Sulphurous mineral water oral therapy: Effects on erythrocyte metabolism

Maria Cristina Albertini a,*, Laura Teodorib, Augusto Accorsia, Abdelaziz Soukric, Luigi Campanellad, Francesco Baldoni e, Marina Dachaf

a Istituto di Chimica Biologica "G. Fornaini", Università degli Studi di Urbino, Urbino 61029, Italy
b Unità di Biotecnologie, Sezione di Toxicologia e Scienze Biomediche, ENEA-Casaccia, Roma, Italy
c Facolté des Sciences Ain-chock, Université Hassan II, BP: 5366 Maarif, Casablanca, Morocco
de Dipartimento di Chimica, Università "La Sapienza", Roma, Italy
f Dipartimento di Medicina Interna, II Università di Roma "Tor Vergata", Roma, Italy
g I.C.R., Università Campus-Biomedico di Roma, Roma, Italy

Article history:
Received 29 November 2007
Accepted 11 August 2008

A R T I C L E  I N F O

Keywords:
Sulphurous mineral water
Climatic idropinic treatment
Spas
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
Spring therapy

A B S T R A C T

The ingestion of water containing hydrogen sulphide (H2S) is common in spring sulphurous mineral water (SMW) therapy. We hypothesized that observed detrimental effects are related to the alteration of erythrocyte metabolism caused by H2S. To verify our hypothesis, we treated 20 healthy volunteers with SMW and evidenced an increase of methemoglobin concentration, an inhibition of both erythrocyte glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydrogenase (G6PDH) activities. To investigate the mechanism of H2S effect on GAPDH activity, an in vitro study was performed by incubating both erythrocytes from 12 healthy volunteers and purified GAPDH with buffered [35S]-H2S labelled sulphurous water. The interaction between H2S and NAD+ (H) was also investigated. The results indicate that a direct reaction between GAPDH and H2S does not occur and the observed decrease of GAPDH activity is to ascribe to the reaction between H2S and NAD+(H). This may lead to GAPDH inhibition by two ways, namely (i) cellular NAD+(H) reduced availability and (ii) catalytic site blockade. In conclusion, our results show that among the detrimental effects of SMW administration are erythrocyte GAPDH and G6PDH activity inhibition and increased methemoglobin concentration. A mechanism to explain the occurrence of these biochemical events is also proposed.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

Modern medical research appears to confirm the validity of the use of sulphurous spring waters as therapeutic and preventive remedies for a large variety of diseases affecting the respiratory tract, skin, liver, intestine, gynaecological apparatus and osteo-articular system (Cristalli et al., 1996; Nasuti et al., 2004; Staffieri et al., 1998; Matz et al., 2003; Frosch, 2007; Albertini et al., 2007). We found positive effects on animal models: sulphurous mineral water (SMW) oral treatment have anti-oxidant properties (Albertini et al., 1996, 1999a) and no pathological changes were observed on biochemical oxidative enzymatic markers and hematohormone in humans healthy volunteers undergoing the same oral treatment (Albertini et al., 1999b). However, adverse events such as general or neurological disturbances like asthenia, malaise etc., often observed during the first six days of treatment have been also reported (Talenti, 1970; Nappi et al., 1993). So far, the effect of H2S on various clinical pathology parameters has been restricted to acute studies (Ibadova et al., 1997; Costantino et al., 2003; Lopalco et al., 2004) and investigations on hematological and serum enzyme alterations as effects of sub chronic exposure to low concentration of H2S are strongly needed together with information concerning the potential target organ system of H2S.

We focused our attention on the effects of low H2S concentrations on erythrocyte metabolism observed during two weeks of sulphurous water oral treatment and their relationship with the occurring adverse events.

Several studies on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have highlighted the (reversible) inhibition of the (active) cytosolic form upon binding to the inner cytoplasm membrane surface, through erythrocyte (RBC) band 3 glycoprotein. The binding of GAPDH to band 3 depends on the integrity of the latter. This feature is of high interest: RBC band 3 has already been described as a senescent antigen corresponding either to band 3 degradation products or band 3 clustering molecules, thus representing a sensitive marker of RBC alteration undergoing degradation or clusterization (Mallozzi et al., 1995; Galli et al., 1998; Low, 1986; Rogalski et al., 1989). GAPDH, per se, is not rate limiting for the human RBC glycolytic pathway (it can be inhibited up to 90% without affecting

* Corresponding author. Tel.: +39 0722 305 288; fax: +39 0722 305 324.
E-mail address: mc.albertini@hotmail.it (M.C. Albertini).
glucose consumption) (Maretzki et al., 1989), but its activity depends on the NAD+/NADH ratio (Momsen, 1981; Ninfali et al., 1984). Different compounds, depending upon their chemistry, affect GAPDH activity via different mechanisms such as direct oxidation of NADH, stimulation of the reversible membrane–cytoplasm translocation (i.e. by band 3 association and dissociation), phosphorylation, or induction of NADP(H) linkage to the enzyme (McDonald and Moss, 1993; Dimmerle and Brune, 1992, 1993; Molina et al., 1992; Kots et al., 1992; Kawamoto and Caswell, 1986; Minetti et al., 1996; Mohr et al., 1996; Soukri et al., 1996; Tanna and Endo, 1990). To understand if the inhibition of GAPDH was indeed due to the NAD and NADH linkage to the enzyme, we designed an “in vitro” study incubating healthy RBC and human purified GAPDH with buffered sulphurous water.

A mechanism to explain GAPDH inhibition is eventually proposed, and the possibility to prevent some of the adverse events related to sulphurous water oral therapy, is also discussed.

2. Materials and methods

2.1. In vivo treatment

After informed consent, healthy volunteers (20 subjects) were treated with 200 ml of SMW every day for 14 days. Blood samples were collected before, at one week, and at two weeks of treatment, for GAPDH, glucose-6-phosphate dehydrogenase (GAPDH) enzyme activities and GAPDH band 3 binding properties investigation (as described below). The subjects were carefully selected and followed up by the “Dipartimento di Medicina Interna II” of the University of Roma “Tor Vergata”. Written informed consent was obtained from all participants. Patient management was performed according to the Italian law on the Privacy (A3 and A4 D.lgs n. 196, 2003).

2.2. In vitro treatment

Fresh whole venous blood obtained from 12 healthy control volunteers was collected and placed in heparinized tubes. To remove the white cells and platelets containing buffy coat, RBC were washed three times in physiological buffered saline solution PBS (0.13 mM NaCl, 5.4 mM KCl, 0.8 mM CaCl2, 0.1 mM MgCl2) and 150 mM NaCI at pH 7.6. Packed RBCs were resuspended with an equal volume of PBS and diluted 1:20 with the different treatments: PBS alone as control; PBS + 4.95 mg/l H2S and PBS + 4.95 mg/l H3S + nicotinamide (1 mM). Phosphodiesterase (2 U/mg), Alkaline phosphatase (P AL, 3 ml/mg) and nicotinamide were added after 60 min of incubation as described in the figure legends. Prior free GAPDH activity measurements, RBCs were washed and lysed (see below: paragraph 2.7). The enzymes (phosphodiesterase and PAL) were added to the hemolysate (see Fig. 2 – Treatments). Analogously, commercial purified human GAPDH (0.85 µg/µL) (Sigma–Aldrich, Milan, Italy) was treated with the same solution conditions then RBCs. To study the reversibility of the effect, the removal of H2S was performed, after 1 h, by washing RBC with PBS.

2.3. Radioisotopes

The radioisotopes used for “in vitro” experiments are [35S]-Sulphur (in toluene, 18.5–92.5 Gbq/milliliter; 0.5–2.5 Ci/milliliter), and [4-3H]-NADH (1.0 µCi/ml; 37 MBq/mL with a specific activity of 2.10 Ci/mmol; 117 MBq/mg) were from Amersham (UK).

2.4. Sulphurous solutions

Sulphurous mineral water used for the in vivo experiments has been supplied by a local thermal centre (from Macerata Feltria, Pesaro–Urbino, Italy). The sulphurous solutions for the in vitro experiments were prepared either from the same SMW used for the in vivo experiments, diluted in PBS (solution A) or from a H2S containing phosphate saline solution (solution B). The latter was generated from 0.64 mg S8 and 2 mg NaBH4 in a final volume of 500 µl methanol. Solutions A and B were diluted to a final H2S concentration of 0.12 and 0.13 mM, respectively. Dithionitrobenzene was used to evaluate SH content of the different solutions, as described by Beutler (1984).

2.5. Labelling treatment conditions and electrophoresis analysis

A labelled sulphurous solution was also prepared with radioactive S8 as previously described for solution B. For technical reasons, [35S]-Sulphur was used as follows: solution I prepared with PBS and [35S]-H2S (0.02 µCi); solution II prepared with PBS, SMW and [35S]-H2S (13.4 µCi); solution III prepared with PBS, SMW and [35S]-H3S (0.25 µCi).

Labelled 4-[3H]-NADH solutions were prepared as follows: PBS control + 4-[3H]-NADH 5 µCi (solution IV); SMW solution (0.12 mM, solution A) + 4-[3H]-NADH 5 µCi (solution V); SMW solution (0.12 mM, solution A) + 4-[3H]-NADH 5 µCi + nicotinamide 1 mM (solution VI); H2S solution (0.13 mM, solution B) + 4-[3H]-NADH 5 µCi (solution VII); H2S solution (0.10 mM, solution B) + [4-[3H]-NADH 5 µCi (solution VIII); H2S solution (0.05 mM, solution B) + [4-[3H]-NADH 5 µCi (solution IX); H2S solution (0.025 mM, solution B) + [4-[3H]-NADH 5 µCi (solution XI); H2S solution (0.025 mM, solution B) + [4-[3H]-NADH 5 µCi + nicotinamide 1 mM (solution XII).

Labelled solutions are summarised in Table 1.

RBC and commercial purified human GAPDH were treated either with [35S]-H2S or 4-[3H]-NADH containing solutions. Samples were subjected to electrophoresis in denaturing conditions in a 5% acrylamide Laemli gel (15 µg/lane of hemolysate or 3 µg/lane of purified GAPDH) and in non-denaturing conditions in 5% acrylamide Laemli gel (20 µg/lane of hemolysate and 30 µg/lane of purified GAPDH). For the analysis with the Phosphor Imager (Molecular Dynamics, Sunnyvale, CA) [35S]-H2S labelled gels were dried and exposed for 3 h, while [4-[3H]-NADH labelled gels were dried and exposed for two days.

2.6. Western-blot analysis

Cells incubated under different conditions were parked by centrifugation (3000 rpm or 800g for 10 min at 4 °C) and lysed by the addition of 20 volumes of ice-cold 5 mM sodium phosphate buffer, supplemented with 0.1 mM phenylmethylsulfonyl fluoride at pH 7.8. Ghosts were prepared by repeated washings with the same buffer and centrifuged at 20,000g for 40 min at 3 °C for 30 min until pale yellow. Protein concentrations were determined by the procedure of Bradford (Bradford, 1976), using bovine serum albumin as a standard. The samples were then electrophoresed and subsequently transferred (300 mA) to a nitrocellulose membrane (Hybond TM–C extra, Amersham, UK) and incubated overnight at 4 °C with the first antibody solution (1:3000) rabbit anti-cytochrome band 3 (Sigma–Aldrich, Milan, Italy). The nitrocellulose membrane was then incubated with the second antibody solution (1:5000) Protein–G horseradish peroxidase conjugate (Sigma–Aldrich, Milan, Italy) for 1 h at room temperature. Cytoplasmic band 3 was evidenced by a light emitting enhanced chemiluminescence system (ECL, from Amersham, UK) on X-ray film.

2.7. Evaluation of free and membrane-bound GAPDH

Free GAPDH activity was assayed spectrophotometrically. One volume of packed RBC was diluted with 20 volumes of ice-cold distilled water, and the hemolysate was used for the spectrophotometric enzyme assay (Beutler, 1984). Commercial human erythrocyte GAPDH (from Sigma–Aldrich, Milan, Italy) 2.4 µg/ml was also used. Phosphodiesterase (2 U/mg), or alkaline phosphatase (PAL, 3 µm/mg) or nicotinamide (Nic, 1 mM) were added after 60 min of incubation.

Membrane bound GAPDH was measured by loading erythrocyte membranes on 8.5% SDS–polyacrylamide Laemli gel electrophoresis (SDS–PAGE) as described by Laemmli (1970). Coomassie blue stained gels were used for GAPDH band intensity quantification by an Ultroscan XL Enhanced Laser Densitometer (LKB, Bromma, Sweden). The results were expressed in arbitrary optical density (OD) units.

2.8. Hemolysis determination

Aliquots of the “in vitro” H2S treated erythrocyte suspensions were centrifuged and the supernatants were used to measure hemoglobin concentration by direct spectrophotometric determination at 412 nm using a Perkin–Elmer Lambda 25 UV/VIS Spectrometer (Perkin–Elmer, Milano, Italy).

2.9. Methemoglobin determination

One hundred microliters of whole fresh blood were lysed in 9 ml of distilled water plus 0.1 ml of detergent solution (2.55 g of Na2B4O7 and 33 ml of Triton X-100 in 100 ml). The hemolysate is divided into two equal volumes (A and B). The absorbance in 1 ml of A was measured spectrophotometrically at 630 nm (D1). Ten microliters of 10% potassium cyanide solution were added and the optical density was again measured after mixing (D2). Ten microliters of 10% potassium ferricyanide solution were added to 1 ml of B, and after 5 min the optical density was measured at the same wavelength (D3). Ten microliters of 10% potassium cyanide solution were added to B and after mixing a final reading was made (D4). All the measurements were made against a blank (the detergent solution used to lyse the blood) in the same proportion as present in the samples. Methemoglobin (%) concentration was calculated as follows:

\[
\text{Methemoglobin} (%) = \left( \frac{D1 - D2}{D3 - D4} \right) \times 100
\]

Total hemoglobin concentration (g/100 ml) was evaluated as described in our previous works (Albertini et al., 1999b; Nagel, 1978).
2.10. Spectrofluorometric analysis of tryptophan GAPDH emission

The purified GAPDH used for the measurement of GAPDH activity, was also used for the evaluation of tryptophan emission (at 340 nm) during excitation at 295 nm and transferring emission at 470 nm (NADH emission). The evaluation was performed using a Perkin–Elmer Lambda 25 UV/VIS Spectrometer (Perkin–Elmer, Milano, Italy).

2.11. Statistical analysis

The results were analysed by using the Student’s t-test.

3. Results

3.1. In vivo study

The results of the in vivo experiments demonstrated a significantly decrease of GAPDH and G6PDH activities and a related significant methemoglobin concentration increase in red blood cells from healthy volunteers undergoing SMW treatment for 14 days (Fig. 1a).

![Graph showing G6PDH and GAPDH activity, methemoglobin concentration, and band 3 degradation over days of treatment]

Fig. 1. Healthy subjects (n = 20) were treated with mineral sulphurous water, experiments were performed before the treatment (0), after 7 days (7) and after 14 days (14) as reported in the x axis. (a) Sulphurous mineral water induces GAPDH (●; expressed as IU/gHb), G6PDH (Δ; expressed as IU/gHb) enzyme activities inhibition and increased intracellular methemoglobin concentration (○; expressed as gMet/100 mL). (b) Sulphurous mineral water induces increasing GAPDH membrane binding after 7, but not after 14 days of treatment. After each treatment, erythrocyte membranes were prepared and electrophoresed in 8.5% SDS polyacrylamide gel in denaturing conditions (see material and methods) for GAPDH densitometry evaluation. A representative example of a coomassie blue stained gel is reported in the left panel. The densitometry values, as percentage of total protein (GAPDH at 0 days was 15.8 ± 1.24; at 7 days was 17.6 ± 1.22; at 14 days was 16.5 ± 1.64. The values are the mean of 20 different measurements derived from the samples obtained from each healthy subject treated) are reported in the right panel. The results were significantly different after 7 days of treatment (p < 0.05 “0 vs 7) demonstrating that the treatment increased GAPDH membrane binding properties. (c) Sulphurous mineral water induces band 3 degradation after 14 days of treatment. Western blot of band 3 protein, evidenced a degradation profile (a new band resulting from band 3 degradation appears) after 14 days of treatment.
The effects of sulphurous water on GAPDH binding property to band 3 erythrocyte membranes were also investigated. The Western-blot results are reported in Fig. 1b. As shown in the left panel, the strip corresponding to GAPDH increased after seven days of treatment, as a consequence of an increased membrane binding property, as also evidenced in the right panel, where the densitometry profile, expressed as percentage value of GAPDH to total protein loaded per lane is reported. Since the binding of GAPDH to cell membrane is related to a reversible electrostatic inhibition of the enzyme activity (Mallozzi et al., 1995; Galli et al., 1998; Low, 1986; Rogalski et al., 1989; Maretzki et al., 1989), thus, the increased binding property observed here can be ascribed to the reduced activity of the enzyme. However, as we proceed with the treatment, GAPDH membrane binding decreases as demonstrated in Fig. 1b (right panel, densitometry profile of GAPDH) at the 14th day of treatment. The fact that, in spite of its decreased binding, GAPDH was still inhibited, suggested that the decreased GAPDH binding to band 3 at this time point, was ascribed to the degradation of the erythrocytes band 3. Indeed a band 3 degradation profile was observed (a new band resulting from band 3 degradation is shown in Fig. 1c). Thus, 14 days SMW treatment, alongside with the GAPDH and G6PDH, inhibition produced a degradation of RBC band 3.

To elucidate how SMW interacts with GAPDH enzyme activity, we reproduced an “in vitro” system. Erythrocytes from healthy donors (Fig. 2a) and commercial human purified GAPDH (Fig. 2b) were both incubated with buffered SMW (solution A, as described in Section 2). The results indicated that SMW is able to inhibit GAP-

![Graph](image-url)

**Fig. 2.** Effect of sulphurous mineral water (H2S 0.12 mM, solution A) on erythrocyte (a) and purified human GAPDH (b) enzyme activities. Phosphodiesterase (2 U/mg), Alkaline phosphatase (PAL, 3 mU/mg) and Nicotinamide (Nic, 1 mM) were added after 60 min of incubation. The enzymes (Phosphodiesterase and PAL) used during the erythrocyte treatments were added to the hemolysate prior GAPDH activity measurement. As shown in the figures, almost all the treatments and the removal of sulphurous mineral water as well (- Sulf, PBS washed RBC) can revert the inhibitory effect of sulphurous mineral water on GAPDH activity ($p < 0.01$). Conversely alkaline phosphatase does not affect GAPDH activity. The result values are the mean of at least 5 different experiments.
DH activity “in vitro” both on erythrocytes and on purified GAPDH already after 60 min (for the results of the other treatments see next paragraph).

3.2. H2S is the agent responsible of GAPDH inhibition

In order to investigate if the GAPDH inhibitory agent of SMW was really the H2S compound, we reproduced an “in vitro” H2S solution, as described in Section 2 (Table 1) and repeated the in vitro incubations with erythrocytes from healthy subjects and with purified GAPDH. The results indicated that the inhibitory agent was indeed H2S and that, in the experimental conditions used, 1 mM nicotinamide has a protective effect (Table 2).

3.3. Posttranslational GAPDH modification

To investigate the mechanism responsible for the inhibition of GAPDH activity, we analysed the possible GAPDH posttranslational modification involved. GAPDH inhibition can involve either its cofactor (NADH oxidation, NAD^+ linkage to the enzyme) or modification involved. GAPDH inhibition can involve either its GAPDH activity, we analysed the possible GAPDH posttranslational modification involved. GAPDH activity, the latter being known as protecting the enzyme from ADP-ribosylation. Conversely, SMW GAPDH inhibition was unaffected by the addition of PAL (Fig. 2a and b).

3.4. H2S interacts with GAPDH

Using non-denaturing gel electrophoresis, we were able to demonstrate that [35S]-H2S SMW reacted only with hemoglobin (since sulfhemoglobin, Hb-[35S]-H2S, was formed), but neither erythrocyte GAPDH nor purified GAPDH were considerably labelled (Fig. 3a). In the same experimental conditions as before,

The “in vitro” effects of H2S on GAPDH activity: the H2S reproduced experimentally, has the same inhibitory effect than SMW indicating that H2S is the inhibitory agent

3.2. H2S is the agent responsible of GAPDH inhibition

In order to investigate if the GAPDH inhibitory agent of SMW was really the H2S compound, we reproduced an “in vitro” H2S solution, as described in Section 2 (Table 1) and repeated the in vitro incubations with erythrocytes from healthy subjects and with purified GAPDH. The results indicated that the inhibitory agent was indeed H2S and that, in the experimental conditions used, 1 mM nicotinamide has a protective effect (Table 2).

3.3. Posttranslational GAPDH modification

To investigate the mechanism responsible for the inhibition of GAPDH activity, we analysed the possible GAPDH posttranslational modification involved. GAPDH inhibition can involve either its cofactor (NADH oxidation, NAD^+ linkage to the enzyme) or ADP-ribosylation and phosphorylation processes (McDonald and Moss, 1993; Dimmeler and Brune, 1992, 1993; Molina et al., 1992; Kots et al., 1992; Kawamoto and Caswell, 1986; Minetti et al., 1996; Mohr et al., 1996; Soukri et al., 1996; Tanuma and Endo, 1990). Furthermore, to assess whether the process was a posttranslational covalent modification or not, the analysis of reversibility of the inhibition was also relevant: a covalent modification does not take place directly on this enzyme nor on a related enzyme. We also investigated the effect of different compounds known to restore GAPDH activity either by dephosphorylating the enzyme (alkaline phosphatase: PAL) (Kawamoto and Caswell, 1986; Soukri et al., 1996) or by ADP–ribose cleavage (phosphodiesterase) (Kots et al., 1992). Our results demonstrated that adding phosphodiesterase and nicotinamide restored GAPDH activity, the latter being known as protecting the enzyme from ADP ribosylation. Conversely, SMW GAPDH inhibition was unaffected by the addition of PAL (Fig. 2a and b).

3.4. H2S interacts with GAPDH

Using non-denaturing gel electrophoresis, we were able to demonstrate that [35S]-H2S SMW reacted only with hemoglobin (since sulfhemoglobin, Hb-[35S]-H2S, was formed), but neither erythrocyte GAPDH nor purified GAPDH were considerably labelled (Fig. 3a). In the same experimental conditions as before,

The “in vitro” effects of H2S on GAPDH activity: the H2S reproduced experimentally, has the same inhibitory effect than SMW indicating that H2S is the inhibitory agent

### Table 1

<table>
<thead>
<tr>
<th>Solutions</th>
<th>SMW (mM)</th>
<th>[35S]-H2S (µCi)</th>
<th>H2S (mM)</th>
<th>4-[3H]-NADH (µCi)</th>
<th>Nicotinamide (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.12</td>
<td>0.13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.12</td>
<td>13.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.12</td>
<td>0.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.12</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.12</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.13</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>0.13</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>0.05</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>0.05</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>0.025</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>0.025</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The solutions used during the treatments

**Table 2**

The “in vitro” effects of H2S on GAPDH activity: the H2S reproduced experimentally, has the same inhibitory effect than SMW indicating that H2S is the inhibitory agent

<table>
<thead>
<tr>
<th>GAPDH activity (AOD/min)</th>
<th>Purified human erythrocyte GAPDH (2.4 µg/mL)</th>
<th>H2S concentration of the solutions used (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolysate RBCs 1:20(v/v)</td>
<td>66 ± 6.8</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>Solution A</td>
<td>16 ± 2</td>
<td>50 ± 5.1</td>
</tr>
<tr>
<td>Solution B</td>
<td>12.4 ± 2.5</td>
<td>35 ± 4.1</td>
</tr>
<tr>
<td>Solution A + 1 mM</td>
<td>48 ± 5.1</td>
<td>85 ± 5.9</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>55 ± 5.4</td>
<td>70 ± 5.9</td>
</tr>
<tr>
<td>Nicotinamide + 1 mM</td>
<td></td>
<td>0.13</td>
</tr>
</tbody>
</table>

H2S solutions used were either from a dilution of SMW in PBS (solution A) or experimentally generated in PBS (solution B), as described in Section 2. Nicotinamide can revert the H2S inhibitory effect on GAPDH activity.

Results are the mean ± S.D. of at least five different experiments.

*P < 0.05: significance vs. the control value.*
but using [4-3H]-NADH (in the presence and in the absence of 1 mM nicotinamide) instead of [35S]-H2S, we obtained detectable-la-
belled GAPDH when the purified enzyme was used and when nic-
otinamide was not present (Fig. 3 b). Moreover, the same
experiments were performed under denaturing conditions without
producing any labelled protein (data not shown).

3.5. H2S reacts with nicotinamide and NAD+/H

The interaction of H2S with nicotinamide was then further
investigated by using absorbance spectra analysis (Fig. 4) with
which a decreased absorbance at 260 nm was observed just after
adding H2S to nicotinamide (see 0 min, in Fig. 4a) or NAD+/H
(Fig. 4a and c) solutions. The same decrement of absorbance was
also observed after 30, 60 and 90 min of incubation. The evaluation
of SH groups in the same solution was also performed showing a
correspondent decreased concentration.

4. Discussion

The use of sulphurous spring mineral water therapy has been a
tradition for centuries because of its therapeutic properties. Re-
ports on any possible therapeutic benefit resulting from SMW
treatment have been exclusively based on data that refer sensa-
tions of improved health in subjects complaining of various disor-
ders with often complex and/or heterogeneous aetiology. Some
authors also found that sulphurous SMW therapy can provide ben-
eficial effects in chronic inflammatory disorders with an immunol-
ogy pathogenesis, by inhibiting the immune response at a local
level. Only recently, the validity of spring curative therapies has
been approached by advanced scientific research methods. Any-
how, the fact that some authors also observed adverse events dur-
ing health spa cure must be taken into account (Talenti, 1970;
Nappi et al., 1993; Ibadova et al., 1997; Costantino et al., 2003;
Lopalco et al., 2004). Most of these adverse events are neurological
or general as: asthenia, malaise, etc. On the light of these observations, we investigated the possible effects that sulphurous water can generate on erythrocyte metabolism to give a scientific explanation, at least in part, to the reported adverse symptoms.

Herein, we present a study of two weeks SMW in vivo treatment of healthy volunteers. Blood samples before, after one and two weeks of treatment were collected. We reported the inhibition of erythrocyte GAPDH and G6PDH activities, confirming our preliminary results (Albertini et al., 1999b). Since GAPDH binds the erythrocyte membrane by the band 3 cytosolic domain (Mallozzi et al., 1995), we demonstrated that its binding property increased during sulphurous water treatment (Fig. 1b). Band 3 has been described as an antigen which undergoes clusterization or alteration as a consequence of cell senescence, so its aging or damaging may mimic antigen cleaning out (Low, 1986). The presence of unstable hemoglobin that was found to form during our treatment (Fig. 1) was probably related to GAPDH and G6PDH enzyme activity inhibition. In fact, these latter are both involved in the methemoglobin-reducing reaction as a biochemical carriers between NADH, NADPH and methemoglobin, explaining the increased methemoglobin concentration observed (Fig. 1a) and the possible impairment in methemoglobin-reducing system (Nagel, 1978; Kinoshita et al., 2007; Kuma et al., 1972a; Kuma and Inomata, 1972b). The clinical follow up (which is not a part of this study) demonstrated that after one week of treatment most of the volunteers (70% of the total) reported some adverse events (e.g. asthenia and cephalic) and one subject had to interrupt the treatment. Our hypothesis is that these adverse symptoms may be ascribed, at least in part, to the observed biochemical effects reported above, namely decreased erythrocyte GAPDH and G6PDH activity and increased methemoglobin concentration.

We then tried to investigate how H2S interferes with GAPDH activity. GAPDH can be impaired by different compounds which, depending upon their chemistry, exert effects via different mechanisms such as direct oxidation of NADH, stimulation of the reversible membrane–cytoplasm translocation, or induction of NAD+(H) linkage to the enzyme (McDonald and Moss, 1993; Dimmel and Brune, 1992, 1993; Molina et al., 1992; Kots et al., 1992; Kawamoto and Caswell, 1986; Minetti et al., 1996; Mohr et al., 1996; Soukri et al., 1996; Tanuma and Endo, 1990; Batthyany et al., 2006). To get insight into GAPDH inhibition and GAPDH binding properties during sulphurous water oral treatment, some in vitro experiments were performed by incubating both erythrocytes and purified GAPDH with buffered sulphurous mineral water. During a 2 h incubation of RBC from healthy subjects and purified enzyme with sulphurous buffer, GAPDH activity was inhibited while erythrocyte methemoglobin concentration was increased. When the same experiments were done in the presence of nicotinamide, removal of sulphurous buffer or addition of phosphodiesterase, GAPDH was reactivated (Fig. 2). Indeed, when alkaline phosphatase was added, GAPDH activity was unaffected demonstrating that a phosphorylating process does not occur. The H2S action is reversible, suggesting that the interaction of SH groups with GAPDH active site is involved, and nicotinamide is related to the inhibitory mechanism.

In order to identify the inhibitory GAPDH agent of SMW, we prepared H2S solutions (see Table 1 in Section 2) and incubated either RBC or purified GAPDH. Indeed, as shown in Table 2, the same inhibitory effect of thermal mineral sulphurous water (with protection by nicotinamide) on GAPDH was observed.

The incubation of buffered [35S]-H2S labelled sulphurous water with purified GAPDH or isolated RBC demonstrated the absence of
direct interaction of H$_2$S with the enzyme (Fig. 3a and b). On the other hand the non covalent interaction between NADP+ and GAPDH is demonstrated by the incubation of the purified enzyme with 4-[H]-NADP+ (Fig. 3d). When H$_2$S was incubated either with nicotinamide, or NAD$^+$, the absorbance around 260 nm decreased, as SH concentration did (Fig. 4). These experiments may indicate that a direct interaction between GAPDH and H$_2$S does not occur, and that the reaction between H$_2$S and NAD$^+$ can be responsible for the decrease in GAPDH activity. To explain the effects observed on GAPDH, we hypothesized a reaction mechanism between nicotinamide and NAD$^+$, i.e. SH groups generated from an H$_2$S solution (as the SMW) can interfere on the nicotinamide ring causing a decreased absorbance at 260 nm. This phenomenon is specific for the nicotinamide ring since we observed the same effects either on NAD$^+$ and nicotinamide. Furthermore, it seems possible that SH groups can be added to nicotinamide ring and subsequently interact with GAPDH –SH group by a substitution mechanism. Finally, we attempted to describe a hypothetical mechanism of action of the SH groups generated in H$_2$S solutions with NAD$^+$ and nicotinamide rings. The addition of SH groups generated from H$_2$S solution to the ring and a subsequent substitution with a SH group from the catalytic domain of GAPDH seem to occur (Fig. 5). The model of action proposed considers three different molecules (NAD$^+$, NADH and nicotinamide) sharing the same chemical structure of the ring.

These data, taken together with those observed in our previous studies (Albertini et al., 1996, 1999a,b, 2007), support the hypothesis that the ingestion of SMW can interfere with GAPDH activity by limiting the availability of cellular NAD$^+$. These data can also give supplemental information on the adverse events and, at least in part, explain the clinical symptoms observed. Furthermore, it appears possible to use the assay of erythrocyte GAPDH to monitor the risk to undergo adverse events.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Funding source: This study did not receive any sponsorship and the funds came from the Faculty of Pharmacy of the University of Urbino.

References