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Sulfurous thermal waters stimulate the osteogenic differentiation of human mesenchymal stromal cells – An *in vitro* study

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ABSTRACT

Strategies aimed at delaying the onset of bone tissue degeneration and the resulting skeletal fragility are key to decrease the risk of bone fracture correlated to ageing. The therapeutic properties of sulfurous thermal waters (STWs), rich in hydrogen sulfide (H_2S), have been claimed for centuries. However, the direct regulation of bone cells by STWs has not been investigated yet. Here we aimed at analyzing the effect of STWs on cultured human mesenchymal stromal cells (hMSCs) derived from bone tissue. Two concentrations of STWs from 2 health spa centers in Italy (here named STW-1 and STW-2) containing, respectively, high and moderate quantities of H_2S, were added to the culture media. Cytotoxicity and osteogenic differentiation were evaluated. We provided first evidence that treatment of hMSCs with STWs results in a sharp increase in intracellular H_2S content, coherent with the different concentrations of H_2S, thereby reveling that STWs-released H_2S is internalized by cells. STWs treatment significantly induced osteogenic differentiation of hMSCs. In particular, mineral apposition was increased with a similar pattern by the two STWs, while mRNA expression of osteogenic markers (BSP, OC, RUNX-2, OPN) was differently affected. Only STW-2 induced a significant, dose-dependent increase in these gene expression. These findings support the rationale for the use of STWs as a complementary treatment of bone wasting diseases.

1. Introduction

Musculoskeletal diseases are one of the major global health burdens for individuals and society. They are associated to ageing [1–5] and are predicted to exponentially increase given that the aged population is expected to rise to more than 2 billion by 2050 [6]. Among the most prevalent musculoskeletal disorders is osteoporosis, a systemic disease characterized by low bone mass and deterioration in the micro-architecture of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. It is estimated that 50 % of women and 20 % of men aged over 50 years will experience an osteoporosis-related fracture and that these percentages will at least double in the next 20 years due to the changing population demographics [7]. Osteoporosis arise from an increased bone resorption by the osteoclasts, which is no longer compensated by bone formation by osteoblasts [8]. This is caused by several mechanisms induced by ageing, including a decreased number of osteoprogenitor cells [9], the decreased mineralizing capacity of senescent bone cells [10], the inhibition of the proliferation [11], and the shift from osteogenic toward adipogenic lineage in mesenchymal stromal cells (MSCs) [12].

Targeting these processes to delay the onset of bone loss and skeletal fragility is a key milestone to avoid the high risk of fracture associated with osteoporosis and the early need of pharmacological treatment.

In this perspective, the bioactive and therapeutic effects of thermal water can provide a complementary opportunity of treatment. Thermal water originates in the deep layer of the soil and is physically and

Abbreviations: ALP, alkaline phosphatase; AR-S, alizarin red stain; BSP, bone sialoprotein; CBS, cystathionine-β-synthase; CollI, collagen I; CollXV, collagen XV; CTH, cystathionine-γ-lyase; CTRL, control; D, day; EDTA, ethylenediaminetetraacetic acid; FACS, flow cytometry; FBS, fetal bovine serum; FW, forward; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; GCP, good clinical practices; H-min, high mineralizing; hMSCs, human mesenchymal stromal cells; H_2S, hydrogen sulfide; LDH, lactate dehydrogenase; L-min, low mineralizing; MEM, minimal essential medium; mMSCs, murine mesenchymal stromal cells; Min, mineralization; MSCs, mesenchymal stromal cells; N/A, not applicable; NaHS, sodium hydrosulphide; N-min, non mineralizing; OC, osteocalcin; OPN, osteopontin; PCR, polimerase chain reaction; PBS, phosphate-buffered saline; REV, reverse; RT, room temperature; RUNX-2, Runt Related Transcription Factor 2; SMAD1, Smad Family Member 1; STWs, sulfurous thermal waters; STW-1, sulfurous thermal water n1; STW-2, sulfurous thermal waters n2; WNT-16, Wnt Family Member 16; WISP1, Wnt1-inducible-signaling pathway protein 1

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chemically characterized depending on the temperature, geographical localization and geological layer it crosses. Among thermal waters are sulfurous, salso-bromo-iodic, bicarbonate, bicarbonate-sulfate waters. Medicinal use of thermal water (balneotherapy, thalassotherapy, hydrotherapy) arise from ancient Egyptian, Greek, Roman, Celtic, Hebrew [13] and is currently recognized by several areas of medicine. One of the major clinical application is for the treatment of pathologies affecting the skin; in the musculoskeletal system it is used to delay the onset of muscle soreness and recovery from intense exercise [14]; relieve symptoms of rheumatoid arthritis, osteoarthritis and other joint problems [15,13]; modulate bone metabolism as well as reduce somatic complaints [16]. Despite the wide clinical application, the knowledge associated to the clinical properties of thermal water is mainly empiric given that only a few *in vitro* studies on the function of keratinocytes and fibroblasts exists [17–19]. Among thermal waters, sulfurous thermal water (STWs) are characterized by the presence of hydrosulfide ion, sulfide anions and most abundantly hydrogen sulfide (H_2S). STWs were broadly used in the treatment of rheumatism and osteoarthritis [20–22], colitis [23], skin disorders [24], limb spasticity and pain [25], and several pathologies involving ear and hearing function [26–29], respiratory tract infections [30], as well as for inducing a stress-response effect. Overall, the therapeutics effects of STWs have been ascribed to analgesic, antioxidant, antibacterial, anti-inflammatory properties. In particular, STWs have antioxidant effect on human neutrophils [31] and anti-inflammatory properties on human monocytes [32]. Coherently with the therapeutics effect of STWs, H_2S has been shown to hold analgesic, antioxidant, antibacterial and anti-inflammatory properties [33–35].

However, the potential of STWs to directly regulate bone cells function and to use STWs in complementary medicine for the treatment of bone wasting diseases has never been investigated. A growing body of evidence shows that H_2S has therapeutic potential in age-associated diseases [36]. Moreover, we have recently contributed to demonstrate its relevance in the osteogenic differentiation of MSCs and anabolic function in bone. In particular, we have shown that: the H_2S producing-enzymes are expressed in human MSCs (hMSCs) and osteoblasts and are transcriptionally regulated during osteogenesis [37,38]; that H_2S exogenous stimulation induces osteogenic differentiation of hMSCs and murine MSCs (mMSCs) [39,40]; that H_2S donors can be used, similarly to other osteoinductive molecules, to increase the osteogenicity of bone implants [41,42], and most importantly that therapeutic intraperitoneal administration of H_2S prevents the onset of bone loss in the mouse model of osteoporosis [39].

Objective of this study was to investigate whether the treatment of hMSCs with STWs increase intracellular H_2S levels and modulate their osteogenic differentiation, thereby exploring the potential use of STWs as complementary treatment of bone wasting diseases.

2. Material and methods

2.1. Materials and reagents

In this study, two STWs (here named STW-1 and STW-2) were obtained from 2 health spa centers in Italy and evaluated. Table 1 reports the chemical composition of STW-1 and STW-2, as provided by the centers.

2.2. Cell isolation and culture

The study has been approved by the Institutional Ethic Committee (CE AVEC 779/2018/Sper/IOR) and has been conducted in accordance with the Helsinki Declaration (Fortaleza, October 2013), with the principles of the ICH-GCP, as well as with all the national and international legislation applicable to clinical research. In particular, all surgical procedures and the harvesting of human tissues were performed at the Rizzoli Orthopedic Institute after having obtained patients' informed consent (women and men aged 71 ± 8). hMSCs were isolated by using a mechanical and a Ficoll-density gradient isolation protocol from bone chips of tibial plateau obtained by patients undergoing surgical knee replacement [37]. Cells were cultured in α-MEM (minimal essential medium) 15 % FBS (fetal bovine serum) in 37 °C, 5 % CO_2 and 95 % O_2 and medium was replaced twice per week; they were expanded until passage 2, when they were harvested by treatment with trypsin/ethylenediaminetetraacetic acid (EDTA) solution 0.25 % (Biochrom, Berlin, Germany) and seeded for the study of osteogenesis and cell viability.

Table 1
Chemical composition of the STWs used in the study.

Chemicals, Concentration (mg L − 1)	STW-1	STW-2
Hydrogen sulfide	119 mg/l	14.6 mg/l
Hydrosulfide ion (HS-)	20 mg/l	N/A
Hydrogen sulfide not ionized	99 mg/l	N/A
Sulphates	1445 mg/l	67 mg/l
Calcium	706 mg/l	126.1 mg/l
Magnesium	55.1 mg/l	29.5 mg/l
Bicarbonates	552 mg/l	462 mg/l
Nitrites	< 0002 mg/l	< 0.01
Nitrates	< 1 mg/l	< 1 mg/l
Bromides	< 0.5 mg/l	N/A
Iodide	< 0.5 mg/l	N/A
Chlorides	92.7 mg/l	106 mg/l
Sodium	87.4 mg/l	73.2 mg/l
Ammonium ion	1.36 mg/l	1.7 mg/l
Potassium	4.6 mg/l	4 mg/l
Lithium	0.049 mg/l	0.101 mg/l
Strontium	N/A	0.750 mg/l
Aluminum	N/A	< 0.004 mg/l
Iron	< 0.01 mg/l	< 0.01 mg/l
Silica	25.4 mg/l	16.74 mg/l

2.3. Osteogenic differentiation and alizarin red staining

A total of five donors of hMSCs were employed in this set of experiments; each analysis was performed in duplicate. Osteogenic cultures of hMSCs at passage 3 were established to assess the amount of mineral apposition in control (CTRL) *vs* STWs-treated hMSCs. hMSCs were seeded at 5×10^4 cells/cm^2 in 12 wells plate in α-MEM 15 % FBS. The day after the seeding, they were stimulated and then cultured for 14 days in osteogenic medium with or without treatment with STWs. CTRL cells were treated with the common osteogenic medium (α-MEM 20 % FBS supplemented with 100 nM dexamethasone, 100 μM ascorbic acid and 10 mM β-glycerophosphate). STWs-treated hMSCs were treated with an osteogenic medium containing 33 % (STW-1_{low} or STW-2_{low}) or 66 % (STW-1_{high} or STW-2_{high}) of the two STWs. Notably, STW-1 and STW-2 were diluted with a concentrated osteogenic culture medium according to the procedure represented in Suppl. Fig. 1, to ensure that every sample received an equal concentration of FBS and of each component of the culture medium. Culture medium and stimulation were replaced twice *per* week. At day 14 (D14) Alizarin Red S (AR-S) (Sigma Aldrich) staining was performed to assess the presence and extent of mineralization. Cells were stained with 40 mM AR-S for 20 min after being fixed for 15 min at room temperature (RT) in formaldehyde (Kaltek, Padova, Italy) 10 % phosphate buffered saline (PBS) and washed twice with PBS, as detailed in our previous work [38]. A spectrophotometric analysis with TECAN Infinite® 200 PRO (Tecan Italia S.r.l., Cernusco Sul Naviglio, Italy) was performed to quantify the mineral apposition as previously described [37] and to classify each donor as for its osteogenic behavior. The result of the test is an average of 177 readings for each well. If the average was below the detection threshold of 0.3, cells were considered as non-mineralizing hMSCs (N-min) [37]; above this threshold, cells were considered low (L-Min) or high mineralizing (H-min) when the average readings

were, respectively, below 0.9 or above 0.9. Photos (100 x magnification) of AR-S staining were taken using Nikon Instruments Europe BV (Amstelveen, the Netherlands).

The same analysis was performed on cultures with or without STWs at D7 cultured in non-osteogenic medium for Lactate dehydrogenase (LDH) assays.

2.4. Cytotoxicity assay

A total of three donors of hMSCs were employed in this set of experiments. hMSCs were seeded at 5×10^4 cells/cm^2 in 12 wells plate. STWs were added to α-MEM 15 % FBS depleted of phenol red to obtain medium containing 33 % (STW-1_{low} or STW-2_{low}) and 66 % (STW-1_{high} or STW-2_{high}) of STWs. CTRL wells were diluted, similarly to STWs, with tap water to obtain a medium containing 33 % ($CTRL_{low}$) and 66 % ($CTRL_{high}$) of tap water. Duplicates were seeded to establish the positive CTRLs. The use of tap water to dilute the medium of culture was used only in this experiment, as we could not prepare concentrated media depleted of phenol red. Cells were analyzed at D1 and D7. For analyses at D7, medium was changed two times/week. 30 min before each assay, Triton X-100 was added to medium of one $CTRL_{low}$ and $CTRL_{high}$ well to induce cellular cytotoxicity (positive CTRL of the assay; CTRL + $_{low}$, CTRL + $_{high}$). Lactate dehydrogenase (LDH) assay (Roche) was performed following manufacturers' protocols as detailed elsewhere [40] on medium harvested at D1 and D7 of treatment (quadruplicates from each wells were analyzed). A spectrophotometer (TECAN Infinite® 200 PRO) was used to detect the colorimetric reaction on supernatants by reading absorbance at 492 and 620 nm. The difference between measurement (492 nm) and reference measurement (620 nm) was calculated for each sample. CTRL, CTRL + and cells treated values were then subtracted of the value of the wells containing just medium. STW-1_{low} and STW-2_{low} relative absorbance data were compared to $CTRL_{low}$ and CTRL + $_{low}$; while STW-1_{high} and STW-2 $_{high}$ were compared to CTRL $_{high}$ and CTRL + $_{high}$. The percentage of cytotoxicity was calculated as following: (Treated XYZ – CTRL) / (CTRL + - CTRL-) * 100. Cells were then fixed in formaldehyde 10 % FBS and stained with toluidine blue (Sigma Aldrich). Photos (100 x magnification) of cell morphology were taken using Nikon Instruments Europe BV (Amstelveen, the Netherlands).

2.5. RNA analyses

A total of five hMSCs donors (in duplicates) were employed to assess gene expression during osteogenic stimulation at passage 3. hMSCs were seeded at 5×10^4 cells/cm^2 in 12 wells plate in α-MEM 15 % FBS and cultured for 14 days in osteogenic medium with or without treatment with STWs, as described in Section 2.3. 1 ml of RNA pure solution (Euroclone, Milan, Italy) was used to lyse the cells before performing the chloroform-phenol-ethanol extraction protocol and the purification from genomic DNA by treatment with DNase I (DNA-free Kit, Ambion, Austin, TX, USA), according to manufacturer instructions. cDNA synthesis was performed by using SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen) on 2720 Thermal cycler (Applied Biosystem, Life Technologies) at 25 °C for 10 min, 42 °C for 60 min, 85 °C for 5 min and 4 °C for 30 min. mRNA expression was assessed by Real-time polymerase chain reaction (PCR) analysis by using the SYBR Premix Ex Taq (TaKaRa Biomedicals, Tokyo, Japan). Primers were purchased from Life Technologies Italia (primers sequences are reported in Table 2). The real-time PCR analyses were run on LightCycler Instrument (Roche) as following: one cycle at 95 °C for 10 s and 45 cycles at 60 °C for 20 s and at 95 °C for 5 s. Standard melting curve analyses were performed at 95 °C for 10 s, 65 °C for 15 s and 95 °C in one-degree increments, for confirming the specificity of the PCR products. PCR products were relatively quantified with the comparative C_T method, comparing to the housekeeping mRNA expression of glyceraldehyde-3 phosphate dehydrogenase (GAPDH).

2.6. Flow cytometry analysis

A total of four hMSCs donors were employed to assess intracellular H_2S levels. Here we used a fluorescent probe based on nucleophilic substitution-cyclization, named WSP-5, previously designed by Peng et al. and tested for H_2S detection in aqueous solution and in cell imaging [43]. Analyses were performed in non-fixed samples at two time points (15 min and 1 h after stimulation). Cells at passage 3 were harvested by treatment with trypsin/EDTA solution 0.25 % (Biochrom), divided in tubes with 3×10^5 cells each and washed in PBS 1 ×. Afterwards, cells were incubated 30 min at 37 °C in buffer BS (composition: HEPES 20 mM, NaCl 120 mM, KCl 2 mM, $CaCl_2x2H_2O$ 2 mM, $MgCl_2x6H_2O$ 1 mM, glucose 5 mM) with 50 μM WSP-5 (Cayman Chemical, Ann Arbor, Michigan, USA) and 100 μM Hexadecyltri-methylammonium bromide (Sigma Aldrich). Cells were washed in PBS 1x and incubated with buffer BS with 100 μM Hexadecyltri-methylammonium bromide (Sigma Aldrich) and STW-1 and STW-2 at 33 % and 66 %. As a control of the positive intracellular stain we used the treatment with 200 μM sodium hydrosulphide (NaHS; Thermo Fisher Scientific New Jersey, USA), a fast releasing H_2S donor widely used in our previous studies. Cells positivity to WSP-5 was assessed by flow cytometry (FACS) analysis performed on FACS canto II (BD bioscience, San Jose, CA, USA): 224 voltage (FITCH); Threshold 33303.

2.7. Statistical analysis

GraphPad Prism 7 (La Jolla, CA) was used for statistical analysis to compare CTRL cells with treatments. The presence of outliers was checked by ROUT (Q = 1 %) test and were removed from each data set, when present. D'Agostino & Pearson normality test was performed to analyze the normality of our data. We performed One-way ANOVA and Dunnett's multiple comparisons test, for matched comparisons (data set of FACS analysis and LDH assays), or Holm-Sidak's multiple comparisons test, for un-matched comparisons (data set of real-time PCR), when data were sampled from an ideal Gaussian distribution. Otherwise, we performed Friedman and Dunn's multiple comparisons test, for matched comparisons (data set of AR-S stain), and Wilcoxon matched-pairs signed rank test (data set of AR-S performed in non-osteogenic cultures). Significance was attributed when $p < 0.0001$ (****), $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.05$ (*). Data will be kept for at least ten years and can be requested to the corresponding author.

3. Results

3.1. Treatment with STWs increases the intracellular H_2S levels

H_2S is one of the major components of STWs. H_2S up-take by cells is known to occur by simple diffusion and does not require facilitation by membrane channels [44,45]. However, whether STWs treatment can increase intracellular H_2S levels has never been investigated. Here we set up an innovative flow cytometric analysis, based on the use of a fluorescent probe specific for H_2S (WSP-5), to quantitively measure the intracellular H_2S levels after STWs treatment. H_2S-donor NaHS was used as positive control. Cells from four independent donors were assayed at 15 min and 1 h after treatment and individually displayed in Fig. 1**A** and **B** to attest the reproducibility of the assay. Data at 15 min and 1 h gave similar results, thus were pooled to analyze the differences in average and median (Fig. 1**C**). Fig. 1**A** and **B** report the overlaid histogram showing CTRL cells *vs* STW-treated cells (A) and CTRL cells *vs* NaHS-treated cells (B) [37]. Coherently with the known fast release of H_2S by NaHS in the buffer, we detected a substantial increase in the intracellular H_2S levels reaching the saturation of detection within 15 min. Both concentrations of STW-1 significantly increased intracellular H_2S levels by 5 fold ($p < 0.0001$) and reached the saturation of the probe, thus we did not detect any dose-dependent differences in the intracellular H_2S levels (Fig. 1**C**a,b). Conversely, STW-2 induced a

Table 2
List of primers sequences.

Gene	Protein		5′-Sequence-3′	Product length (bp)	Gene ID
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase	FW	CGGAGTCAACGGATTTGG	218	NM_002046
		REV	CCTGGAAGATGGTGATGG		
ALP	Alkaline phosphatase	FW	GGAAGACACTCTGACCGT	152	NM_000478
		REV	GCCCATTGCCATACAGGA		
OC	Osteocalcin	FW	CAGCGAGGTAGTGAAGA	148	NM_199173
		REV	TCCTGAAAGCCGATGTGG		
RUNX-2	Runt Related Transcription Factor 2	FW	GGAATGCCTCTGCTGTTATG	105	NM_001024630.4
		REV	AGACGGTTATGGTCAAGGTG		
BSP	Bone sialoprotein	FW	CAGTAGTGACTCATCCGAAG	158	NM_004967
		REV	CATAGCCCAGTGTTGTAGCA		
WNT16	Wnt Family Member 16	FW	GCCAGTTCAGACACGAGAGA	140	NM_057168
		REV	TGCAGCCATCACAGCATAAA		
Col I	Collagen I	FW	CCTGGATGCCATCAAAGTCT	170	NM_000088
		REV	CGCCATACTCGAACTGGAAT		
OPN	Osteopontin	FW	ATGATGGCCGAGGTGATAG	119	NM_00058
		REV	GCTTTCCATGTGTGAGGTG		
COLL XV	Collagen XV	FW	AAGCCGTCACCTACACTCAA	228	NM_001855
		REV	CACCATCCACAGAATGAACC		
SMAD1	SMAD Family Member 1	FW	CACCCGTTTCCTCACTCTCC	257	NM_005900
		REV	TCCTCATAAGCAACCGCCTG		
WISP1	WNT1-inducible-signaling pathway protein 1	FW	ACACGCTCCTATCAACCCAAG	103	NM_003882
		REV	CATCAGGACACTGGAAGGACA		

FW: forward primer; REV: reverse primer.

lower and dose-dependent increase in intracellular values of H_2S by 2 (STW-2_{low}) and 2.8 (STW-2_{high}) fold ($p < 0.001$) (Fig. 1**C**a,b). As opposed to NaHS and STW-1, STW-2 induced heterogeneous response among donors (Fig. 1**A**). Notably, STW-2 contains about 12 % the H_2S concentration of STW-1 (Table 1), showing that the uptake of H_2S that we detected in hMSCs is correlated with its abundance within STWs.

3.2. Treatment with STW-2 does not affect cell viability, while treatment with high concentration of STW-1 induces acute but transient cell cytotoxicity

Cell viability was measured at D1 and D7 of culture by LDH assay. STW-2 did not induce any cell cytotoxicity throughout the experiment (Fig. 2**A**a–d). Conversely, STW-1_{high} induced a transient cytotoxicity at D1 of culture (Fig. 2**A**b, 50 %, $p < 0.0001$). Despite this noticeable cytotoxicity, the majority of cells survived at D7 (Fig. 2**A**d) and mineralized (Fig. 4). STW-1_{low} induced a mild cytotoxic effect only at D7 (Fig. 2**A**c; 10 %, $p < 0.05$); however, this did not affect the osteogenic differentiation of cells. Interestingly, we observed a different susceptibility among donors to the cytotoxicity of STW-1 as in 1/3 donors we found cells overall viable at both concentrations. Fig. 2**B** shows cell morphology upon staining with toluidine blue, in cells treated with STW-1_{high} at D1 and D7 as compared to CTRL cells. Pictures show that, despite a substantial decrease in cellularity, the majority of cells remains viable with no gross signs of cytotoxicity.

3.3. Treatment with STWs increases the mineral apposition by hMSCs

H_2S was shown to induce osteogenic differentiation of MSCs and bone anabolism [46,41,42,40,37]. Here, we performed osteogenesis of hMSCs in the presence or absence of STWs to investigate whether the intracellular H_2S increase would stimulate the osteogenic differentiation of these cells. The analysis was performed at D14 of culture in cells from five independent donors. Fig. 3**A** shows the average, the standard deviation and each duplicate value of mineral apposition of donors analyzed (CTRL *vs* STW-1 *vs* STW-2). In the absence of STWs treatment (CTRL group) the majority of hMSCs falls within low-mineralizing (L-min, 69 %) and the non-mineralizing (N-min; 13 %) range. In contrast, stimulation with both STWs induced a marked increase in mineralization; as a result, hMSCs were predominantly found in the high-mineralizing range: STW-2_{low} (60 %) and STW-2_{high} (80 %), STW-1_{low} (100 %). Only for STW-1_{high} the majority of hMSCs were in the L-min range (60 %). The analysis of the average mineral apposition in the five biological replicates confirmed that all treatments with STWs induced a statistically significant increase in mineralization (Fig. 3**B**c,d). In particular, we found that STW-1_{low} increased mineral apposition of CTRL by 2.1 fold (Fig. 3**B**c); STW-2_{low} and STW-2 $_{high}$ increased mineral apposition compared to CTRL by 2.3 fold (Fig. 3**B**d). Despite an overall reduced cellularity in the well, the treatment with STW-1 $_{high}$ was still able to induce a significantly increased (1.1 fold, Fig. 3**B**c) mineral apposition compared to CTRL. Fig. 3**B**a,b shows representative figures of AR-S positive stain of CTRL *vs* STW-1 *vs* STW-2 treated cells, to qualitatively appreciate the high stimulation of STW-1_{low}, STW-2_{low} and STW-2_{high} as compared to CTRL cells and the low stimulation of STW-1_{high} as compared to CTRL cells (only a few regions in the wells clearly shows a substantially increased positivity to the stain and are shown in the insert).

Interestingly, in the STW1_{high} group, mineral apposition was noticed serendipitously (and confirmed by AR-S staining) during the experiments performed to analyze cell viability conducted in absence of osteogenic media (representative figure Fig. 4**A**). CTRL cells falls in the N-min range while STW-1_{high} falls in the L-min range (Fig. 4**B**). The presence of mineral apposition only in the group of STW with the highest H_2S and mineral content suggest that these concentrations may be sufficient to stimulate mineral deposition.

3.4. Treatment with STW-2 increases the expression of osteogenic markers by hMSCs

Next, we analyzed the mRNA expression of several osteogenic genes at D14 of culture in 5 independent donors in duplicates. While data on mineral apposition showed similar results, our analysis of mRNA expression clearly evidenced a different pattern of stimulation between the two STWs. In particular, STW-2 increased the mRNA expression of BSP, OC, RUNX- 2 and OPN (Fig. 5**B** panels a,c,d,g), and showed an increased trend of mRNA expression of WNT16, SMAD1 and WISP1 (Fig. 5**B** panels k,e,i,j). Conversely, STW-1 showed an increased trend of mRNA expression of WNT16 and OPN (Fig. 5**A** panels e,g); a decreased trend of RUNX-2 (Fig. 5**A** panel d); and similar expression of BSP, OC and SMAD1 (Fig. 5**A** panels a,c,i). No genes were significantly upregulated by STW-1. Moreover, both STW-1 and STW-2 downregulated ALP, COL I and COLLXV (Fig. 5**A**, panels b, f, h; B panels f, h) mRNA

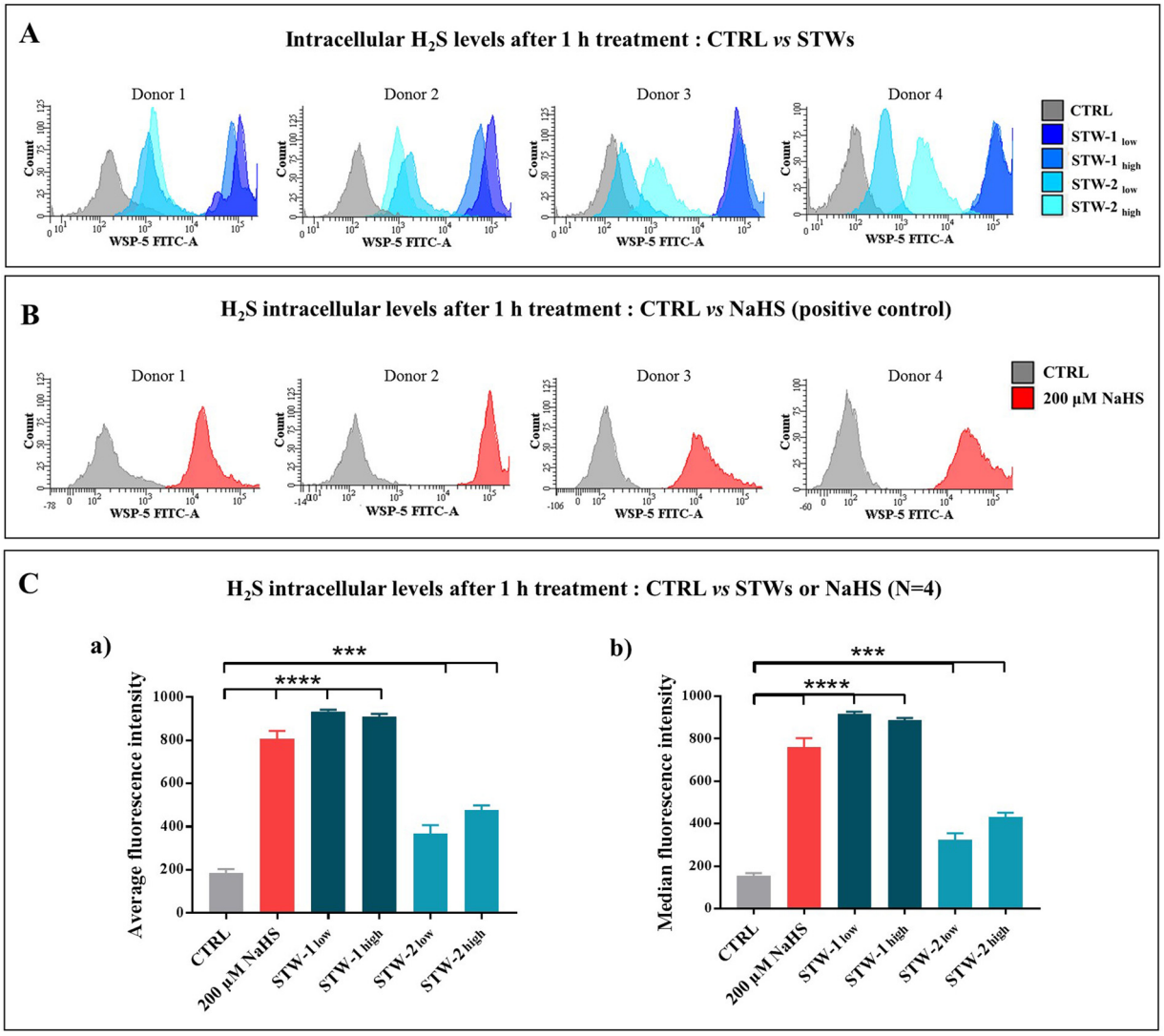

Fig. 1. Intracellular H_2S levels in hMSCs treated or not treated with STW-1 and STW-2. A) Histograms showing the reproducibility and differences in the WSP-5 intensities among 4 donors after STWs treatments. **B)** Histograms showing the reproducibility and differences in the WSP-5 intensities among 4 donors after NaHS treatments. **C)** Graphs showing the average and median comparing WSP-5 intensities in STWs *vs* NaHS *vs* CTRL cells (N = 4 independent experiments). One-way ANOVA and Dunnett's multiple comparisons test were performed for the statistical analysis (*** $p < 0.001$; **** $p < 0.0001$). Low indicates the presence of 33 % of STWs; high indicates the presence of 66 % of STWs. WSP-5 indicates the fluorescent probe detecting H_2S. NaHS is a fast releasing chemical donor of H_2S.

expression. STW-2 up-regulated WISP1 mRNA expression (Fig. 5**B**, panels j) while STW-1 down-regulated WISP1 (Fig. 5**A**, panels j). In Table 3 are reported the fold increase/decrease and significances. Overall, these data demonstrate that STW-2 but not STW-1 increased mRNA expression of osteogenic markers.

4. Discussion

Our findings first demonstrate a stimulation of osteogenic differentiation in hMSC by STWs *in vitro*.

Hydrotherapy and balneotherapy have long been employed as complementary treatment for musculoskeletal diseases [47,48]; the beneficial effects for these therapies arise from a combination of physical therapy, thermal treatment and the biochemical cues provided by ions-rich waters, which show analgesic, antioxidant and anti-inflammatory properties [31,32]. Among thermal waters, STWs hold a special interest to biomedical research due to the high content in H_2S, a molecule playing a prominent biological role in the homeostasis of several organs [49,50]. In this work, we postulated that thermal water rich in H_2S could increase the osteogenic differentiation of hMSCs in a way similar to the pharmacological and chemical treatments we employed in our previous studies [46,41,42,40,37]. This is the first experimental *in vitro* research conducted on cells derived from bone tissue and treated with STWs. We compared two STWs from northern Italy health spa centers (here named STW-1 and STW-2), characterized by different amounts of H_2S and other ions and elements. In particular, STW-1 was obtained from one the of the springs with the highest levels of H_2S in Italy, over 100-times higher than the threshold of 1 mg/liter which is used to define waters as 'sulfurous'. On the other hand, STW-2 contains concentrations of H_2S closer to the range used by others and us in preclinical studies [46,51].

As a first step, we sought to investigate whether the exposure of hMSCs to STWs would increase intracellular H_2S levels. Numerous probes detecting H_2S have been developed and applied for detecting and elucidating the role played by H_2S in several biological systems [52,53]. Here we used a fluorescent probe detecting H_2S whose efficiency was previously demonstrated only in aqueous solution and in cell imaging [43]. We demonstrated for the first time that fluorescent probes may be applied in flow cytometry to provide evidence of the intracellular H_2S levels after administration of H_2S donors. This method

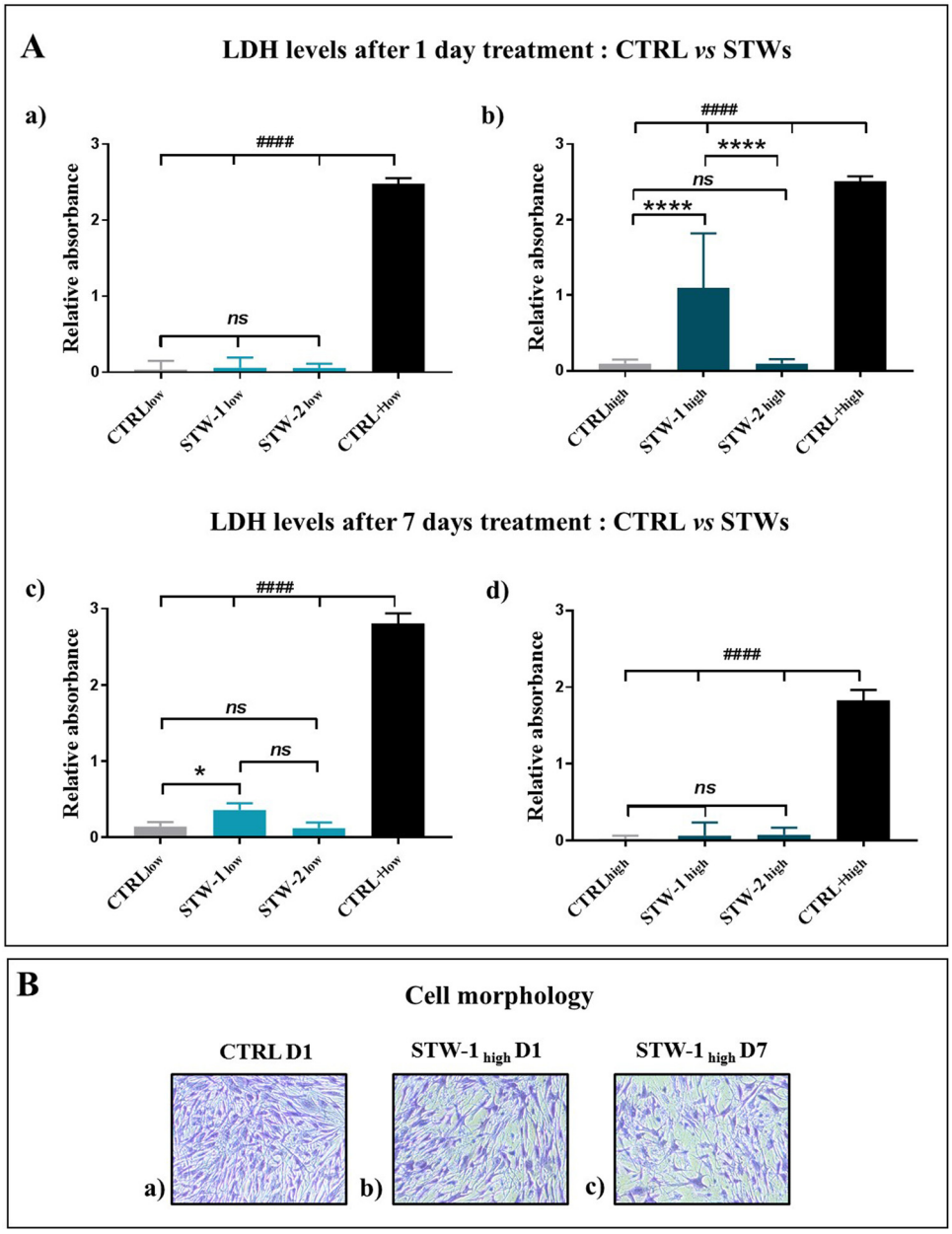

Fig. 2. LDH release by hMSCs treated or not treated with STW-1 and STW-2. A) Graphs showing the relative absorbance of CTRL, CTRL + and hMSCs treated with STW-1 and STW-2 at the indicated concentration (Panel a,c low; panel b,d high; panel a,b D1, panel c,d D7). Data are expressed as the mean ± SD of four independent experiments in quadruplicates. One-way ANOVA and Dunnett's multiple comparisons test, for matched comparisons were performed for statistical analysis (*** $p < 0.001$; **** $p < 0.0001$ *vs* CTRL; #### $p < 0.0001$ *vs* CTRL+). Low indicates the presence of 33 % of STWs; high indicates the presence of 66 % of STWs. **B)** Representative images of toluidine blue stain comparing CTRL *vs* STW-1 $_{high}$ (magnification 100x) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

could expand our tools to investigate the relationship between intracellular H_2S levels and the biochemical effects induced by H_2S. Our data confirmed that stimulation with STWs increases intracellular H_2S levels, thereby STWs behave as an alternative type of H_2S donor. Consistent with the different H_2S content, the assay revealed that the intracellular H_2S levels were higher after STW-1 treatment compared to STW-2. Moreover, we demonstrated that is possible to modulate intracellular H_2S levels by using different dilutions of STWs, as shown by STW-2 treatment, where we did not reach the highest level of fluorescence detection.

Then, we investigated whether the increased intracellular H_2S levels, would stimulate osteogenesis in hMSCs. Our data showed that both the lower H_2S and ionic concentration of STW-2 and the higher H_2S and ionic concentration of STW-2 were able to induce a marked increase in the mineral apposition by hMSCs. This is consistent with the reported evidence of induction of mineral apposition in hMSCs by H_2S-donors administered within the micromolar range [46,51]. The high concentrations of H_2S and ions contained in STW-1 did not further increase mineralization, showing that the osteogenic effect of STWs has probably reached its plateau.

STWs also regulated the expression of some of the genes most closely related to osteogenic differentiation, albeit STW-2 was substantially more effective than STW-1. The analysis of mRNA expression evidenced that STW-2 significantly increased the expression of the osteogenic markers BSP, OC, RUNX-2, OPN. Interestingly, it was previously reported that a treatment with H_2S donors leads to the up-regulation of RUNX-2 and OC mRNA in mMSCs [51,39]; moreover, an up-regulation of BSP mRNA was found in hMSCs after H_2S treatment [46]. The significant down-regulation of ALP and COLL XV could be the results of a high state of differentiation as high levels of mineralization and differentiation have been linked to down-regulation of the expression of these genes [37,54]. Conversely, STW-1 only showed a trend toward up-regulation of BSP, OC, RUNX-2, OPN, thus revealing that the chemical components of STWs are important mediators of the osteogenic effect and differently impact on gene expression. In this context, it is important to highlight that H_2S is not the only chemical element, which distinguish the two STWs used in this study. Compared to STW-2, STW-1 has 22 fold higher levels of sulphates; 5.6 fold higher

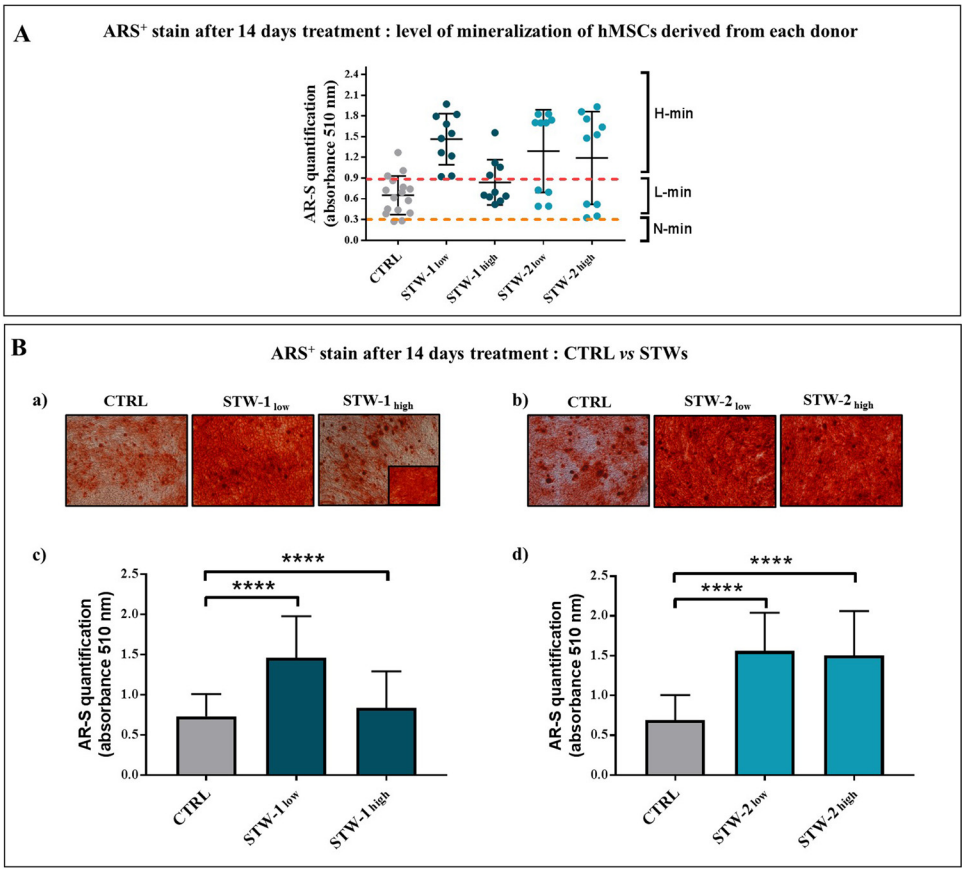

Fig. 3. Mineral apposition by hMSCs treated or not treated with STW-1 and STW-2 during osteogenic cultures. A) Graph showing mean, SD and the single distribution of each donor (in duplicates) in the three levels of mineralization: non-mineralization (N-min), low mineralization (L-min) and high mineralization (H-min). **B) Panel a-b**, Representative images of AR-S stain in STW-1 *vs* CTRL (a) or STW-2 *vs* CTRL (b); magnification 100x; **Panel c-d**, Graphs showing quantification of mineral apposition. Data are expressed mean ± SD of six independent experiments. Friedman and Dunn's multiple comparisons test, for matched comparisons were performed for the statistical analysis (**** $p < 0.0001$). Low indicates the presence of 33 % of STWs; high indicates the presence of 66 % of STWs.

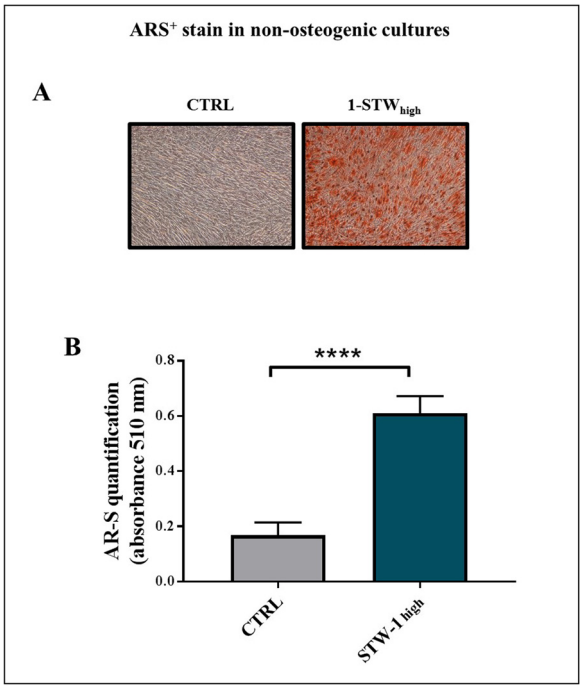

Fig. 4. Mineral apposition by hMSCs treated or not treated with STW-1 and STW-2 during non-osteogenic cultures. A) Representative images of AR-S stain in STW-1_{high} *vs* CTRL (magnification 100x); **B)** Graphs showing quantification of mineral apposition. Data are expressed as median ± SEM of three independent experiments. Wilcoxon matched-pairs signed rank test, for matched comparisons, was performed for the statistical analysis (**** $p < 0.0001$). High indicates the presence of 66 % of STWs.

levels of calcium; 1.9 fold higher levels of magnesium; 1.2 fold higher levels of bicarbonates and sodium; 1.1 fold less levels of chlorides; 1.3 fold less levels of ammonium; 1.2 fold higher levels of potassium; 1.5 fold higher levels of silica. Several of these elements play important roles in bone homoeostasis and regeneration. Particularly, calcium ions have a strong effect on the proliferation, osteogenic differentiation and mineralization of hMSCs [55] and on calvarial cells [56]; magnesium ions released from bone fixation devices promoted bone regeneration [57] also by tuning immunomodulation [58]; silica ions released from mesoporous silica nanospheres induced the osteogenic differentiation of hBMSCs by activating bone-related gene and protein expression [59].

Magnesium has also been found to inhibit the osteogenic differentiation of hMSCs [60,61]. Therefore, we cannot rule out potential synergistic or antagonist effects of the different ions towards the overall effect on osteogenesis. Further studies will be needed to dissect the contribution of individual ionic species contained in STWs to understand the mechanism behind the differential biological effect we detected.

Finally, our study evidenced that different STWs have different effect on cell viability. hMSCs remained viable through the overall period of treatment when treated with STW-2. Conversely, STW-1 at the highest concentration induced a transient high cytotoxicity on hMSCs and STW-1 at the lowest concentration induced a low cytotoxicity. It is conceivable that at the high concentration some of the elements composing STW-1 contribute to the transient cytotoxicity observed in these experiments.

While our findings are consistent with the previously established role of H_2S in osteogenesis, the experimental design of this study holds limitations that should be acknowledged when discussing the significance of the data; in particular, we did not compare thermal waters containing or not containing H_2S and therefore we cannot rule out a contribution of other ionic species to these results.

Our data overall demonstrate that appropriate concentrations of

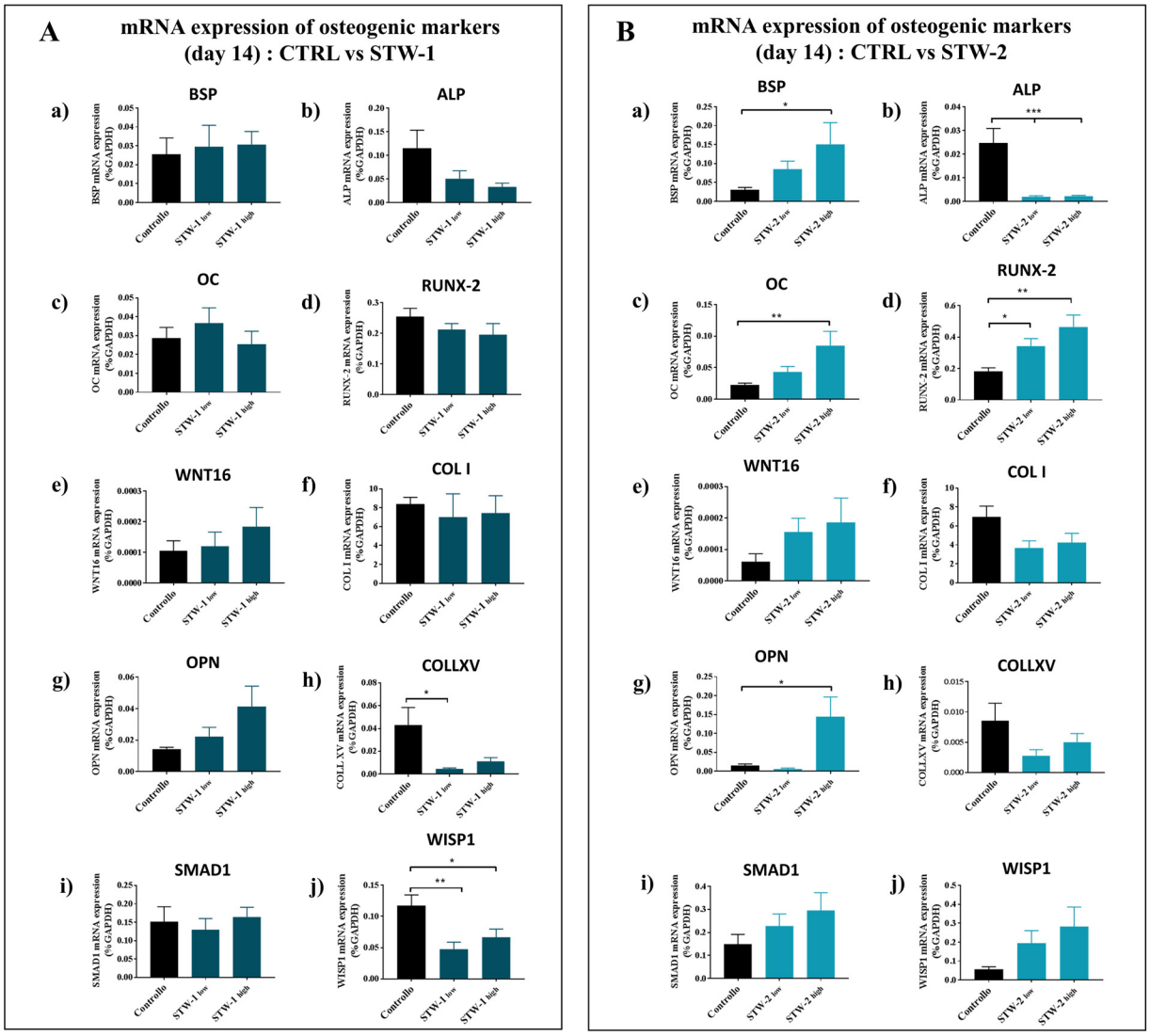

Fig. 5. mRNA expression of osteogenic markers by hMSCs treated or not treated with STW-1 and STW-2 in osteogenic medium. A) Graphs of mRNA expression of CTRL *vs* STW-1; **B)** Graphs of mRNA expression of CTRL *vs* STW-2. Data are expressed as mean ± SEM of five independent experiments in duplicates. One-way ANOVA and Holm-Sidak's multiple comparisons test, for un-matched comparisons were performed for the statistical analysis (*$p < 0.05$; **$p < 0.01$; *** $p < 0.001$). Low indicates the presence of 33 % of STWs; high indicates the presence of 66 % of STWs.

STWs can be used to induce the osteogenic differentiation of hMSCs. Therefore, we can propose the use of STWs among the complementary therapeutic strategies aimed to delaying the onset of bone tissue degeneration, skeletal fragility and bone fracture. STWs balneotherapy, hydrotherapy, thalassotherapy are recognized as a cost-effective alternative to pharmaceutical products in a variety of disorders and have been recently proposed as a strategy to promote an healthy ageing. Moreover, water-based exercises are considered an effective approach to prevent bone loss due to the lower risks of traumatic fracture, less stress and impact to joints and most importantly the loading effect on skeleton which slows down the rate of bone loss [62]. The findings of the present work support the rationale for the use of STWs in complementary medicine for the treatment of bone wasting diseases by combining the beneficial effect of water-based physical therapy with the biochemical cues provided by STWs. However, they raise the question of whether H_2S contained in STWs may be able to reach the inner organs and regulate their functions. H_2S uptake by cells it is known to occur by simple diffusion and does not require receptors or facilitation by membrane channels [44,45]; as a consequence, it is conceivable that bathing in STWs may induce H_2S to penetrate the skin and act at the cellular level in the underlying tissues. Indeed, this has been demonstrated by subcutaneous detection of H_2S in mice: H_2S was shown to diffuse through the skin of live mice, previously submerged in STW, by using an electrometric probe [63]. Moreover, balneotherapy in STWs reduced the features of cartilage degeneration in an osteoarthristis mice model in the left knee [21]. These findings are consistent with some clinical observations on humans; the combination of bathing and drinking of STWs was shown to increase plasma thiol levels and to decrease inflammatory cytokines and cell matrix proteases in osteoarthritis patients [64] and STWs balneotherapy reduced pain in the hand, improved grip strength of both hands and the overall quality of life in patients with osteoarthritis of the hand [65]. These evidences combined with our findings lay the ground for studying STWs-based prevention of bone loss in animal models and clinical trials.

5. Conclusions

Our data demonstrate that STWs increased intracellular H_2S levels and the osteogenic differentiation of hMSCs. STWs treatment, even at the lower H_2S and ion content, induced a marked increase in mineral apposition. By showing direct regulation of the anabolic function in bone cells, these findings open up new perspectives on clinical

Table 3
List of fold increase or decrease and statistical significance in mRNA expression.

Gene	STW	Group of comparison	Increase /Decrease	Fold, significance
OC	STW-2	STW-2_{high} *vs* CTRL	Increase	3.7 fold $P < 0.01$
RUNX-2	STW-2	STW-2_{low} *vs* CTRL	Increase	1.9 fold $P < 0.05$
	STW-2	STW-2_{high} *vs* CTRL	Increase	2.5 fold $P < 0.01$
BSP	STW-2	STW-2_{high} *vs* CTRL	Increase	5 fold $P < 0.05$
OPN	STW-2	STW-2_{high} *vs* CTRL	Increase	9.8 fold $P < 0.05$
COLL XV	STW-1	STW-1_{low} *vs* CTRL	Decresase	8.9 fold $P < 0.05$
WISP1	STW-1	STW-1_{low} *vs* CTRL	Decrease	2.5 fold $P < 0.01$
	STW-1	STW-1_{high} *vs* CTRL		1.8 fold $P < 0.05$
ALP	STW-2	STW-2_{low} *vs* CTRL	Decrease	12.8 fold $P < 0.0001$
	STW-2	STW-2_{high} *vs* CTRL		11 fold $P < 0.001$

applications of STWs in complementary medicine of bone wasting diseases.

Author's contribution

FG and LG conceived and designed this study. GF enrolled the patients and provided the tibial plateau for cell isolation. LG carried out the experiments and collected data. FG, LG conducted data analysis and interpreted the data. Manuscript original draft preparation by FG, LG. Manuscript review and editing by GF, BG.

Declaration of Competing Interest

The Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.110344.

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