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ORIGINAL ARTICLE

Antioxidative effects of sulfurous mineral water: protection against lipid and protein oxidation

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Objectives: To investigate the antioxidative properties of sulfurous drinking water after a standard hydropinic treatment (500 ml day^{-1} for 2 weeks).
Subjects/Methods: Forty apparently healthy adults, 18 men and 22 women, age 41–55 years old. The antioxidant profile and the oxidative condition were evaluated in healthy subjects supplemented for 2 weeks with (study group) or without (controls) sulfurous mineral water both before (T0) and after (T1) treatment.
Results: At T1, a significant decrease ($P<0.05$) in both lipid and protein oxidation products, namely malondialdehyde, carbonyls and AOPP, was found in plasma samples from subjects drinking sulfurous water with respect to controls. Concomitantly, a significant increment ($P<0.05$) of the total antioxidant capacity of plasma as well as of total plasmatic thiol levels was evidenced. Tocopherols, carotenoids and retinol remained almost unchanged before and after treatment in both groups.
Conclusions: The improved body redox status in healthy volunteers undergoing a cycle of hydropinic therapy suggests major benefits from sulfurous water consumption in reducing biomolecule oxidation, possibly furnishing valid protection against oxidative damage commonly associated with aging and age-related degenerative diseases.
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Keywords: hydropinic treatment; sulfurous mineral water; hydrogen sulfide; thiols; oxidative markers; antioxidant profile

Introduction

Sulfur (S) is an interesting nonmetallic element representing about 0.25% of our total body weight (Taylor and Williams, 1995; Beinert, 2000). As a part of the amino acids methionine, cysteine and taurine, S performs a number of functions in enzyme reactions and protein synthesis. It is necessary for the formation of collagen, the protein found in connective tissue, and keratin, which is fundamental for the maintenance of the skin, hair and nails. Taurine is found in bile acids used in digestion and mucopolysaccharides may contain chondroitin sulfate, which is important for joint tissues. S is part of other important body chemicals such as insulin, which helps regulate carbohydrate metabolism, and glutathione (GSH), the principal antioxidant in cells.

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For all these reasons, mineral water employed in thermal medicine, containing S in the format of sulfate ($SO_4^{2-} > 200\,mg\,l^{-1}$) and/or hydrogen sulfide ($H_2S > 1\,mg\,l^{-1}$), has a long history of use in the treatment of various clinical conditions, from dermatological to muscle/skeletal disorders (Sukenik *et al*., 1999; Gupta and Nicol, 2004). In the past few years, it has been documented that some S-based therapies might play an important role in antioxidant strategies against oxidative damage commonly associated with aging and age-related degenerative diseases (Casetta *et al*., 2005; Sachidanandam *et al*., 2005). For example, Bellometti *et al*. (1996) observed that a cycle of mud therapy with sulfurous mineral water consumption increased serum antioxidant defenses in patients suffering from osteoarthritis through the increment of both ceruloplasmin and transferrin levels, and the reduction of the lipid peroxidation product, malondialdehyde (MDA). Accordingly, Caraglia *et al*. (2005) evidenced the antioxidant effect of mud therapy in mice with osteoarthritis showing a significant decrease in the production of endogenous NO, which, on the one hand, reacts with superoxide anion to form the pro-oxidant agent peroxy-

nitrite, and on the other, inhibits cartilage matrix synthesis and promotes its degradation (Scher *et al*., 2007). At the same time, sulfur bath therapy may cause a significant decline in peroxide concentrations and superoxide dismutase activities, as well as a reduction in homocysteine plasma levels in patients with degenerative osteoarthritis (Ekmekcioglu *et al*., 2002; Leibetseder *et al*,. 2004).

Together with mud and bath therapies, therapies involving the drinking of water containing S (hydropinic treatments) are also employed in thermal medicine, especially for their action on gastroenteric and hepatic functions; until now, however, the response of the antioxidant defense system to orally ingested sulfurous waters has been poorly documented. The aim of the present study is to investigate the antioxidative properties of a standard cycle of hydropinic therapy in healthy volunteers receiving sulfurous mineral water for a period of 2 weeks with respect to a group of control subjects who regularly drank commercial mineral water. Participants were evaluated both before (T0) and after (T1) treatment to monitor their antioxidant profile as well as their lipid and protein oxidation markers. It is well-known that the balance between pro-oxidant agents and antioxidant molecules is not perfect even under nonpathological conditions; thus a certain degree of oxidative damage to biomolecules also occurs in healthy subjects. From this point of view, sulfurous water may be valuable in preserving and enhancing antioxidant status.

Materials and methods

Subjects and study design

Forty subjects (18 men and 22 women, ages 41–55 years) were recruited to participate in this study after giving informed consent. All participants were in good health as determined by a medical history questionnaire, physical examination and clinical laboratory tests. All subjects fulfilled the following eligibility criteria: (1) no history of cardiovascular, hepatic, gastrointestinal or renal disease; (2) no antibiotic or supplemental vitamin and/or mineral use for at least 4 weeks before the beginning of the study and (3) nonsmoker. The study protocol was in accordance with the Helsinki Declaration of 1975, as revised in 1983.

The subjects were randomly divided into two groups. The study group ($n=20$) received 500 ml day^{-1} of sulfurous mineral water from the Thermal Center of Saturnia (Grosseto, Italy) for 2 weeks, which has a sulfuric degree of 14.5 mg l^{-1}, as reported in Table 1. To avoid H_2S loss, water was consumed within 1 h after the opening of the bottle. In the control group ($n=20$), subjects drank regularly natural mineral water from local food markets, which did not contain H_2S. Participants were asked to continue their usual diet; therefore, changes in the serum lipid levels during this short time period were not taken into account. At the end of the hydropinic therapy, subjects underwent a final medical examination to exclude any toxic effect of H_2S-rich water consumption.

Table 1 Chemical and physical characteristics of the mineral water from Saturnia

Temperature	°C	36.9
pH (25°C)	—	6.25
Conductivity (25°C)	μS cm^{-1}	2996
Hardness	°f	204
Fixed residue at 180°C	mg l^{-1}	2990
Sulfuric degree	mg l^{-1}	14.5
CO_2	mg l^{-1}	674
Ca^{2+}	mg l^{-1}	598
Mg^{2+}	mg l^{-1}	134
Na^{+}	mg l^{-1}	63.7
K^{+}	mg l^{-1}	9.3
HCO_3	mg l^{-1}	675
F^{-}	mg l^{-1}	1.9
Cl^{-}	mg l^{-1}	71.4
NO_2^{-}	mg l^{-1}	<0.01
P_2O_5	mg l^{-1}	<0.01
SO_4^{2-}	mg l^{-1}	1469
NO_3^{-}	mg l^{-1}	<0.1
NH_4^{+}	mg l^{-1}	26.8
Iron	mg l^{-1}	<0.01
SiO_2	mg l^{-1}	20.7

Blood sampling

Blood samples were collected from each subject in heparinized tubes both before (T0) and after (T1) treatment. At T1, the time interval from the last consumption of thermal water and the blood sample collection was 24 h. Tubes were immediately centrifuged at 2500 r.p.m. for 10 min and plasma aliquots were stored at −80°C until assayed. The following parameters were monitored during the study: hydroperoxides, MDA, protein carbonyls and advanced oxidation protein products (AOPPs) as markers of oxidative stress; total thiols (–SH) and liposoluble vitamins (α-, δ- and γ-tocopherol, retinol, lutein, lycopene, α- and β-carotene) as nonenzymatic antioxidants; and finally, the total antioxidant capacity (TAC) of plasma that takes into account both lipophilic and hydrophilic antioxidant components.

Hydroperoxide determination

Hydroperoxides were evaluated in plasma samples using a commercial kit from Diacron s.r.l. (Grosseto, Italy). In this test, plasmatic hydroperoxides, in the presence of iron (that is released from plasma proteins by an acidic buffer), are able to generate alkoxyl and peroxyl radicals, according to Fenton's reaction. Such radicals, in turn, are able to oxidize an alkyl-substituted aromatic amine (that is dissolved in a chromogenic mixture), thus transforming them to a pink-colored derivative photometrically quantified at 505 nm. The intensity of the developed color is directly proportional to the concentration of hydroperoxides, according to Lambert–Beer's law. Results are expressed in mg of H_2O_2 dl^{-1}. The linearity range of the test is between 4 and 40 mg H_2O_2 dl^{-1}, the intra-assay coefficient of variation is 2.1%, while the inter-assay is 3.1%. Reference values of healthy subjects are between 20 and 24 mg H_2O_2 dl^{-1} (Cesarone *et al*., 1999).

Malondialdehyde determination
Malondialdehyde plasmatic levels were evaluated by reverse-phase high-performance liquid chromatography (HPLC) as described previously (Agarwal and Chase, 2002). Briefly, sample derivatization was carried out by adding 50 μl 0.05% butylated hydroxytoluene solution, 400 μl 0.44 mol l^{-1} H_3PO_4 solution and 100 μl 42 mmol l^{-1} thiobarbituric acid to 50 μl 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 μl of butanol was added to extract the MDA–thiobarbituric acid complex. Tubes were vortexed and then centrifuged at 10 000 *g* to separate the two phases. Twenty microliters of the sample was removed from the butanol layer and placed into an HPLC injector for analysis without evaporation. The assay was performed using an Alltima C_{18} column (4.6 × 250 mm, 5 μm, from Alltech, Milan, Italy) equipped with a guard column Alltima C_{18} (4.6 × 7.5 mm, 5 μm). The eluent phase was methanol/buffer (40:60, v/v), buffer consisting of 50 mmol l^{-1} KH_2PO_4, pH 6.8. The flow rate was 1 ml min^{-1}. UV detection was carried out at 532 nm, and the fluorescence detector was set at an excitation wavelength of 515 nm and emission wavelength of 553 nm. All the organic solvents were pure HPLC grade from Carlo Erba (Milan, Italy). The HPLC instrument was from Jasco Corporation (Tokyo, Japan).

Carbonyl assay
The spectrophotometric analysis of plasmatic carbonyls was based on the reaction of dinitrophenylhydrazine with protein carbonyls to form protein hydrazones (Levine *et al*., 2000). Carbonyl content was calculated from the peak absorbance (355–390 nm) of dinitrophenylhydrazine-treated samples using the molar extinction coefficient of dinitrophenylhydrazine (22 000 M^{-1} cm^{-1}). Protein content was calculated from a bovine serum albumin standard curve dissolved in guanidine hydrochloride and read at 280 nm.

Advanced oxidation protein product assay
Plasmatic levels of AOPP were measured by spectrophotometry and calibrated with chloramine-T, which in the presence of potassium iodide (KI), absorbed at 340 nm (Witko-Sarsat *et al*., 1996). The reaction mixture was formed by 200 μl plasma diluted 1:5 in 20 mmol l^{-1} phosphate-buffered saline (PBS), 10 μl 1.16 mmol l^{-1} KI and 20 μl acetic acid; the absorbance was immediately read at 340 nm on a microplate reader (Bio-Rad Laboratories, Milan, Italy) against a blank containing 200 μl of PBS instead of plasma. AOPP concentration was expressed in μmol l^{-1} of chloramine-T equivalents.

Determination of tocopherols, retinol and carotenoids
Plasma levels of liposoluble antioxidants were measured by reversed-phase HPLC following deproteinization with ethanol and extraction with hexane (Aebischer *et al*., 1999). After centrifugation, the organic layer was removed and evaporated; the residue was dissolved in 400 μl of a mixture of acetonitrile/tetrahydrofuran/methanol (68:22:7, by vol.) and 100 μl were injected into the HPLC system. The assay was performed using an Alltima C_{18} column (4.6 × 250 mm, 5 μm, from Alltech) equipped with a guard column Alltima C_{18} (4.6 × 7.5 mm, 5 μm). The eluent phase was acetonitrile/tetrahydrofuran/methanol (68:22:7, by vol.) adjusted to 100 (v/v) with 1% ammonium acetate; the flow rate was 1.5 ml min^{-1}. UV and fluorescent detectors were programmed according to absorption, excitation and emission wavelengths of each molecule. All the organic solvents were pure HPLC grade from Carlo Erba. The HPLC instrument was from Jasco Corporation.

Determination of plasmatic –SH groups
The total thiol groups were evaluated by the use of 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, followed by the development of a colored complex that can be measured photometrically at 405 nm (Hu, 1994). Plasmatic values range from 400 to 600 μmol l^{-1}.

Total antioxidant capacity determination
Total antioxidant capacity was evaluated in plasma samples by using a commercial kit from Diacron s.r.l. The method measures ability of plasma to reduce ferric ions and is based on the ability of a solution of ferric ions, which bind to a particular chromogen, to decolorize when they are reduced from ferric to ferrous ions (Benzie and Strain, 1996). Values are obtained by comparing the absorbance change at 505 nm in the test reaction mixture with mixture containing ferrous ions in known concentration. Absorbance changes are linear over a wide concentration range in 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%. The plasmatic antioxidant power is expressed in μmol l^{-1} of vitamin C; the normal value in healthy subjects is approximately 2200 μmol l^{-1}.

Statistics and data processing
Results are expressed as mean ± s.d. Statistical analysis was carried out using the *t*-test for paired data (to evaluate T0 vs T1) or unpaired data (to evaluate the study group vs control group). Probability values of <0.05 were accepted. Statistics and graphs were obtained using Microcal Origin 6.0 software (Microcal Software Inc., Northampton, MA, USA).

Results

The oxidative condition of healthy subjects, supplemented for 2 weeks with (study group) or without (controls)

sulfurous mineral water both before (T0) and after (T1) treatment, was evaluated. Data are summarized in Table 2. At T0, no significant differences were found between study and control groups with regard to the plasmatic levels of both oxidative stress biomarkers (hydroperoxides, MDA, carbonyls and AOPP) and nonenzymatic antioxidants (tocopherols, carotenoids, retinol and total thiols). Accordingly, TAC values were comparable in the two groups. At T1, these parameters remained almost unchanged ($P=$NS) with respect to the baseline in controls receiving nonsulfurous mineral water; on the contrary, a significant decrease ($P<0.05$) in lipid (MDA) and protein (carbonyls and AOPP) oxidation markers was observed in the study group after the 2-week treatment with sulfurous water (Figures 1a–c), while hydroperoxide levels were not affected. With regard to the antioxidant defense system, no variations in the plasmatic levels of tocopherols, carotenoids and retinol were detected in the study group at T1 when compared to the baseline; however, a significant increment ($P<0.05$) in total thiol levels was evidenced after the hydropinic treatment, with a concomitant increase ($P<0.05$) in the TAC, as indicated in Figures 2a and b. Concerning the final medical examination, no toxic effects of H_2S-rich water consumption were observed in the study group throughout the hydropinic treatment.

Discussion

The pivotal importance of S in the biosynthesis of vital cofactors and biomolecules (Taylor and Williams, 1995; Beinert, 2000) and its involvement in complex reaction mechanisms such as the sulfation of glycoproteins in the lining of the gastrointestinal tract to build mucous membranes or sulfation of galactosyl ceramides to form sulfatides needed for myelin formation are well-known; often, sulfation is a key step in the detoxication of unwanted or excess metabolites (Mulder and Jakoby, 1990). All these aspects may have clinical relevance for the interpretation of

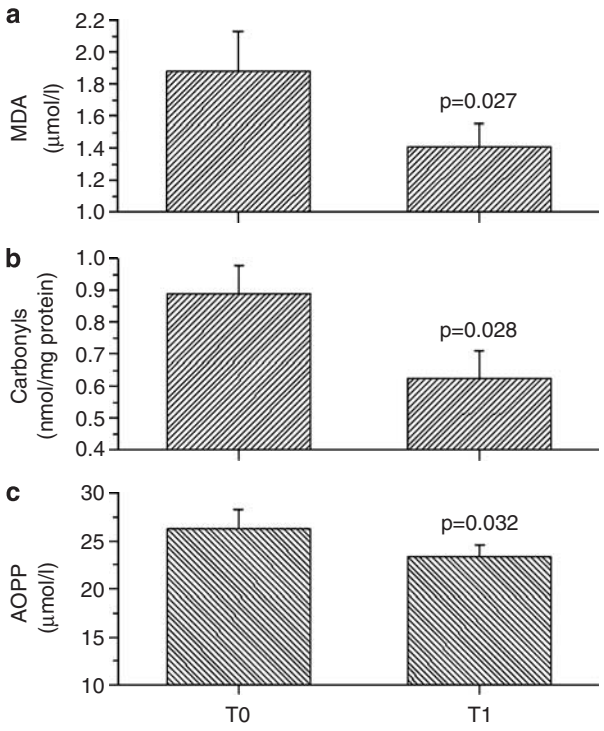

Figure 1 Significant decrease in malondialdehyde (MDA) (**a**), carbonyl (**b**) and advanced oxidation protein product (AOPP) (**c**) plasmatic levels in healthy subjects treated for 2 weeks with sulfurous mineral water (500 ml day^{-1}). $P<0.05$ T1 vs T0, *t*-test for paired data.

Table 2 Oxidative stress biomarkers and antioxidant profile in healthy volunteers before (T0) and after (T1) treatment with (study group) or without (control group) sulfurous water

	Study group		*Control group*	
	T0	*T1*	*T0*	*T1*
Hydroperoxides (mg H_2O_2 dl^{-1})	23.9±2.6	23.8±3.0	23.6±2.7	23.8±2.9
MDA (µmol l^{-1})	1.89±0.25	1.41±0.14[a,b]	1.87±0.22	1.85±0.21
Carbonyls (nmol/mg proteins)	0.89±0.09	0.63±0.08[a,b]	0.91±0.09	0.93±0.08
AOPP (µmol l^{-1})	26.3±2.0	23.4±1.2[a,b]	27.6±2.1	27.0±1.9
TAC (µmol l^{-1})	2184±49	2284±55[a,b]	2179±45	2187±51
–SH groups (µmol l^{-1})	533±42	570±38[a,b]	525±39	532±43
Retinol (µmol l^{-1})	3.5±0.4	3.6±0.4	3.4±0.3	3.5±0.5
α-Tocopherol (µmol l^{-1})	23.7±1.3	23.5±1.0	24.07±1.4	23.8±1.3
δ-Tocopherol (µmol l^{-1})	0.098±0.008	0.095±0.008	0.097±0.008	0.099±0.008
γ-Tocopherol (µmol l^{-1})	0.49±0.05	0.046±0.06	0.52±0.06	0.49±0.05
Lutein (µmol l^{-1})	0.38±0.05	0.37±0.05	0.40±0.05	0.39±0.05
Lycopene (µmol l^{-1})	0.73±0.14	0.75±0.15	0.72±0.12	0.74±0.13
α-Carotene (µmol l^{-1})	0.080±0.011	0.086±0.013	0.084±0.012	0.083±0.011
β-Carotene (µmol l^{-1})	0.43±0.07	0.44±0.08	0.45±0.07	0.42±0.06

Abbreviations: AOPP, advanced oxidation protein products; MDA, malondialdehyde; TAC, total antioxidant capacity.
[a]Significantly different from T0 ($P<0.05$, *t*-test for paired data).
[b]Significantly different from controls ($P<0.05$, *t*-test for unpaired data).

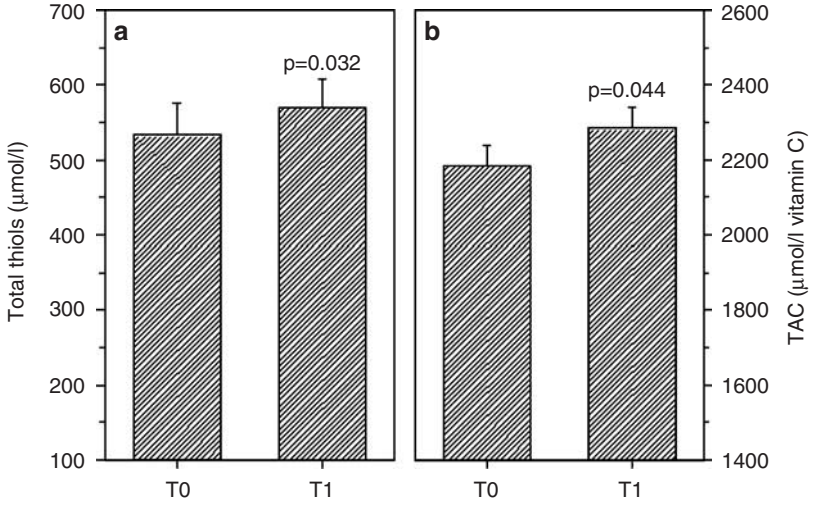

Figure 2 Significant increment in total thiols plasmatic levels (**a**) and total antioxidant capacity (**b**) in healthy volunteers receiving 500 ml day^{-1} of sulfurous drinking water for a period of 2 weeks. $P<0.05$ T1 vs T0, *t*-test for paired data.

the beneficial effects evidenced during S-based thermal treatments such as mucolytic, expectorant, antiphlogistic and antiseptic actions together with the improvement of gastrointestinal functions.

Less known is the role of S contained in thermal waters in oxidative stress reactions; in particular, the effects of drinking therapies involving H_2S-rich waters on the human redox status has been poorly investigated. Interestingly, recent papers have shown that the exogenous administration of the H_2S donor, NaHS, to rats with myocardial injury may reduce the accumulation of plasmatic lipid peroxidation markers such as MDA and conjugated dienes; at the same time, H_2S may inhibit the formation of protein carbonyls induced *in vitro* by hypochlorous acid (Geng *et al*., 2004; Whiteman *et al*., 2005). In accordance with these findings, in this study we observed a significant decrease in both lipid and protein oxidation products, namely MDA, AOPP and carbonyls, in plasma samples from healthy volunteers subjected to a cycle of hydropinic therapy with H_2S-rich water (500 ml day^{-1} for 2 weeks). MDA is the principal and most studied product of polyunsaturated fatty acid peroxidation (Moore and Roberts, 1998). This aldehyde is a highly toxic molecule and should be considered more than just a marker of lipid peroxidation; in fact, its interaction with nucleic acids and proteins has often been referred to as potentially mutagenic and atherogenic (Del Rio *et al*., 2005). On the other hand, AOPP levels are a measure of highly oxidized protein concentration (especially albumin) and correlate with plasma level of both dityrosine and pentosidine (an advanced glycation end product) as indices of oxygen-mediated protein damage (Witko-Sarsat *et al*., 1998). Finally, carbonyls are early markers of protein oxidation due to metal-catalyzed oxidative modifications of amino-acid residues such as lysine, arginine, proline and histidine (Stadtman and Levine, 2003).

The protective effects of sulfurous water administration against the free-radical damage of lipids and proteins were linked to a significant increment in the TAC of plasma that takes into account both lipophilic and hydrophilic antioxidant components. As the levels of tocopherols, carotenoids and retinol almost remained unchanged before and after treatment, the increase in TAC values was possibly related to the significant increase in total –SH levels, which include both protein (principally albumin) and nonprotein (cysteine and GSH) thiol groups.

In this context, a very interesting article was published during the draft of this paper, showing that endogenously produced H_2S can protect neurons from oxidative injury by increasing the intracellular concentrations of GSH, the principal antioxidant in cells (Kimura *et al*., 2006). This effect depends on the ability of H_2S to increase the activity of γ-glutamylcysteine synthetase and to upregulate cystine transport, resulting in an increment in GSH levels. At the moment, it is not possible to demonstrate the direct connection between H_2S-rich water ingestion and the increase in intracellular GSH levels; in fact, no blood cells were collected at T0 and T1. Nevertheless, it is possible to hypothesize (Figure 3) that the increment in endogenous H_2S following the ingestion of sulfurous water may lead to an increase in intracellular GSH levels, which in turn is released from tissues to maintain plasmatic thiols in their reduced and functional forms. In addition, H_2S itself might be involved in the reduction of thiols, thus being directly implicated in redox reactions as an antioxidant.

Literature reports that endogenously produced H_2S can be hydrolyzed to hydrosulfide and sulfide ions; however, even if in aqueous solution, about one-third of H_2S remains undissociated at pH 7.4 and can rapidly diffuse through tissues (Wang, 2002). Indeed, H_2S is permeable to plasma membranes as its solubility in lipophilic solvents is approximately fivefold

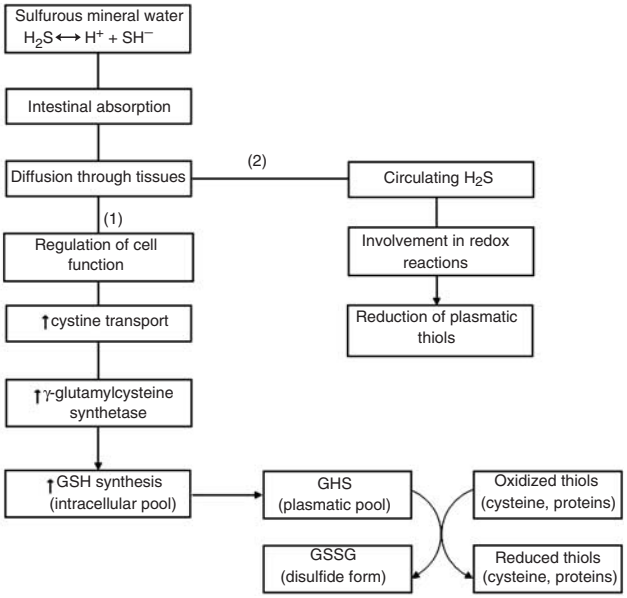

Figure 3 Possible relationships between orally ingested H_2S, glutathione (GSH) synthesis and plasmatic thiols. (1) H_2S may upregulate cystine transport and increase the activity of γ-glutamylcysteine synthetase, resulting in an increment in intracellular GSH levels which in turn is released from tissues to maintain plasmatic thiols in their reduced form. (2) H_2S itself may be involved in the reduction of plasmatic thiols, thus being directly implicated in redox reactions as an antioxidant.

greater than in water; for this reason, H_2S is now considered a gasotransmitter able to induce specific cellular responses (Jeong *et al*., 2006; Oh *et al*., 2006; Rinaldi *et al*., 2006). To date, the rate of H_2S absorption in the gastrointestinal tract after oral ingestion of sulfurous water is not documented, nor it is clear in which forms this compound is actually bioavailable. Studies along these lines are currently in progress; at the same time, it would be of value to investigate how long the effects of sulfurous water consumption last after suspension of the therapy and if an acute treatment (that is, 1 day) generates the same results. In this context, possible increments in intracellular GSH levels should be investigated as well as the balance of GSH and oxidized glutathione (GSSG), which may reflect changes in redox signaling and control.

In conclusion, our findings of improved body redox status in healthy volunteers undergoing a cycle of hydropinic therapy suggest major benefits from sulfurous water consumption in reducing biomolecule oxidation, possibly furnishing valid protection against oxidative damage commonly associated with aging and age-related degenerative diseases.

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