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Protective effect of sulfurous water in peripheral blood mononuclear cells of Alzheimer's disease patients

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ABSTRACT

Aims: One of the main features of sulfurous water (SW) is the presence of hydrogen sulfide (H_2S), which confers its antioxidant activity. Since oxidative stress plays an important role in Alzheimer's disease (AD) we hypothesize that SW could have a protective effect in these patients.
Material and methods: A therapeutic *in vitro* approach of SW was performed in peripheral blood mononuclear cells (PBMCs) of AD patients and in age-matched healthy non-demented controls using one modification of the comet assay (to measure oxidative DNA damage) and the MTT assay (as an indicator of cell viability). Hydrogen peroxide and homocysteine were used to induce oxidative DNA damage, and vitamin C, Trolox and N-acetylcysteine were selected as antioxidants of reference to compare SW treatment results.
Key findings: SW did not increase *per se* the oxidative DNA damage of PBMC. Furthermore, SW protected them against enhanced oxidative stress in AD and control populations after pro-oxidant stimuli, with similar results to those observed when using the antioxidants of reference. Nevertheless, SW was the only treatment that could avoid the loss of viability of PBMC for all pro-oxidant stimuli in both populations, suggesting that H_2S could confer to SW a more antioxidant capacity than other known antioxidants.
Significance: The protective effect of SW was proved for the first time not only in DNA stability but also in cell viability preservation in AD, indicating that further research in other *in vitro* and *in vivo* models could lead to include SW as a possible therapy for AD.

1. Introduction

World population aging has led to an increased prevalence of some aging-related diseases in the last years, such as dementias [16]. Alzheimer's disease (AD) is the major cause of dementia in the world elderly population and is one of the top ten causes of death in the USA [35]. People with AD have different symptoms: general cognitive decline, loss of memory or changes in behavior among others. Physiologically there are two well-established hallmarks in the pathology: amyloid plaques and neurofibrillary tangles in the brain. But there are other important aspects related to AD, such as an elevated oxidative stress (OS) level.

OS can be defined as the imbalance of pro-oxidant agents against antioxidants in favor of the former [48] and it plays a key role not only in the aging but also in the pathogenesis and development of AD [1,49]. Elevated OS level in the brain of AD patients could be due to two main causes: amyloid beta oligomers and an enhanced susceptibility to pro-oxidant stimuli in AD brains because of decreased levels of natural antioxidants [4]. Furthermore, recent studies have included the presence of metals as potent catalysts in the formation of free radicals and reactive oxygen species (ROS) [42].

However, OS is not only present in the brain of AD patients but also in peripheral tissues [19], including body fluids such as serum, plasma and urine [10,28,38]. Regarding peripheral tissues cells, most of the studies are focused in peripheral blood cells [25,29,36,54]. Peripheral blood mononuclear cells (PBMCs) are a good model of AD to study OS due to their physiological and biochemical similarities with neurons [30,56] and the ease of sample collection. Thus, different OS biomarkers have been investigated in the last years, including direct intracellular ROS levels [29], expression and activity levels of different OS-related enzymes [9], protein and lipid oxidative damage [53,54] and DNA oxidative damage [25,36].

Abbreviations: ANOVA, analysis of variance; AD, Alzheimer's disease; CASP, Comet Assay Software Project Lab; CO_2, carbonic anhydride; DMSO, dimethyl sulphoxide; DNA, deoxyribonucleic acid; EDTA, ethylene diamine tetraacetic acid; GSH, glutathione; HCY, homocysteine; hOGG1, human 8-hydroxyguanine DNA-glycosylase; HP, hydrogen peroxide; LSD, least significant difference; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAC, N-acetyl-L-cysteine; NaCl, sodium chloride; NaOH, sodium hydroxide; OS, oxidative stress; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; ROS, reactive oxygen species; RPMI-1640, Roswell Park Memorial Institute medium; SEM, standard error of the mean; SW, sulfurous water; TRIS, tris(hydroxymethyl) aminemetane; TRITON X-100, polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether; TROLOX, soluble analog of vitamin E.

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Comet assay is one of the most used methodologies to determine DNA oxidative damage in PBMC due to its sensitivity. Since it was developed by Singh et al. [50] it has been modified in several ways, being a widespread technique that can be useful also in genotoxicity studies as well as in new antioxidants testing both *in vitro* and *in vivo*. One of the most used modifications is the addition of DNA lesion-specific repair enzymes to the methodology which recognize particular damaged bases and create additional breaks. Along with the widely used Endo III and FPG repair enzymes it has been recently described that human 8-hydroxyguanine DNA-glycosylase (hOGG1) is more specific for oxidative DNA damage and may be a more useful alternative for testing the effects of novel compounds when using this technique [51].

The presence of OS in peripheral tissues gives researchers an opportunity to test different antioxidant treatments that might be applied in the future to improve the patient's conditions. Thus, several compounds of different nature have been tested both *in vitro* and *in vivo*, in example: vitamins C and E or N-acetyl-cysteine [22,23,26,31] and even some clinical trials have been developed in AD patients with dissimilar results. However, no studies of the antioxidant effects of sulfurous water (SW) in AD are available in the literature.

Medicinal–mineral SW has been used in traditional medicine since the Romans but nowadays their properties have been scientifically proved [27,32]. Recently, antioxidant capacity has also been demonstrated in SW [6,8,43]. The main component in SW that confers its antioxidant activity is hydrogen sulfide (H_2S) which reacts with up to four different ROS and enhances GSH formation [7,44].

Taking into consideration these arguments, we hypothesize that SW should have protective effects in DNA of isolated PBMC from AD patients.

2. Materials and methods

2.1. Study population

All the patients were recruited in a consecutive manner. We examined 25 individuals with AD (8 males and 17 females), aged from 66 to 93 years diagnosed by the Dementias Unit of the Geriatrics Service at the San Carlos Clinical Hospital in Madrid (Spain). The AD diagnosis was carried out according to DSM-IV and NINCDS-ADRDA [34], the mini-mental state test [17] and also included clinical history (Camdex), physical examination, neurochemistry parameters and neuroimaging procedures [46]. Since the antioxidant effect of SW could be beneficial not only in AD but also in aging, we compare AD population results to a control population. 25 non-AD individuals were selected as healthy controls, and matched by age, sex and life style. All subject of the sample lived in the same geographic area at the time of the study. Blood samples from control population were obtained from San Francisco de Asís Hospital in Madrid (Spain).

For both groups systemic exclusion criteria were: vascular dementia, inflammatory, autoimmune, hematologic, or neoplastic disease; diabetes mellitus, thyroid disease, alcohol addiction, acute or chronic infection; history of hepatic or renal failure, myocardial infarction, severe arthrosis, pathological biochemistry and/or hematological parameters, without any form or pharmacologic treatment.

Each participant or a legal guardian signed an informed consent form detailing the analysis and handing of the data. The study was approved by the Ethic Committee of Superior Council of Scientific Investigation in Spain.

2.2. Blood sample collection

First morning peripheral blood samples using EDTA as anticoagulant were collected by venipuncture from individuals included in the study of both AD and control populations. In order to isolate the peripheral blood mononuclear cells (PBMCs) Histopaque 1077 was used following the manufacturer instructions after diluting the blood sample 1:2 with PBS. Afterwards PBMCs were resuspended in phenol red-free RPMI-1640 medium. Before performing the antioxidant and pro-oxidant treatments, cell viability was assessed by trypan blue exclusion method obtaining a viability of >90% in all samples.

2.3. Reagents

Histopaque 1077, Trolox, N-acetyl-cysteine, Homocysteine, NaCl, Na_2EDTA, Trizma base, Triton X-100, DMSO, NaOH and ethidium bromide were purchased from Sigma-Aldrich (Spain). Ascorbic acid was purchased from Fluka (Switzerland). Hydrogen peroxide was purchased from Foret (Spain). Absolute ethanol and NaCl were purchased from Panreac (Spain). Normal melting point agarose and low melting point agarose were purchased from Bio-Rad (Spain). MTT assay commercial kit was purchased from Promega (USA). hOGG1 was purchased from New England Biolabs Inc. (UK).

2.4. Sulfurous mineral–medical water

Sulfurous mineral–medical water (SW) from spring of Platea (Calatayud, Spain) was kindly provided by SUCARSE S.L. and its physico-chemical properties are shown in Table 1. SW aliquots were always kept at −80 °C and thawed at room temperature when needed. pH of SW was measured after thawing each aliquot and before each experiment by using standard pH test strips (Sigma-Aldrich) with a measuring range of 6.0–7.7, and a resolution of 0.3–0.4 pH units.

2.5. Antioxidants and pro-oxidant agents

To compare the effects of SW on PBMC from both populations, three non-enzymatic antioxidants were selected based on their different mechanisms of action. Ascorbic acid (AA) was selected as a hydrophilic antioxidant due to the wide range of free radicals that it can scavenge; Trolox was chosen because it is the soluble analog of α-tocopherol (vitamin E, lipophilic antioxidant) and N-acetyl-cysteine (NAC) was selected as the SH group donor that leads to GSH formation. NAC was used only in the comet assay because the performing of MTT assay was focused in testing different mechanisms of action and NAC acts as the same way than SW as GSH formation inducer.

On the other hand, two different pro-oxidant agents were used to generate oxidative damage in the PBMC: hydrogen peroxide (HP) and homocysteine (HCY). HP can increase the oxidative stress to cells by enhancing superoxide anion production and inactivating thiol groups of some enzymes [11,20] and it was used in both comet assay and MTT assay. On the other hand, HCY stimulates PH production in metal-mediated reactions and producing peroxynitrites [40] and it was used only in MTT assay.

2.6. Cell treatments

Once PBMCs were isolated three different treatments were carried out. In order to confirm that SW and the selected antioxidants did not generate oxidative damage *per se*, the first treatment consisted of the

Table 1
Physico-chemical properties of sulfurous water from Platea (Calatayud, **Spain**). Authorized reproduction by SUCARSE S.L.

pH	6.7	NO_2^-	<0.02 mg/L
CO_2	21.0 mg/L	NO_3^-	<5 mg/L
H_2S	8.83 mg/L	SO_4^{2-}	3910 mg/L
Ca^{2+}	650.5 mg/L	NH_4^+	0.1 mg/L
Mg^{2+}	466.5 mg/L	S^{2-}	8.31 mg/L
Na^+	2460 mg/L	Iron	0.72 mg/L
K^+	15.5 mg/L	SiO_2	39.9 mg/L
HCO_3^+	276 mg/L	Sr^{2+}	11.28 mg/L
F^-	3.1 mg/L	Li^+	0.48 mg/L
Cl^-	3290 mg/L	Mn^{2+}	0.09 mg/L