SULPHUROUS THERMAL WATER INCREASES THE RELEASE OF THE ANTI-INFLAMMATORY CYTOKINE IL-10 AND MODULATES ANTIOXIDANT ENZYME ACTIVITY

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The beneficial effects of hot springs have been known for centuries and treatments with sulphurous thermal waters are recommended in a number of chronic pathologies as well as acute recurrent infections. However, the positive effects of the therapy are often evaluated in terms of subjective sense of wellbeing and symptomatic clinical improvements. Here, the effects of an S-based compound (NaSH) and of a specific sulphurous thermal water characterized by additional ions such as sodium chloride, bromine and iodine (STW) were investigated in terms of cytokine release and anti-oxidant enzyme activity in primary human monocytes and in saliva from 50 airway disease patients subjected to thermal treatments. *In vitro*, NaSH efficiently blocked the induction of pro-inflammatory cytokines and counterbalanced the formation of ROS. Despite STW not recapitulating these results, possibly due to the low concentration of S-based compounds reached at the minimum non-toxic dilution, we found that it enhanced the release of IL-10, a potent anti-inflammatory cytokine. Notably, higher levels of IL-10 were also observed in patients' saliva following STW treatment and this increase correlated positively with salivary catalase activity ($r^2 = 0.19$, $^*p<0.01$). To our knowledge, these results represent the first evidence suggesting that S-based compounds and STW may prove useful in facing chronic inflammatory and age-related illness due to combined anti-inflammatory and anti-oxidant properties.

The beneficial effects of hot springs have been known for centuries. Treatment with sulphurous thermal waters has been recommended in a number of chronic pathologies as well as acute recurrent infections. However, the positive effects of the therapy are often evaluated in terms of subjective sense of wellbeing and symptomatic clinical improvements, while the effects of thermal waters on the immune, nervous and endocrine systems are still to be well elucidated. In addition, each different thermal water is unique in terms of composition and properties, and thus *ad hoc* studies may be required to elucidate its specific effects on cells and systems. In the last decade, numerous studies have been focused on understanding the molecular and cellular mechanisms involved in hot spring therapeutic effects (1). Besides the well documented effects on upper and lower respiratory airways, such as antibacterial and mucolytic activity

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and trophic effects on respiratory mucosa (2, 3), many other functions have been attributed to sulphurous water, such as hypocholesterolemic activity (4). In the past few years it has also 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 disease, such as osteoarthritis and chronic inflammatory diseases (5-7). As expected, based on the known effect of oxidative stress on immune responses (8), *in vitro* studies have shown that H_2S and sulphurous thermal water can inhibit proliferation of normal lymphocytes and of T cells obtained from patients with chronic immunomediated diseases. H_2S can also inhibit interleukin 2 and interferon gamma release from T helper 1 lymphocytes. In addition, it has been shown that hydrotherapy can modulate peripheral leukocyte ratios and the proliferative response of T lymphocytes (9). It should also be taken into consideration that since many immune processes are ion dependent, including peroxide scavenging, oxygen-dependent cytotoxicity, and antigen presentation (7, 11), the availability of certain ions such as Zn^{2+}, Fe^{2+} and boron in waters from different thermal locations may well influence the immune response, as has been shown for bacterial clearance (11, 12). However, whether or not thermal waters affect the immune response due to the increased ion supply is still unknown. In addition, hydrogen sulphide has also long been known as a toxic gas. At high concentration, H_2S complexes with the Fe^{3+} of mitochondrial cytochrome oxidase, consequently inhibiting cellular oxidative metabolism. Recently, it has emerged that H_2S is endogenously generated from cysteine in reactions catalyzed by cystathionine-β synthetase (CSB) and cystathionine γ-lyase (CSE) (1, 13, 14). H_2S, similarly to nitric oxide, a gaseous compound with a pleiotropic biological activity, plays a role as a neuromodulator in the central and peripheral nervous system (15, 16). Furthermore, the best characterized physiological effect of H_2S to date is the relaxation of vascular smooth muscle cells, generated by H_2S-mediated K_{ATP} channel opening (17). Based on these findings, it has been suggested that H_2S can be a signalling molecule and its physiological/therapeutic effects can overcome its already known properties.

Much evidence underlines a reduced activity of the immune system to face noxious stimuli and the increased oxidative stress in elderly subjects; two situations that have obvious implications in chronic inflammatory diseases. ROS and NOS exert a double role in respect to the immune system, and play an important role in killing foreign organisms and in acute inflammation. In addition, ROS and NOS up-regulate pro-inflammatory cytokines, chemokines and chemokine receptors (11, 18-21) and may alter the immune balance towards a pro-inflammatory, tissue damaging phenotype (22, 23). Therefore, correct control of the redox profile may prevent the generation of a pro-inflammatory status. Immune cells are endowed with efficient systems that control ROS/RNS generation. Three enzymes, working in concert, are delegated to maintain a fine balance by reducing ROS levels. First, Superoxide Dismutase (SOD) catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen (24). Then, catalase and seleno-dependent glutathione peroxidase (GPx) catalyze the degradation of H_2O_2 to water. It is very important that SOD and GPx/catalase work subsequently to avoid H_2O_2 accumulation, since it is potentially the most toxic and powerful ROS. In addition, myeloperoxidase released by degranulation from neutrophils and monocytes reacts with H_2O_2 in the extracellular compartment.

Here, we investigated the effects of a specific sulphurous thermal water (STW) on the inflammatory and anti-oxidant enzyme responses *in vitro* and *in vivo* in order to understand whether it may help age-related illness through the ability to modulate the immune system and the redox profile.

MATERIALS AND METHODS

Leukocyte purification

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy blood donors (courtesy of the Centro Trasfusionale, Spedali Civili di Brescia, Brescia, Italy) by Ficoll gradient (Ficoll-Paque™ Plus, GE Healthcare, Israel). Highly enriched blood monocytes were obtained from PBMCs by immunomagnetic separation using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotech, Germany) as previously described (25).

Cell culture and treatments

In the experiments that were performed with NaSH

(Sigma-Aldrich St.Louis, Mo, USA), unstimulated monocytes (CTR), monocytes treated with 100 ng/ml LPS (*Escherichia coli* 055:B5, Sigma-Aldrich St.Louis, Mo, USA) or 2.5 mM NaSH alone and cells pre-incubated with 100 ng/ml LPS (1 h) and then stimulated with 2.5 mM NaSH were cultured at $1x10^6$/ml in conditioned RPMI 1640 1% FBS for 24 h at 37°C.

Other experiments were carried out with Sirmione Thermal Water (STW). Monocytes (CTR) and LPS-stimulated monocytes ($1x10^6$/ml) were cultured for 24 h in conditioned RPMI 1640 1% FBS alone or in RPMI 1640 1% FBS and Sirmione Thermal Water (STW) at the following dilutions 1:10, 1:5, 1:2.5 and then incubated at 37°C overnight. To prepare conditioned medium, RPMI 10X (Sigma-Aldrich St.Louis, Mo, USA) was dissolved in STW and then brought to volume with sterile, apirogen water. Conditioned medium (286 mOSM) was buffered with $NaHCO_3$ 2g/l and Hepes 2.3 g/l and added with Folic Acid 1mg/l (all Sigma-Aldrich products). Supernatants were collected and analyzed by ELISA while apoptosis was measured by propidium iodide (PI) staining according to the manufacturer's instructions (Invitrogen, Eugene, Oregon, USA) and analyzed by flow cytometry.

Pellets were harvested in 80 µl of lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride, 0.5µg/µl leupeptin, 5 µg/µl aprotinin and 1 µg/ml pepstatin. Samples were sonicated and centrifuged at 15,000 g for 30 min at 4°C. The resuspended pellets were used for Western blot experiments and for measurement of the antioxidant enzyme activities.

ELISA

Human TNFα, IL-1β, IL-6, IL-12 p70, IL-8/CXCL8, CCL5/Rantes, IL-10 protein levels in supernatants were measured by DuoSet® sandwich ELISA as described elsewhere (DuoSet® ELISA Development System, R&D System, Minneapolis, USA) (26). Quantikine human IL-10 High Sensitive assay kit was used in salivary samples.

Subject enrolment and saliva preparation

Fifty patients (32 males and 18 females), average age 54.1±8.3 SD, were enrolled at San Vigilio Spa, Sirmione, Italy. The majority of enrolled subjects, who benefited from the thermal treatment, suffered from chronic sinusitis, rhino-pharyngitis, bronchitis or otitis in the absence of any concomitant acute infection. Thermal treatment consisted of 12 days of one or more of the following: aerosol, inhalations, micronized shower and humage. Saliva samples were collected before (T0) and after (T1) the thermal treatments. Thirty-six healthy subjects (20 males and 16 females) with and average age of 38.8±9.6 SD, were also enrolled (indicated as CTR). The protocol of the study was approved by the Bioethical Committee for studies on Human Subjects at the ASL Brescia. Approval n. 49/2011 and a written consent was obtained from all subjects. The demographic, clinical and thermal treatment information are reported in Table I.

Unstimulated saliva samples were collected between meals. Subjects were first asked to rinse their mouth for three time using water. Then they were told to sit comfortably and to spit into plastic tubes (50 ml) until the volume of saliva was reached approximately 2 ml. Finally samples were stored in small aliquots at -20°C (27). Two cohorts of subjects were chosen: controls (CTR) and patients who had decided to undergo different types of thermal treatments because they were suffering from diseases that affect, in most cases, the upper airways. In these cases the saliva was collected before (T0) and after (T1) the thermal treatments, which lasted for 12 days.

Before the determination of protein amount, the proteins were precipitated by adding cold acetone pre-chilled at -20°C to the saliva sample (sample: acetone= 1:9) and leaving the mixture at -20°C overnight. After spinning at 14,000 g for 20 min and washing the pellets with cold acetone, supernatants were removed to obtain the pellets. Eighty µl of lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride, 0.5µg/µl leupeptin, 5 µg/µl aprotinin and 1 µg/ml pepstatin was added to pellets. Protein contents were determined by a conventional method (BCA protein assay Kit, Pierce, Rockford, IL, USA). The protein concentration of the patients and of the healthy subjects (indicated as CTR) was comprised in the 1.7-2.6 µg/µl interval. The protein concentration in the patients did not change before (T0) and after the thermal treatment (T1).

Western blot analysis

Cells were harvested in 80 µl of lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 µM NaCl, 5 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride, 0.5µg/µl leupeptin, 5 µg/µl aprotinin and 1 µg/ml pepstatin. Samples were sonicated and centrifuged at 15,000 g for 30 min at 4°C. Protein contents were determined by a a conventional method (BCA protein assay Kit, Pierce, Rockford, IL, USA). Twenty mg of protein extracts were electrophoresed on 12% SDS-PAGE, and transferred to nitrocellulose paper (Amersham-GE Life Sciences Healthcare, Milan, Italy). Filters were incubated at room temperature overnight with primary antibodies in 5% non-fat dried milk (Euroclone CELBIO, Milan, Italy). The antibodies used for this study were: antibodies that recognize the two isoforms of superoxide dismutase, Cu-Zn superoxide dismutase (anti-SOD1 antibody, 1:200, Santa Cruz Biotechnology Inc., Heidelberg, Germany) and

Mn-superoxide dismutase (anti-SOD2 antibody, 1:200, Sigma Aldrich, St Louis, MO, USA) and anti α-tubulin antibody (1:2000, Sigma Aldrich, St Louis, MO, USA). The secondary antibodies (Dako, Glostrup, Denmark) and a chemiluminescence blotting substrate kit (Amersham-GE Life Sciences Healthcare, Milan, Italy) were used for immunodetection.

Superoxide dismutase (SOD) activity

Superoxide dismutase catalyzes the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide. The O_2^- substrate for SOD is generated indirectly in the oxidation of epinephrine at alkaline pH by the action of oxygen on epinephrine. As O_2^- builds in the solution, the formation of adrenochrome accelerates because O_2^- also reacts with epinephrine to form adrenochrome (measured at 480 nm). SOD reacts with the O_2^- formed during the epinephrine oxidation and therefore slows down the rate of formation of the adrenochrome as well as the amount that is formed.

SOD total activity was measured using a buffer (G buffer) that contained 0.05 M glycine, 0.1 M NaCl and 0.1 M NaOH, ph 10.34. Five µl of sample and 5 µl of epinephrine (Sigma Aldrich, St Louis, MO, USA) were added to 190 µl of buffer. The reaction was monitored in a 96-well plate reader by measuring the decrease of absorbance at 480 nm.

Catalase activity

Optimal conditions for the assay were as follows: 5 µl of sample were incubated in 95 µl of substrate (65 µM hydrogen peroxide in 6.0 mM sodium potassium phosphate buffer ph 7.4 (Sigma Aldrich, St Louis, MO, USA) at 37°C for 60 s. One unit of catalase decomposes 1 µmol of hydrogen peroxide/1 min under these conditions. The enzymatic reaction was stopped by addition of 100 µl of 32.4 mM ammonium molybdate [$(NH_4)_6$ Mo_7O_{24} • $4H_2O$ (Sigma Aldrich, St Louis, MO, USA)] and the yellow complex of molybdate and hydrogen peroxide was measured at 405 nm.

Glutathione peroxidase (Gpx) activity

GPx activity was determined using hydrogen peroxide as substrate. Ten µl of sample were mixed with 190 µl of a reaction mix containing 50 mM sodium phosphate buffer with 0.4 mM EDTA, ph 7.0, 1.0 mM sodium azide solution, β- NADPH (1.0 mg vial of β- NADPH, reduced form, tetrasodium salt, Sigma stock No. 201-201), glutathione reductase (GR) (100U/ml Sigma Prod No. G-3664) and 200 mM glutathione reduced (GSH). The absorbance at 340 nm was monitored until constant and then 5 µl of 0.042% hydrogen peroxide were added. The decrease in A_{340} was recorded for approximately 5 min. The data were expressed as nmol NADPH oxidized to $NADP^+$/mg protein/min by using the molar extinction coefficient of 6.2 at 340 nm.

Reactive oxygen species detection

Detection of intracellular ROS was performed as follow: monocytes from healthy donator, grown on 10-cm^2 dishes, were centrifuged and resuspended in pre-warmed buffer (HBSS) containing the probe (2',7'-dichlorofluorescein (DCF) (Molecular Probes, Invitrogen, USA) to provide a final working concentration of 5 µM dye for 30 min at 37°C. The cells were then washed 3 times with HBSS and were finally resuspended in 300 µl of PBS. Chemically reduced and acetylated forms of 2',7'- dichlorofluorescein (DCF) are nonflourescent until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell. Oxidation of this probe can be detected by monitoring the increase in fluorescence with a flow cytometer Partec PASII (Partec, GmbH, Munster, Germany). For each sample, data from 20,000 events were recorded in list mode, displayed on logarithmic scales and analyzed using FlowJo software.

NADPH oxidase activity

It is known that NADPH oxidase converts molecular oxygen to superoxide through the oxidation of NADPH to NADP plus H^+. NADPH has an absorbance spectrum at 340 nm, and the reduction in absorbance at 340 nm is proportional to the decrease in NADPH through its consumption by the NADPH oxidase. It was shown that NADPH has an absorption coefficient of 6.22 $mM^{-1}cm^{-1}$ at 340 nm, which was used to calculate the amount of NADPH consumed during the assay

Thirty-five µl of buffer [(Dulbecco's phosphate buffered saline pH 7.4 (Sigma Aldrich, St Louis, MO, USA)] and 10 µl of sample were transferred into a 96-well plate. Each sample was carried out in duplicate. Immediately before the plate was read in the plate reader, 0.1 mM NADPH was added to each well (50 µl final volume), and the decrease in absorbance of NADPH, because it was converted to NADP, was measured at 340 nm every 15 s for 2 min.

Data were expressed in µM NADP/µg protein/min, and was calculated using the equation C=A/(ExL), where C is the concentration of NADP reduced by the NADPH oxidase, A is the absorbance (the gradient of the initial rate of reaction), E is the extinction coefficient of NADPH (6.22 $mM^{-1}cm^{-1}$) and L is the pathway length (1 cm).

Statistical evaluation

Results were given as mean ± standard error mean values. Statistical significance of differences was determined by mean values of ANOVA, followed by Bonferroni test.

Significance was accepted for a * p value < 0.05.

RESULTS

Effects of hydrogen sulphide on cytokine release and antioxidant enzyme capacity in human monocytes

To evaluate the effect of NaSH on the immune response and antioxidant profile, monocytes from 8 different healthy donors were challenged with a pro-inflammatory stimulus represented by Gram negative lipopolysaccharide (LPS) in the presence or absence of NaSH. Preliminary concentration-curve experiments evaluating cell viability and cytokine release were run in order to identify the appropriate concentration of NaSH. The exposure to NaSH at concentrations ranging 0.1 mM-2.5 mM did not induce significant cell death in monocytes, as evaluated in terms of PI positive cells, while inducing a dose-dependent increase of the pro-inflammatory chemokine CXCL8 (data not shown) (14). Therefore, subsequent experiments were run with the concentration of 2.5 mM (28). Fig. 1 shows that NaSH increased two-fold the release of the chemotactic cytokine CXCL8 (NaSH 3.07±0.98 *vs* CTR 0.74±0.34 * $p< 0.01$) but was ineffective on the other cytokines tested. When added 1 h following LPS, NaSH strongly reduced the release of TNF-α (Fig. 1B), IL-6 (Fig. 1D) and IL-10 (Fig. 1E). We also observed decreased levels of CXCL8 (Fig. 1A) and IL-1β (Fig. 1C), although these were not

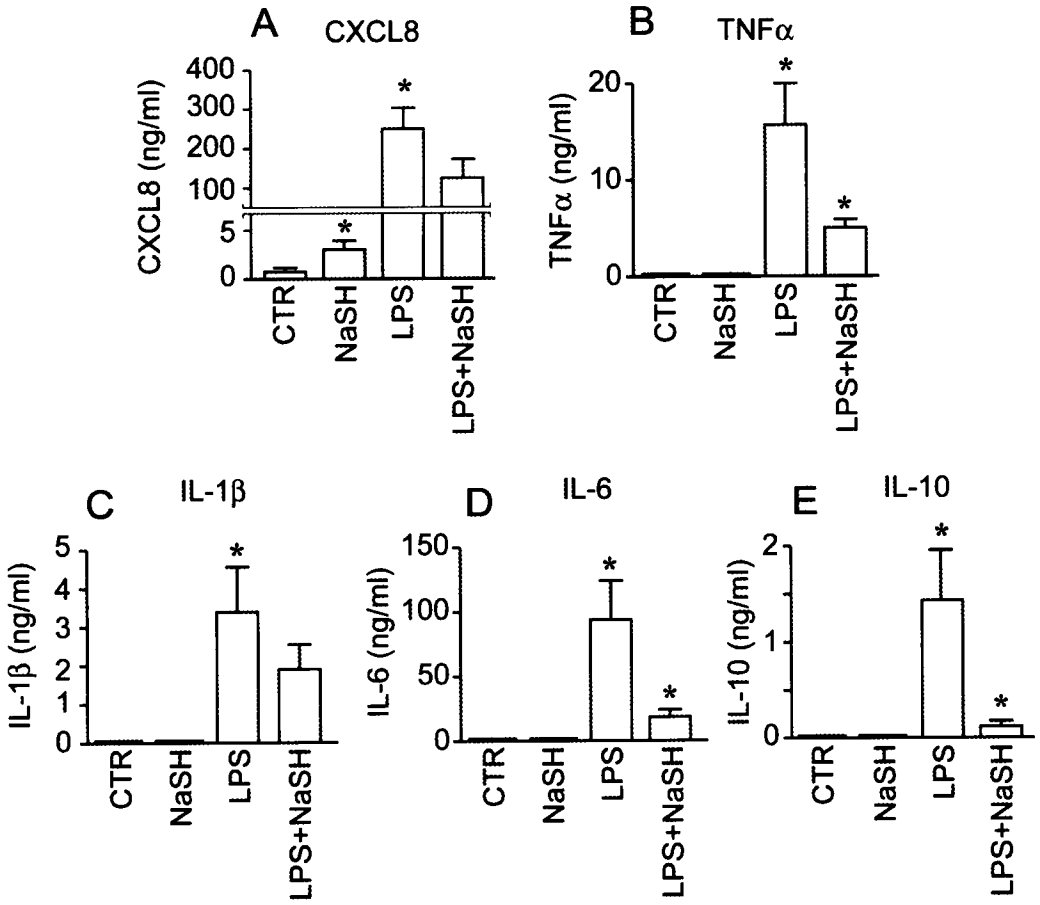

Fig. 1. *Effects of NaSH on cytokine production in human monocytes. Monocytes from 8 different healthy donors were exposed to 2.5 mM NaSH alone or in combination with 100 ng/ml LPS and 24 h later processed for measuring cytokine release. **A)** CXCL8 (statistical significance: CTR vs NaSH * $p<0.05$ and CTR vs LPS * $p<0.0001$). **B)** TNF-a (statistical significance: CTR vs LPS * $p< 0.0001$, NaSH vs LPS * $p< 0.0001$ and LPS vs LPS+NaSH *$p<0.001$), **(C)** IL-1b (statistical significance: CTR vs LPS * $p<0.001$ and NaSH vs LPS * $p<0.001$), **(D)** IL-6 (statistical significance: CTR vs LPS * $p<0.001$, NaSH vs LPS * $p<0.001$ and LPS vs LPS+NaSH * $p<0.001$) and IL-10 (statistical significance: CTR vs LPS * $p<0.001$, NaSH vs LPS * $p<0.001$ and LPS vs LPS+NaSH * $p<0.05$).*

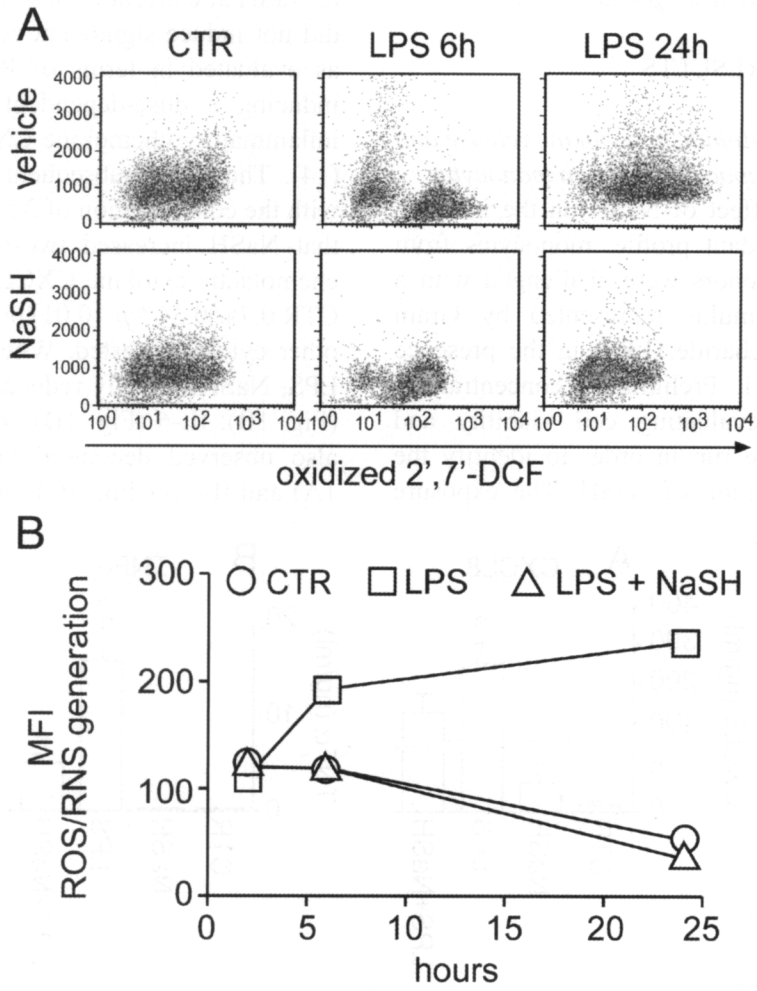

Fig. 2. *Effects of NaSH on ROS production in human monocytes. Freshly isolated human monocytes were exposed to 2.5 mM NaSH alone or in combination with 100 ng/ml LPS and 2, 6 and 24 h later processed for measuring ROS/RNS generation and AOE activity. Representative scatter image of oxidized 2',7'-DCF fluorescence (**A**) and quantitative analysis expressed as Mean Fluorescence Intensity (MFI) (**B**). Results are representative of three independent donors.*

statistically significant. We also addressed the release of the anti-inflammatory cytokine TGF-β, which was found undetectable in all experimental conditions (data not shown).

Next, we investigated the effect of NaSH on LPS-induced ROS/RNS generation (Fig. 2). As expected, LPS exposure induced the production of ROS/RNS over time as assessed by accumulation of oxidized 2',7'- dichlorofluorescein (DCF) (Fig. 2A upper panels and 2B open squares). However, the presence of NaSH completely reverted this effect (Fig. 2A lower panels and 2B open triangles).

Given the well known role of free radical species in developing and sustaining the pro-inflammatory status, the effects of NaSH were investigated on the cellular ROS/RNS generation as well as on their detoxification cascade. In particular, the activity of NADPH oxidase, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) enzymes was measured in protein extracts of LPS-stimulated monocytes. As expected, LPS induced a statistically significant increase (LPS *vs* CTR * $p<0.0001$) of NADPH oxidase activity already at 2 h (Fig. 3A). Such enhancement was sustained up to

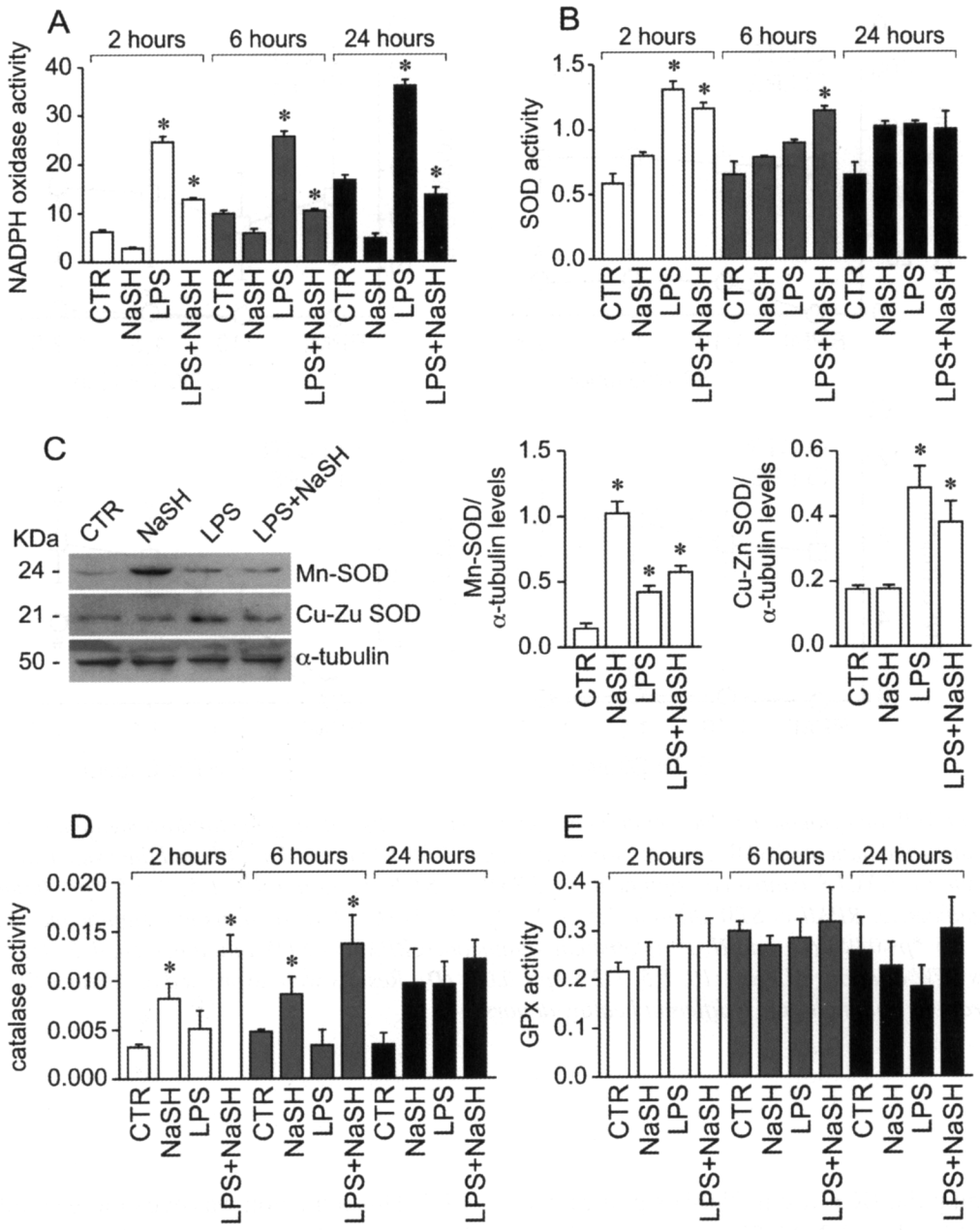

Fig. 3. *Effects of NaSH on pro and antioxidant enzyme activities in human monocytes. Monocytes from 8 different healthy donors were exposed to 2.5 mM NaSH alone or in combination with 100 ng/ml LPS and 2, 6 and 24 h later were processed for NADPH oxidase (statistical significance 2 h: CTR vs LPS * p< 0.0001 and LPS vs LPS+NaSH * p< 0.0001, 6 h: CTR vs LPS * p< 0.0001 and LPS vs LPS+NaSH * p< 0.0001, 24 h: CTR vs LPS * p< 0.0001 and LPS vs LPS+NaSH * p< 0.0001) (**A**), total SOD (statistical significance 2 h: CTR vs LPS * p<0.0001 and CTR vs LPS+NaSH * p< 0.001; 6 h: CTR vs LPS+NaSH * p< 0.05) (**B**), catalase (statistical significance 2 h: CTR vs NaSH * p value< 0.05 and CTR vs LPS+NaSH * p value< 0.001; 6 h: CTR vs NaSH * p value< 0.05 and CTR vs LPS+NaSH * p value< 0.001) (**D**) and GPx activity (**E**). Protein extracts were processed for western blot analysis with anti-Mn-SOD and anti-Cu-Zn SOD antibodies and band intensity was quantified by densitometry (**C**). Results are expressed as Mean ± SE of at least 3 independent donors (statistical significance: Mn-SOD: CTR vs LPS and LPS vs LPS+NaSH * p value< 0.0001 CTR vs NaSH * p value< 0.05; Cu-Zn-SOD: CTR vs NaSH * p value< 0.0001 and LPS vs LPS+NaSH * p< 0.05)*

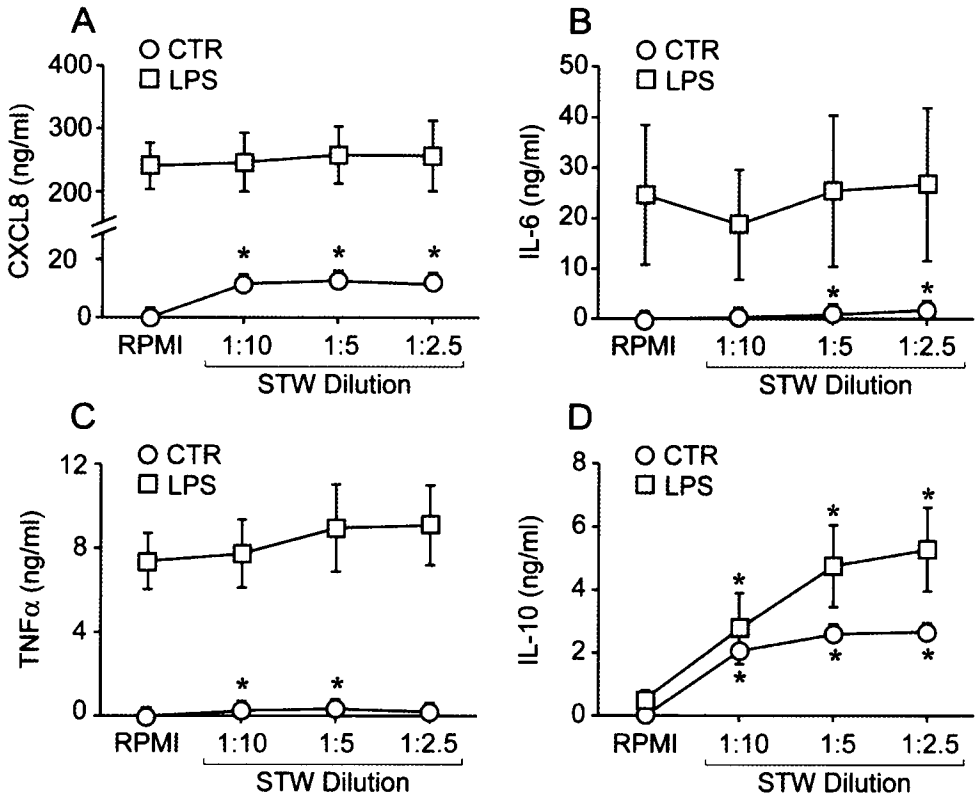

Fig. 4. *Effects of STW on cytokine production in human monocytes. Cells cultured in standard medium or in conditioned medium containing the indicated STW amounts were exposed to LPS 100 ng/ml for 24 h and then supernatants analyzed for cytokine release CXCL8 (statistical significance: RPMI vs STW dilution 1:10, 1:5, 1:2.5 *p< 0.0001)(**A**); IL-6 (statistical significance: RPMI vs STW dilution 1:5, 1:2.5 *p< 0.05) (**B**); TNF-a (statistical significance RPMI vs STW dilution 1:10, 1:5 *p<0.05) (**C**) and IL-10 (statistical significance RPMI vs STW dilution 1:10, 1:5, 1:2.5 *p< 0.001; RPMI+LPS vs STW dilution + LPS) 1:10, 1:5, 1:2.5 *p< 0.05) (**D**). Results were expressed as Mean ± SE of 6 different culture preparations obtained from 6 different healthy donors*

24 h, confirming the DCF result. NaSH significantly prevented LPS-induced NADPH oxidase activity at all times examined (LPS *vs* LPS+NaSH * $p<0.0001$). In addition, SOD activity was increased by all conditions, especially at earlier time-points (Fig. 3B). Consistent with this finding, all treatments could increase the expression of mitochondrial Mn-SOD, although to a different extent, while NaSH alone or in combination with LPS also induced the expression of cytosolic Cu-Zn-SOD (Fig. 3C). Interestingly, at 2 and 6 hours of treatment, NaSH significantly increased the activity of the superoxide anion detoxifying enzyme (catalase) as compared to LPS. This induction was even more significant when NaSH was administered to cells in combination with LPS (Fig. 3D). As shown in Fig. 3E, none of the treatments affected the activation of GPx.

Effects of STW on cytokine production and antioxidant enzyme activity in human monocytes

Thermal waters are characterized by a specific composition in terms of trace elements which makes their effects unique. STW is characterized, beside H_2S, by additional ions such as sodium chloride, bromine and iodine. To compare the effects of complete STW with those described for NaSH

Table I. *Demographic, clinical and thermal treatment information.*

PATIENTS	AGE	SEX	PATHOLOGY	THERMAL TREATMENTS
1	41	M	Chronic sinusitis	Micronized shower, inhalation, aerosol
2	53	M	Chronic sinusitis	Micronized shower, inhalation, aerosol
3	43	M	Rhino-pharyngitis	Micronized shower, inhalation, aerosol
4	57	M	Chronic sinusitis	Micronized shower, inhalation, aerosol
5	68	M	Bronchitis	Inhalation, aerosol
6	62	M	Chronic sinusitis	Micronized shower, inhalation, aerosol
7	55	F	Chronic sinusitis	Micronized shower, inhalation, aerosol
8	46	F	Chronic sinusitis	Micronized shower, inhalation, aerosol, humage
9	46	M	Rhino-pharyngitis	Micronized shower, inhalation, aerosol
10	46	M	Rhino-pharyngitis	Inhalation, aerosol
11	70	F	Rhino-pharyngitis	Inhalation, aerosol
12	47	F	Rhino-pharyngitis	Inhalation, humage
13	56	M	Rhino-pharyngitis	Micronized shower, inhalation, aerosol
14	50	F	Rhino-pharyngitis	Inhalation, aerosol
15	65	M	Rhino-pharyngitis	Inhalation, aerosol
16	42	F	Chronic sinusitis	Micronized shower, inhalation, aerosol
17	59	F	Otitis	Micronized shower, inhalation, aerosol
18	40	F	Rhino-pharyngitis	Micronized shower, inhalation, aerosol
19	54	M	Bronchitis	Inhalation, humage
20	49	F	Chronic sinusitis	Micronized shower, inhalation, aerosol
21	59	M	Otitis	Micronized shower, inhalation, aerosol
22	50	F	Chronic sinusitis	Inhalation, aerosol
23	48	F	Chronic sinusitis	Micronized shower, inhalation, aerosol
24	49	F	Chronic sinusitis	Micronized shower, inhalation, aerosol
25	42	M	Chronic sinusitis	Micronized shower, inhalation, aerosol
26	61	F	Chronic sinusitis and arthritis	Micronized shower, inhalation, aerosol, mud
27	65	M	Chronic sinusitis	Micronized shower, inhalation, aerosol
28	51	M	Rhino-pharyngitis	Micronized shower, inhalation, humage
29	59	M	Rhino-pharyngitis	Micronized shower, inhalation, aerosol
30	59	M	Rhino-pharyngitis	Micronized shower, inhalation, aerosol
31	59	M	Chronic sinusitis	Inhalation, aerosol
32	62	M	Otitis	Micronized shower, inhalation, aerosol
33	51	F	Otitis	Inhalation, aerosol
34	41	M	Chronic sinusitis	Micronized shower, inhalation, aerosol, humage
35	50	M	Otitis	Micronized shower, inhalation, aerosol
36	54	M	Chronic sinusitis	Micronized shower, inhalation, aerosol
37	57	M	Chronic sinusitis	Inhalation, humage
38	49	M	Rhino-pharyngitis	Micronized shower, inhalation,
39	41	M	Chronic sinusitis	Micronized shower, inhalation, aerosol
40	71	F	Rhino-pharyngitis	Inhalation, aerosol
41	63	M	Rhino-pharyngitis	Inhalation, humage
42	46	F	Chronic sinusitis	Micronized shower, inhalation, aerosol, humage
43	59	M	Rhino-pharyngitis	Inhalation, aerosol, humage
44	64	M	Chronic sinusitis	Micronized shower, aerosol
45	55	F	Chronic sinusitis	Micronized shower, inhalation, aerosol
46	63	M	Chronic sinusitis	Inhalation, humage
47	54	F	Chronic sinusitis	Inhalation, humage
48	57	M	Chronic sinusitis	Micronized shower, inhalation, aerosol
49	66	M	Rhino-pharyngitis	Micronized shower, inhalation, aerosol
50	51	M	Rhino-pharyngitis	Inhalation, aerosol, humage

M: male; F: female

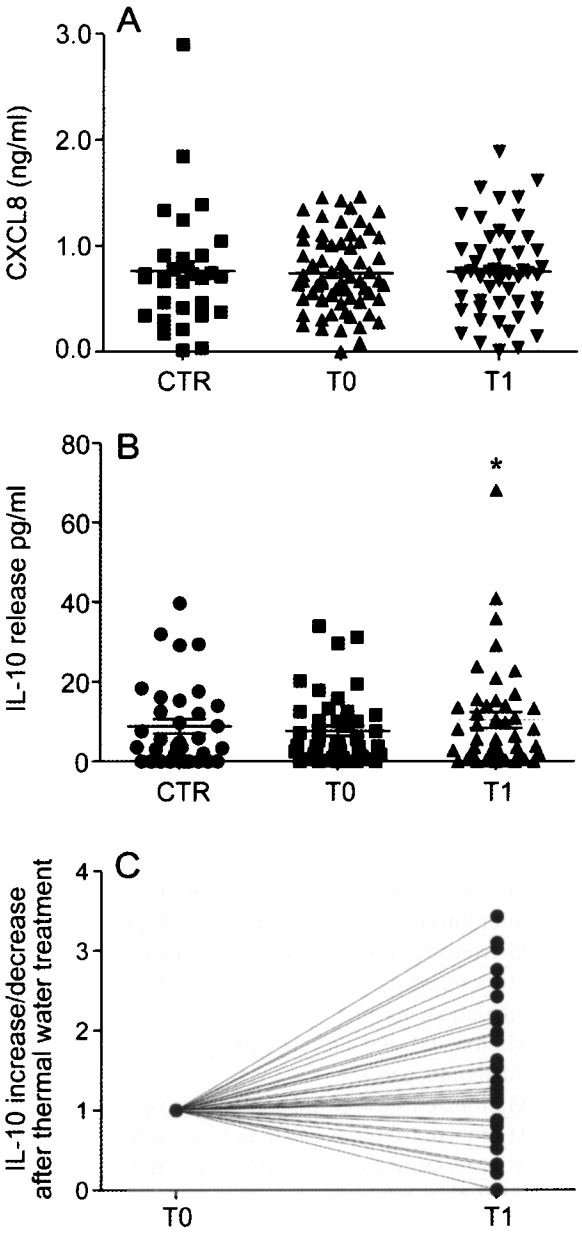

Fig. 5. *Effects of a 12-day STW therapy on salivary release of IL-8 and IL-10. Salivary samples derived from 36 healthy controls and 50 patients before (T0) and after (T1) the 12-days STW therapy were processed for the measurement of IL-8 (**A**) and IL-10 (statistical significance T1 vs T0 * $p<0.01$) (**B**). Fold increase/decrease of IL-10 after STW treatment (**C**).*

in previous experiments, we repeated monocyte stimulation in presence of different dilutions of STW (1:10; 1:5; 1:2.5; 1:1.25. See materials and methods for dilution preparation). We found that monocytes were healthy and viable after 24-h culturing in all STW dilutions except for the lowest one (1:1.25), which was thus eliminated from subsequent analysis (not shown). First, we evaluated whether STW itself could stimulate the release of pro- and anti-inflammatory cytokines. STW at all dilutions induced the release of CXCL8, IL-6 and TNF-α in a dose dependent manner (Fig. 4 A, B, C). However, its presence could not significantly influence the release of these pro-inflammatory cytokines induced by LPS (Fig. 4 A, B, C). However, STW induced the release of the anti-inflammatory cytokine IL-10 both in basal conditions and in the presence of LPS (Fig. 4D). When investigating the effects of STW on the activity of antioxidant enzymes, we found that STW exposure did not alter either the basal level of AOE activity, or that induced by LPS stimulus (data not shown).

Effects of STW on cytokine release and antioxidant enzyme capacity in patients' saliva

The effects of STW were evaluated on 50 patients affected by chronic upper airway diseases (Table I). The majority of the enrolled subjects, who benefited from the thermal treatment, suffered from chronic sinusitis, rhino-pharyngitis, bronchitis or otitis. Thermal treatment foresaw 12 days of two or more of the following applications: aerosol, inhalations, micronized shower and humage. Saliva samples were collected before (T0) and after (T1) thermal treatments. Thirty-six healthy subjects were enrolled as controls (CTR). Saliva samples were processed for the measurement of cytokine amount and antioxidant enzyme activities. The levels of CXCL8 were similar in controls and patients, and were not modified by the treatment (Fig. 5A). Twelve-day STW treatment increased the salivary release of IL-10 in 23 patients out of 41 (8 unchanged, 10 decreased) (Fig. 5B and C).

Interestingly, we found that patients affected by upper airway diseases showed higher SOD activity and lower GPx activity in comparison with healthy subjects, corresponding to a clear pro-oxidant profile and suggesting an impairment in the ROS detoxification cascade (Fig. 6 A, C). At variance, catalase activity did not significantly differ between control and patients at T0 because the values of the

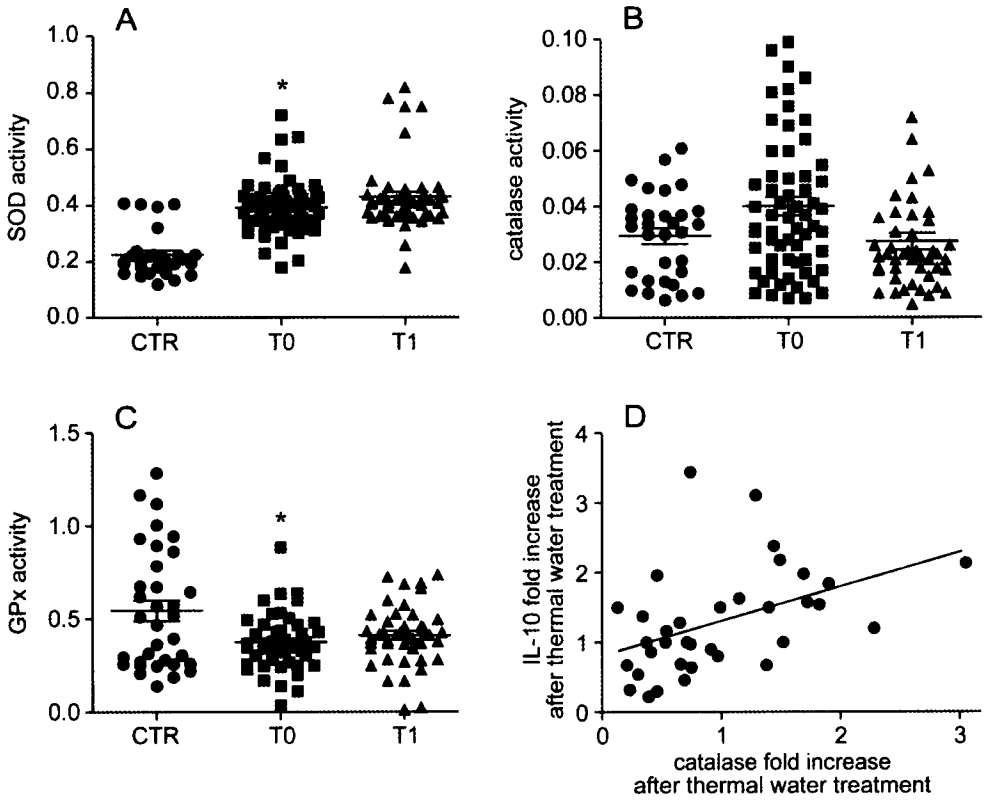

Fig. 6. *Effects of a 12-day STW therapy on AOE activity in patients' saliva. Salivary samples derived from 36 healthy control and 50 patients before (T0) and after (T1) the 12-day STW therapy were processed for the measurement of SOD activity (statistical significance CTR vs T0 * p< 0.0001) (**A**), catalase activity (**B**) and GPx activity (statistical significance CTR vs T0 * p< 0.001) (**C**). Correlation between IL-10 fold increase and catalase fold increase after STW treatment (y= 0.4747x+ 0.836, r^2 = 0.19 *p<0.01)*

enzyme were found very diffused in both groups (Fig. 6B). Despite 12 days of STW treatment not significantly altering this trend (Fig. 6 A-C), we found a positive correlation between salivary IL-10 and catalase activity (r^2= 0.19, * p<0.01, y= 0.4747x +0.836) (Fig. 6D).

DISCUSSION

The present study investigates the effects of S-based compounds (NaSH and STW) on the inflammatory and anti-oxidant enzyme activities in primary human monocytes and in saliva from airway disease patients. Our results indicate that these compounds may prove useful in facing chronic inflammatory and age-related illness due to combined anti-inflammatory and anti-oxidant properties.

The relationship between inflammation and ROS production is multi-faceted. On one hand, ROS production in response to pattern recognition receptor stimulation and pro-inflammatory cytokines is an indispensable weapon of the immune system for pathogen killing. On the other hand, it is becoming increasingly clear that ROS production plays a pro-inflammatory role by activating redox-regulated transcription factors (NF-kB and AP-1) and inducing the release of cytokines such as IL-1, IL-6, CXCL8, RANTES/CCL5 (8, 29). More recently, ROS has been proposed to play a role also in inflammasome activation (20). Therefore, a correct control of the redox profile may prevent the generation of chronic inflammation.

When investigating the effects of S-based compounds on cytokine release by monocytes, we found two different and apparently opposing scenarios depending on the concomitant presence/absence of LPS, a prototypical pro-inflammatory stimulus. Alone, both NaSH and STW induced the release of moderate amounts of pro-inflammatory mediators. In particular, both induced the neutrophil-attracting chemokine CXCL8, and STW also induced low levels of TNF-α and IL-6. Despite this difference, which may well depend on the peculiar ion and oligoelement composition of STW, it is tempting to speculate that in some contexts S-based compounds may exert a mild pro-inflammatory effect which, according to the "Hormesis theory" (30), would alert the immune system to react promptly against intervening infections. In this scenario, our results could support the effectiveness of thermal water treatments also in prevention of inflammatory diseases.

By contrast, when administered to monocytes in combination with LPS, NaSH efficiently blocked the release of pro-inflammatory cytokines and counterbalanced the formation of ROS, suggesting a more anti-inflammatory action. ROS reduction correlated with an increase in both catalase and SOD activity, likely linked to the induction of cytoplasmic Cu-Zn SOD protein, and with a decrease in NADPH oxidase activity. In the presence of LPS alone, SOD activity was similarly increased, but catalase activity remained close to basal levels until later time points, suggesting an accumulation of H_2O_2 which is very stable and plays key roles in both pathogen killing and tissue damage (31). STW did not repeat the above-mentioned results, possibly due to the lower concentration of S-based compounds reached at the minimum non-toxic dilution (H_2S 0.2 nM, SO_4 0.72 nM) in comparison with NaSH 2.5 mM. Despite this, the finding that STW enhanced the release of the anti-inflammatory cytokine IL-10 may similarly suggest the capacity to induce a shift towards milder pro-inflammatory conditions.

Notably, an increase of IL-10 was also observed in saliva of more than half of the patients subjected to STW treatment, substantiating, at least in part, the well-known anti-inflammatory properties of thermal waters. However, the treatment could not revert the pro-oxidant profile observed in these patients compared to healthy controls. It may be that this latter effect, as well as the lack of IL-10 increase in some patients, depends on our experimental protocol, given that standard therapy foresees at least one cycle of three 12-day treatments. Furthermore, differences in the release of anti-inflammatory cytokines may also be due to the individual responsiveness of each patient. Taken together, these results suggest that inflammation can be modulated more promptly in comparison to a pre-existing pro-oxidant status. Despite this, we found a positive correlation between salivary IL-10 and catalase activity, suggesting the existence of an antioxidant and anti-inflammatory trend. Such a correlation has been previously described in the literature (32). IL-10 is a well-known anti-inflammatory antioxidant cytokine which has been described to inhibit the release of ROS in monocytes. On the contrary, antioxidants were reported to quench the release of pro-inflammatory mediators by up-regulating IL-10 (32).

All in all, our results point to an anti-inflammatory effect of STW due to the fine tuning of the immune balance, especially mediated by an increase of the anti-inflammatory cytokine IL-10 and consequent regulation of antioxidant enzyme activities.

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REFERENCES

1. Navarra P, Dello Russo C, Mancuso C, Preziosi P, Grossman A. Gaseous neuromodulators in the control of neuroendocrine stress axis. Ann NY Academy Sci 2000; 917:638-46.
2. Albertini R, Bird M, Doerrer N, Needham L, Robison S, Sheldon L, Zenick H. The use of biomonitoring data in exposure and human health risk assessments. Environ Health Persp 2006; 114(11):1755-62.
3. Ottaviano G, Marioni G, Staffieri C, Giacomelli L, Marchese-Ragona R, Bertolin A, Staffieri A. Effects

of sulfurous, salty, bromic, iodic thermal water nasal irrigations in nonallergic chronic rhinosinusitis: a prospective, randomized, double-blind, clinical, and cytological study. Am J Otolaryngol 2011; 32(3):235-39.
4. Schoppen S, Perez-Granados AM, Carbajal A, Oubiña P, Sánchez-Muniz FJ, Gómez-Gerique JA, Vaquero MP. A sodium-rich carbonated mineral water reduces cardiovascular risk in postmenopausal women. J Nutr 2004; 134(5):1058-63.
5. Casetta I, Govoni V, Granieri E. Oxidative stress, antioxidants and neurodegenerative diseases. Curr Pharm Des 2005; 11(16):2033-52.
6. Sachidanandam K, Fagan SC, Ergul A. Oxidative stress and cardiovascular disease: antioxidants and unresolved issues. Cardiovasc Drug Rev 2005; 23(2):115-32.
7. Braga PC, Sambataro G, Dal Sasso M, Culici M, Alfieri M, Nappi G. Antioxidant effect of sulphurous thermal water on human neutrophil bursts: chemiluminescence evaluation. Respiration 2008; 75(2):193-201.
8. Sozzani S, Bosisio D, Mantovani A, Ghezzi P. Linking stress, oxidation and the chemokine system. Eur J Immunol 2005; 35(11):3095-98.
9. Yamaguchi N, Shimizu S, Izumi H. Hydrotherapy can modulate peripheral leukocytes: an approach to alternative medicine. Adv Exp Med Biol 2004; 546:239-51.
10. Savina A, Amigorena S. Phagocytosis and antigen presentation in dendritic cells. Immunol Rev 2007; 219:143-56.
11. Rishi P, Kaur P, Virdi JS, Shukla G, Koul A. Amelioratory effects of zinc supplementation on Salmonella-induced hepatic damage in the murine model. Dig Dis Sci 2008; 53(4):1063-70.
12. Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood 2003; 102(3):783-8.
13. Moore PK, Bhatia M, Moochhala S. Hydrogen sulfide: from the smell of the past to the mediator of the future? Trends Pharmacol Sci 2003; 24(12):609-11.
14. Mirandola P, Gobbi G, Sponzilli I, Pambianco M, Malinverno C, Cacchioli A, De Panfilis G, Vitale M. Exogenous hydrogen sulfide induces functional inhibition and cell death of cytotoxic lymphocytes subsets. J Cell Physiol 2007; 213(3):826-33.
15. Abe K, Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. J Neurosci 1996; 16(3):1066-71.
16. Dello Russo C, Tringali G, Ragazzoni E, Maggiano N, Menini E, Vairano M, Preziosi P, Navarra P. Evidence that hydrogen sulphide can modulate hypothalamo-pituitary-adrenal axis function: *in vitro* and *in vivo* studies in the rat. J Neuroendocrinol 2000; 12(3):225-33.
17. Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H(2)S as a novel endogenous gaseous K(ATP) channel opener. EMBO 2001; 20(21):6008-16.
18. Saccani A, Saccani S, Orlando S, Sironi M, Bernasconi S, Ghezzi P, Mantovani A, Sica A. Redox regulation of chemokine receptor expression. Proc Natl Acad Sci USA 2000; 97(6):2761-6.
19. Sato M, Miyazaki T, Nagaya T, Murata Y, Ida N, Maeda K, Seo H. Antioxidants inhibit tumor necrosis factor-alpha mediated stimulation of interleukin-8, monocyte chemoattractant protein-1, and collagenase expression in cultured human synovial cells. J Rheumatol 1996; 23(3):432-8.
20. Zhou R, Yazdi AS, Menu P, Tschopp J. A role for mitochondria in NLRP3 inflammasome activation. Nature 2011; 469(7329):221-5.
21. Yamamoto S, Shimizu S, Kiyonaka S, et al. TRPM2-mediated Ca2+influx induces chemokine production in monocytes that aggravates inflammatory neutrophil infiltration. Nat Med 2008; 14(7):738-47.
22. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23(11):549-55.
23. Weigert A, Brune B. Nitric oxide, apoptosis and macrophage polarization during tumor progression. Nitric Oxide 2008; 19(2):95-102.
24. Maier CM, Chan PH. Role of superoxide dismutases in oxidative damage and neurodegenerative disorders. Neuroscientist 2002; 8(4):323-34.
25. Bosisio D, Vulcano M, Del Prete A, et al. Blocking TH17-polarizing cytokines by histone deacetylase inhibitors *in vitro* and *in vivo*. J Leukocyte Biol 2008; 84(6):1540-8.
26. Riboldi E, Daniele R, Cassatella MA, Sozzani S,

Bosisio D. Engagement of BDCA-2 blocks TRAIL-mediated cytotoxic activity of plasmacytoid dendritic cells. Immunobiology 2009; 214(9-10):868-76.

27. Fanali C, Inzitari R, Cabras T, Pisano E, Castagnola M, Celletti R, Manni A, Messana I. alpha-Defensin levels in whole saliva of totally edentulous subjects. Int J Immunopathol Pharmacol 2008; 21(4):845-9.
28. Rinaldi L, Gobbi G, Pambianco M, Micheloni C, Mirandola P, Vitale M. Hydrogen sulfide prevents apoptosis of human PMN via inhibition of p38 and caspase 3. Lab Investig 2006; 86(4):391-7.
29. Carta S, Tassi S, Pettinati I, Delfino L, Dinarello CA, Rubartelli A. The rate of interleukin-1beta secretion in different myeloid cells varies with the extent of redox response to Toll-like receptor triggering. J Biol Chem; 286(31):27069-80.
30. Toussaint O, Remacle J, Dierick JF, Pascal T, Frippiat C, Royer V, Chainiaux F. Approach of evolutionary theories of ageing, stress, senescence-like phenotypes, calorie restriction and hormesis from the view point of far-from-equilibrium thermodynamics. Mech Ageing Dev 2002; 123(8):937-46.
31. Tian L, White JE, Lin HY, et al. Induction of Mn SOD in human monocytes without inflammatory cytokine production by a mutant endotoxin. Am J Physiol1998; 275(3):C740-7.
32. Haddad JJ, Fahlman CS. Redox- and oxidant-mediated regulation of interleukin-10: an anti-inflammatory, antioxidant cytokine? Biochem Biophys Res Comm 2002; 297(2):163-76.