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REVIEW PAPER

A comprehensive analysis to understand the mechanism of action of balneotherapy: why, how, and where they can be used? Evidence from in vitro studies performed on human and animal samples

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Abstract

Balneotherapy (BT) is one of the most commonly used complementary therapies for many pathological conditions. Its beneficial effects are related to physical and chemical factors, but the exact mechanism of action is not fully understood. Recently, there has been an increased interest in the use of preclinical models to investigate the influence of BT on inflammation, immunity, and cartilage and bone metabolism. The objective of this comprehensive analysis was to summarize the current knowledge about the in vitro studies in BT and to revise the obtained results on the biological effects of mineral waters. Special attention has been paid to the main rheumatological and dermatological conditions, and to the regulation of the immune response. The objective of this review was to summarize the in vitro studies, on human and animal samples, investigating the biological effects of BT. In particular, we analyzed the properties of a thermal water, as a whole, of an inorganic molecule, such as hydrogen sulfide in different cell cultures (keratinocytes, synoviocytes, chondrocytes, and peripheral blood cells), or of the organic component. The results corroborated the scientific value of in vitro studies in demonstrating the anti-inflammatory, antioxidant, chondroprotective, and immunosuppressive role of BT at the cellular level. However, the validity of the cell culture model is limited by several sources of bias, as the differences in experimental procedures, the high heterogeneity among the available researches, and the difficulties in considering all the chemical and physical factors of BT. We would like to stimulate the scientific community to standardize the experimental procedures and enhance in vitro research in the field of BT.

Keywords Balneotherapy · Mineral waters · Hydrogen sulfide · Cell cultures · Keratinocytes · Chondrocytes

Introduction

Balneotherapy (BT) is a complementary therapy that generally employs mineral and/or thermal waters from natural springs, peloids (mud), and other traditional remedies, for the treatment of different pathological conditions (dermatological, rheumatological, gastroenterological conditions, pulmonary diseases, cardiovascular, gynecological, metabolic, neurological, psychiatric, and endocrine disorders) (Contoli et al. 2013; Fioravanti et al. 2017; Forestier et al. 2017; Guidelli et al. 2012; Katz et al. 2012; Matsumoto 2018; Naumann and Sadaghiani 2014; Tenti et al. 2015).

A large number of clinical studies have reported the beneficial effects of this approach for the prevention, treatment, and rehabilitation of various rheumatic disorders, such as osteoarthritis (OA) (Espejo-Antúnez et al. 2013; Fioravanti et al. 2015; Forestier et al. 2010; Király et al. 2019; Masiero 2008; Masiero et al. 2018), fibromyalgia (FM) (Fioravanti et al. 2018; Naumann and Sadaghiani 2014; Ozkurt et al. 2012), low-back pain (Karagülle and Karagülle 2015; Tefner et al. 2012; Yücesoy et al. 2019), rheumatoid arthritis (RA) (Brosseau et al. 2002; Santos et al. 2016), and other chronic inflammatory rheumatic diseases (Cozzi et al. 2018). Current results support a positive effects of BT on pain, function, and quality of life with a significant reduction in symptomatic drug consumption (Antonelli et al. 2018; Matsumoto et al. 2017); furthermore, the clinical efficacy lasts over time, until 6–9 months after a cycle of treatment (Fioravanti et al. 2015;

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Forestier et al. 2010; Fortunati et al. 2016). Finally, this approach showed a favorable cost/effectiveness profile (Ciani et al. 2017).

The use of mineral waters and, in particular of the sulfurous waters, represents a useful complementary therapy in dermatological patients (Huang et al. 2018; Péter et al. 2017; Szabó 2007). Recently, growing evidence disclosed the potential therapeutic properties of BT in psoriasis and atopic or contact dermatitis (Huang et al. 2018; Péter et al. 2017).

The beneficial effects of mineral waters are related to their physical and chemical properties, such as temperature, salt composition and concentrations, osmotic pressure, and electric conductivity (Fioravanti et al. 2011; Morer et al. 2017). These particular characteristics make difficult to understand the mechanisms of action of BT and to analyze the biological role of the different components which constitute a whole mineral water (Fioravanti et al. 2011; Morer et al. 2017). Indeed, thermal waters are very sophisticated systems consisting of a mixture of different organic and inorganic compounds. Traditionally, they were classified on the basis of their inorganic composition, but, recently, their organic fraction has been demonstrated as highly bioactive, contributing to the medicinal effects of BT (Szabó and Varga 2019).

In the last years, there has been an increased interest in the use of preclinical models (animal or in vitro studies) to investigate the biological effects of BT on inflammation, immunity, and cartilage and bone metabolism.

To date, just a limited number of controlled animal studies have been carried out in this field. In particular, some authors demonstrated the anti-inflammatory, antinociceptive, antioxidant, and chondroprotective properties of BT, principally reducing cytokines and free radical production, and decreasing the mechanical hyperalgesia and edema in different murine models of arthritis or psoriasis (Abu-al-Basal 2012; Bajgai et al. 2017; Britschka et al. 2007; Cozzi et al. 2004; Tékus et al. 2018; Zivná et al. 2012). Some aspects of these studies are disputable and could represent a source of bias. Indeed, comparisons among the various studies are difficult principally because of the difference in type and length of interventions, and, in certain cases, the combinations of treatment modalities. The problem in working with animals is mainly related to economical problems, to establish a specific model for a particular disease and to find the most suitable application routine of BT or the protocol of mud treatment (Tékus et al. 2018). Furthermore, the restraint conditions at which the animals are subjected for the treatments may exert stress reactions that can influence the experimental outcomes (Scheich et al. 2017; Tékus et al. 2016).

These considerations stimulate to identify new preclinical studies employing cell culture models to avoid possible bias related to the use of animals.

The objective of this comprehensive analysis was to revisit the role of the in vitro studies in BT and to summarize the obtained results on the biological effects of mineral waters. Special attention has been paid to the main rheumatological and dermatological conditions, which take any advantage by BT, and to the regulation of the immune response.

In vitro studies on balneology: why, how, and where

Why

In vitro research represents the starting point in biological and medical investigation, and it is conducted using components of an organism (tissues or cells) that have been isolated from their usual biological surroundings and used to emulate different aspects of human body functions; this permits a simpler, more rapid, detailed, and convenient analysis than what can be done with whole organisms (Johnson et al. 2016; Miller and Spence 2017). The myriad of in vitro experimental models offers a diverse variety of experimental approaches, which can provide empirical data potentially unobtainable from whole-animal studies, also reducing the probability of bias when animal usage tests or clinical trials are performed (Miller and Spence 2017).

In vitro studies were normally used to evaluate the effects and/or the mechanism of action of mechanical (such as hydrostatic pressure, mechanical compression, ultrasound, magnetic or electromagnetic fields) or chemical factors (cytokine, growth factors, adipokines, drugs, mineral elements, etc.) on cell or tissue morphology and metabolism (Cheleschi et al. 2015; Cheleschi et al. 2018; Cheleschi et al. 2019a; Cheleschi et al. 2019b; Collodel et al. 2013; Fioravanti et al. 2010; Kloesch et al. 2011; Marrazzo et al. 2019; Shams et al. 2018).

In vitro investigation plays a pivotal role to advance research into the physiopathology of a particular disease, and to help the development of potential therapeutic strategies (Johnson et al. 2016).

How

In recent years, lots of advancements have been made in the area of culture systems, which have enhanced functionality and stability of cells in vitro.

Tissue of human or animal origin, monodimensional or tridimensional cell cultures (for instance keratinocytes, chondrocytes, fibroblasts, polymorphonuclear cells), cell suspensions, or co-cultures can be employed. Studies on primary cells are the most used model for in vitro tests because they closely represent the tissue of origin, are not modified, and provide excellent culture systems for studying the physiological or pathological behavior of the native organ in vivo. Standardized and immortalized cell lines have been

also developed to obtain indefinite subcultures with a high experimental repeatability and reproducibility, but without phenotypic characteristics of the in vivo tissue (Hanks et al. 1996; Miller and Spence 2017).

Each culture system presents advantages and disadvantages, and each is particularly suitable for exploring one specific aspect of cell metabolism, according to the aims of the study.

In the field of BT, tissue explants, primary cells, or immortalized lines can be used to investigate the potential biological effects of a single inorganic molecule (Carbajo and Maraver 2017; Viegas et al. 2019; Wallace and Wang 2015), or organic compounds (Gerencsér et al. 2019), or a mineral water as a whole (Fioravanti et al. 2011; Gálvez et al. 2018).

Among the inorganic molecules, which generally constitute the mineral waters, sulfur has currently been recognized as a crucial element with a wide range of functions, mainly when it was found in the form of hydrogen sulfide (H_2S) (Carbajo and Maraver 2017; Viegas et al. 2019; Wallace and Wang 2015). H_2S represents the main active molecule of sulfurous mineral-medicine waters; it is a small gaseous molecule traditionally considered as toxic gas, but, in the last years, scientific opinion has changed as more reports on its biological activity were published, and it is now considered a biologically relevant molecule (Carbajo and Maraver 2017; Wallace and Wang 2015).

H_2S is an endogenous gasotransmitter, and, as such, it can be absorbed by numerous routes; it is able to penetrate the skin and mucosae and can therefore act at the cell level both in the skin and in internal organs (Burguera et al. 2017; Carbajo and Maraver 2017).

Its exogenous and endogenous donors at high micromolar concentrations were generally employed in in vitro research to mimic physiological functions of H_2S at cellular level and to identify its potential mechanism of action (Carbajo and Maraver 2017; Wallace and Wang 2015).

Organic components of thermal waters were demonstrated to have biological effects contributing to the healing mechanisms, but their medical significance is not still fully understood (Varga 2012a). However, in a recent double-blind, randomized controlled trial on knee and hip OA patients, the organic fraction separated from the whole Szigetvár medicinal water and redissolved in tap water determined a major improvement of the studied clinical outcomes than what is observed in tap water alone (Hanzel et al. 2019). This finding explains the so-called Varga's organic hypothesis, which supports that the biological effects of thermal waters are caused by bioactive organic molecules more likely than by the inorganic salt content (Varga 2010; Varga 2012b).

However, due to the raised number and variety of organics, the only method to analyze these organic mixtures, including possible interactions, is the determination of their biological activities in different tests. The most informative results were obtained from the Comet Assay (single-cell microgel electrophoresis for DNA damage) and the Salmonella Ames mutagenicity studies with the different chemical fractions of waters and peloids. Recently, (Varga et al. 2015)) presented a new application of the Salmonella TA strains originally engineered for the Ames mutagenicity test. After the organic extracts were isolated from five Hungarian thermal spa waters, the authors found that 4 of them showed a detectable UV-protective effect in Salmonella TA bacteria, demonstrating for the first time the UV-protective property of organic matter in natural thermal water samples.

However, it is plausible to think that the efficacy of a thermal mineral water is probably related to a complex relationship among a number of different chemical components (Fioravanti et al. 2011; Morer et al. 2017). This consideration leads us to identify the most suitable preclinical model to better investigate the mechanism of action of a mineral water as a whole.

Where

In vitro studies on keratinocyte investigating the effects of BT on skin diseases

Gobbi et al. in 2009 and Mirandola et al. in 2011 (Gobbi et al. 2009; Mirandola et al. 2011) demonstrated the anti-inflammatory effect of exogenous source of H_2S, natrium hydrogen sulfide, a fast-dissolving salt (NaHS), in normal skin–derived immortalized human keratinocyte cultures. After incubation of the cells with NaHS (400 mM, for 6, 12, 18, 24 h) in the presence of a specific mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) inhibitors, a reduced secretion of interleukin (IL)-8, IL-17, and IL-22, as well as a decreased cell proliferation and adhesion, through a downregulation of adhesion molecule expression, was observed, by reducing MAPK/ERK signaling phosphorylation (Table 1).

Furthermore, a group of investigators performed three different in vitro studies in order to evaluate the potential beneficial effects of Comano spa's water (Trentino, Italy), a thermal hypotonic water containing various electrolytes as sodium, calcium, and bicarbonate, on the clinical manifestations of psoriasis (Chiarini et al. 2006a; Chiarini et al. 2006b; Dal Pra et al. 2007). Human psoriatic keratinocytes were incubated with different concentrations of Comano water (totally or partially dissolved in the culture medium) for an experimental period from 3 to 15 days and, then, the medium was collected and the cells processed for further analyses. The results demonstrated a reduced release and expression of vascular endothelial growth factor (VEGF) A, IL-8, IL-6, and cytokeratin (CK)-16 (a marker associated with keratinocyte psoriatic phenotype) in cells treated with all the studied concentrations of mineral water (Table 1).

Table 1 In vitro studies on human samples evaluating the beneficial effects of BT on skin diseases

Authors	Treatment(s)	Experimental model	Mineral water or inorganic or organic components	Pathology	Biochemical parameters	Results
(Gobbi et al. 2009)	30-min preincubation with MAPK/ERK inhibitors (10–30 μM) + NaHS (400 mM) dissolved in the culture medium, for 6, 12, 18, 24 h	Normal skin–derived immortalized human keratinocytes	NaHS	Psoriasis	IL-8, IL-17, IL-22; cell proliferation and adhesion; MAPK/ERK signaling phosphorylation	Reduced IL-8, IL-17, IL-22 secretion, adhesion molecules expression, and MAPK/ERK phosphorylation Reduction of inflammation events typical of psoriatic lesions
(Mirandola et al. 2011)	30-min preincubation with MAPK/ERK inhibitors (10–30 μM) + NaHS (400 mM) dissolved in the culture medium, for 6, 12, 18, 24 h	Normal skin-derived immortalized human keratinocytes	NaHS	Psoriasis	IL-8; MAPK/ERK signaling phosphorylation	Reduced basal and IL-17/IL-22-induced IL-8 expression and secretion; Reduced MAPK/ERK phosphorylation Reduction of inflammation events typical of psoriatic lesions
(Chiarini et al. 2006a)	25%, 50%, or 100% of Comano water dissolved in the culture medium, for 11 days	Human primary epidermal keratinocytes	Comano spa's water (Trentino, Italy), rich in sodium, calcium and bicarbonate	Psoriasis	VEGF-A	Reduced VEGF-A expression and secretion Reduction of VEGF-A-mediated angiogenic, vessel permeabilizing, and chemotactic effects
(Chiarini et al. 2006b)	25%, 50%, or 100% of Comano water dissolved in the culture medium, from 3 to 15 days	Human primary epidermal keratinocytes	Comano spa's water (Trentino, Italy), rich in sodium, calcium and bicarbonate	Psoriasis	IL-6, CK-16, VEGF-A	Reduced IL-6, VEGF, and CK-16 release and expression Reduction of inflammation, and neo-angiogenic phenomena of local psoriatic manifestations
(Dal Pra et al. 2007)	25%, 50%, or 100% of Comano water dissolved in the culture medium, from 11 to 13 days	Human primary epidermal keratinocytes	Comano spa's water (Trentino, Italy), rich in sodium, calcium and bicarbonate	Psoriasis	IL-8, TNF-α	Reduced IL-8 and TNF-α intracellular levels and secretion rates Reduction of inflammation events typical of psoriatic lesions
(Lee et al. 2012)	50% of Yong-gung oncheon thermal spring water dissolved in the culture medium, for 1, 4, 10, and 24 h + LPS (10 μL/mL)	Human keratinocyte cell lines, HaCaT	Thermal spring water (Yong-gung oncheon, Ganghwa-gun, Korea), rich in sulfur, magnesium, calcium and selenium	Skin disease	IL-6, IL-8; CD4+ T cells differentiation	Reduced IL-6 and IL-8 gene and protein expression; attenuated differentiation of CD4+ T cells in Th1, Th2 and Th17 Attenuation of immune skin reactions
(Karagülle et al. 2018)	10% of Bursa and Bolu thermal mineral waters dissolved in the culture medium, for 3 days	Human keratinocyte cell lines, HaCaT	Bursa and Bolu thermal mineral waters (Turkey), rich in silicium, zinc, sodium bicarbonate, and boron	Psoriasis and rosacea	IL-1α, TNF-α, and VEGF	Reduced IL-1α, TNF-α, and VEGF gene expression Reduction of inflammation, and neo-angiogenic phenomena of skin disease manifestations
(Gerencsér et al. 2019)	Organic fraction of Hungarian Kakasszék and Gyopáros Spa waters dissolved in the culture medium for 1 h + UV exposure of 10, 20, 30, 40 and 50 s	Human keratinocyte cell lines, HaCaT	organic-rich extract of Hungarian Kakasszék (65 mg/L) and Gyopáros Spa waters (14 mg/L) (Hungary)	Skin disease	DNA fragmentation rate	Reduced DNA lesions Prevention of adverse effects of solar or artificial UV radiation on the human skin

MAPK mitogen-activated protein kinase, *ERK* extracellular signal–regulated kinase, *NaHS* natrium hydrogen sulfide, *IL* interleukin, *VEGF-A* vascular endothelial growth factor A, *CK-16* cytokeratin 16, *TNF-α* tumor necrosis factor α, *LPS* lipopolysaccharide, *HaCaT* human keratinocyte cell lines, *CD4+* cluster of differentiation 4, *Th* T helper cells

Human keratinocyte cell lines, HaCaT, were used by Lee et al. (2012) to analyze the immunomodulatory or anti-inflammatory effects of a thermal spring water, from the Yong-gung oncheon (Ganghwa-gun, Korea), rich in sulfur, magnesium, calcium, and selenium, against the typical signs of inflammatory skin diseases. The Authors showed a suppressed expression of inflammatory cytokines (IL-6, IL-8) and an attenuated differentiation process of subsets of CD4+ T cells, into Th1, Th2, and Th17 cells, after 1, 4, 10, or 24 h of treatment with 50% concentration of spa spring water (Table 1).

HaCaT lines were also employed to an in vitro test of Bursa and Bolu waters, two traditional and historical thermal mineral waters of Turkey (Karagülle et al. 2018). Three days of incubation of the cells with 10% concentration of the two tested waters significantly reduced the gene expression of IL-1α, tumor necrosis factor (TNF)-α, and VEGF. The obtained results proved the anti-inflammatory and angiogenic properties of these spa waters in skin diseases such as rosacea and psoriasis (Table 1).

Recently, the effect of Hungarian Kakasszék spa (medicinal) water (KSZ) and Gyopáros spa water (GYP), containing a high variability of organic components, in preventing the adverse effects of solar or artificial UV radiation on the human skin was analyzed (Gerencsér et al. 2019). The organic fractions of each water were prepared using a procedure involving isolation of organics on Amberlite XAD macroreticular adsorbent resins. After 1 h incubation of HaCaT cells with organic-rich extract of KSZ or GYP with or without different timings of UV irradiation exposure, GYP isolate incubation resulted to be able to prevent DNA lesions of keratinocytes induced by UV exposure (Table 1).

In vitro studies on fibroblast-like synoviocytes, chondrocytes, and osteoblasts investigating the effects of BT on joint disorders

Fibroblast-like synoviocyte cultures, derived from RA and OA patients, and chondrocyte cell line C-28/I2 were employed by Kloesch et al. (2010; 2012a; b) to study the molecular mechanism of action of H_2S, by using its exogenous source, NaHS. These cells constitutively expressed and secreted large quantities of IL-6 and IL-8. Furthermore, their treatment with different concentrations of NaHS (0.030–1 mM, for a maximum of 12 h) transiently reduced the constitutive expression of IL-6 and IL-8 and the activation of MAPK/ERK signaling, as well as those induced by IL-1β (5 ng/mL, 1 h) (Kloesch et al. 2010, 2012a, b). On the contrary, high concentration of NaHS (above 0.5 mM) demonstrated opposite effects both on the expression of cytokines and cyclooxygenase (COX)-2 and on the activation of MAPK/ERK proteins (Kloesch et al. 2012a, b). These results explained the beneficial effect of H_2S on inflammatory processes involved in the pathophysiology of RA and OA, underling the importance of sulfur baths as possible therapeutic effect in such kind of diseases, taking into consideration H_2S exposure in terms of timing and concentration (Table 2).

Growing evidence underlines the relevance of H_2S and its exogenous sources as anti-inflammatory and anti-catabolic agents in human OA chondrocyte and synoviocyte cultures (Table 2).

In 2012, (Fox et al. 2012) studied the ability of human primary chondrocytes and mesenchymal progenitor cells to synthesize H_2S in response to pro-inflammatory mediators stimulation (IL-1β, IL-6, TNF-α, and lipopolysaccharide (LPS)), and their response to the exogenous slow-releasing H_2S source (GYY4137). Endogenous H_2S produced by the cells and the treatment with different concentrations of GYY4137 (50–500 mol/L for 18 h) significantly reduced cell death and oxidant-induced mitochondrial dysfunction, caused by inflammatory cytokines, via protein kinase B (Akt)/phosphoinositide 3-kinase (PI3K)-dependent signaling.

Li et al. (2013) assessed the effect of GYY4137 (0.1–0.5 mM) on LPS (10 μg/mL)-caused release of inflammatory mediators from human arthritis synoviocytes and articular chondrocytes. After 18 h of treatment, GYY4137 demonstrated a prominent anti-inflammatory effect decreasing the production of nitrite (NO_2^-), prostaglandin E2 (PGE2), TNF-α, and IL-6 from both cell types, reducing the levels and catalytic activity of inducible nitric oxide synthase (iNOS) and COX-2, and limiting nuclear factor (NF)-κB activation induced by LPS.

Equivalent results were obtained in a study on human OA chondrocytes stimulated with IL-1β, used as prototype pro-inflammatory cytokine to reproduce the "OA-like effect" (Burguera et al. 2014). The results of the research proved the ability of NaHS and GYY4137 (0.05–1 mM, for 24 or 48 h) to significantly limited nitric oxide (NO), PGE2, and IL-6 released by the cells and at protein level, as well as the gene expression of NOS2, COX-2, prostaglandin E synthase (PTGES), IL-6, and NF-κB nuclear translocation activated by IL-1β stimulus (5 ng/mL). Furthermore, these Authors firstly demonstrated the anti-catabolic activity of these compounds through the downregulation of metalloproteinase (MMP)-13 in the supernatant and at protein level.

These data were confirmed by Ha et al. (2015) in a study on human OA chondrocyte cultures treated with NaHS (0.06–1.5 mM) in the presence or not of IL-1β (10 ng/mL) stimulus for 24 h. The compound markedly reversed the effects of IL-1β on the gene expression of COX-2, MMP-13, and NOS and on their production in the supernatant. In addition, NaHS inhibited the activation of the ERK/IκBα/NF-κB pathway which was induced by IL-1β.

In the same year, Sieghart et al. (2015) investigated the effects NaHS (0.06–1 mmol/L), in OA fibroblast-like synoviocytes stimulated with IL-1β (10 ng/mL). The

Table 2 In vitro studies on human and animal samples evaluating the beneficial effects of BT on joint disorders

Authors	Treatment(s)	Experimental model	Mineral water or inorganic or organic components	Pathology	Biochemical parameters	Results
(Kloesch et al. 2010)	NaHS (0.030–1.0 mM) dissolved in the culture medium, for 1, 3, 6, 12 h + IL-1β (5 ng/mL) for 1 h	RA human fibroblast-like synoviocytes	NaHS	Rheumatoid Arthritis	IL-6; P38/MAPK/ERK signaling activation/deactivation	Reduced IL-6 expression and activation of MAPK/ERK signaling (low NaHS concentrations) Increased IL-6 expression and activation of MAPK/ERK signaling (high NaHS concentrations) Reduction of inflammatory events of RA
(Kloesch et al. 2011)	NaHS (1.0 mM) dissolved in the culture medium, for 1, 3, 6, 12 h	RA and OA human fibroblast-like synoviocytes	NaHS	Rheumatoid Arthritis and Osteoarthritis	IL-6, IL-8, COX-2; MMP-2, MMP-3, MMP-14; P38/MAPK/ERK protein expression	Increased IL-6, IL-8, COX-2 and p38/MAPK/ERK expression Increase of inflammatory events of RA and OA
(Kloesch et al. 2012a; Kloesch et al. 2012b)	NaHS (0.125 and 1.0 mM) dissolved in the culture medium, for 15, 30, 45 and 60 min + MAPK inhibitors (1 and 5 μM) + IL-1β (5 ng/mL) for 1 h	Human chondrocyte cell line (C-28/I2)	NaHS	Rheumatoid Arthritis	IL-6, IL-8; P38/MAPK/ERK and NF-kB signaling activation/-deactivation	Reduced IL-6 and IL-8 expression and activation of p38/MAPK/ERK and NF-kB signaling Reduction of inflammatory processes of arthritis
(Fox et al. 2012)	IL-1β, IL-6 and TNF-α (5 ng/mL) for 6, 12 and 18 h + GYY4137 (50–500 mol/L) dissolved in the culture medium, for 12 h	Human primary articular chondrocytes	GYY4137	Rheumatoid Arthritis	Cell death; Mitochondrial membrane potential	Reduced cell death and oxidant-induced mitochondrial dysfunction Limitation of inflammation in chronic inflammatory diseases
(Li et al. 2013)	GYY4137 (0.1–0.5 mM) dissolved in the culture medium, for 18 h + LPS (10 μg/mL)	Human primary arthritis synoviocytes and chondrocytes	GYY4137	Rheumatoid arthritis	IL-6, TNF-α, PGE2, COX-2; NO, iNOS; NF-kB signaling activation/deactivation	Reduced IL-6, TNF-α, PGE2 and NO production, COX-2 and iNOS catalytic activity, and NF-kB activation Reduction of inflammatory processes of arthritis
(Burguera et al. 2014)	NaHS and GYY4137 (0.05–1 mM) dissolved in the culture medium, for 24 or 48 h + IL-1β (5 ng/mL)	Human primary OA chondrocytes	NaHS and GYY4137	Osteoarthritis	IL-6, PGE2, PTGES, COX-2; NO, NOS2; MMP-13; NF-kB signaling activation	Reduced IL-6, PGE2, and NO release and protein level, IL-6, PTGES, COX-2, and NOS2 gene expression, and NF-κB nuclear translocation Reduction of inflammatory and degrading processes of OA damage
(Ha et al. 2015)	NaHS (0.06–1.5 mM) dissolved in the culture medium, for 24 h + IL-1β (10 ng/mL)	Human primary OA chondrocytes	NaHS	Osteoarthritis	COX-2, iNOS, MMP-13; ERK/IκBα/NF-κB signaling activation	Reduced COX-2, iNOS, MMP-13 release and gene expression; Inhibited ERK/IκBα/NF-κB activation Reduction of degrading processes of OA damage
(Sieghart et al. 2015)	NaHS (0.06–1 mmol/L) dissolved in the culture medium, for 1 h + IL-1β (10 ng/mL)	Human primary OA fibroblast-like synoviocytes	NaHS	Osteoarthritis	IL-6, IL-8; MMP-2, MMP-14; MAPK and Akt1/2/PI3K protein phosphorylation	Reduced IL-6 and IL-8 secretion, MMP-2 and MMP-14 gene expression, and MAPK phosphorylation; Increased Akt1/2 phosphorylation Reduction of inflammatory and degrading processes of OA damage
(Vela-Anero et al. 2017)	NaHS or GYY4137 (200 or 1000 μM) dissolved in the culture medium, for 21 days + IL-1β (5 ng/mL)	Human OA cartilage disks	NaHS and GYY4137	Osteoarthritis	MMP-3, MMP-13; Col2a1, glycosaminoglycans, aggrecans	Reduced MMP-3 and MMP-13 production, and increased Col2a1, glycosaminoglycans, and aggrecans synthesis Reduction of degrading processes of OA damage
(Fioravanti et al. 2013)	25%, 50%, or 100% of Vetriolo thermal water dissolved in the culture medium, for 48 h	Human primary OA chondrocytes	Vetriolo thermal water (Trentino Alto Adige,	Osteoarthritis	NO, iNOS; Cell viability and apoptosis; Morphological assessment	25%, 50% of Vetriolo water increased survival recovery rate, reduced NO levels, iNOS expressions, and apoptosis %;

Table 2 (continued)

Authors	Treatment(s)	Experimental model	Mineral water or inorganic or organic components	Pathology	Biochemical parameters	Results
	+ IL-1β (5 ng/mL)		Italy), strongly acidic sulfate, rich in calcium, magnesium and iron			Enhanced morphological characteristics Reduction of degrading processes of OA damage
(Xu et al. 2011)	NaHS (100 μM) dissolved in the culture medium, for 4 h + (H_2O_2) (400 μM)	Murine osteoblast-like cell line (MC3T3-E1)	NaHS	Osteoporosis	Viability, proliferation and apoptosis; NO, ALP, SOD, NADPH oxidase p38/ERK1/2/MAPKs activation	Increased viability, cell proliferation, ALP and SOD activities; Decreased apoptosis, NO release and NADPH oxidase activity, and p38/ERK1/2/MAPKs activation Proliferative and antioxidant effects against osteoporosis damage
(Gambari et al. 2014)	NaHS (50–300 μM) dissolved in the culture medium, for 72 h to 6 days	Human differentiated osteoclasts	NaHS	Osteoporosis	Osteoclasts differentiation; ROS production, NRF2, KEAP1, NQO1, and PRDX1	Decreased osteoclast differentiation, intracellular ROS levels; Upregulated NRF2 protein expression and nuclear translocation, and increased antioxidant gene expression Antioxidant effects against osteoporosis damage
(Lv et al. 2017)	GYY4137 (100 μM) dissolved in the culture medium, for 4 h + (H_2O_2) (400 μM)	Murine osteoblast-like cell line (MC3T3-E1)	GYY4137	Osteoporosis	Viability, proliferation, Runx2, and apoptosis; NO, ALP, and SOD ERK1/2 activation	Increased viability, cell proliferation, ALP and SOD activities, and Runx2 gene expression; Decreased apoptosis, NO release, and ERK1/2 activation Proliferative and antioxidant effects against osteoporosis damage
(Liu et al. 2017)	NaHS (400 μmol/L) dissolved in the culture medium, for 12 h	Rat primary osteoblasts	NaHS	Osteoporosis	Osteoblast proliferation and mineralization; Apoptosis; KATP protein expression	Decreased cell proliferation, and increased the number of apoptotic cells, osteoblast mineralization, and KATP protein expression Reduction of osteoporosis damage

NaHS natrium hydrogen sulfide, *IL* interleukin, *RA* rheumatoid arthritis, *MAPK* mitogen-activated protein kinase, *ERK* extracellular signal-regulated kinase, *OA* osteoarthritis, *COX-2* cyclooxygenase 2, *MMP* metalloproteinase, *C-28/I2* human chondrocyte cell line, *NF-κB* nuclear factor-κB, *GYY4137* exogenous slow releasing H_2S, *LPS* lipopolysaccharide, *TNF-α* tumor necrosis factor α, *PGE2* prostaglandin E2, *NO* nitric oxide, *iNOS* inducible NO synthase, *PTGES* prostaglandin E synthase, *IκBα* inhibitor of κB, *Akt* protein kinase B, *PI3K* phosphoinositide 3-kinase, *Col2a1* collagen type II alpha 1 chain, *ALP* alkaline phosphatase, *SOD* superoxide dismutase, *NADPH* nicotinamide adenine dinucleotide phosphate, *ROS* reactive oxygen species, *NRF2* nuclear factor erythroid 2-related factor 2, *Keap1* Kelch-like ECH-associated protein 1, *NQO1* NADPH quinone dehydrogenase 1, *PRDX1* peroxiredoxin 1, H_2O_2 hydrogen peroxide, *MC3T3-E1* murine osteoblast-like cell line, *Runx2* runt-related transcription factor 2, *KATP* ATP-sensitive potassium channels

Authors observed that 1 h of NaHS treatment reduced spontaneous and IL-1β-induced secretion of IL-6, IL-8, and RANTES, the expression of MMP-2 and MMP-14, and the phosphorylation of several MAPK proteins. On the contrary, sulfide source increased the phosphorylation of pro-survival factor Akt1/2, suggesting the ability of H_2S to partially antagonize IL-1β stimulation via selective manipulation of the MAPK and the PI3K/Akt pathways.

Later, a similar research was conducted on OA cartilage extracts co-cultured with IL-1β (5 ng/mL) and NaHS or GYY4137 (200 or 1000 μM) for 21 days (Vela-Anero et al. 2017). At the end of the treatment, the histological and immunohistochemical analyses of the samples demonstrated a reduction of catabolic processes and a stimulation of cell anabolism. Indeed, a decrease in glycosaminoglycan destruction and MMP-3 and MMP-13 production caused by IL-1β, in addition to an increased synthesis of collagen type II alpha 1 chain (Col2a1) and aggrecans, was observed in NaHS or GYY4137-treated cells.

All these findings provide new information about the anti-inflammatory, antioxidant, and anti-catabolic properties of H_2S and of its exogenous sources in in vitro cultures. H_2S seems to act as a chondroprotective agent by regulating relevant factors implicated in OA pathogenesis and progression, and counteracting IL-1β pro-inflammatory signals that lead to cartilage destruction.

Furthermore, in 2013, Fioravanti et al. (2013) studied the potential beneficial effect of Vetriolo thermal water (Trentino Alto Adige, Italy), a highly mineralized water, strongly acidic sulfate (SO_4^-), rich in calcium, magnesium, and iron, in human OA chondrocytes cultivated in the presence of IL-1β (5 ng/mL). To better appreciate the properties of mineral water, it has been tested at different concentrations (100%, 50%, 25%), directly dissolved in the culture medium. The Authors showed a significant survival recovery rate, a reduction in NO levels, and expression of iNOS, as well as an enhancement of morphological characteristics of the cells, altered by IL-1β, in chondrocytes treated with 50% and 25% Vetriolo thermal water; these data demonstrated the chondroprotective role of Vetriolo mineral water.

On bone-derived cells, only a limited number of in vitro studies were performed to investigate the properties of exogenous sources of H_2S (Table 2).

Firstly, Xu et al. (2011) showed the proliferative, antioxidant, and anti-inflammatory effects of 4 h of treatment with H_2S donor, NaHS (100 μM), in hydrogen peroxide (H_2O_2) (400 μM)-stimulated murine osteoblast-like cell line. The Authors observed an increased cell viability, cell proliferation (by enhancing alkaline phosphatase activity), and reduced apoptosis, caused by H_2O_2, after NaHS incubation. Furthermore, the H_2S source increased superoxide dismutase activity, while it decreased reactive oxygen species (ROS) production, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, and NO and TNF-α release, probably via p38 and ERK1/2 MAPKs. These results were later confirmed by Lv et al. (2017), in an analogous experimental study, examining the effect of GYY4137 (100 μM) added at the culture medium for 4 h in the presence of H_2O_2 (400 μM).

These findings are in agreement with what is observed in a research performed on human differentiated osteoclasts (Gambari et al. 2014). After an incubation period ranging from 72 h to 6 days in presence of NaHS (50–300 μM), Gambari et al. (2014) found a significant dose-dependent decrease in osteoclast differentiation and intracellular ROS levels, and an upregulation of nuclear factor erythroid 2-related factor 2 (NRF2) activity, related to an increased transcription of the downstream antioxidant genes. Thus, the Authors suggested the key role of NRF2 as a possible mediator of inhibitory effects of NaHS.

In 2017, Liu et al. (2017) pretreated rat primary osteoblast cultures with 400 μmol/L NaHS for 30 min, followed by an incubation in DMEM, with high glucose concentration (HG), for 12 h, before the analysis of cell proliferation, apoptosis, and mineralization. NaHS significantly prevented osteoblast injury induced by HG, through decreasing the rate of cell proliferation, increasing the number of apoptotic cells, and blocking the HG-induced osteoblast mineralization inhibition, via activating ATP-sensitive potassium (KATP) channels.

In vitro studies on lymphocytes, neutrophils, and eosinophils investigating the effects of BT on immune response

The protective effects of H_2S and of its exogenous sources on cellular immune response was firstly investigated by Rinaldi et al. (2006) in a study on purified human neutrophils, eosinophils, or lymphocytes which were treated with NaHS at concentrations ranging from 0.23 to 3.66 mM for 24 h. The Authors found an increased short-term survival of neutrophils delaying the onset of apoptosis, while no changes in lymphocytes or eosinophils were observed. The pro-survival effect of NaHS was due to its inhibitory activity on caspase-3 cleavage and p38/MAPK phosphorylation at the protein level (Table 3).

A similar experimental protocol was performed, 1 year later, by Mirandola et al. (2007) in human purified peripheral blood lymphocytes. The cells were incubated with different concentrations of NaHS (from 0.20 to 4.0 mM), for a time period of 24 h, to examine its role in regulating cell death and cytotoxicity, and its anti-inflammatory properties. At the end of the treatment, a dramatically decreased proliferation of surviving lymphocyte subsets, CD8+ T and NK cells, as well as a reduced IL-2 production, induced in response to mitogens, were observed (Table 3).

Also, Sulen et al. (2016) investigated the ability of H_2S sources to regulated the activation of signaling transduction pathways implicated in immune response. Human peripheral blood mononuclear cells (PBMCs) isolated from healthy

donors were stimulated with NaHS at concentrations of 10, 100, or 1000 μM for 10 min, and the phosphorylation of p38/MAPK, NF-κB p65, AKT, and cAMP response element-binding protein (CREB) was analyzed with flow and mass cytometry. NaHS induced phosphorylation of p38, AKT, and CREB, but not NF-κB. These results provided a description of a NaHS-induced signal transduction pathway in human primary immune cells that may have relevance for the role of sulfides in inflammation (Table 3).

Furthermore, H_2S donors were used to examine their role in mediating the immune response in inflammatory bowel diseases. At this regard, nanomolar levels of Na_2S and GYY4137 (50–500 nM) were employed to treat primary mouse T lymphocytes (CD3+) and OT-II CD4+ T cells at time points of 4, 10, and 24 h to establish whether endogenous H_2S production is required for T cell activation, in mediating inflammatory response in such kind of diseases (Miller et al. 2012). H_2S donors enhanced T cell activation assessed by CD69 expression, IL-2 expression, and CD25 levels, with a maximum capacity at 300 nM. Besides, activation increased the capacity of T cells to synthesize endogenous amounts of H_2S via increased expression of cystathionine γ-lyase and cystathionine β-synthase. These findings lead to define H_2S as an endogenous and exogenous immunomodulatory molecule in T cells signal (Table 3).

The proliferative activity of H_2S donors was also demonstrated in experiments carried out on peripheral blood lymphocytes isolated from patients with systemic lupus erythematosus (Han et al. 2013). Various concentrations of NaHS (0.25, 0.5, 1, 2, 4, and 8 mM) and GYY4137 (200, 400, 800, 1600 μM) were added to the culture medium of the cells for different time points in order to evaluate the cell viability, cell cycle distribution, and expression of proteins involved in pathological pathways regulating autoimmune response. H_2S donors inhibited the abnormal activation and proliferation of lupus lymphocytes through the AKT/GSK3β pathway (Table 3).

Another in vitro study has been performed in activated human neutrophils isolated from blood of healthy donors, and treated with increasing amounts of the sulfurous thermal water of Acqui Terme, Piemonte, Italy, for 15 min (Braga et al. 2008). The cells were stimulated with N-formyl-methionyl-leucyl-phenylalanine and phorbol-12-myristate-13-acetate before and after incubation with sulfurous water, then the luminol-amplified chemiluminescence methodology was used to investigate ROS and reactive nitrogen species (RNS) release. The results showed that this mineral water significantly reduced the luminol-amplified chemiluminescence induced by the stimulus, on average from 0.94 to 15.5 μg/mL of HS (Table 3).

The same Authors performed a similar experiment treating neutrophils for 15 min with different concentrations of the above mentioned sulfurous water or NaHS (Braga et al. 2010); elastase release was evaluated by spectrofluorimetry, and elastolytic activity of the cells was determined by measuring the diameter of the area of elastinolysis on elastine-agarose gel plates. Sulfurous water, at concentrations ranging from 4.5 to 18 mg/mL, and NaHS, from 2.2 to 18 mg/mL, significantly inhibited elastase release but did not show any direct elastolytic activity. This finding revealed the possible contribution of sulfurous water in controlling the inflammatory processes of upper and lower airway diseases (Table 3).

In 2013, Prandelli et al. (2013) used human primary monocytes to test the beneficial effects of Sirmione thermal water (Lombardia, Italy), very rich in sodium chloride, bromide, and iodide, and of NaHS at concentration of 2.5 mM. Thermal water or NaHS was added to the culture medium of the cells for 24 h in the presence or not of 100 ng/mL of LPS, then the release of pro-inflammatory cytokines and the formation of ROS were evaluated. NaHS efficiently blocked the production of TNF-α, IL-1β, IL-6, IL-12, CXCL8, and CCL5 induced by LPS, and limited ROS formation and antioxidant enzymes activity; Sirmione water did not induced the same results, but only enhanced the release of IL-10, probably due to the low concentration of S-based compounds reached at its non-toxic dilution. The Authors attested the anti-inflammatory and antioxidant properties of S-based compounds against the main manifestation of chronic inflammatory and age-related illness (Table 3).

Discussion

The objective of this review was to summarize the current available information about in vitro studies on human and animal samples investigating the biological effects of thermal mineral waters or of their organic and inorganic components, in order to identify the mechanism of action of BT in different pathological conditions.

Increasing evidence corroborated the anti-inflammatory, antioxidant, chondroprotective, and immunosuppressive role of mineral waters or H_2S on some pilot researches, despite the differences in experimental procedures, treatment modalities, and cell cultures employed.

In particular, in human psoriatic keratinocytes, H_2S donors (NaHS and GYY4137) were demonstrated to exert anti-inflammatory and anti-angiogenic effects, confirming the beneficial properties of sulfur components of mineral waters on psoriatic lesions (Gobbi et al. 2009; Mirandola et al. 2011). H_2S sources seem to counteract the inflammatory processes both in arthritic fibroblast-like synoviocytes and chondrocytes, and in OA chondrocytes (Burguera et al. 2014; Fox et al. 2012; Ha et al. 2015; Kloesch et al. 2010; Kloesch et al. 2012a; Kloesch et al. 2012b; Li et al. 2013; Sieghart et al. 2015). Furthermore, OA cartilage might benefit from the exogenous supplementation of H_2S due to their

Table 3 In vitro studies on human and animal samples evaluating the beneficial effects of BT on immune response

Authors	Treatment(s)	Experimental model	Mineral water or inorganic or organic components	Pathology	Biochemical parameters	Results
(Rinaldi et al. 2006)	NaHS (from 0.23 to 3.66 mM) dissolved in the culture medium, for 24 h + p38/MAPK inhibitors (30–60 μM)	Human purified neutrophils, eosinophils or lymphocytes	NaHS	Inflammatory processes of respiratory tract	Cell viability and apoptosis; P38/MAPK signaling activation/-deactivation	Short-term survival of neutrophils delaying the onset of apoptosis Reduced caspase-3 cleavage and p38/MAPK phosphorylation in neutrophils Accelerate the resolution of inflammatory processes
(Mirandola et al. 2007)	NaHS (from 0.20 to 4.0 mM) dissolved in the culture medium, for 24 h + caspase inhibitors (30 μM)	Human purified peripheral blood lymphocytes	NaHS	Inflammatory processes	Cell viability and apoptosis; IL-2	Decreased proliferation of lymphocyte subsets, CD8+ T and NK cells, and reduced IL-2 production Accelerate the resolution of inflammatory processes
(Sulen et al. 2016)	NaHS (10, 100 or 1000 μM) dissolved in the culture medium, for 10 min	Human peripheral blood mononuclear cells (PBMCs)	NaHS	Inflammatory processes	p38/MAPK, NF-κB p65, AKT and CREB phosphorylation	Induced p38/MAPK, AKT, and CREB phosphorylation Reduction of inflammatory processes
(Miller et al. 2012)	H_2S (50–500 nM) dissolved in the culture medium, for 4, 10, and 24 h	Primary mouse T lymphocytes (CD3+), OT-II CD4+ T cells	H_2S	Inflammatory processes of bowel diseases	CD69, CD25; IL-2; cystathionine γ-lyase, cystathionine β-synthase	Enhanced T cell activation, CD69, and IL-2 expression, and CD25 levels; Increased cystathionine γ-lyase and cystathionine β-synthase expression Reduction of inflammatory processes of bowel diseases
(Han et al. 2013)	NaHS (0.25, 0.5, 1, 2, 4 and 8 mM) and GYY4137 (200, 400, 800, 1600 μM) dissolved in the culture medium, for 0.5, 1, 2, 4, 6, 12, 24, 36, 48 h	Human purified peripheral blood lymphocytes	NaHS and GYY4137	Inflammatory processes of systemic lupus erythematosus	Cell viability, cell cycle distribution; AKT (ser473), GSK3β (ser9), $p27^{Kip1}$ and $p21^{CIP1}$	Increased cell proliferation and S phase distribution of cell cycle; Decreased AKT (ser473), GSK (ser9), and increased p27Kip1 and p21CIP1 expression and phosphorylation Reduction of inflammatory processes of systemic lupus erythematosus
(Braga et al. 2008)	Sulfurous thermal water (different concentrations) dissolved in the culture medium, for 15 min + N-formyl-methionyl-leucyl-phenylalanine/-phorbol-12-myristate-13-acetate	Human purified neutrophils	Sulfurous thermal water (Acqui Terme, Piemonte, Italy), which contains different HS groups concentrations	Inflammatory processes	ROS and RNS	Reduced ROS and RNS release at 0.94 to 15.5 μg/mL of HS Reduction of inflammatory processes
(Braga et al. 2010)	Sulfurous water or NaHS (from 4.5 to 18 mg/mL) dissolved in the culture medium, for 15 min	Human purified neutrophils	Sulfurous thermal water (Acqui Terme, Piemonte, Italy) and NaHS	Inflammatory processes of upper and lower airway diseases	Elastase release; elastolytic activity	Inhibited elastase release Reduction of inflammatory processes
(Prandelli et al. 2013)	Sirmione thermal water or NaHS (2.5 mM) dissolved in the culture medium, for 24 h + LPS 100 (ng/mL)	Human primary monocytes	Sirmione thermal water (Lombardia, Italy), rich in sodium chloride, bromine and iodine	Chronic inflammatory and age-related illness	TNF-α, IL-1β, IL-6, IL-12; CXCL8, CCL5; ROS, antioxidant enzymes	NaHS reduced TNF-α, IL-1β, IL-6, IL-12, CXCL8, CCL5 production, ROS formation and antioxidant enzymes; Sirmione water enhanced IL-10 release Reduction of chronic inflammatory and age-related illness manifestations

NaHS natrium hydrogen sulfide, *MAPK* mitogen-activated protein kinase, *IL* interleukin, *NK* natural killers, *H_2S* hydrogen sulfide, *CD3* cluster of differentiation 3, *CD4* cluster of differentiation 4, *CD69* cluster of differentiation 69, *CD25* interleukin-2 receptor alpha chain, *GYY4137* exogenous slow releasing H_2S, *Akt* protein kinase B, *GSK3β* glycogen synthase kinase-3β, *$p27^{Kip1}$* cyclin-dependent kinase inhibitor 1B, *$p21^{CIP1}$* cyclin-dependent kinase inhibitor 1, *PBMCs* human peripheral blood mononuclear cells, *NF-κB* nuclear factor-κB, *CREB* cAMP response element-binding protein, *ROS* reactive oxygen species, RNS reactive nitrogen species, *LPS* lipopolysaccharide, *TNF-α* tumor necrosis factor α, *CXCL8* chemokine (C-X-C motif) ligand 8, *CCL5* Chemokine (C-C motif) ligand 5

additive antioxidant and chondroprotective properties (Burguera et al. 2014; Fox et al. 2012; Ha et al. 2015; Li et al. 2013; Sieghart et al. 2015). The ability of H_2S donors to limit the oxidative stress damage was also confirmed in human primary or transformed cell lines of osteoblasts and osteoclasts (Gambari et al. 2014; Liu et al. 2017; Lv et al. 2017; Xu et al. 2011).

Moreover, the sulfide compounds seem to regulate inflammation and immune response in purified human peripheral blood neutrophils, eosinophils or lymphocytes (Braga et al. 2010; Miller et al. 2012; Mirandola et al. 2007; Rinaldi et al. 2006; Han et al. 2013; Sulen et al. 2016).

Finally, a limited number of studies attested the beneficial effect of different thermal mineral waters, tested as a whole, in human psoriatic keratinocytes, primary neutrophils and monocytes, and OA chondrocytes (Braga et al. 2008; Braga et al. 2010; Chiarini et al. 2006a; Chiarini et al. 2006b; Dal Pra et al. 2007; Fioravanti et al. 2013; Karagülle et al. 2018; Lee et al. 2012; Prandelli et al. 2013); furthermore, Gerencsér et al. (2019) confirmed the beneficial effects of organic rich extract of selected medicinal waters in protecting human keratinocytes from UV radiation-related DNA damage. The results demonstrated the anti-inflammatory and chondroprotective activities of thermal waters, as well as their antioxidant and immunomodulatory functions.

Taken together, these results demonstrated the simple, detailed and convenient analysis that can be made in in vitro testing, underlining the usefulness of these experimental studies in investigating how thermal waters, their mineral elements or their organic fraction are able to regulate cell metabolism under physiological or pathological stimuli. This approach allows to understand the specific effect of a mineral water or a particular inorganic or organic component, in terms of mechanisms of action and/or toxicity. Besides, the in vitro studies could be used to select the best required concentrations of a specific compound necessary for obtaining optimal biological benefits.

However, the validity of an in vitro study is conditioned by the adoption to the most appropriate experimental setup.

The choice of the suitable cell culture represents an important and essential key point to obtain the best experimental performance in terms of biological response. Although the high experimental repeatability and reproducibility of standardized and immortalized cell lines, primary cultures should be preferred to be carried out in in vitro studies since they are directly derived from the original tissue, and are heterogeneous and significant in terms of being the closest forms of the state of the cells that they represent in physiological or pathological tissues (Isyar et al. 2016). In any case, it is needed to keep in mind that primary cells have a limited lifespan and they stop dividing after a certain number of cell divisions and can be difficult to culture and maintain. The heterogeneity induced by the isolation of the cells from different donors could represent a source of experimental variability. Thus, the identification of the most suitable cell model for a specific pathological state, together with the maintenance of the optimal culture conditions, is required to obtain the best in vitro results, limiting possible bias (Khan and Gasser 2016).

Moreover, when the test is relative to a thermal water as a whole, it is needed to perform an adequate and accurate sterilization, without alter its physical-chemical characteristics, to avoid the possible contamination of the cultures, which can influence or interfere with the performance of the experiments (Lee et al. 2012).

However, the relevance of the preclinical tests needs to be considered with caution, since the changes that occur in the cells do not necessarily reflect in vivo conditions. Indeed, when cells are taken out from their natural environment and transferred into the culture, they are subtracted from a series of information or local and general interferences, and might lose their structural and functional characteristics (Cheleschi et al. 2017; De Palma et al. 2018; Fioravanti et al. 2010; Johnson et al. 2016; Montagne et al. 2017; Ogura et al. 2019; Pascarelli et al. 2015).

Furthermore, it is necessary to underline that the beneficial effects of BT in vivo are partially related to the temperature of the mineral water, while all the above-described in vitro studies have been performed at the physiological temperature of 37 °C, because supraphysiological temperatures could represent a source of bias in terms of cell viability and metabolism (Castander-Olarieta et al. 2019; Kaplan et al. 2003; Mead et al. 2012).

Another limitation of in vitro models in assessing the potential beneficial and/or toxic effects of thermal mineral waters or chemical or organic components is related to the purification and filtration procedures which could remove the natural spring water microbiome, recently considered to be responsible for some positive effects of BT in different diseases (Nicoletti et al. 2019; Pedron et al. 2019; Varga 2019).

The intent of this review served to summarize the current knowledge and advance about the in vitro research in the field of BT, discussing its strength and drawbacks. Taken together, the results of our analysis show that the in vitro studies take the advantage to represent simplified biological systems, useful to improve the knowledge about the potential mechanism of action of mineral water, tested as a whole or as singular mineral element or as organic fraction, at the molecular level. However, the usefulness of the culture models is limited by several sources of bias. Their validity is conditioned by a correct experimental procedure, which should take into consideration the particular and complex composition of mineral waters. Nowadays, analyzing the effects of the only inorganic composition of thermal waters is not enough, but it is necessary to perform a complex chemical analysis which should also consider their organic fraction that may play a role in the therapeutic efficacy or in other biological mechanisms as

toxicity (Varga 2016). Another important critical point is related to the high heterogeneity existing among the available scientific studies that makes urgent the need to standardize the experimental procedures. Finally, the in vitro research does not allow to consider all the chemical, organic, and physical factors, as heat or microbiome influence, responsible for the clinical efficacy of BT. A possible solution to overcome some of these criticisms could be to investigate different treatment groups: (1) negative control, (2) positive control, (3) group of treatment with thermal water as a whole, (4) group of treatment with inorganic ingredients, and (5) group of treatment with organic extract from the original water (Varga 2016).

On the basis of these considerations, we would like to stimulate the following reflection: the studies on in vitro models could open the way to the scientific progress in the field of BT, but there is still a lot to do especially to engage the scientific community and to develop new research projects.

The future experimental strategies could also help us, in clinical practice, to identify the best personalized approach for each patient and for each pathological condition.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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