

## Quantitative Assessment of Antibacterial, Antioxidant and Cytoprotective Properties of Sulphurous Spring Water



### Biochemistry

**KEYWORDS :** Sulphurous spring water, Antibacterial, Antioxidant, Cytoprotection, Dermal fibroblasts.

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### ABSTRACT

*Sulphur-containing spring water, which is generally rich in minerals and metallic elements, is traditionally considered beneficial for diseases of dermal origin. We investigated sulphur-containing spring water from the Ildong Jeil springs in Korea for antibacterial properties as well as antioxidant and cytoprotective effects on primary human dermal fibroblasts. Sulphurous spring water from Ildong Jeil springs significantly restricted bacterial growth compared to that of spring water with no sulphur content. We also recorded significant increases in total cellular anti-oxidant activity in primary human dermal fibroblasts cultured in media reconstituted in sulphurous spring water. Although, procollagen gene expression was not enhanced significantly, culture medium prepared using sulphurous spring water protected dermal fibroblasts from tBOOH (tertiary butyl hydroperoxide)-induced cytotoxicity. Our data provides evidence that sulphur-containing spring water has beneficial effects on primary human dermal fibroblasts substantiating its medicinal properties on human skin.*

### INTRODUCTION

Bathing in spring water has been advocated as a treatment for multiple dermatologic conditions such as psoriasis and atopic dermatitis for several decades (Matz, Orion and Wolf, 2003). Spring waters from different sources vary significantly in their chemical composition and physical properties, and thus may differ in their therapeutic mode of action and overall effects (Andreassi and Flori, 1996). Sulphurous spring water (defined as containing > 0.1 mg/L total hydrogen sulphide gas) possibly exerts therapeutic effects as a result of the interaction of the sulphur with the amino acid cysteine and its catabolites present in living cells and neutralization of free oxygen radicals (Matz, Orion and Wolf, 2003 and Lin, Reimer and Carte, 1988). The sulphur in sulphurous water may exist in free, dissolved form or in compound form, such as sulphates and/or hydrogen sulphide gas (Lin, Reimer and Carte, 1988 and Hann, 1996). Sulphur penetration into the skin can trigger physiological responses such as vasodilation in microcirculation, analgesic effects on pain receptors and inhibition of inflammatory immune responses thus play an important role in improving dermal psoriasis (Matz, Orion and Wolf, 2003). Atopic dermatitis is associated with progressive bacterial infection (specifically with *Staphylococcus aureus*); treatment with sulphur containing spring water is believed to enable a reaction between sulphur and the free oxygen radicals in the skin forming disulphur hydrogen and further on pantathionic acid, which has antibacterial and anti-fungal activity (McMurtry, 1913).

Dermal fibroblasts are involved in the production of collagen that is critical for skin tensility (Lee et al., 2007; Cuiyan et al., 2004 and Fujii, Wakaizumi, Ikami and Saito, 2008). The continued exposure of skin to UV radiation, microbes, chemicals and physical aggression along with the natural aging process enhances oxidative stress via production of reactive oxygen species (ROS), resulting in damage of cellular components such as mitochondria, cell membrane, cell wall, DNA etc (Bickers and Athar, 2006 and Sander et al., 2004) culminating in apoptotic or necrotic cell death (Kanupriya et al., 2007 and Chandra, Samali, and Orrenius, 2004). Several studies have clearly shown that the human skin is very sensitive to reactive oxygen species. The "free radical theory" is most widely accepted as the cause of

accelerated skin aging where free radicals lead to inflammation that aggravates skin aging (Harman, 1956). Endogenous antioxidant cellular components include enzymes like catalase, superoxide dismutase and glutathione peroxidase as well as molecules such as vitamin E, vitamin C, glutathione and ubiquinone. Based on the theory of free radicals, many skin care products containing natural and/or artificial antioxidants help reducing or neutralizing free radicals (Jenkins et al., 2002 and Shindo et al., 1994). Thus, the ability of sulphur to interact with and neutralize oxygen radicals may impart an antioxidant property on sulphurous spring water inducing therapeutic and protective effects on skin fibroblasts.

Most claims about the benefits of sulphurous spring water on skin are based on traditional word of mouth accounts ("folk-remedy") and not scientifically qualified or documented (Braga et al., 2008). Hence, in this study we explore beneficial effects of sulphur containing spring water by analyzing (a) antibacterial (b) anti-oxidant (c) cytoprotective effects, as well as (d) influence on collagen gene expression in primary human dermal fibroblasts. To our knowledge this is the first evaluation of spring water with sulphur on primary human skin cells in culture.

### Materials and Methods:

#### Materials

Luria Bertani (LB) broth powder was purchased from Becton Dickinson (BD), dimethyl sulfoxide (DMSO), (3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), tetrabutyl hydroperoxide (tBOOH) from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were from Life Technologies (Logan, UT). Antioxidant assay kit was purchased from Cayman Chemicals (Ann Arbor, MI). RNAiso Plus and rTaq DNA polymerase were obtained from TaKaRa Bio Inc. (Shiga, Japan). iScript cDNA synthesis kit was from Bio-Rad (Hercules, CA).

#### Water source

Sulphurous and non-sulphurous spring water were obtained from the different streams of Ildong Jeil Hot Springs, Kyungki-do, Korea certified by Korea Hot spring Association. Although both types of water were collected from same Ildong Jeil spring,

the streams are different and classified as sulphurous or non-sulphurous spring water based on sulphur content ( $> 0.1$  mg/L total hydrogen sulphide gas). Both types of waters were used after filtering through  $0.45\mu\text{m}$  millipore membrane filter from Millipore (Billerica, MA). As a control, distilled water and filtered tap water from Seoul was used for all experiments. Water components were analyzed by Korea Central Spa Research Institute.

#### Anti-Bacterial growth test

LB broth powder was dissolved individually in sulphurous spring, non-sulphurous spring, tap or distilled waters followed by filtering through a  $0.45\mu\text{m}$  filter (Millipore). The BL21 strain of the bacteria was inoculated separately into 150 ml of LB medium prepared from the four different types of water and were incubated in a shaker incubator at  $26^\circ\text{C}$ . Bacterial growth was monitored by measuring the optical density (OD) at 600 nm every hour.

#### Cell culture

Primary human dermal fibroblast cells were purchased from Life Technologies (Grand Island, NY). They were cultured in DMEM containing 10% FBS, 1% penicillin-streptomycin antibiotic solution, 5% non-essential amino acids and 5% L-glutamine (complete DMEM). DMEM was prepared as above by dissolving DMEM powder in the different waters under investigation and filtered through a  $0.45\mu\text{m}$  filter (Millipore). Cells were maintained under standard culture conditions at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ .

#### Analysis of total anti-oxidant activity in human dermal fibroblasts

Primary human dermal fibroblasts were seeded in 6 well plates at a density of  $1 \times 10^5$  cells per well and cultured in complete DMEM made with distilled water as above. 24 h later, the cells were washed with PBS and incubated in complete media prepared by dissolving the DMEM powder in the different waters under investigation for a further 24 h period in triplicate. Cells were harvested using a cell scraper and collected by centrifugation at  $2000 \times g$  for 10 min at  $4^\circ\text{C}$ . The cell pellet was sonicated on ice in 1ml of ice-cold sonication buffer (5 mM potassium phosphate, pH 7.4, containing 0.9% sodium chloride and 0.1% glucose) and centrifuged at  $10,000 \times g$  for 15 min at  $4^\circ\text{C}$ . Total antioxidant activity in the cell lysates was assessed by the ability to inhibit the oxidation of ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] by metmyoglobin. The amount of oxidized ABTS was monitored by measuring the absorbance at 750 nm. The capacity of antioxidants in the cell lysate to prevent the oxidation of ABTS was compared with that of the antioxidant trolox, a water-soluble tocopherol analogue, and is quantified as molar trolox equivalents. The assay was performed according to the manufacturer's (Cayman chemicals) protocol and absorbance was measured at 750 nm using a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

#### Cell viability

Primary human dermal fibroblasts were seeded in 12 well plates at a density of  $5 \times 10^4$  cells per well in 1ml of complete DMEM made with distilled water. 24 h later, cells were washed with PBS and incubated with complete media prepared by dissolving DMEM powder in the different waters under investigation for a 24 h period in triplicate. Each well was then treated with  $600\mu\text{M}$  tertiary butyl hydroperoxide (tBOOH) and incubated for 6hrs. Cells were washed and incubated with complete media prepared by mixing DMEM powder in different types of water for additional 48hrs. Viability of these treated cells was determined using the MTT assay by reduction of 3-(4,5'-dimethylthiazol-2-yl)-2,5'-diphenyltetrazolium bromide (MTT) to a formazan product measured at 570 nm using a spectrophotometer.

#### Pro-collagen mRNA expression in primary human dermal fibroblasts

Primary human dermal fibroblasts were plated in 100 mm dishes at a density of  $3 \times 10^5$  cells per well in 5mL of complete DMEM media prepared from distilled water. 24 hr later the cells were washed with PBS and incubated in complete medium prepared

by dissolving DMEM powder in different types of water for a further period of 48 hr. Total RNA was then extracted from the cells using RNAiso Plus (TaKaRa Bio, Japan). One microgram of RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad). Levels of pro-collagen and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA were checked by RT-PCR with specific primers using rTaq DNA polymerase (TaKaRa Bio, Japan). The primer sequences for pro-collagen are 5'-CTC-GAGGTGGACACCACCCT-3' (forward) and 5'-CAGCTGGATGGC-CACATCGG-3' (reverse) and 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGTA-3' (reverse). The PCR protocol composed of an initial incubation at  $95^\circ\text{C}$  for 10min followed by 35 cycles of  $95^\circ\text{C}$  for 30s,  $58^\circ\text{C}$  for 30s and  $72^\circ\text{C}$  for 1min and followed by  $72^\circ\text{C}$  for 10min as described previously (Babu et al., 2013).

#### Statistical analysis

Each experiment was performed in triplicates and the data was represented as the mean  $\pm$  SD (standard deviation). Differences between the treatments were examined for statistical significance by the student's T test. A  $p$  value of less than 0.05 was considered as statistically significant.

#### Results:

##### Determination of major and trace elements

The major and trace elements that make up the chemical composition of spring water contribute to its physical and medicinal properties (Matz, Orion and Wolf, 2003). We analyzed the composition of sulphur containing spring water and compared it with that of another stream that had no sulphur from Ildong Jeil springs in South Korea as well as tap water of Seoul (Korea). Spring water with a sulphur content  $> 0.1\text{mg/L}$  was defined as sulphurous spring water as per the guidelines set by the Korea Ministry of Public Administration and Security (MOPAS). Besides the pH of the spring waters being more alkaline than tap water expected, both spring waters varied from tap water in their mineral and salt content as is to be expected. Levels of sodium, bicarbonate and silicates were high in spring water while calcium, manganese and dissolved oxygen are generally more concentrated in tap water to enhance palatability for domestic use (Table 1). Importantly, sulphurous and non-sulphurous spring water differed mainly in levels of sulphur content. The defining factor for total sulphur content ( $\text{S}^2$ ;  $>0.3\text{mg/L}$ ) in sulphur-containing spring water in the Ildong sulphur springs appeared to originate from dissolved hydrogen sulphide ( $0.524\text{mg/L}$  in sulphurous spring water), that was not detected in the other waters (Table 1).

##### Evaluation of anti-bacterial activity

To evaluate antibacterial activity, we cultured bacteria in LB media prepared from sulphurous and non-sulphurous spring waters, Seoul tap water and laboratory distilled water. The growth curve of *E.coli* strain BL21 cultured in media prepared from non-sulphurous spring water receded *E. coli* growth by about  $\sim 15\%$  ( $p < 0.05$ ) when compared to LB media prepared with distilled water (Fig. 1). Importantly, sulphur containing spring water depicted a significant reduction (by  $\sim 30\%$ ,  $p < 0.01$ ), which was significant even when compared to non-sulphurous spring water ( $p < 0.05$ ) (Fig. 1). The antibacterial property could be due to a combination of factors like minerals and the pH of the spring waters. The pH of spring water (pH 8.7) is higher compared to the tap water (pH 7.1), hence we also tested tap water adjusted to pH 8.5. This failed to inhibit the bacterial growth. As bacterial growth was affected to a higher extent in sulphurous spring water compared to the non-sulphurous spring water ( $p < 0.05$ ), the higher sulphur content (and dissolved hydrogen sulphide) may partially contribute towards this effect. Indeed, conversion of hydrogen sulphide to pentathionic acid ( $\text{H}_2\text{S}_5\text{O}_{10}$ ) by skin keratinocytes is thought to synergistically inhibit bacterial growth on the skin (Hann, 1996). These results demonstrate higher antibacterial effects of sulphur containing spring water over spring water that does not contain sulphur or regular tap water.

##### Antioxidant activity of spring water on human dermal fibroblasts

Skin is the largest and most exposed organ which experiences

several chemical, physical and environmental assaults that results in ageing and inflammation which is characterised by oxidative stress (Fujii, Wakaizumi, Ikami and Saito, 2008; Bickers and Athar, 2006 and Mukherjee, Maity, Nema and Sarkar, 2011). As dermal fibroblasts provide a supportive function to the outer keratinocyte layer of the skin by harboring large amounts of collagen, blood vessels and nerve endings, we studied effects of sulphurous spring water on human skin-derived primary fibroblasts. Culturing of primary fibroblasts in DMEM prepared using sulphurous spring water increased the total antioxidant component (TAC) activity of the cell by about 3 times in terms of protein concentration compared to culturing in control media prepared from distilled water ( $p < 0.05$ ) (Fig. 2). We have not investigated the identity of the induced cellular components or the underlying mechanism responsible for this increase in cellular antioxidant activity, however the similar tendencies exhibited by media made of tap water but not of distilled water in treated cells (although not reaching statistical significance) is indicative that minerals/elements that are removed upon distillation partly contribute to this effect (Fig. 2).

#### Effects of sulphurous water on cellular stress response

Persistent oxidative stress as a result of environmental factors, chemicals, radiations (UVA) eventually drive human skin fibroblast cells to death by apoptosis or necrosis (Kanupriya et al., 2007 and Chandra, Samali, and Orrenius, 2004). We tested the ability of sulphurous spring water to protect fibroblasts from stress-induced death. In order to induce oxidative stress, we employed tBOOH, an organic compound that induces cellular death by depleting cellular antioxidative molecules (Braga et al., 2008 and Mukherjee, Maity, Nema and Sarkar, 2011). As shown in the Figure 3, tBOOH treatment of primary human dermal fibroblast cultures induced cell death in about 80% of the cells, but culturing in medium prepared from sulphurous spring water protected about 50% of the cells from death ( $p < 0.001$ ).

#### Effect of spring water on pro-collagen synthesis

Premature skin aging is another area of dermatological concern and is usually associated with the rapid degradation of extracellular matrix composed of collagen in the skin due to various reasons (Mukherjee, Maity, Nema and Sarkar, 2011). Fibroblasts are the major cells which are involved in the synthesis of collagen from pro-collagen, damage to these cells could thus become one of the causes for premature aging. We evaluated if sulphurous water could induce pro-collagen synthesis, however this was not the case (Fig. 4). We therefore conclude that the anti-aging properties attributed to sulphurous spring water may be more of an indirect effect through promoting survival of dermal fibroblasts as viable and healthy cells contribute towards the production of collagen.

#### Discussion:

Spring water based treatment has been practiced for several decades as a therapeutic modality for multiple dermatological conditions and many therapeutic benefits of thermal spring water have been investigated and reported (Matz, Orion and Wolf, 2003; Andreassi and Flori, 1996; Hann, 1996; Shido et al., 1995; Lee et al., 2012 and Castex-Rizzi, Charveron and Merial-Kieny, 2011). Spring water also beneficial affects to the expression of pro-inflammatory cytokines by modulating CD4 T cell differentiation (Lee et al., 2012). In particular, sulphur rich spring water is used commonly for treatment of dermal conditions such as psoriasis and atopic dermatitis (McMurtry, 1913; Gupta and Nicol, 2004; Valitutti, Castellino and Musiani, 1990).

In this study, we investigated the beneficial effects of sulphur-containing spring water in terms of antibacterial, antioxidant and cytoprotective effects. Our data indicated that sulphurous spring water not only had significantly better antibacterial activity compared to that of non-sulphurous spring water. Since the major difference in chemical composition of sulphurous and non-sulphurous spring water used for in this study is the sulphur content, mostly in the form of hydrogen sulphide, this may contribute towards this effect (Table 1). As tap water adjusted to pH 8.5 failed to inhibit the bacterial growth, pH could again not explain the effect indicating that sulphur must be the active an-

tibacterial component in the spring water (Fig. 1). Treatment of human skin-derived primary fibroblasts with sulphurous spring water increased the total antioxidant component (TAC) activity of the cell by about 3 times compared to the control tap water ( $p < 0.05$ ) (Fig. 2). Moreover, primary human dermal fibroblast cultures in medium prepared from filtered sulphurous spring water protected ~50% of the cells from death induction of oxidative stress by tBOOH compared to the cells treated with non-sulphurous spring or tap water which induced cell death in about 80% of the cells (Fig. 3). Thus, based on the ability of sulphur containing spring water to interact with and neutralize oxygen radicals, it is plausible that the anti-oxidant property of sulphur containing spring water may indeed have therapeutic and protective properties on skin fibroblasts.

Premature skin aging is another area of dermatological concern and is usually associated with the production of reactive oxygen species (ROS), resulting in damage of cellular components such as mitochondria, cell membrane, cell wall, DNA etc culminating in apoptotic or necrotic cell death (Bickers and Athar, 2006; Sander et al., 2004, Kuanupriya et al., 2007 and Chandra, Samali, and Orrenius, 2004). Many skin care products comprise of natural and/or artificial antioxidants that help reducing or neutralising free radicals are in market (Jenkins, 2002; Shindo et al., 1994 and Valitutti, Castellino and Musiani, 1990). Therefore, reduction of inflammation and antioxidant effect is more beneficial to anti-aging process (Gupta and Nicol, 2004 and Valitutti, Castellino and Musiani, 1990). Although sulphurous spring water could not directly induce pro-collagen synthesis (Fig. 4) it is plausible promoting fibroblast survival can contribute towards the production of collagen preventing premature aging.

#### Conclusion:

In conclusion, although the therapeutic effects of sulphurous spring water on skin cells can be pleiotropic in nature and act on different cell types that make up the skin, our results demonstrate that dermal fibroblasts are one of the cell type that definitely respond favorably upon exposure sulphurous spring water. The concentration of sulphur in spring water as well as mineral components that individually contribute to these beneficial effects need to be investigated in detail. Nevertheless, we provide scientific evidence that sulphurous spring water possesses antibacterial, antioxidant and cryoprotective qualities on primary dermal fibroblasts, the main cells constituting human skin.

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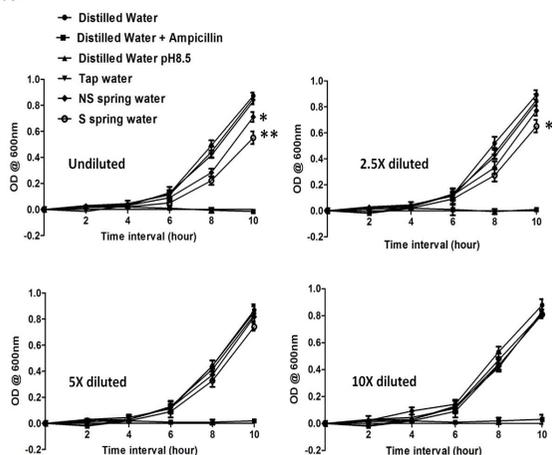
**Table 1: Major and trace elements in the different waters used in this study**

Paramters	Sulphurous Spring water	Non-sulphurous Spring water	Tap water
pH	8.76	9.23	7.12
Temperature <sup>a</sup>	25°C	25°C	24°C
EC <sup>b</sup> (µs/cm)	395	337	50
Component	mg/L	mg/L	Mg/L
T-solids	207	320	86
Total Sulphur <sup>c</sup>	0.3	ND <sup>d</sup>	ND <sup>d</sup>
Na	79	72.3	7.0
K	0.75	1.7	2.2

Ca	6.77	2.65	14.9
Mg	0.09	0.02	3.3
Li	0.26	0.23	0.05
HCO <sub>3</sub>	128.1	73.2	ND <sup>d</sup>
CO <sub>3</sub>	ND <sup>d</sup>	ND <sup>d</sup>	1.7
Free CO <sub>2</sub>	13.203	18	ND <sup>d</sup>
DO <sup>e</sup>	0.05	0,04	11.7
Cl	22.4	20.5	12
SO <sub>4</sub>	11.3	11.8	14
PO <sub>4</sub>	<0.05	<0.05	ND <sup>d</sup>
F	12.1	12	<0.3
SiO <sub>2</sub>	19.2	33.4	ND <sup>d</sup>
Fe	0.03	0.02	ND <sup>d</sup>
Mn	<0.01	<0.01	0.05
Zn	0.02	0.01	0.0028
Cu	<0.01	<0.01	<0.01
Pb	<0.03	<0.03	ND <sup>d</sup>
Al	0.04	<0.02	0.02
Br	<0.05	<0.05	ND <sup>d</sup>
* H <sub>2</sub> S	0.524	ND <sup>d</sup>	ND <sup>d</sup>

aTemperature at time of analysis, bEC- Electrical conductivity, ctotal sulphur content measured, dND- Not detected, eDO- Dissolved Oxygen, \*H<sub>2</sub>S-Hydrogen sulphide, Sulphurous-spring water defined as total sulphur content > 0.1 mg/L by Korea Ministry of Public Administration and Security (MOPAS). T-solid; Total dissolved solid.

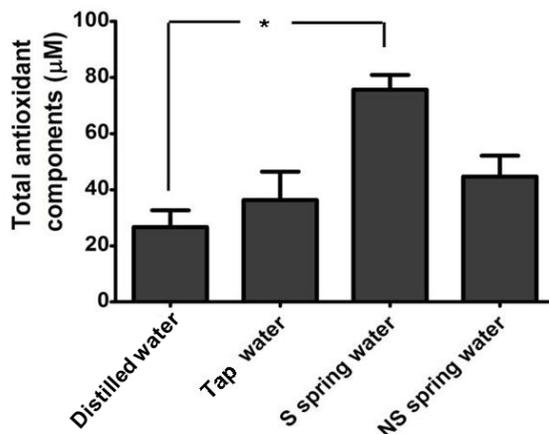
Figure 1. Anti-bacterial property of sulphurous spring water:



E.coli growth curves in Luria broth (LB) media prepared

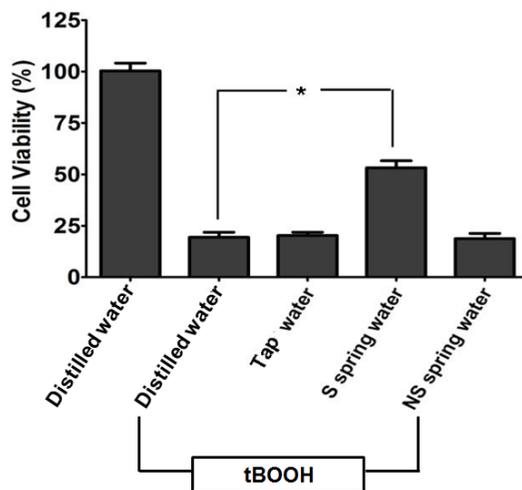
from distilled, tap, sulphurous spring (S) and non-sulphurous (NS) spring waters. LB media with tap water containing ampicillin and pH adjusted to 8.5 served as controls. Data represented here are the means ± SD of three independent experiments. \**p*<0.05 and \*\**p*<0.01, significantly differently when compared to the distilled water treated group.

Figure 2. Induction of antioxidant activity in primary human fibroblasts by sulphurous spring water:

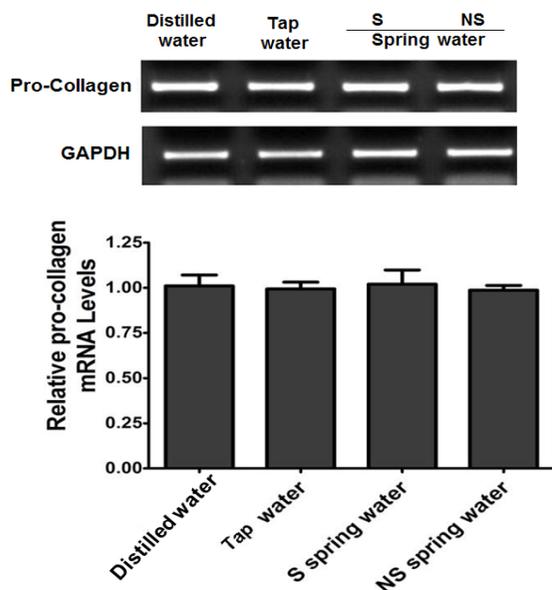


Primary human dermal fibroblasts were cultured for a period of 24 hours in DMEM media prepared from distilled water, tap water, sulphurous (S) spring and non-sulphurous (NS) spring water for a period of 24 hours. Data represent the total antioxidant component (TAC) activity in cell extracts in terms of protein concentration as means ± SD from three independent experiments. \**p*<0.05, when compared to media prepared from distilled water.

Figure 3. Inhibition of tBOOH induced cytotoxicity by sulphurous spring water:



Primary human dermal fibroblasts were cultured in DMEM media prepared from distilled water, tap water, sulphurous (S) spring and non-sulphurous (NS) spring water and cell viability was assessed after exposure to tBOOH for 6 hours. The data here is there present mean ± SD of three independent experiments. \**p*<0.05, as indicated.

**Figure 4: Effect of sulphurous spring water on pro-collagen mRNA expression:**

Primary human dermal fibroblasts were cultured in DMEM media prepared from distilled water, tap water, sulphurous (S) spring and non-sulphurous (NS) spring water for a period of 24 hours. Pro-collagen mRNA levels were analyzed by RT-PCR. *Upper panel*- Representative image of agarose gel stained with ethidium bromide showing the PCR- amplified pro-collagen and GAPDH cDNA; *Lower panel*- bar graph of data in (upper panel) normalized to GAPDH mRNA levels expressed as arbitrary pixel units. The data represents the mean  $\pm$  SD of three independent experiments.

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