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EDITORIAL

SKIN, INFLAMMATION AND SULFUROUS WATERS: WHAT IS KNOWN, WHAT IS BELIEVED

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One could argue that balneotherapy and mud therapy would have not lasted 2,000 years or so if they were not effective. No doubt a long history cannot be taken *per se* as scientific proof of efficacy. Some empiricism is still present in the field: the concept of spa itself is quite confounding, whereas spring waters are used for leisure purposes but also for non-acute patient therapy and late phases of clinical recovery. These confounding elements ultimately feed the opinion of those who aprioristically reject any potential beneficial effect of balneotherapy: instead, it should at least generate questions that deserve scientific answers. Clinical practices sequentially integrating pharmacological therapy with those natural principles for which a sufficient scientific demonstration is available, would probably cut the costs of public health, generating widespread advantages for the community. Recently, it has become evident that mineral waters may have intrinsic pharmacological properties. Of the numerous salts dissolved in thermal waters that might show pharmacological properties, for certain hydrogen sulfide (H_2S) contained in sulfurous waters is the one that has obtained greater scientific attention, to which should be added the extensive scientific effort recently dedicated to H_2S as a cellular gasotransmitter, independently from its natural sources. Dermatology and cosmetics are among the most studied applications of sulfurous waters, around which, however, some empiricism still confounds opinions: we therefore considered that a state-of-the-art focus on this topic might be timely and useful for future studies.

Hydrogen sulfide is a gaseous transmitter

H_2S - generally known as the malodorous rotten eggs smelly gas - belongs to the gaseous transmitter family together with nitric oxide (NO) and carbon monoxide (CO) (1) and has recently generated great scientific interest for its ability to resuscitate small animals (2). Although still far from the more famous NO and CO "cousins", that from 2007 to 2012 have been addressed in approximately 840,000 and 240,000 papers respectively, H_2S has been cited "only" 41,000 times (in nearly 6,100 papers) in the medical and biological scientific community in the same period [from ISI Web of Knowledge, Thomson Reuters].

The first evidence of biological activity of H_2S was reported for the liver and kidney, where the H_2S-

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Table I. *Effects of H2S.*

Effect	activity	target
↓	Proliferation	T cells (CD8, CD4, NK, Th1)
↑	cell death	T cells (CD8, CD4, NK)
↓	IL-2 secretion	T cells (CD8 and CD4)
↓	proliferation, adhesion	keratinocytes
↑	cell death	keratinocytes
↓	CoCl2 hypoxic stress	keratinocytes
↓	IL-1β, IL-6, IL-8, VEGF secretion	keratinocytes
↓	IL-17 and IL-22-mediated activation	keratinocytes
↓	LPS-induced activation	macrophages, endothelial cells
↓	IL-6 secretion	fibroblasts

In vivo effects

Effect	activity	target
↓	dandruff, scaling, dryness	skin
↓	pruritus	skin
↓	fungal, bacterial and parasite growth	skin
↓	radiation exposure side effects	skin
↑	would healing	skin
↓	acanthosis, hyperkeratosis	skin
↓	vessel dilation	derma
↓	ERK activation and IL- secretion	epidermis

generating enzymes were studied in detail starting 30 years ago (1). Since then it has become evident that H_2S plays several other biological roles and functions involving neurological, cardiovascular, inflammatory and cancer diseases, which is no surprise, as H_2S is endogenously produced in most cells. Endogenous H_2S production is dependent on the complex of catabolic pathways of the sulfured amino acids and of glutathione reduction (GSH), and is strictly related to the control of the redox state of the cell. In particular, H_2S is made by pyridoxal-phosphate-dependent enzymes, including cystathionine-c-lyase (CGL, CSE), cystathionine-β-synthase (CBS) and 3-mercaptopyruvate sulfurtransferase (3-MST), during cysteine metabolism (1, 3). The putative quantification of the exact free and bioavailable sulfide levels in blood and tissues is probably from 50 to 150 μM; however, substantial differences in the absolute values have been reported. There are relevant differences in the methods and protocols used to quantify sulfuric acid and, in general, a number of sulfurous molecules acting as H_2S donors have been described (3).

The anti-inflammatory properties of H_2S-rich waters on the skin have been known for centuries (4), however, its molecular mechanisms are just starting to be understood, with growing attention to keratinocytes. The specific interest for skin derives from its potentially simple translation to patient care. This review focuses on the role of H_2S on skin in general and keratinocytes in particular, as a framework for the topical complementary treatment of specific skin diseases with H_2S/sulfurous waters.

Sulfurs in skin therapy

Most of the scientific literature on H_2S effects on cell viability, proliferation, activation, cytokine secretion and adhesion, refers to macrophages, granulocytes, fibroblasts, smooth muscle cells, myoblasts, cardiomyocytes, neuroblastoma cells, neurons, astrocytes, hepatocytes, endothelial cells, lymphocytes, intestinal epithelial and colon cancer cells (3). As far as skin is concerned, sulfurs are able to penetrate the skin and a sulfur-rich balneotherapy, known to be effective in the treatment of psoriasis (5), may be useful also in other T-cell-mediated autoimmune diseases of the skin.

Sulfur has a long history of topical use: hydrogen

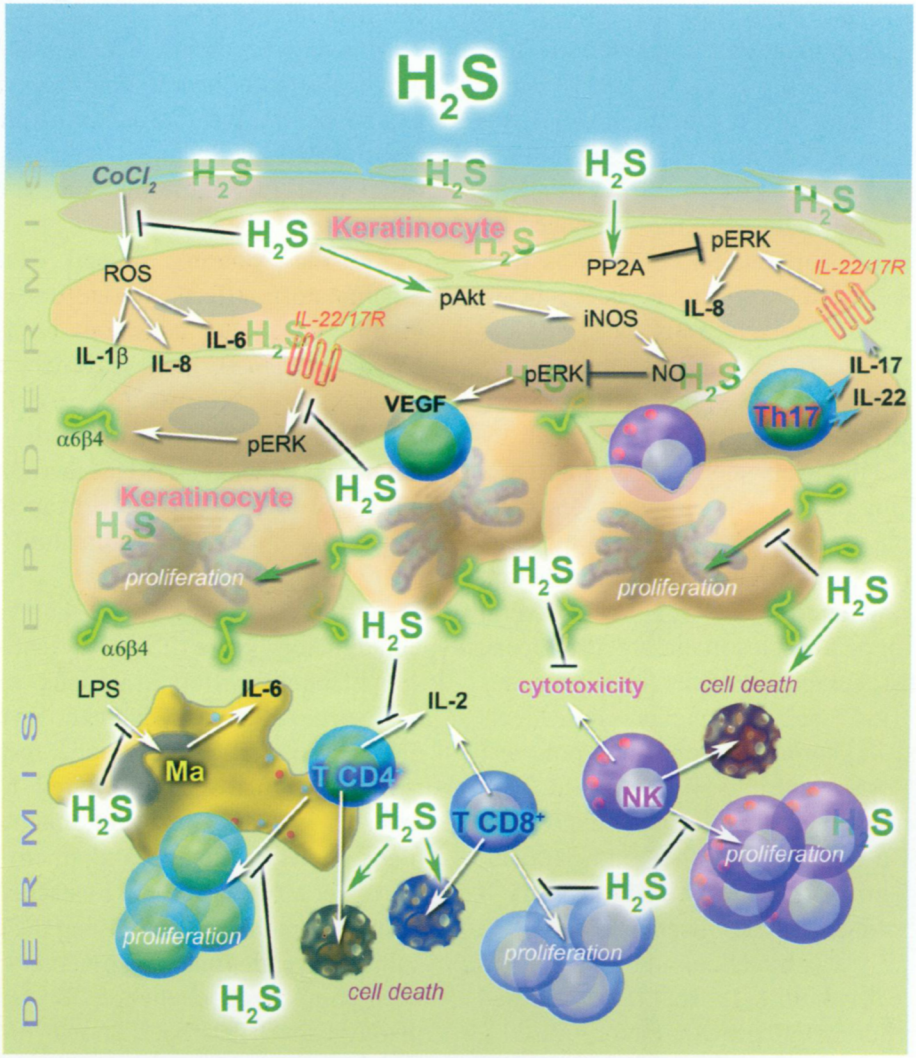

Fig. 1. *H_2S signaling on keratinocyte and lymphocyte activation, proliferation and inflammatory cytokine secretion in autoimmune skin diseases. Macrophages (Ma), T lymphocytes (CD8+, CD4+ and CD4 Th17 subset), natural killer (NK) and keratinocytes of dermis and epidermis layers are shown. Stimulation and inhibition signaling pathways are indicated by an arrow and by a black line (flat head), respectively.*

sulfide is present in a variety of skin care products, including soaps and cleansers (6), and is used to reduce dandruff and decrease scalp scaling and dryness (7). It has recognized fungicidal, bactericidal and antipruritic properties, and therefore it is used for the therapy of mite infestations in animals. Sulfur was even proposed as an antidote for acute exposure to radioactive material (8). Indeed, it has been demonstrated that sulfurs favor wound healing, acts as a keratolytic agent and can induce various histological modifications in the skin (including hyperkeratosis, acanthosis, and dilation of dermal vessels).

Cytokines in skin inflammatory diseases

Psoriasis is now considered a genetically programmed, immune-mediated, skin-specific inflammatory disease, in which intralesional T lymphocytes trigger keratinocytes to proliferate and perpetuate the disease process (9). Epidermal T cells are mainly $CD8^+$, whereas dermal T lymphocytes are a mixture of $CD4^+$ and $CD8^+$ cells, with a $CD4^+$ predominance (10, 11). Recent studies on the cellular pathogenesis of psoriasis revealed the involvement of specific classes of $CD4^+$ T lymphocytes. In particular, it has been demonstrated that the Th17/

Th1 and Th17/Treg ratios strongly change in psoriasis, with a predominant accumulation of Th17 cells and reduction of Treg.

In psoriatic plaque, the presence of Th1-type cytokines, including interferon (INF)-g, interlukin (IL)-2 and tumour necrosis factor (TNF)-a has been demonstrated (9). Relevant cytokines produced by the keratinocytes of psoriatic lesions, such as IL-6 and IL-8, have been shown to induce keratinocyte proliferation (11). The more recently described IL-15, IL-17, IL-19, IL-20, IL-22 and IL-23, that also are found in psoriatic lesions and which also influence keratinocyte proliferation, are mainly produced by cells of hematopoietic origin such as dendritic (DC), macrophages, lymphocytes and granulocytes (12). Studies on the role of interleukins secreted by Th17 cells (IL-17 and IL-22) revealed a complex interaction between T cells and keratinocytes (11, 12) in several inflammatory skin pathologies including acanthosis, atopic dermatitis, cutaneous lupus erythematosus (13). Increased IL-17 and IL-22 expression levels have been found in psoriatic lesions and epidermal hyperplasia (14, 15); both IL-17 and IL-22 have been shown to induce keratinocyte gene expression of antimicrobial β-defensins S100A8 and S100A9, all up-regulated in psoriatic lesions (11, 15). IL-8 released by keratinocytes has been shown to be induced by IL-17 via extracellular-signal-regulated kinase (ERK) activation (11, 16, 17). Released IL-8, vascular endothelial growth factor (VEGF), and TNF-α promote neoangiogenesis, neutrophil and lymphocyte extravasation, perpetuating skin inflammation and dermal infiltration of inflammatory cells.

Another relevant pathogenic factor that must be considered in skin inflammatory pathologies is hypoxia, which impairs would healing and mediates dermic injury in various diseases, such as pressure, diabetic and venous ulcers (18). Hypoxia mediates dermic injury by promoting the expression of important pro-inflammatory mediators as prostaglandins, that are synthesized through the activation of keratinocyte cyclooxygenases (19).

In summary, a rationale therapeutic approach to inflammatory skin pathologies should limit activation, cytokine secretion and proliferation of keratinocytes and immune cells, in particular $CD4^+$ and $CD8^+$ lymphocytes.

H_2S and inflammation

It is now becoming evident that H_2S exerts non-univocal effects on inflammation. For example, it has been reported that administration of NaHS, a fast releasing H_2S donor, induces an inflammatory reaction in liver and lung, and the extravasation of leukocytes in the lung (3).

On the contrary, several data support anti-inflammatory effects of sulfurs: their ability to inhibit leukocyte adhesion to gastric mucosal blood vessels (3); their scavenger activity for pro-inflammatory oxidants (3, 20); their attenuation of lipopolysaccharide-induced formation of inflammatory mediators in macrophages (21); their inhibition of IL-6 secretion from fibroblasts in the synovial membrane of rheumatoid arthritis patients (22).

Sulfurous spa waters are known for their anti-inflammatory properties. Different waters (most of which contain sulfurs in various combinations with halogens or other ions) exert known beneficial effects on mucosal surfaces of the upper respiratory airways, and are normally administered by inhalation or aerosol (4, 23, 24), restoring mucociliary clearance, physiological drainage and micro-environment of the upper airways and the Eustachian tubae (4). The molecular pathways underlying the role of hydrogen sulfide in inflammatory processes have therefore been a research challenge for balneotherapy for many years. It has been known for more than two decades that sulfur thermal waters have inhibitory effects on the proliferative response of T cells (25). In a pioneering study, Valitutti et al. analyzed *in vitro* the phenotype and proliferation of peripheral blood mononuclear cells (PBMC) from ten patients treated with a 14-day cycle of inhalatory therapy for upper respiratory disorders and six patients taking mud therapy for osteoarticular diseases. Although inhalatory or mud therapy did not induce alterations in lymphoid subset distribution (as defined by CD2, CD3, CD4, CD8, CD19 surface antigen expression), PBMC were highly sensitive to sulfur-enriched water in *in vitro* assays. The anti-proliferative effects of sulfurs were dose-dependent and more evident on anti-CD3, rather than PHA, stimulation. Moreover, Valitutti and coworkers hypothesized that the inhibitory effects of low dose sulfurs on lymphocyte proliferation was Th1-memory-restricted because

exogenous IL-2 and/or INF-γ were able to revert it. Much more recently, we studied the effects of hydrogen sulfide on human lymphocyte subset viability, proliferation and cytokine secretion (26). Hydrogen sulfide induced a caspase-independent cell death of human lymphocytes, with differential effects among lymphocyte subsets: $CD8^+$ T lymphocytes and NK cells were far more sensitive to the toxic effects of sulfides than $CD4^+$ T cells. However, while high doses of NaHS (2 mM) killed more than 80% of T cells, a residual significant fraction (50%) of NK still survived. Both surviving CD4 and CD8 T cells showed a decreased proliferative capacity in response to PHA and IL-2. Moreover, NaHS-surviving NK cells showed a decreased spontaneous cytotoxicity against tumor target cells and neither CD8 nor CD4 T cells secreted significant levels of IL-2 during activation. As previously reported, monocytes were resistant to sulfurs (26) while caspase-dependent neutrophil cell death is delayed by NaHS via p38 activation (27).

H_2S and the cytokine secretion of keratinocytes

The exact role of H_2S in inflammation is controversial since both pro- and anti-inflammatory effects have been documented. However, the role of sulfurs on keratinocyte activation and inflammatory cytokine secretion is emerging. Recently, it was reported that hydrogen sulfide can protect keratinocytes against chemical hypoxia-induced inflammatory response. Cobalt chloride ($CoCl_2$), a well-known mimetic agent of hypoxia/ischemia, induces oxidative stress and inflammation. NaHS inhibits the inflammatory response of keratinocytes to cobalt chloride exposure. In particular, it has been demonstrated that NaHS significantly attenuates $CoCl_2$-stimulated interleukin IL-6, IL-8 and IL-1β secretions from human keratinocyte-derived HaCaT cells (28). Yang et al. speculated that NaHS could act by promoting elimination of ROS produced during hypoxia. Although a definitive *in vivo* demonstration is lacking, it has been shown that NaHS is able to eliminate superoxide anions and H_2O_2 both by direct interaction (3) and, indirectly, via the potent endogenous antioxidant scavenger GSH (1, 3). The anti-inflammatory signaling of sulfuric acid on skin can be also obtained by the inhibition of cytokine secretion by other cell types (such as macrophages, fibroblasts, endothelial cells), that play a role in the architectural changes of skin structure in psoriasis and other immune-based diseases. In particular, H_2S has been shown to attenuate lipopolysaccharide-induced formation of inflammatory mediators in macrophages and vascular endothelial cells (21) and, in addition, it also inhibits IL-6 secretion from the fibroblasts of the synovial membrane in rheumatoid arthritis patients (22). Cytokines highly expressed by keratinocytes of psoriatic skin, such as IL-6 and IL-8, have been shown to induce keratinocyte proliferation and promote the recruitment of both neutrophils and T lymphocytes, thus greatly contributing to the pathophysiology of psoriasis (11). Recently, we have demonstrated both *in vitro* and *in vivo* that hydrogen sulfide not only reduces basal expression and secretion of IL-8, but also interferes with IL-17 and IL-22-induced IL-8 production by impairing ERK phosphorylation levels (11).

As far as the mechanism by which NaHS may impair ERK phosphorylation on keratinocytes, we observed that (29) hydrogen sulfide rapidly down-regulates phospho-ERKs levels by blocking MAPK kinases (MEK)-1/2 activation. Moving upstream along the MAPK signalling axis, we also demonstrated that NaHS signalling converges to serine/threonine protein phosphatases-1 and 2A, that are known to activate the Raf/MEK/ERK pathway by inducing the dephosphorylation of Raf-1 at ser 259 (an inhibitory phosphorylation site). Very recently, Merighi et al. reported that sulfurs may reduce VEGF secretion of human skin-derived keratinocyte cell lines (NCTC). The mechanism by which NaHS impairs VEGF transcription and protein secretion involves NO production (30). In particular, these Authors demonstrated that NaHS, by activating the classical membrane phospholipid breakdown singalling pathway, generated an Akt-dependent induction of inducible nitric oxide synthase (iNOS) expression followed by NO production. These increased levels of NO, in turn, reduced the activation levels of the MAPK signaling pathway, preventing VEGF transcription in an ERK-1/2-dependent way.

H_2S and keratinocyte growth

Human and mouse keratinocytes express α6β4 (laminin-5 receptor) integrin that contributes to their anchorage to the basal membrane *in situ*. Moreover,

α6β4 promotes keratinocyte migration and activates signaling pathways that are synergistic with those of growth factor receptors (31). More in general, α6β4 is necessary for tumor cell invasion, cooperates with *c-met* in the transformation of rodent fibroblasts and is necessary to maintain the tumorigenic phenotype of carcinoma cells. Recently, we demonstrated that NaHS is able to inhibit the expression of α6β4 in keratinocytes and can be considered relevant in the control of keratinocyte growth and adhesion. In fact, NaHS-induced reduction of β4-integrin expression in the NCTC cell line can explain sulfur-induced cell death as well as sulfur-mediated inhibition of keratinocyte clonal growth and cell adhesion (29). The NaHS-mediated interference with the Ras/ ERK-1/2 kinase, with the consequent down-regulation of ERKs phosporylation observed after sulfur treatment is responsible for α6β4 inhibition.

According to previous studies, keratinocytes of hyperplastic epidermis express high levels of pERK with the classical nuclear localization, while keratinocytes of normal skin show low pERK levels with a main cytoplasmic localization. By *in vivo* experiments, we have shown that topic treatment of psoriatic lesions with NaHS water solutions at sulfur concentrations similar to that used for balneotherapy, was sufficient to reduce ERK levels in intralesional keratinocytes and, as expected, to reduce their IL-8 production, as well (11, 29).

Purified sulfur has been used as a therapeutic agent to relieve the side effects of radiation therapy in the treatment of cervical cancer. The effects of sulfur on cell proliferation, cell division, and apoptosis in immortalized human skin and oral keratinocytes (HaCaT and IHOKs) and in two oral SCC cell lines (HN4, HN12) was studied (32). H_2S induced the morphological and biochemical changes characteristic of apoptosis, as indicated by sub-G1 phase accumulation, increase in annexin$^+$/PI$^+$ cells, chromatin condensation and DNA fragmentation. The down-regulation of Bcl-2, cyclins D1, D2, and E, and the up-regulation of p53, CDK inhibitor p21, and p16 have been proposed as the most relevant molecular mechanism of hydrogen sulfide-mediated anti-proliferative effects (32). However, several studies have recognized H_2S as a rescuer from hypoxia-induced cell damage (1, 3, 33, 34), and a similar anti-apoptotic activity may also take place on keratinocytes (28).

It is remarkable that topical administration of H_2S promotes dermal wound healing, whereas genetic ablation of CSE attenuates it. Angiogenesis is crucial in the early stages of wound healing and the administration of H_2S to endothelial cells in culture stimulates cell proliferation, migration and tube formation. Moreover, sulfurs stimulate blood vessel growth and branching *in vivo* (35). Angiogenetic mediators such as VEGF and angiopoietin-2 play significant roles in the pathophysiology of psoriasis and may even account for the maintenance of chronic inflammation: thus one could expect unfavorable effects from the topical treatment of psoriatic lesions with sulfurs or sulfur-rich waters due to its intrinsic pro-angiogenic activity. We try a reconciliation of this apparent paradox in the next paragraph.

Fast vs slow H_2S releasing agents

One interesting property of fast H_2S donors as NaHS salts, is the low risk of severe secondary effects due to accumulation in unrelated tissues and cells. In fact, at physiological pH values, NaHS rapidly dissociates in Na+ and HS- ions that are rapidly converted into gaseous H_2S. This lipophilic gas is then eliminated by breathing (1). Due to their rapid pharmacokinetics, these sulfur donors tend to induce acute biological effects. On the contrary, slow HS-releasing donors can diffuse and accumulate in other tissues, inducing a chronic HS-mediated stimulation with toxicity and relapse effects. However, it must be said that low HS-releasing compounds, which should be used at lower concentration than NaHS, limit smelling (that can represent a serious obstacle for a topical therapy), are easier to handle and are generally more stable than NaHS or gaseous H_2S-enriched solutions. Several disadvantages exist for most of the HS-releasing compounds: the backbone of GYY4137 (36) that remains after H_2S release, the *N*-benzoylthiobenzamides that acylate biological molecules carrying nucleophilic groups and the S-diclofenac (37) have biological activities unrelated to H_2S release. A new generation of HS-releasing compounds are synthesized (thioglycine and l-thiovaline) with a non-toxic and inert carrier scaffolds (38).

Several studies have demonstrated the cytoprotective effects of H_2S at micromolar

concentrations, whereas exposure of cells to higher (millimolar) H_2S concentrations tends to be cytotoxic due to free radical generation, calcium mobilization, glutathione depletion, and induction of mitochondrial cell death pathways.

Balneotherapy with naturally H_2S-rich waters, can be reproduced in part using as surrogate the NaHS-enriched phosphate saline solution. The concentration of HS^- and S^{2-} in spring sulfur water vary considerably, spanning from 0.5 to 20mg/L of equivalent H_2S. In general, spa sulfurous water for irrigation, inhalation, bathing and drinking based-therapies contain more than 1mg/L of H_2S (11). However, the actual type(s) and concentration(s) of H_2S donors should be determined at each source, and the role played by temperature and additional mineral components of spring water should also be considered. In some studies the net effects of highly concentrated spring water *vs* tap water have been investigated, excluding that additional benefit of balneotherapy observed in patients with psoriasis and atopic dermatitis are due to the salinity (NaCl *in primis*) of the liquids (39).

Mud, gel and other semisolid supports have been generated and used for massage and shampooing treatment. Clinical evaluation of the anti-inflammatory activity of mud-pack has been investigated and general reduction of inflammatory cytokines (IL-6) levels have been reported (40).

CONCLUSIONS

Balneotherapy is certainly a relaxing and restorative experience – that can be well considered as part of the therapy – which, however, represents a confounding element in the distinction between "pharmacological" and placebo effects. Thus the immediate translation into clinical studies of the basic scientific findings on H_2S effects cannot be made. It is well known, in fact, that placebo effects are of particular relevance when diseases such as psoriasis and dermatitis are investigated. However, animal studies might be designed for this purpose, for which appropriate models are immediately available. The question is whether or not there is sufficient scientific evidence now supporting the integration of sulfur waters in the therapy of specific skin diseases, when appropriate (Fig. 1). Given the potential benefits for the patients, the low-intensity caring and the savings in health costs, this question should arise spontaneously. However, despite the basic scientific data on sulfurs, the belief that balneotherapy (including sulfur water therapies) will never reach the level of scientific demonstration of therapeutic efficacy (40), and should therefore be kept out of the medical field *tout court,* is generally diffuse. We did not meta-analyze here the available scientific literature in the balneotherapy field, which is, by the way, much broader than currently believed. Instead, we took the most active component of thermal waters (the sulfur ion) revisiting some of its robustly demonstrated biological effects. As a conclusion, we believe that a special research effort could be reasonably dedicated to bring to the same table of discussion what is now known on the cellular effects of H_2S and what has been observed for centuries about the clinical effects of sulfur balneotherapy on some dermatological conditions. No doubt prejudices of various origin(s) shadow the potential link between the two ends of this line: animal studies, we believe, should now be implemented to close the circle.

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