ABSTRACT

Introduction: According to the scientific literature, sulfur contained in various foods and medicines is almost completely absorbed in the gastrointestinal tract. However, the role of sulfur containing mineral waters as a dietary source of sulfur still remains unrevealed. The focus of this study was the effect of sulfurous mineral water from two public fountains on the territory of Varna town on the expression rates of enzymes involved in the processes of sulfonation in human intestinal cells.

Methods: Human intestinal epithelial cells were incubated with different percentage content of sulfurous mineral water in the growth medium. Cell viability was measured by the MTT test, and three concentrations of mineral water were chosen for gene expression analyses. The cells were treated with water samples with different residence times after filling: 24 hours, 3 and 7 days. Messenger RNA levels of two sulfotransferases isoforms (SULT1A1 and SULT1A3) and 32-phosphoadenosine-5-phosphosulfate synthase 1 (PAPSS1) were measured.

Results: Significantly increased mRNA levels of both sulfotransferases were measured in cells treated with 24-hours samples from the two fountains. This stimulatory effect was dependent on the percentage content of mineral water in the culture medium. Despite the stimulated expression of sulfotransferases, higher volume concentrations of 24-hour samples of mineral water significantly inhibited the gene expression of PAPSS1. It could be suggested that the H2S, as a major active compound in the mineral water, has the potential to modulate the gene expression of the investigated enzymes.

Conclusion: Sulfurous mineral water should be considered an important dietary source of sulfur. We believe that the presented results would be of interest to society and health professionals.

Keywords: sulfurous mineral water, intestinal cells, sulfonation, human metabolism

INTRODUCTION

The healing properties of mineral waters have been known for thousands of years. Even in the age of advanced science and technologies, interest in this valuable gift of nature is not diminishing. Nowadays, more and more people in the so-called Western societies are turning to alternative and complementary therapies, including hydro- and balneotherapy. So, we focused our research on the mechanisms behind the beneficial effects of mineral waters.

Though small in territory, Bulgaria is extremely rich in thermal mineral springs, due to the geographical location, the favourable climate and diverse landscape, almost all types of mineral water may be found in the territory of the country, including the sulphur containing mineral waters [1].

The focus of our study was the mineral water from the Varnabasin, which is a part of the Malm-Valanginian water-bearing horizon [2] and which is known to be actively used since Roman times for balneology and as a drinking remedy. Because of its chemical characteristics and mineral content, this water is classified as sulfurous [3]. Recently detailed physicochemical characteristics of the water were made, and new data were obtained about the content of biologically active compounds such as potassium, dissolved sulfides and free hydrogen sulfide (S2, SH-, H2S), selenium, and chromium [4]. Furthermore, the first results from a human interventional study were obtained, revealing some of the molecular mechanisms in support of empirical knowledge about the beneficial effects of Varna mineral water when applied as a drinking remedy. After a two-month interventional period, the sulfurous mineral water (SMW) improved the body’s redox status and reduced levels of inflammatory markers in healthy volunteers. Furthermore, in the same study, improved effectivity of kidney function was estimated by decreased plasma creatinine levels and increased glomerular filtration [5, 6].

The beneficial effects of SMWs have been attributed to the presence of sulfur, mainly in the form of hydrogen sulfide (H2S) [7, 8]. Sulfur is a macronutrient with important metabolic functions. In addition to maintaining redox status at the cellular and extracellular levels, it
is involved in maintaining the native conformation of proteins and their posttranslational modification [9]. Furthermore, sulfur is a substrate in the reactions of sulfonation which are related to the addition of a sulfonate group to a substrate increasing its water solubility. Except in posttranslational modification of endogenous proteins, the reactions of sulfonation are also an important part of Phase II of the metabolism of many xenobiotics, including medicines and food supplements. These reactions are catalysed by a super enzyme family named sulfotransferases (SULTs), widely distributed in human tissues [10, 11]. At least seven isoforms, including 1A1 and 1A3, are reported to be widely expressed in the gastrointestinal tract [10, 12]. SULT1A1 is described as very sensitive to the environment and highly adaptive to the type and concentration of the available substrates [13]. Because of its active site plasticity, this isoform is known to have a broad substrate specificity and is reported to be involved in the metabolism of numerous drugs, toxins, phenol compounds, including plant polyphenols [14, 15]. On the other hand, SULT1A3 prefers monoamine substances such as dopamine and is known to be the major isoform metabolising catecholamins and serotonin in extrahepatic tissues [16].

The universal donor of the sulfonategroup and a mandatory co-substrate for sulfonation reactions is 3′-phosphoadenosyl-5′-phosphosulfate (PAPS). In the human organism, PAPS is synthesized by a bifunctional enzyme with both ATP sulfurylase and adenosine 5′-phosphosulfate (APS) kinase activity [17]. Two isoforms of human PAPS synthase (PAPSS) are identified – PAPSS1 and PAPSS2. The first one has been found to be expressed in most tissues, while the latter is the dominant isoform in the liver, bones and cartilage [18].

According to scientific reports, certain biologically active compounds in plant-derived foods could affect transcriptional factors in intestinal epithelial cells resulting in up-regulation of the expression of detoxification enzymes [19]. Therefore, the utilization of dietary sulphur might be an important factor in modulating the sulfonation activity in tissues during the metabolism of drugs and of other xenobiotics, hence affecting their biological activity. A significant number of studies has been devoted to the beneficial effects of sulfur containing compounds in plant derived foods. Moreover, it is believed to the beneficial effects of sulfur containing compounds from foods. The aim of the study was to investigate the effect of SMW from the Varna basin on the gene expression of enzymes involved in sulfonation processes in human intestinal epithelial cells.

### Materials and Methods

#### Mineral water sampling

The samples of mineral water from two public fountains in the city of Varna, Dom Mladost (DM) and Aquarium (A), were taken in clean plastic bottles with a capacity of 0.5 L. The samples were stored at T 4°C for a period of 24 hours, 3 days or 7 days prior to analyses. Immediately prior to analysis, 50 milliliters of each sample were separated, filtered through a 0.2 μm-pore-size filter and analyzed. All analyses were carried out at room temperature.

#### Cell culture

Human intestinal epithelial cells HIEC-6 (ATCC® CRL-3266™) were cultured in Opti-MEM™ I Reduced Serum Medium (Gibco), supplemented according to manufacturer’s directions, with fetal bovine serum (FBS, Sigma-Aldrich) to a final concentration of 2%, 10 mM GlutaMAX, (Gibco) to a final concentration of 5%, 10 ng/mL epidermal growth factor (Genaxxon) to a final concentration of 0.1%, and penicillin/ streptomycin mixture (Sigma-Aldrich, Germany) to a final concentration of 1%. Cells were cultured in 75cm² flasks at 37°C in a humidified chamber with a 5% CO₂ atmosphere.

#### Cell viability assay

The viability of mineral water-treated cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The assay is based on the ability of viable cells to reduce the yellow MTT to purple insoluble formazan. Cells were seeded at 6.5x10⁴ density in 12-well plates. Following 24 h of incubation, the culture medium was removed, and cells were incubated with fresh medium with varying percentage content of mineral water in the culture medium (v/v): 1%, 2%, 4%, 8%, 16% for 24 hours. At the 20th hour of incubation, the culture medium was removed, and 1 mL of dimethyl sulfoxide was added to each well to lyse the cells and dissolve the reduced MTT. Finally, the intensity of the dissolved formazan crystals (purple color) was quantified using a Synergy 2 plate reader (BioTek) at a wavelength of 550 nm. The relative cell viability was calculated as the percentage of untreated cell viability, which was considered 100%. All treatments were performed in triplicate. Data were presented as mean±SEM.

#### Reverse transcription

HIEC-6 cells were incubated for 24 hours with selected percentage content of mineral waters (2, 4, 8%) from two public fountains DM and A with different degree of storage - after 24 hours, 3 days and 7 days from the sampling. Total RNA was isolated using Ron’s Fast Tri reagent (Bioron) following the manufacturer’s protocol. The concentration of isolated RNA was measured on Synergy 2 plate reader (BioTek). RNA (100 ng) was reversely transcribed with Revertaid™ First Strand cDNA Synthesis Kit (Thermo Scientific), applying oligo (dT)18 priming strategy according to the manufacturer’s requirements. cDNA synthesis was performed on Gene Amp PCR
7500 thermal cycler (Applied Biosystems). Reaction conditions in final volumes of 10 µL were 60 min incubation at 42°C and then termination of the process at 70°C for 5 min. After synthesis, cDNA was diluted by adding of 30 µL nuclease-free water to each sample.

**Real-time quantitative polymerase chain reaction**

The nucleotide sequences of the primers for real-time PCR analyses are presented in Table 1. The primers were designed using the Real-Time PCR gene expression design tool with free Internet access and were synthesized by Sigma-Aldrich and Integrated DNA Technologies. As a template for real-time PCR, 0.39 µL of cDNA was amplified in a 5 µL final volume. The final primers’ concentration was 300 nM. Amplification was performed using AccuPower®2XGreenStarTMqPCR Master Mix (Bioneer) containing ROX fluorescent dye. Reactions were performed on 96 well plates. Reaction parameters were the following: enzyme activation and denaturation at 95°C/10 min; 40 cycles of amplification at 95°C/15 sec, annealing at 60°C/1 min; followed by melting curve analyses. Analysis was performed on ABI PRISM 7500 (Applied Biosystems).

Gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method [21], and levels of mRNA were presented as relative units (RU). The effect of SMW on the gene expression of interest was assessed by comparison with the untreated cells (controls), where the mRNA levels were considered to be equal to 1. RPL-0 was used as an endogenous control gene.

**RESULTS**

The effect of Varna SMW from two public fountains on the cell viability was analysed and compared (Fig. 1). It was observed that at the three lowest values of percentages content in the culture medium, SMW did not significantly affect the cell viability. The cell viability dropped sharply at 8 % SMW content. However, the cell viability remained above 80%, even at the highest percentage content of SMW. Based on these results, all applied concentrations of SMW were considered non-toxic [22]. Three values of SMW content in culture medium were chosen for further analyses: 2, 4 and 8 %. The last-mentioned concentration was chosen with the idea to introduce in the nutritional medium a higher concentration of H2S and have relatively preserved cell viability.

Fig. 1. Effect of SMW of Dom Mladost and Aquarium public fountains from Varna basin on the viability of HIEC-6. Data are presented as mean ± SD of three independent experiments; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, versus the control group (untreated cells).

In order to monitor the effect of hydrogen sulphide levels on the expression of target genes and bearing in mind that it is a volatile gas, the cells were incubated with water samples with a varying time of residence: 24 hours (24h), 3 days (3d), and 7 days (7d) after sampling and treated with 0 (controls), 2, 4 and 8% content of SMW in the culture medium.

Analysis of the gene expression showed a significant increase in mRNA levels of SULT1A1 in cells treated with 24 hours samples of SMW from both fountains (Fig. 2, A and B). This stimulatory effect was strongly emphasized depending on the percentage content of mineral water in the culture medium. Decreased expression of SULT1A1 was estimated in cells treated with samples from both fountains on the 3d and 7th day of water sampling (Fig. 2 A and B).

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**Table 1. Primers nucleotide sequences:**

<table>
<thead>
<tr>
<th>Genes description</th>
<th>Nucleotide sequence (5’-3’)</th>
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| **RPLP0**         | AGC CCA GAA CAC TGG TCT C (Forward)  
                      ACT CAG GAT TTC AAC GGT GCC (Reverse) |
| **SULT1A1**       | CGG CAC TAC CTG GGT AAG C (Forward)  
                      CAC CCG CAT GAA GAT GGG AG (Reverse) |
| **SULT1A3**       | GGA ACC CTC AGG GCT GGA G (Forward)  
                      CGT CCT TTG GGT TTC GGG (Reverse) |
| **PAPSS1**        | GGG AGC CTG TGC AAG AAA GT (Forward)  
                      GGT GAC ATT GGT TGC TCT CTG (Reverse) |

**Statistical analysis**

Statistical analyses were performed using Microsoft Excel Office software and GraphPad Prism 5.0. Unpaired two-tailed Student’s t-tests were used to compare the differences between treated cells with the controls. All samples were measured in triplicate, and the mean values were used for analyses. The data are presented as mean ± standard deviation (SD). The p value of less than 0.05 was considered a significant difference between the groups.
Fig. 2. Effect of SMW on gene expression of SULT1A1. Gene expression levels were calculated as relative units (RU) compared to untreated control cells, where the gene expression was considered to be equal to 1. Data are presented as mean ± SD; *p<0.05 increased levels of mRNA versus controls; #p<0.01 decreased levels of mRNA versus controls.

Similar to these results were the effects of Aquarium fountain SMW on SULT1A3 expression (fig. 3 A). As presented in figure 3 B, decreased mRNA levels of this gene were estimated in the cells treated with 2 % samples SMW from DM fountain after 24 h of residence. The samples of SMW after 3 and 7 days of residence did not affect the expression of the SULT1A3 gene, and the mRNA levels measured in these cells were close to that observed in untreated controls (Fig. 3).

Fig. 3. Effect of SMW on gene expression of SULT1A3. Gene expression levels were calculated as relative units (RU) compared to untreated control cells, where the gene expression was considered to be equal to 1. Data are presented as mean ± SD; *p<0.05 increased levels of mRNA versus controls; #p<0.01 decreased levels of mRNA versus controls.

The effect of SMW on mRNA levels of PAPSS1 is presented in figure 4. The treatment with 24-hour samples from the Aquarium fountain (fig. 4, A) had a significant stimulating effect on the gene expression in cells treated with 2 % SMW content in the nutrition medium. In all other cells treated with samples from the Aquarium fountain, the mRNA levels of PAPSS1 were lower or similar to those measured in untreated controls. A similar effect was observed for cells treated with DM samples after 24 h or 3 days of water residence (fig. 4, B). On the other hand, in cells treated with 4 and 8 % SMW from this source after 7 days of sampling, the expression of the PAPSS1 gene was stimulated significantly (fig. 4, B).
DISCUSSION

The focus of our study was the SMW from two public fountains in the city of Varna, which are freely available and used every day by thousands of people. In recent years the scientific interest in SMW has increased because of the belief that H2S contained in this type of water is the main biologically active compound with a key role in their therapeutic potential [7, 8]. Furthermore, we were interested in whether SMW as a dietary source of sulfate moiety could affect the phase II enzymes involved in processes of sulfonation in human enterocytes. Many scientific studies have been dedicated to the effects of active compounds from natural nutrition as sources on detoxifying enzymes [23, 24]. However, none of these studies have considered the mineral waters as a dietary source of biologically active compounds. Low-mineralized waters such as Varna SMW should be considered an essential nutritional factor in everyday diet.

Considering the fact that H2S is a volatile gas and that the H2S/soluble sulfides ratio changes after a certain period of time, we explored the effect of SMW on gene expression of the target enzymes at different time points of water storage. In a previous study, we analysed the physicochemical composition of the water from two fountains at different time points of sampling [4]. The study of selected parameters in dynamics - after 24 hours, 3 and 7 days of sampling showed significant content of H2S in the 24 hours-samples from both fountains but much higher in DM samples. Further analyses showed a change in the concentration of dissolved sulphides and H2S, which decreased significantly in Aquarium samples after the 3rd day of sampling [4] but still remained high enough in DM samples (unpublished data). For comparison, H2S and soluble sulfides measured in 24-hour samples from both fountains were 0.04±0.004 and 1.9±0.2 [mg/L], respectively.

For a long time, our understanding of H2S was based on its toxicity as an environmental pollutant. Since it was recognized as an endogenous signal molecule, many studies focused on its role in various physiological processes in the human body. In physiological concentrations, H2S has beneficial effects on various metabolic functions such as energy metabolism, redox status and anti-inflammatory response [25]. The potential of H2S donors to induce phase-II metabolizing enzymes in vivo and in vitro has also been reviewed [26]. However, SULTs were not included in these studies, and thus the effect of H2S from exogenous sources on the gene expression of these enzymes remained unrevealed.

The aim of the present study was to investigate the effect of SMW from the Varna basin on the gene expression of enzymes involved in sulfonation processes. The first step of this study was to explore the effects of SMW on cell viability by applying an MTT assay on human intestinal epithelial cells (fig. 1). All applied concentrations were considered non-cytotoxic as the reduction of cell viability was less than 20 % [22]. Based on this test, we determined SMW concentrations in the nutrition medium for gene expression analyses - 2, 4 and 8 %.

Gene expression analyses showed that after 24 hours of residence, SMW significantly stimulated SULTs gene expression in HIEC-6. This effect was strongly exhibited in the cells treated with the highest percentage of SMW. Furthermore, the expression levels of SULT1A3 in the cells treated with DM samples were approximately 12 times higher than in the cells treated with samples from the Aquarium fountain (fig. 2, A and B, respectively). Apparently, this effect was concentration dependent, and it could be attributed to the biologically active compounds introduced in the culture medium with the mineral water.

The expression of the two SULTs genes sharply diminished in the cells treated with SMW samples after 3 and 7 days (fig. 2 and 3). The mRNA levels of SULT1A1 in the cells treated with DM samples were approximately 12 times higher than in the cells treated with samples from the Aquarium fountain (fig. 2, A and B, respectively). Apparently, this effect was concentration dependent, and it could be attributed to the biologically active compounds introduced in the culture medium with the mineral water.

It is known that SULTs exert their function with the participation of PAPS as a universal donor of sulfate and a mandatory co-substrate for sulfonation reactions. The low availability of PAPS is shown to be a limiting factor for sulfotransferases activity [27]. Therefore, the rate of PAPS
synthesis might be an important factor in regulating the sulfonation processes in tissues during the metabolism of drugs and xenobiotics, hence affecting their biological activity. Our results showed that gene expression of the PAPSS1 (fig. 4) did not follow the trend observed for SULTs. It is interesting to note that, despite the stimulated expression of sulfotransferases (fig. 2 and 3), higher volume concentrations of SMW content after 24 hours of sampling significantly inhibited the gene expression of PAPSS1 (fig. 4). We know that 24 hours after sampling of SMW the concentration of H2S stays high, and one possible explanation of these diverse effects could be the different sensitivity of the above-mentioned enzymes to H2S.

Because the highest concentration of hydrogen sulfide and other active components dissolved in SMW was established in the 24-hour samples from this fountain [4], and considering the sulfonation mechanism, it should be expected SMW to have similar effects on the expression of SULTs and on PAPS synthases. However, 24-hour samples of SMW had divergent effects on these two types of enzymes-stimulated expression of SULTs, while expression of the PAPSS1 was inhibited or unaffected compared to the controls. The only observed exception was the increased mRNA levels of PAPSS1 in the cells treated with the lowest percentage volume of 24-hours SMW (fig. 4 A). In summary, it could be suggested that H2S has the potential to modulate the gene expression of the investigated enzymes. Furthermore, it seems that the expression of SULTs was stimulated in cells treated with a higher concentration of mineral water, independently of the PAPS synthase expression.

To the best of our knowledge, no similar studies on the effects of mineral waters on enzymes involved in xenobiotics metabolism are reported so far in the available scientific literature. Therefore, it is difficult to compare the results of the present study with other similar ones. On the other hand, many studies are focused on the effects of biologically active compounds from plant derived foods, such as polyphenols and organosulfides, on the expression and activity of Phase 2 enzymes of xenobiotics metabolism including sulfotransferases [23, 24]. The potential of organosulfides from various edible plants to induce the expression of phase II xenobiotic metabolizing enzymes has been associated with the activation of the Keap1/Nrf2 antioxidant response pathway. [28]. No data about the similar effect of organosulfides from foods on the expression and activity of PAPS synthases were reported so far.

**CONCLUSIONS**

Sulfurous mineral water should be considered an important dietary factor contributing to the sulfur metabolism in humans. Data obtained are the first scientific evidence of a direct link between sulfurous mineral water and metabolic challenges in the enzymes involved in sulfur utilization and metabolism. From a scientific point of view, the presented findings would be a starting point for a new research line focusing on the contribution of mineral waters to the metabolic supply of active components. Furthermore, we believe that the results obtained would have a great social and health care impact. Bearing in mind the role of the investigated enzymes in xenobiocits metabolism, the findings in this study may be of interest to health professionals regarding the consumption of sulfur-containing mineral waters by people on drug and hormone replacement therapies.

**Abbreviations:**

SULT1A1 – sulfotransferase 1A1
SULT1A3 – sulfotransferase 1A3
PAPSS1 – 32 - phosphoadenosine-52 - phosphosulfate synthase 1
mRNA – messenger RNA
H2S – hydrogen sulfide
SMW - sulfurous mineral water
HIEC 6 – human intestinal epithelial cells
DM – Dom Mladost fountain
A – Aquarium Fountain

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