means of these seven variables only it is possible to cluster controls and MS R-R patients.

one-way ANOVA between each of the dependent variables and the subject's pathology (considered as independent variable). When a variable reported a p-value equal or smaller than 0.01 we considered it as significant. In the rest of the work, we used the remaining 17 variables, as reported by Table II.

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):

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

Here |W| is the determinant of the within group variance matrix, while |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 subject classification in the two different groups. By means of a MANOVA analysis, we performed a linear combination of the available variables, in order to group the subjects using a single parameter. Table III reports the 7 variables that showed a Λ value lower than 0.01.

RESULTS

Primary endpoint: long-term effects of ozone auto-hemotherapy

The NIRS can measure only a relative concentration (*i.e.* a concentration change) of a chromophore; therefore, all the signals were scaled to the zero reference value at the beginning of the recording. Starting from the 5th window (about 20 min after the end of re-injection), the O_2Hb showed a marked increase. The O_2Hb concentration in the windows from 5th to 8th was statistically higher than that in the first window (Student's *t*-test; $p < 0.05$). The concentration of the HHb decreased from the beginning of the protocol and starting from the 3rd window it was lower than at the beginning ($p < 0.05$). Then, the HHb concentration decreased until the end of the monitoring. Fig. 2 reports the average concentration changes of the O_2Hb and HHb during the entire protocol.

The TOI decreased during the blood drawing (2nd window) and then it progressively recovered its baseline value during the monitoring (Fig. 3A). The CYT-

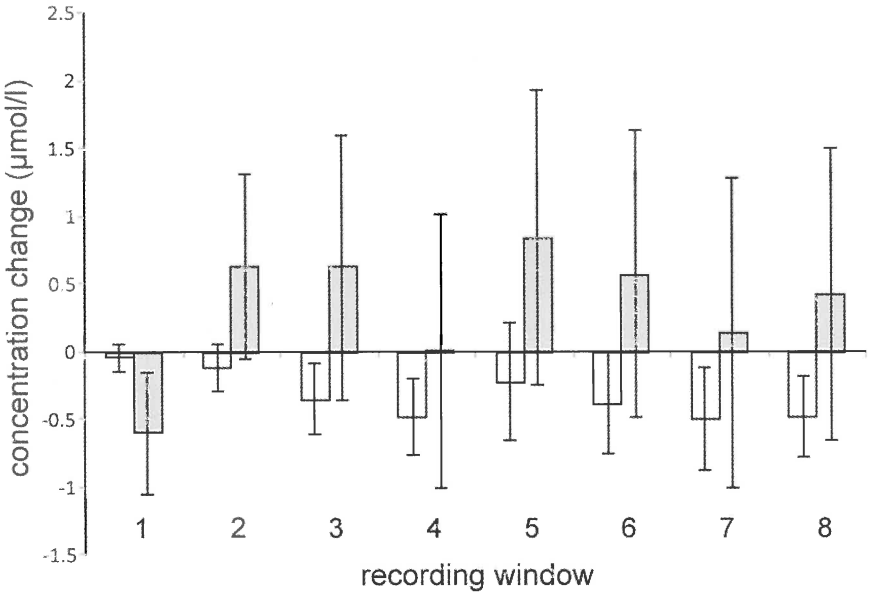

Fig. 2. *Concentration changes of O_2Hb (gray bars) and of HHb (white bars) in the eight observation windows. These graphs are relative to the time average of the signal in the respective windows for all the subjects. The vertical bars represent the standard error. An increase of O_2Hb is observed starting from the 5th window, wherease the HHb decreases along the windows.*

c concentration showed an overt increase in the 7th window (one hour from the end of the monitoring) (Fig. 3B). This increase is not linked to the increase in the oxygenation level, as the O_2Hb increased starting from the 5th window. In the next subsection, relative to the secondary endpoint, we will further observe the importance of the CYT-c changes in MS patients.

The average values of the cerebral blood flow velocity in the middle cerebral arteries are depicted by Fig. 4A (right side) and Fig. 4B (left side). Both graphs show a moderate increase in the blood flow velocity, which is slightly higher in the left side. However, none of the blood flow value was significantly different from the baseline value of the 1st window.

Secondary endpoint: long-term effects of ozone autohemotherapy

In this case, we aimed at documenting specific changes in the metabolic pattern of MS R-R patients when compared to healthy controls. We considered only the NIRS data, because the blood flow velocities did not show any difference between the two groups.

In order to discover any difference in the response of the two groups, we investigated 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 subjects (i.e. MS and controls) and the variance generated by the different observation windows. The p-values obtained were all less than 0.01 and therefore we rejected the three different null hypotheses (i.e. all patients belonged to the same population, all the observation windows belonged to the same population and there was no relation between patient group and window).

The MANOVA showed that the dimension of the space containing the group means was equal to one, *i.e.* the subjects belonged to two groups and it was possible to differentiate the subjects by a single canonical variable. The representation of the subjects in function of the first two canonical variables is reported in Fig. 5. It can be observed that the subjects are neatly separated. Therefore, by using 7 NIRS-derived variables (reported by Table III), it is possible to differentiate the MS patients from the control because they show a different metabolic pattern.

Of the 7 variables that resulted as discriminant,

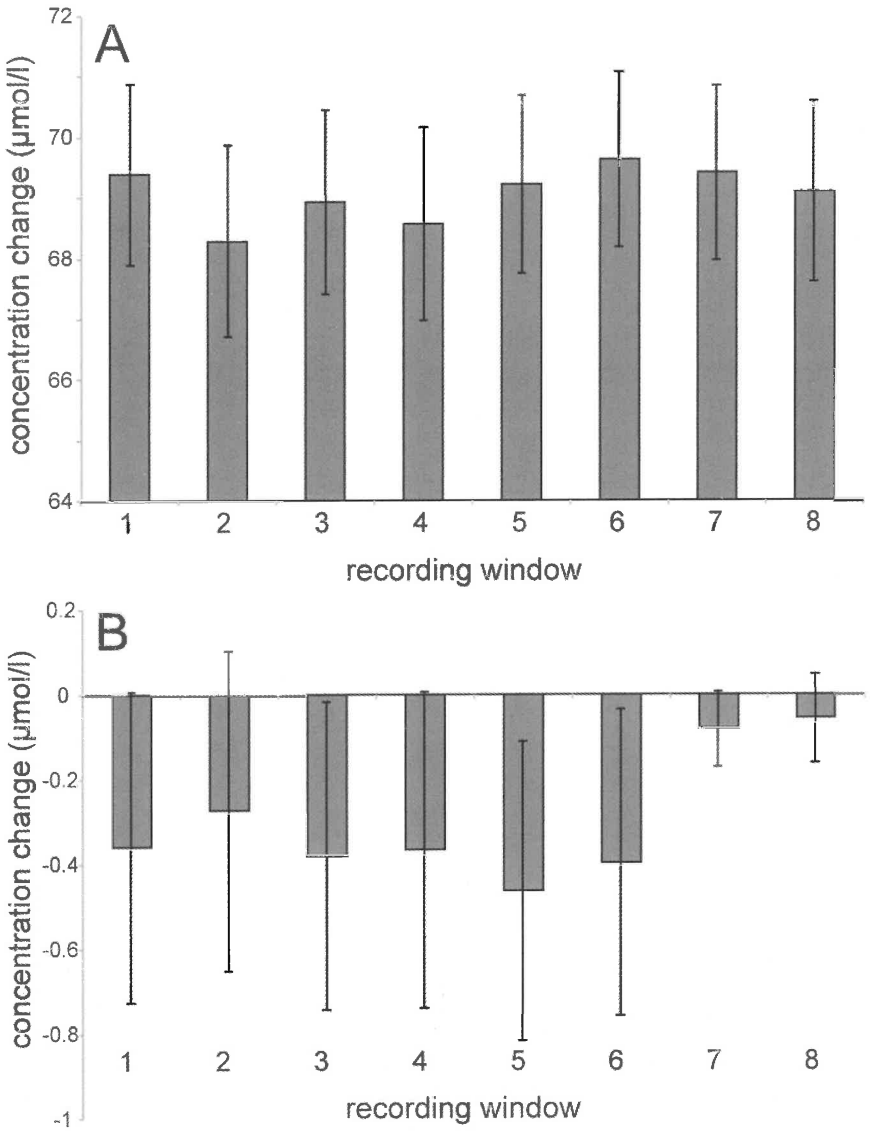

Fig. 3. *Concentration changes of TOI (panel **A**) and of CYT-c (panel **B**) in the eight observation windows. The TOI decreases during blood drawing (2nd window) and then raises back to the baseline levels. The CYT-c markedly increases in the 7th window (1 hour from the end of the blood reinjection).*

three were computed in the time domain and four in the time-frequency domain (Table III). The three discriminant variables computed in the time domain were the O_2Hb and CYT-c concentrations during blood drawing (2nd window) and the CYT-c concentration at the end of the monitoring (8th window), after 1.5 hours from blood reinfusion. The four remaining 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 in the 6th window 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.

DISCUSSION

In this study, we showed the long-term effects at cerebral level of the ozone autohemotherapy on a group of 50 subjects. To demonstrate our prima-

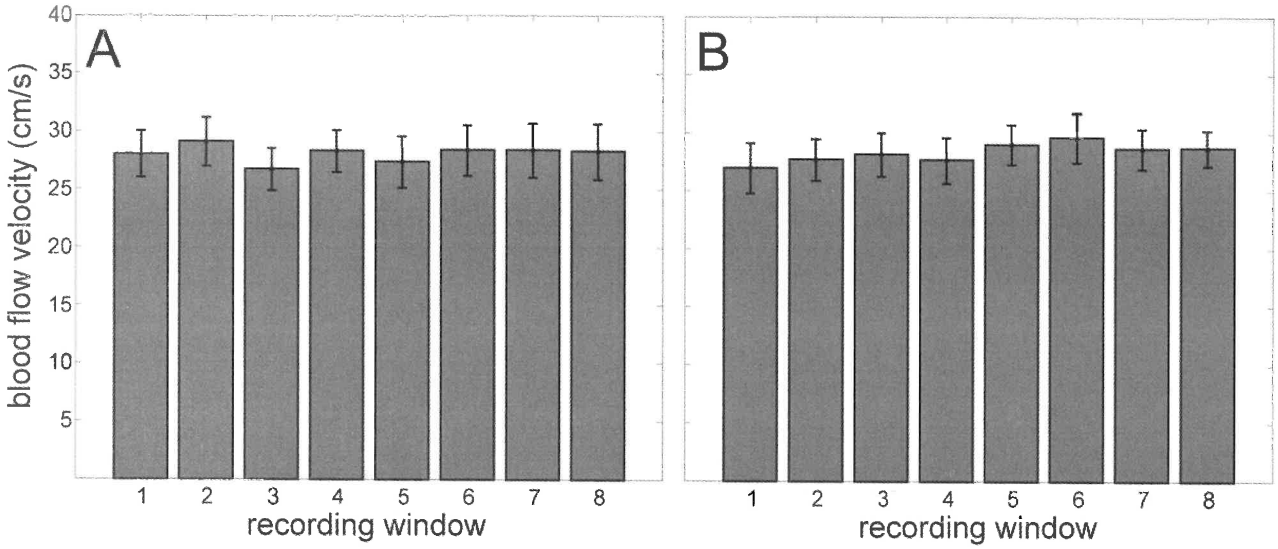

Fig. 4. *Changes in the blood flow velocity in the middle right (panel **A**) and left (panel **B**) cerebral artery in the eight observation windows. No significant changes are observable.*

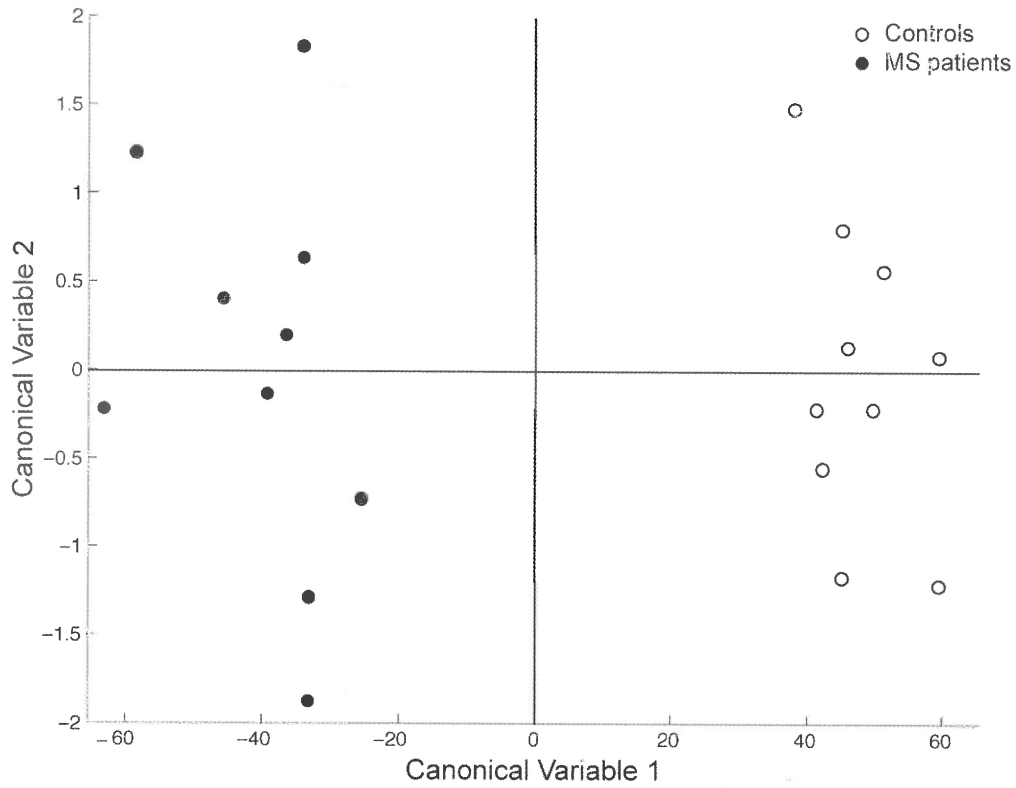

Fig. 5. *Representation of the controls (white circles) and of the MS R-R patients (black circles) in the plane of the first and second canonical variables as represented by the MANOVA. The controls are clearly separated from the MS patients. One single variable (the 1st canonical variable) is sufficient to discriminate among the two groups.*

Table IV. *Mean value of the 7 discriminant variables of Table III in the group of controls and MS patients (mean value ± standard deviation).*

Variable	Controls	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

All variable were statistically different between the two groups (Student's t-test; $p < 0.05$).

ry endpoint, we monitored the subjects for nearly 3 hours by using NIRS and TCD. This led us to jointly monitor metabolic changes in the brain tissue (NIRS) and the blood flow velocity in the cerebral circulation (TCD). Both techniques, NIRS (16) and TCD (14), have already been used jointly to better assess the metabolic and cerebrovascular functionality in a non-invasive way (24, 25). Both techniques monitor a functional response (11, 13, 26) which, in this experimental protocol, could help to better understand the effect at cellular level of the ozone, mainly in MS subjects.

In 2004, Clavo et al. found that repeated ozone autohemotherapy increased the cerebral blood flow systolic velocity by 15% after one week (3 therapies in a week) (27). Our TCD data did not show evidence of significant variation of the cerebral blood flow during the protocol. However, the overall amount of ozone injected was less that 1/3rd of what Clavo *et al.* used on a weekly basis. Nevertheless, we observed a moderate, even though non-significant, increase of the blood flow velocity in the left side. As a hypothesis, this higher increase in the left side could be related to side dominance of the subjects, since about 84% of the subjects were right handed. However, further analysis is needed to assess the hemodynamic changes in the cerebral circulation after ozone autohemotherapy.

There are no studies that we are aware of showing evidence of the oxygen concentration in the brain cortex after ozone autohemotherapy. The most relevant metabolic effect is the marked increase of the CYT-c an hour after the end of the blood reinjection. This result is in accordance with studies regarding aging, in which it was shown that administration of O_2/O_3 mix ameliorated the age-related cerebrocortical alterations, including the complex IV (CYT-c) activity (28).

Regarding our secondary endpoint, we assessed the different metabolic pattern in response to ozone autohemotherapy of a group of MS R-R sufferers compared to controls. Our results demonstrate that the ozone autohemotherapy had 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 MS and our *in-vivo* data confirm this finding (29). 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 (9). 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 (6). 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 in the MS patients because of their starting conditions of hyper-oxidative stress and lower CYT-c levels.

In our sample groups, the average age of the MS patients was equal to 25.6±2.7 years, whereas the average age of the controls was significantly higher (65.4±3.7 years). However, in our opinion, this difference does not bias the comparison. In fact, it was shown that the CYT-c impairments causing oxidative stress in neurodegenerative pathologies (30) are also present, even though to a very low extent, in the mitochondria of all the cells as age progresses (31). Therefore, we compared the MS group to a control group that could already have baseline lower CYT-c activity. Even in this worst-case condition, we observed that MS patients had initial lower levels and final higher changes in the CYT-c activity as a consequence of ozone autohemotherapy.

We used a supervised (MANOVA) analysis in order to better understand whether the independent and non-collinear variables were actually descriptive of our sample population. Results indicated that the selected variables were discriminant between the two groups. Therefore, we believe that the NIRS technique could be suitable for 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 and TCD recordings for the quantitative assessment of the hemodynamic and metabolic changes occurring in the cerebral cortex of patients undergoing ozone autohemotherapy. Given the complexity and the variable nature of these signals, time and time-frequency analysis, and clustering techniques are suitable tools in physiological and neuroscience experimental protocols.

The ozone administration could be very useful in treating neurological disorders, since we showed that there was a marked effect on the activity of the cytochrome-c-oxidase, the impairment of which is common in inflammatory and degenerative neurological diseases. The Authors are currently assessing the clinical efficacy of ozone autohemotherapy in the treatment of MS, in a multi-centric study that should be concluded by the end of 2015. As a perspective, the aim is to observe whether MS patients undergoing ozone autohemotherapy show a reduced number of annual relapses, an improvement in the functional scores, and stabilization of the number of white matter lesions (as documented by MRI).

ACKNOWLEDGEMENTS

This work was partly supported by the Italian Society for Oxygen/Ozone Therapy (SIOOT).

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