CO_2 balneotherapy: effect on free radicals release and total antioxidant status in peripherical arterial occlusive disease

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ABSTRACT

In this study we wanted to evaluate the oxygen free radicals release in patients with peripheral occlusive disease and the possible positive effects of immersion of legs and feet in carbon dioxide (CO_2)-enriched water. Twenty-five patients with peripheral arterial occlusive disease (stage II of Fontaine) and fifteen healthy controls participated in this study. Blood samples were collected into heparinised tubes for determination of total antioxidant status (TAS) and reactive oxygen metabolites (ROMs), after a treatment that was kept five times a week over two weeks. The treatment's standard conditions were: 30 minutes liying down and 10 minutes standing up than lower legs of all patients were immersed in CO_2 enriched water at 34 °C for 20 minutes at dept 35 cm. The results show positive effects of CO_2 enriched-water balneotherapy, in particular on microcirculation due to an increase in cutaneous blood flow leading to a decrease in oxidative stress in patients with arterial occlusive disease validated by blood analysis.

Keywords: CO_2 enriched-water, Total antioxidant status, Reactive oxygen metabolites, peripheral arterial occlusive disease.

INTRODUCTION

CO_2 balneotherapy has long been applied clinically to improve cardiovascular symptoms in European countries. Records of clinical observation about the effects of CO_2 bath on human subjects have been accumulated and the list of effects now includes bradycardia, slight changes in blood pressure, and hypertermia of skin exposed (Hartmann et al., 1997a). Several studies have analysed the oxidative status of animal submitted to acute stress induced by CO_2 inhalation or immersion, where induces bradycardia inhibited by beta-adrenoceptor bl, suggesting that CO_2 absorbed through the skin surface could effect cardiac synaptic nerve activity (Sato et al., 2009), (Hashimoto and Yamamoto, 2004). Carbon dioxide acts a peripheral vasodilatation in blood vessels following inhalation or immersion (Diji and Greenfield, 1960), (Duling, 1973). Intracorporal variations in CO_2 concentrations affect both blood flow, tissue perfusion, and the capacity of hemoglobin to release oxygen (Mohrman and Regal, 1988), (Ohta et al., 1989). CO_2 bathing has an old history and is thought to be effective in the treatment of peripheral vascular disorder (Radawski et al., 1972). While the effects of CO_2 enriched water application has been repeatedly investigated, the present study was designed to investigate specific effects of serial carbon dioxide application on plasma free radicals release. The relationship between oxidative stress and vascular disease progression has recently attracted fresh attention due to the possibility of measuring free radicals (Incandela et al., 2001). The aim of this study was to evaluate oxygen free radicals release in patients with peripheral occlusive disease treated with CO_2 enriched-water. From our knowledge, our study is the first concerning oxidative status markers and CO_2 balneotherapy.

MATERIAL AND METHODS

There were 25 patients (mean age 58, 49-71 years) with peripheral arterial occlusive disease (stage II of Fontaine) and 15 healthy controls, all male who participated in this study. All were healthy, with no family or personal history of diabetes, and normal thyroid, hepatic and renal function. Special care was taken to exclude anyone taking drugs, smokers or drinkers. Both groups were similar in age and body mass index. We use a new carbon dioxide (CO_2) enriched water (1553 mg CO2 per Kg water), from Rabbi SPA (Rabbi Fonti, Trento, Italy). After 30 minutes liying down and 10 minutes standing up, lower legs of all patients were immersed in CO_2 enriched water at 34 °C for 20 minutes at dept 35 cm; these standardized conditions were keept five times a week over two weeks.

After a 12-h overnight fast**,** 10 mL of venous blood was aseptically drawn from the antecubital vein. The samples were collected into heparinised tubes for determination of total antioxidant status (TAS) and reactive oxygen metabolites (ROMs) . Samples were refrigerated (4°C) for 1 h until separation by centrifugation at 1500 *g* for 15 min at 4°C, and immediately used for determinations.

The experimental protocol was approved by the Ethics Comitee and complied with the Declaration of Helsinki.

Inclusion Criteria

The study population comprised male and female patients. Supplementing the drug regimen of subjects with new agents or introducing new treatments was avoided during the study period.

Exclusion Criteria

Exclusion criteria included intact mestrual cycle; radiculopaty or femoral neuralgia; or presence of more serious medical, rheumatological, genitourinary, or dermatological disorders that represent a contraindication to balneotherapy.

Reactive oxygen metabolites (ROMs)

Reactive oxygen metabolites (ROMs) were evaluated on heparinised plasma samples by using the ‘‘d-ROM test’’ (Diacron International s. r. l., Grosseto, Italy). In this test, plasmatic ROMs (mainly hydroperoxides), in presence of iron (that is released from plasma proteins by an acidic buffer, the R_2 reagent of the kit), are able to generate alkoxyl and peroxyl radicals, according to the Fenton's reaction. Such radicals, in turn, are able to oxidize an alkyl-substituted aromatic amine (that is dissolved in a chromogenic mixture, the R_1 reagent of the kit), thus transforming them into a pink derivative photometrically quantified at 505 nm. The intensity of the developed colour is directly proportional to the concentration of ROM, according to the Lambert– Beer's law.

Photometrical results from d-ROMs test have been validated by integrating electrochemical data with the findings obtained in parallel with Electron Spin Resonance (ESR) spectroscopy, the most fit experimental technique for revealing and identifying the presence of radicals in a biological sample.

The results of d-ROM test are expressed in arbitrary units called ‘‘Carratelli Units’’ (CARR U). It has been experimentally established that 1 CARR U corresponds to 0.08 mg/100 mL H_2O_2. The linearity range of d-ROM test is between 50 and 500 CARR U; for values up to 500 CARR U, a sample dilution is required .

Reference values of healthy subjects are between 250 and 300 CARR U. Values between 301 and 320 should be considered as "border line", while conditions of slight, medium, and high oxidative stress are defined, respectively, by values of 320–360, 360–400, and >400 CARR U. (Trotti et al., 2001).

The d-ROMs test has been successfully used either in human or veterinary medicine showing useful in the identifying and monitoring oxidative stress, even after antioxidant (Cesarone et al., 1999) or specific (Digiesi et al., 2000) treatments.

Compared to other conceptually similar available tests aimed to assess oxidative status in plasma samples, the d-ROMs test values were shown to directly and significantly correlate with plasma 8-isoprostane levels ($r=0.68$; $p<0.05$) in healthy subjects. Moreover, a highly significant positive correlation was also found between the d-ROMs test and the FOX-2 assay, the well-established test for the measurement of plasma lipid hydroperoxides, both in controls and in hemodialysed patients.

Briefly, according to the end-point analysis, the absorbances were measured (UVICAM, Cambridge, UK) after incubating a solution of 5 μL of serum, 1 mL of R_2 and 10 μL of R_1 at 37°C for 75 min. A blanck reagent using distilled water instead of serum, and a standard, were included for each series of assays. The absorbance of the reagent blank was subtracted from those of the standard and the samples. The concentration of ROMs were as follows:

(Abs. sample/Abs. standard) x [standard]

where Abs. are the absorbances and [ ] is the standard concentration.

The analytical accuracy was evaluated using a control serum with assigned value, provided by the manufacturer. In agreement with the findings of previous studies (Incandela et al., 2001), (Carratelli et al., 2001) the intra-assay coefficient of variation (CV) calculated on 20 samples of fresh serum was 2.2%, and the inter-assay CV on 20 samples of frozen serum was 3.7%.

All the results were in the linearity range of the test and did not require any dilution of the samples.

Antioxidant status (TAS)

Total Antioxidant Status (TAS) of serum was measured with the RANDOX NX2332 test (RANDOX Laboratories Ltd, Ardmore, UK) (Erel, 2004). This assay, which kit includes phosphate buffer (5 mmol/L, pH 7.4), met-myoglobin (6.1 μmol/L) and hydrogen peroxide (250 μmol/L), is based on incubation of ABTS® (2,2 azinobis 3-ethylbenzthiazolinesulfate) with met-myoglobin and hydrogen peroxide, which may lead to the formation of ABTS-derived radicals.

These have a stable blue-green colour at the wavelength of 600 nm. Antioxidants present in the sample weaken the colour in proportion to their concentration. The results of TAS are expressed as mmol/L.

Total Antioxidant Status assay measures the peroxyl-scavenging capacity of a biological fluid. In a blood plasma sample, such a capacity has been hypothesised to be ascribable to a number of relevant antioxidants, mainly sulfhydryl groups (mostly albumin), urate, ascorbate, α-tocopherol, and bilirubin. However, it was further estimated that 25% to 35% of the measured TAS is provided by uncharacterized antioxidants (Wang et al., 1997).

Briefly, the initial absorbance was recorded. Then, after three minutes, it was measured again for each sample at 37 °C and the wavelength of 600 nm, using a common spectrophotometer (UVICAM, Cambridge, UK).

Means were compared by the unpaired t-test or one-way analysis of variance (ANOVA). Data are presented as means $\pm$ SD. Correlations were assessed according the Tukey-Kramer multiple comparison test and the Mann-Whitney non-parametric test (*U*-test). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Figure 1 shows data concerning ROMs and figure 2 shows data concerning TAS from three groups: healthy controls, group A (before balneotherapy), group B (after balneotherapy). Before balneotherapy, ROMs were higher in patients than in controls and TAS were lower in patients than in controls ($p<0.05$). After balneotherapy, in patients ROMs decreased from 351.8 + 5.66 SD U.Carr to 303.72 + 3.00 SD U.Carr ($p<0.05$). Moreover, at the mean time, in patients TAS increased from 0.63 + 0.014 μmol/L to 1.03 + 0.031 μmol/L ($p<0.05$).

DISCUSSION

In the present study we firstly comparatively evaluated the oxidative balance in a group of patients with peripheral arterial occlusive disease (stage II of Fontaine) *vs.* a group of normal subjects by measuring both the oxidative status (d-ROMs test) and the total antioxidant status (TAS), before and after CO_2 enriched water balneotherapy.

Some evidence supports the idea that CO2 baths might represent an efficient therapeutic means in the rehabilitation of coronary heart disease, myocardial infarction and stroke, and in the treatment of chronic venous insufficiency, certain inflammatory diseases, and functional disturbances (Savin et al., 1995), (Hartmann et al., 1997b). CO_2 acts in different manners, increased skin blood flow during CO2-water immersion facilitates heat transfer from the body to the surrounding water (Ito et al., 1989),(Nishimura et al., 2002) in addition, CO2 inhibits the activity of cold receptors and facilitates the activity of warm-sensitive receptors in the skin (Ricci et al., 2004), affecting the firing rates of preoptic thermosensitive neurons (Dubick et al., 1999). Preoptic thermosensitive neurons are activated in response to peripheral or central temperature stimuli. The greater thermal sensation produced by CO2-water immersion might be effected through increased cutaneous blood flow or peripheral temperature stimuli induced by CO_2 enriched water balneotherapy (Lau et al., 1991). Topically applied, it has different effects, especially its hyperemic effect on the skin. CO_2 might impare skin microcirculation and arterial macrocirculation. Controlled animal experiments have demonstrated that both cutaneous and muscular blood flow and oxygen tension increased during immersion (Komoto et al., 1988). CO2 baths might represent an efficient therapeutic means in the rehabilitation of coronary heart disease, myocardial infraction and stroke, and in the treatment of chronic venous insufficiency, certain inflammatory disease and functional disturbance (Irie et al., 2005), (Toriyama et al., 2002). CO_2 bath, besides, led to improved peripheral oxygen utilization in patients with cardiovascular disease, and produced long-term improvement in blood-flow characteristics by reducing hematocrit and blood viscosity. In view of these findings we

decided to investigate the effects of immersion in CO_2 enriched-water on free radicals release (ROMs) and total antioxidant capacity (TAS) before and after balneotherapy.

The evaluation of ROMs is an important new physiologic parameter that can be associated with other noninvasive tests to study the microcirculation and its evolution and improvement with systemic or topical medical treatment (Ricci et al., 2004). In vivo measuring oxygen derived free radicals activity has always been a problem due to the extremely short half life of oxygen radicals. Other measurement are aimed at the total antioxidant capacity that is lower after oxidative stress

The ROMs and TAS tests provide a simply and practical method (Trotti et al., 2001), to identify subjects with high level of oxidative stress and to demonstrate the systemic effect of treatment.

Such investigations, become possible thanks to new measurement technique.

CO_2 enriched-water has an immediate effect on oxidative balance, confirmed quantitatively and qualitatively in the present study.

In addition, we observed several effects of CO_2 enriched-water bathes (Figure 2). Therapeutic activities of CO_2 enriched-water bathes are explained by a synergism between many factors. The first one are related to immersion it-self: hydrostatic pressure and Archimedes force inducing orthosympathetic inhibition and muscular relaxation. The second ones depend on the pharmacological properties of carbon dioxide acting directly on the blood vessels of the skin, causing vasodilatation and heat sensation (Yamamoto and Hashimoto, 2007). The effects of immersion on CO_2 enriched-water on cutaneous circulation, and vasomotion were measured by laser Doppler flowmetry. Serial carbon dioxide application can be clinically effective in patients with arterial obstruction in the lower extremities, and also could be a scavanger for free radicals generation.

Patients with arterial occlusive disease suffer from temporary lack of oxygen in the legs, caused by narrowing of arteries, resulting in ischemic and followed by reperfusion. Free radicals are involved in atherosclerotic disease, but not in occlusive disease (Dubick et al., 1999). Moreover free radicals

release increased following angioplasty in patient with stenotic, but not occlusive peripheral vascular lesions .

It has been demonstrated that antioxidant vitamins A,C and E would seem uniquely situated to reduce cardiovascular events by improving endothelial function by reducing the concentration of reactive oxygen metabolites in the vessel wall.

From our data there is a reason to believe that an increase in scavenging activity is beneficial in occlusive arterial diseases, and that this activity can reduce the systemic and local inflamatory response seen after ischemia-reperfusion injury (Lau et al., 1991).

The scanvenger could be the CO_2 enriched-water bathes (Hashimoto and Yamamoto, 2004). In our study we have shown a decrease in oxidative stress after two weeks of CO_2 enriched-water balneotherapy in patients with arterial occlusive disease. In cocnlusion the increase in cutaneous blood flow which perisist throughout the period in CO_2 enriched-water bathes, is interpreted as an increase in microcirculation. However the scavenging effect on oxygen derived free radicals can explain part of the effectiveness of thus longely empirical treatment (Resch and Just, 1994), (Hartmann et al., 1991).

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FIGURES LEGEND

Figure 1

Group A is before balneotherapy and Group B is after balneotherapy.
Data show represents Reactive Oxygen Metabolites (ROMs) expressed as ± SD (U.Carr)

Figure 2

Group A is before balneotherapy and Group B is after balneotherapy

Data show represents Total Antioxidant Status (TAS) expressed as ± SD (mmol/L)