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## Antioxidative effects of sulphurous water from Macerata Feltria Thermal resort in patients with osteoarthritis

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### TITOLO

Effetti antiossidanti dell'acqua sulfurea delle Terme di Macerata Feltria in pazienti con osteoartrosi

### KEY WORDS

Osteoarthritis, oxidative stress, sulphurous mineral water, antioxidant effects

### PAROLE CHIAVE

Osteoartrosi, stress ossidativo, acqua sulfurea, effetti antiossidanti

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### Summary

It has been widely demonstrated that reactive oxygen species are implicated as the main causative factors of osteoarthritis (OA), a degenerative joint disease characterized by a progressive degradation of the articular cartilage. Sulphur thermal therapies are employed in the treatment of OA since ancient times; however, their mechanisms of action in the control of OA are only partially known. In the present study, we undertook a preliminary investigation aimed at evaluating the antioxidative effects of a cycle of mud-therapy with sulphurous water in combination with hydrophysiotherapy in fifteen patients affected by degenerative OA from the Thermal Resort of Macerata Feltria. Subjects were evaluated both before (T0) and after (T1) therapy thus to monitor their antioxidant profile as well as some indices of oxidative damage. Interestingly, at the end of the thermal treatment we evidenced a significant increment of plasma total antioxidant capacity and total thiol levels, with a concomitant decrement of both lipid and protein oxidation markers. Our findings of improved body redox status in OA patients undergoing sulphur thermal therapy suggest major benefits from sulphurous water in reducing bio-molecule oxidation, possibly furnishing a valid protection against oxidative injury commonly associated with aging and age-related degenerative diseases.

### Riassunto

E' stato ampiamente dimostrato che le specie reattive dell'ossigeno sono importanti fattori causali dell'osteartrosi (OA), una patologia degenerativa caratterizzata dalla progressiva degradazione della cartilagine articolare. Le terapie termali a base di zolfo sono impiegate da lungo tempo nel trattamento dell'OA; tuttavia i loro meccanismi d'azione nel controllo di questa patologia sono solo parzialmente conosciuti. In questo lavoro abbiamo investigato gli effetti antiossidanti di un ciclo di fangoterapia con acqua sulfurea abbinata a idropinoterapia in 15 pazienti affetti da OA degenerativa presso il Centro Termale di Macerata Feltria. I soggetti sono stati valutati prima (T0) e dopo (T1) la terapia così da monitorare il loro profilo antiossidante ed alcuni indici di danno ossidativo. Alla fine del trattamento termale abbiamo evidenziato un aumento significativo sia della capacità antiossidante totale del plasma che dei livelli di tioli totali; contemporanea-

mente è stata osservata una diminuzione significativa dei livelli plasmatici di alcuni marcatori di ossidazione lipidica e proteica. Il miglioramento dello stato redox in pazienti con OA sottoposti a terapia termale evidenzia come l'acqua sulfurea porti beneficio nel ridurre l'ossidazione delle biomolecole, così da fornire una valida protezione verso il danno ossidativo comunemente associato all'invecchiamento e alle patologie degenerative ad esso correlate.

## Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by a progressive degradation of the articular cartilage, which is associated with chronic pain and significant disability. The underlying mechanisms of cartilage matrix degradation are not completely understood but it has been widely demonstrated that reactive oxygen species (ROS) are implicated as the main causative factors (1-3). As long as ROS production is under the control of the cellular antioxidant defences, ROS can be considered as regulatory factors of cartilage homeostasis, playing a crucial role in the regulation of some normal chondrocytic activities such as cell activation, proliferation and matrix remodelling (4). However, when ROS production exceeds the antioxidant capacities of the cell, an oxidative stress condition occurs, leading to structural and functional cartilage damage. In fact, ROS, namely superoxide anion, hydrogen peroxide and hydroxyl radical, are capable of oxidising and, subse-

quently, damaging numerous components of the joint, including collagen, proteoglycans and hyaluronan (5). Antioxidant dietary or antioxidant supplementation might prevent or slow-down cartilage degradation in joint diseases; however, to date, epidemiological studies examining the role of antioxidants, especially tocopherols, in human OA are few and contradictory (6).

Sulphur thermal therapies, including mineral waters and mud baths, are largely employed in the treatment of rheumatic diseases such as OA, thus integrating the standard pharmacological tools. The mechanisms of action of thermal treatments based on sulphur-rich waters in the control of OA are only partially known, but it is plausible that both sulphates ( $\text{SO}_4^{2-}$ ) and hydrogen sulphide ( $\text{H}_2\text{S}$ ) play a key role in the prevention of cartilage degeneration. Indeed, on one hand, cartilage proteoglycans are highly sulphated and  $\text{SO}_4^{2-}$  supplementation might be beneficial for the de novo synthesis of matrix components

and cartilage health, taking into account that sulphate pools in humans are among the smallest of all species, making them especially susceptible to physiologically relevant small changes (7). On the other hand,  $\text{H}_2\text{S}$  is considered a gas-transmitter that can protect cells from oxidative stress by increasing the activity of  $\gamma$ -glutamylcysteine synthetase and up-regulating cysteine transport, thus resulting in an increment of intracellular glutathione (GSH), the major antioxidant in cells (8, 9).  $\text{H}_2\text{S}$  is also a reducing agent that readily reacts with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (10), possibly being an important redox controlling molecule like other small thiol groups such as cysteine and GSH. As a consequence,  $\text{H}_2\text{S}$  might have an important role in the antioxidant strategies to protect cartilage components against oxidative injury.

In this context, we undertook a preliminary investigation aimed at evaluating the influence of a cycle of mud-therapy with sulphurous water in combination with hydro-pinotherapy (i.e. orally ingested

sulphurous water) on the oxidative status of fifteen patients affected by degenerative OA. Subjects were evaluated both before (T0) and after (T1) therapy thus to monitor their antioxidant profile as well as some lipid and protein oxidation markers.

## Materials and methods

### Subjects

A group of 15 osteoarthritis patients (M=5, F=10, age 65-80 years old), diagnosed on the basis of their clinical symptoms and X-ray examination, were recruited to participate in this study after giving informed consent. Patients with active ischemic heart disease, uncontrolled diabetes mellitus, severe hypertension or central nervous system diseases were excluded. None of the subjects received vitamin and/or mineral supplementation for at least 4 weeks before the beginning of the study. All patients continued to receive their regular medications, including various painkillers and nonsteroidal anti-inflammatory drugs, without any change in the type or dose of their medications during the thermal cures.

### Study design

Subjects underwent a total of 12 mud pack treatments, with daily

frequency, in the thermal resort "Pitinum Thermae" (Macerata Feltria, Italy). The fine soil paste (mud) derived from a mineral water that, for its chemical and physical characteristics, could be considered like sulphurous sulphate bicarbonate calcic water (Table 1). Mature thermal mud was daily applied for 20 min at 46-48°C, followed by a thermal bath in sulphurous water at 37°C. In addition to mud therapy, subjects daily drank 300 ml of sulphurous mineral water having a sulphidric degree of 53.4 mg/l (Ta-

ble 1). To avoid H<sub>2</sub>S loss, water was consumed within 1 hour from the opening of the bottle.

Patients were studied at two experimental time points: before (T0) and after 12 days (T1) of thermal treatments. At each time, venous heparinized blood samples were taken, tubes were centrifuged at 2500 rpm for 10 min and plasma aliquots were stored at -80°C until assayed. The following parameters were monitored during the study: malondialdehyde (MDA) and advanced oxidation protein products (AOPP)

**Table 1 - Chemical and physical characteristics of the mineral water from Pitinum Thermae (Macerata Feltria, Italy)**

Temperature	C°	12.6
(H <sup>+</sup> )	pH	6.65
Conductivity at 20°C	µs/cm	3320
Fixed residue at 180°C	mg/l	3260.0
Oxidability	mg/l O <sub>2</sub>	2.6
Free CO <sub>2</sub>	mg/l CO <sub>2</sub>	190.0
Silica	mg/l SiO <sub>2</sub>	23.0
Bicarbonates	mg/l HCO <sub>3</sub>	587.3
Chlorides	mg/l Cl	125.0
Sulphate	mg/l SO <sub>4</sub>	1750.0
Sodium	mg/l Na	130.0
Potassium	mg/l K	13.0
Calcium	mg/l Ca	620.0
Magnesium	mg/l Mg	152.8
Free iron	mg/l Fe	0.090
Fluorine	mg/l F	1.00
NH <sub>4</sub>	mg/l NH <sub>4</sub>	4.5
Total phosphate	mg/l P	Trace
Sulphidric degree	mg/l H <sub>2</sub> S	53.4
Strontium	mg/l Sr	10.0
Litium	mg/l Li	0.15

as markers of lipid and protein oxidation, respectively; total thiols (-SH) as non-enzymatic antioxidants, the biological antioxidant power (BAP) as index of the total antioxidant capacity (TAC) of plasma.

#### *Determination of MDA*

MDA plasmatic levels were evaluated by reverse-phase HPLC as previously described (11): sample derivatization was carried out by adding 50  $\mu$ l 0.05% butylated hydroxytoluene solution, 400  $\mu$ l 0.44 mol/l  $H_3PO_4$  solution and 100  $\mu$ l 42 mmol/l thiobarbituric acid (TBA) to 50  $\mu$ l plasma. Tubes were vortexed and then heated for 1 h at 100°C. Following derivatization, samples were placed on ice for 5 min and 250  $\mu$ l butanol were added for extraction of the MDA-TBA complex. Tubes were vortexed and then centrifuged at 10000 g to separate the two phases. 20  $\mu$ l were removed from the butanol layer and placed into the HPLC injector for analysis without evaporation. The assay was performed using a Alltima C18 column (4.6 x 250 mm, 5  $\mu$ m, from Alltech, Milan, Italy) equipped with a Alltima C18 guard column (4.6 x 7.5 mm, 5  $\mu$ m). The eluent phase was methanol:buffer (40:60, v/v), buffer consisting of 50 mmol/l  $KH_2PO_4$  pH 6.8. The flow rate was 1 ml/min. UV detection was carried out at 532 nm, the fluorescence detector

was set at an excitation wavelength of 515 nm and emission wavelength of 553 nm. All the organic solvents used were pure HPLC-grade from Carlo Erba, Milan, Italy. The HPLC instrumentation was from Jasco Corporation, Tokyo, Japan.

#### *AOPP assay*

Plasmatic levels of AOPP were measured by spectrophotometry and calibrated with chloramine-T that in the presence of potassium iodide (KI) absorbed at 340 nm (12).

The reaction mixture was formed by 200  $\mu$ l plasma diluted 1/5 in 20 mmol/l PBS, 10  $\mu$ l 1.16 mmol/l KI and 20  $\mu$ l acetic acid; the absorbance was immediately read at 340 nm on a microplate reader (Bio-Rad Laboratories, Milan, Italy) against a blank containing 200  $\mu$ l of PBS instead of plasma. AOPP concentration was expressed in  $\mu$ mol/l of chloramine-T equivalents.

#### *Colorimetric determination of plasmatic -SH groups*

Total -SH groups were evaluated using a commercial kit distributed by Diacron s.r.l., Grosseto, Italy. The method is based on the capacity that plasmatic -SH groups have to react with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), followed by the development of a colored

complex that can be measured photometrically at 405 nm (13).

#### *Colorimetric determination of BAP*

The total antioxidant capacity in plasma samples was evaluated by the use of the "BAP test" (Diacron s.r.l., Grosseto, Italy). The assay measures the combined effect of non-enzymatic defenses in biological fluids and can be useful in providing a putative index of the ability to resist oxidative damage. The method measures the ferric reducing ability of plasma (14) and it is based on the ability of a ferric ion solution, binding to a particular chromogen, to decolorize when the ferric ions are reduced to ferrous ions. The values are obtained by comparing the absorbance change at 505 nm in the test reaction mixture with mixtures containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range with the antioxidant mixture, including plasma, and with solutions containing one antioxidant in purified form (vitamin C). Intra- and inter-assay coefficients of variation are less than 5.5%. Plasmatic antioxidant capacity is expressed in  $\mu$ mol/l of vitamin C. Reference values of healthy subjects are >2220  $\mu$ mol/l of vitamin C; condition of border-line, slight deficiency, high and very high deficiency are defined, re-

spectively, by values of 2200-2000, 2000-1600, 1600-1400, <1400  $\mu\text{mol/l}$  of vitamin C.

#### Statistics and data processing

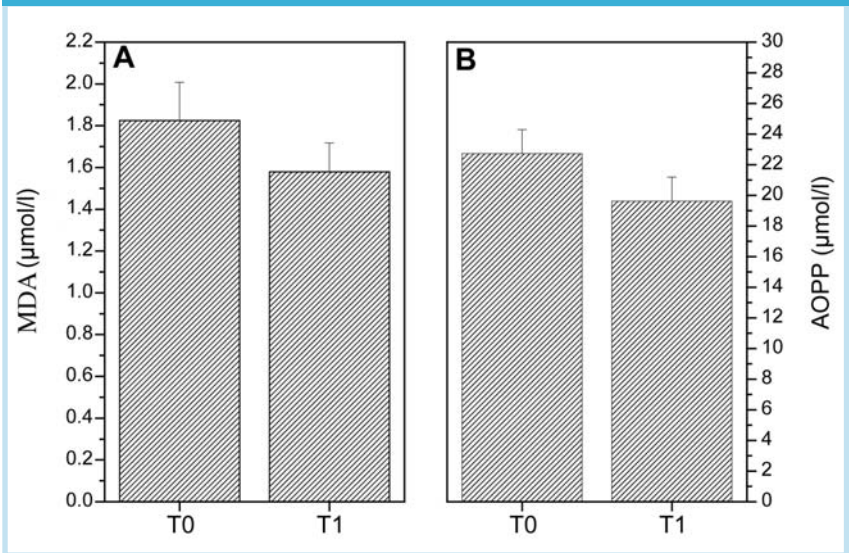
Results are expressed as mean  $\pm$  standard deviation. The statistical analysis was carried out using the Wilcoxon test and probability values of <0.05 were accepted. Statistics and graphs were obtained using the software Microcal™ Origin 6.0 (Microcal Software, Inc., Northampton, Ma, USA).

#### Results

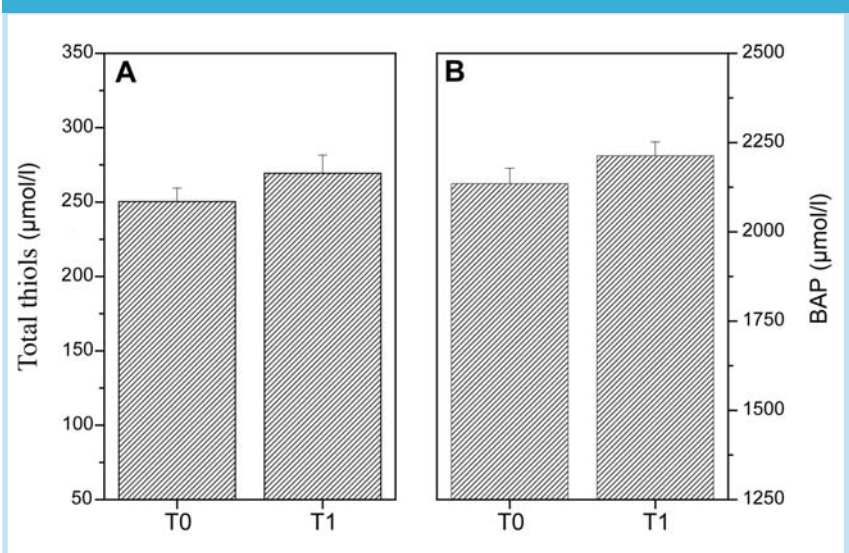
A significant decrement of both lipid and protein oxidation markers was observed at the end of the thermal treatment (T1) with respect to T0 (Fig. 1). In detail, MDA plasmatic levels decreased from  $1.82 \pm 0.18$  (T0) to  $1.58 \pm 0.14$   $\mu\text{mol/l}$  (T1) ( $p < 0.05$ ) (Fig. 1 A); at the same time, AOPP levels decreased from  $24.7 \pm 2.3$  (T0) to  $21.4 \pm 2.1$   $\mu\text{mol/l}$  (T1) ( $p < 0.05$ ) (Fig. 1 B).

As regards the antioxidant profile, total plasmatic thiol levels increased from  $250 \pm 9$  (T0) to  $269 \pm 12$   $\mu\text{mol/l}$  (T1) ( $p < 0.05$ ) (Fig. 2 A); accordingly, the total antioxidant capacity of plasma as evaluated by "BAP test" ranged from  $2134 \pm 43$  (T0) to  $2212 \pm 38$   $\mu\text{mol/l}$  vitamin C (T1) ( $p < 0.05$ ) (Fig. 2 B).

**Figure 1 - Significant decrement of both lipid (MDA) (panel A) and protein (AOPP) (panel B) oxidative stress biomarkers in plasma samples from osteoarthritic patients undergoing sulphurous thermal therapy ( $p < 0.05$  T1 vs T0)**



**Figure 2 - Significant increment of total thiols (panel A) and total antioxidant capacity (BAP) (panel B) in plasma samples from osteoarthritic patients undergoing sulphurous thermal therapy ( $p < 0.05$  T1 vs. T0)**



## Discussion

Osteoarthritis is the most common type of articular degenerative pathology, especially among elderly people, interesting bones and joints. In the last years, numerous studies investigated the possible role of ROS in the etiology and pathogenesis of OA (1-4), demonstrating that oxidative stress is linked to chondrocyte senescence and cartilage aging through a mechanism involving chondrocyte telomere instability and catabolic changes in cartilage matrix structure and composition (5). Consequently, new efforts to prevent the development and progression of OA may include strategies and interventions aimed at reducing oxidative damage in articular cartilage.

Sulphur thermal therapies are employed in the treatment of OA since ancient times; however, the mechanisms of action of thermal cures based on sulphur-rich waters in the control of OA are only partially known. Interestingly, Caraglia et al. (15) recently evidenced a significant decrease of the production of endogenous oxidant agents, such as nitric oxide, in OA mice undergoing mud therapy with sulphurous water. Accordingly, we previously found a significant decrement of both lipid and protein oxidation products in plasma samples from healthy volunteers undergoing a cycle of hydropinic therapy with

H<sub>2</sub>S-rich water (500 ml/day for two weeks), suggesting major benefits from sulphurous water consumption in reducing bio-molecule oxidation (16).

In the light of these results, the present study was aimed at investigating the antioxidative effects of a cycle of mud therapy with sulphurous water in combination with hydropinotherapy, in subjects affected by degenerative OA. Positively, we evidenced that, with respect to the basal evaluation before cures (T<sub>0</sub>), the thermal treatment led to a significant reduction of plasma lipid (MDA) and protein (AOPP) oxidation markers, thus protecting circulating bio-molecules against free radical injury. This protective effects could be due at least in part to the significant increment of the total antioxidant power of plasma that takes into account both lipophilic and hydrophilic antioxidant components, as well as to the significant increase of total -SH levels, which include both protein (principally albumin) and non-protein (cysteine and GSH) thiol groups.

To date, the rate of H<sub>2</sub>S absorption from skin or gastrointestinal tract during sulfur-based cures is not documented, nor it's clear in which forms this compound is really bio-available. However, it is allowed to hypothesize that the increment of endogenous H<sub>2</sub>S following the treatments with sulphurous waters (mud therapy and hydropinother-

apy) leads to an increase of intracellular GSH levels (8, 9) which in turn is released from tissues to maintain plasmatic thiols in their reduced and functional forms. In addition, H<sub>2</sub>S itself might be involved in the reduction of thiols, thus being directly implicated in redox reactions as antioxidant. The accuracy of both possibilities are currently under investigation.

In conclusion, we can affirm that sulphur-based thermal therapies may represent a suitable support in the control of OA thus to integrate and complete the pharmacological treatments; indeed, both sulphates (SO<sub>4</sub><sup>2-</sup>) and hydrogen sulphide (H<sub>2</sub>S) may play a key role in the prevention of cartilage degeneration: the first, as trophic factor for the synthesis of matrix components; the second, as protective agent against cartilage component oxidation.

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