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Research Paper

Do Mineral Waters Have Any Chondroprotective Effects? Evidence From *in vitro* Studies

Medical Science

KEYWORDS : Balneotherapy, Chondrocyte, Cartilage, Mineral Water, Hydrogen Sulphide, Osteoarthritis

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ABSTRACT

The objective of this review was to summarise the information on the possible chondroprotective effects of mineral waters or mineral components on chondrocyte or cartilage cultures. We conducted a search of the literature in april 2015 using PubMed, PED-ro, Scopus, and Web of Science (period examined was 2000–2015). A possible chondroprotective role of mineral waters or mineral components was demonstrated by some pilot studies in chondrocyte cultures. Many authors investigated the activity of different hydrogen sulphide (H_2S) donors in normal or osteoarthritic (OA) chondrocytes stimulated by Interleukin (IL)-1β or lipopolysaccharide (LPS). The incubation with exogenous H_2S sources inhibits the release of Nitric oxide (NO), Prostaglandin (PG)-E2, Metalloproteases (MMPs), Tumor Necrosis Factor (TNF)-α, IL-6, IL-8 and regulates the expression of genes in OA pathogenesis and progression by reducing Nuclear Factor (NF)-kB activation. One study evaluated the role of a highly mineralized water in human OA chondrocytes exposed to IL-1β. The mineral water significantly reduced the NO production, the expression of NO synthase (NOS) and the apoptosis induced by IL-1β. The data presented showed a chondroprotective activity of mineral waters or a singular mineral element and elucidate their potential mechanism of action at molecular level. These reports represent an important contribution to understanding the clinical efficacy of balneotherapy in OA.

Introduction

Balneotherapy is one of the most commonly used non-pharmacological approaches for rheumatic diseases (RDs) in many European and Middle Eastern countries. [1] Thousands of years of history and the abundance of spa resorts in many European countries have undoubtedly contributed to the popularity of these therapies. However, despite its long history and popularity, balneotherapy is still the subject of debate, and its role in modern medicine is still not clear. [2,3] Balneotherapy uses natural thermal mineral waters, whose definition is based on the sum of the cations Na^+, K^+, Ca^{2+} and Mg^2 and the anions of $SO4^{2-}$, Cl^- and $HCO3^-$. It was presumed that most mineral ingredients would be absorbed through the skin, which is an active immune organ and may play an important role in the mechanism, but to date, this theory has not yet been confirmed. [4] In recent decades, more evidence have been published confirming the therapeutic effects of baths or mud-baths, especially in osteoarthritis (OA). [5-10] The results of the randomized clinical trials suggest a positive effect on pain, functional capacity and quality of life in OA. The studies aimed to assess the medium- long- term effect found that, the clinical efficacy of balneotherapy does last over time, until 6 months after the treatment. However, the mechanism by which mineral waters improve the symptoms of OA is still not fully understood. [11]

Destruction of articular cartilage is a common feature in OA; the structural breakdown of Proteoglycans (PG) and collagen is believed to be the result of an imbalance between anabolic and catabolic activities of chondrocytes. Chondrocytes synthesize an Extracellular Matrix (ECM) of PG, collagen, and other non-collagen proteins, which constitutes a dense tissue that is able to support *in vivo* the effects of the mechanical load. [12] Chondrocytes also have a rich enzymatic set (metalloproteases (MMPs), cathepsins, and serine proteases) able to degrade the ECM components. The metabolic activity of these cells is regulated by several mediators, such as cytokines, chemokines, hormones, and growth factors, produced locally by the chondrocytes themselves and also by neighboring tissues. [12] Chondrocyte functions are influenced by the composition of ECM and of the extracellular environment (O_2 tension, ionic concentration, pH, and so on). [13-15]

In vitro studies on chondrocytes or cartilage cultures represent biological systems which allow us to evaluate the effects and/or the mechanisms of action of physical or chemical factors on cartilaginous metabolism.

The objective of this review was to summarise the currently available information on possible chondroprotective effects of mineral waters or mineral components on chondrocyte or cartilage cultures.

Methodology

We conducted a search of the literature in april 2015 by systematically looking through PubMed, PED-ro, Scopus, Web of Science, (the period examined was 2000-2015) using the terms "chondrocyte" and/or "cartilage" in combination with "mineral water", "hydrogen sulphide", "osteoarthritis".

We included only the papers published in English.

Results

A possible chondroprotective role of mineral waters or mineral components was demonstrated by some pilot studies on chondrocyte cultures. Li *et al.* [16] studied the role of hydrogen sulphide (H_2S) using a slow releasing H_2S donor, GYY4137, in human normal chondrocytes stimulated by lipopolysaccharide (LPS). Treatment with GYY4137 (0.1-0.5 mM) for 1 hr prior the stimulation with LPS significantly decreased the levels of pro-inflammatory mediators, Nitric Oxide (NO), Prostaglandin E2 (PGE2), Tumor necrosis Factor (TNF)-α and Interleukin (IL)-6, in the culture medium. Furthermore, GYY4137 reduced the levels and catalytic activity of NO Synthase (NOS) and Cyclooxigenase 2 (COX-2). Also, since the activation of Nuclear Factor (NF)-kB is directly responsible for the upregulation of several proinflammatory cytokines and enzymes, including NOS, COX-2, TNF-α and IL-6. [17] The authors investigated this signaling route as a possible mechanism of action for H_2S. GYY4137 re-

duced, in a significant manner, the LPS-induced NF-kB activity and activation.

Another sulphur-releasing molecule, Natrium Hydrogen Sulphide (NaHS), a fast dissolving salt, was investigated in human chondrocytes cell line C-28/I2 culture. [18] These cells constitutively secrete large amounts of IL-6 and IL-8; furthermore, stimulation of cells with IL-1β lead to a dramatic up-regulation of these genes. Because the mitogen-activated protein kinase (MAPK) are well known stress-activated, kinases implicated in the expression of many genes responsible for inflammatory and immune response, [19,20] Kloesch *et al.* [18] in this study compared the anti-inflammatory property of NaHS with two well known MAPKs inhibitors (SB203580 and U0126). Constitutive and IL-1β induced IL-6 and IL-8 expression were evaluated after treatment of C-28/I2 cells with SB203580 and U0126 or with NaHS (0.125 and 1.0 mM). The results showed that constitutive as well as IL-1β-induced IL-6 and IL-8 expression were partially and transiently blocked by the treatment of cells with both MAPK inhibitors and NaHS. These data seem to be important in evaluating the beneficial functions of MAPK inhibitors and H_2S in immune–pathophysiological processes and in the possible therapeutic effect of sulphur baths.

Burguera *et al.* [21] studied the possible activity of H_2S in human OA chondrocytes stimulated with IL-1β used as a prototype pro-inflammatory cytokine to reproduce the "OA-like effect". In this study, two exogenous sources of H_2S, NaHS and GYY4137 were added to cultures of IL-1β -stimulated human articular chondrocytes isolated from OA tissue. Concentrations of the H_2S sources ranged from 0.05 to 1 mM and their effects on several markers of inflammation and cartilage degradation were investigated. After incubation with H_2S donors the authors demonstrated a significant reduction of NO, PGE2, IL-6 and MMP-13 released in culture medium by the cells stimulated with IL-1β and evaluated by quantitative real-time (qRT)-PCR. This was achieved by down-regulation of relevant genes involved in the synthesis routes of these molecules, namely NOS, COX-2, prostaglandin E synthase (PTGES), IL-6 and MMP-13. Additionally, the H_2S compounds used in the present model seem to reduce the translocation of NF-kB to the chondrocytes nuclei, although not to prevent it completely.

In 2015 April Ha *et al.* [22] published the results obtained from-human OA chondrocytes cultured and pre-treated with H_2S (0.06-1.5 mM) with or without IL-1β (10 ng/ml). H_2S markedly reversed the effects of IL-1β on the gene expression of COX-2, MMP-13 and NOS and on the production of MMP-13, PGE2 and NO. In addition, H_2S inhibited the activation of the extracellular signal-regulated kinase (ERK)/IκBα/NF-κB pathway which was induced by IL-1β.

Collectively, these results showed that supplementation with exogenous H_2S sources can regulate the expression of relevant genes in OA pathogenesis and progression, counteracting IL-1β pro-inflammatory signals that lead to cartilage destruction in part by reducing NF-kB activation.

Fioravanti *et al.* [23] studied the possible chondroprotective role of Vetriolo's thermal water (VW) known for over 150 years for its therapeutic properties in the treatment of OA. [24] This is a highly mineralized water, strongly acidic sulfate, rich in calcium, magnesium and iron. The study aimed to evaluate the *in vitro* effects of VW at concentrations of 25%, 50%, and 100% on human OA chondrocytes incubated in the presence or absence of IL-1β during 48 h of culture, in order to evaluate the cartilage metabolism using biochemical and morphological tests. For this purpose, OA chondrocytes were cultivated in Deionized Water (DW) (DW-DMEM, controls), or in one of three different VW-DMEM media, in which DW had been totally (100%) or in part (50% or 25%) substituted with VW. The results showed that VW alone at 25% or 50% concentration did not affect the viability of cultured OA chondrocytes, and determined a significant survival recovery rate in cultures stimulated with IL-1β. NO levels were low both in DW-DMEM cultures and in those reconstituted with 25% or 50% of VW, and were significantly increased by IL-1β. VW at 25% or 50% concentration significantly ($P<0.001$) reduced the NO production induced by IL-1β. The data of the NO levels were confirmed by the immunocytochemistry assay for inducible Nitric Oxide Synthase NOS. Furthermore, the authors confirmed the pro-apoptotic effect of IL-1β and demonstrated a protective effect of VW at 25% or 50% concentration. The results concerning biochemical data were further confirmed by the morphological findings obtained by a transmission electron microscope, in agreement with other morphological study. [25,26] OA chondrocytes cultivated in presence of IL-1β displayed an altered ultrastructure with numerous vacuoles in the cytoplasm, where rough endoplasmic reticulum, Golgi bodies and mitochondria were strongly reduced; in the cultures reconstituted with VW 20% and 50% the ultrastructural features of chondrocytes were similar to that observed in control cells. In conclusion, this study demonstrated that VW alone at 25% or 50% inhibits the negative effects of IL-1β in chondrocytes cultures. The observed effects of VW seem attributable to the presence of iron, however at present we cannot exclude the effects of other mineral components such as sulphate, calcium or magnesium.

In a recent experience Sieghart *et al.* [27] investigated the effects of exogenous H_2S on fibroblast-like synoviocytes (FLS), which are key players in OA pathogenesis being capable of producing pro-inflammatory cytokines and matrix degrading enzymes. To address this issue primary FLS derived from OA patients were stimulated with IL-1β and treated with the H_2S donor NaHS. Cellular responses were analysed by ELISA, qRT-PCR, phospho-MAPKs array and Western blotting. NaHS treatment reduced both spontaneous and IL-1β -induced secretion of IL-6, IL-8 and RANTES in different experimental settings. In addition, NaHS treatment reduced the expression of MMP-2 and MMP-14. Since expression of IL-6 and several MMPs partially depends on the activation of the MAPKs pathway, [19] the authors investigated the effects of NaHS on IL-1β -induced activation of MAPKs. The analysis revealed that IL-1β induced in a statistically significant manner the phosphorylation of several MAPK. Treatment with NaHS reduced the IL-1β-induced phosphorylation of multiple kinases, whereas it increased phosphorylation of pro-survival factor Akt1/2. Moreover, the authors investigated, in three-dimensional extracellular matrix micromasses, cellular structure and synovial architecture. When cultured in spherical micromasses, FLS intentionally established a synovial lining layer-like structure; stimulation with IL-1β altered the architecture of micromasses leading to hyperplasia of the lining layer which was completely inhibited by concomitant exposure to NaHS. These data suggest that H_2S partially antagonizes IL-1β stimulation via selective manipulation of the MAPK and the PI3K/Akt pathways, may encourage development of novel drugs for treatment of OA.

Effect of thermal mineral sulphur waters on various mediators of cartilage degradation or inflammation (Table 1).

Conclusions

The efficacy of hot springs in OA have been known for century and recently confirmed by various randomized clinical trials, [5-8,10] however the beneficial effects of this therapy are often considered in terms of the subjectiveness of wellbeing. Scientific experimental reports about the mechanisms of action of mineral waters are rare and controversial. The aim of this review was to summarise the currently available information obtained by *in vitro* studies on the possible chondroprotective effects of mineral waters.

In vitro studies on chondrocytes, cartilage or synoviocytes cultures are simplified biological systems that allow us to evaluate the effects and/or the mechanism of action of a physical (mechanical compression, hydrostatic pressure, ultrasound, magnetic or electromagnetic fields, etc) or chemical stimulus (cytokines, growth factors, drugs, mineral elements, etc). [28-32]

The chondroprotective action of any kind of stimulus depends upon its capacity to shift the equilibrium between phenomena of degradation and that of repair, in favor of the last.

The *in vitro* models, present an intuitive series of limits. [33,34] *In vitro* models only reproduce a small part of the physiopathology of chondrocytes and cartilage, as these cells and the ECM are removed from their natural environment and therefore are subtracted from a series of information and local and general interferences. The information and interferences are also mutable from one moment to the next and are therefore difficult to reproduce *in vitro*. In fact, in *in vivo* conditions, there are influences of an imprecise number of hormonal substances and of local mediators, only partially understood, which can act on the level of the normal or pathological chondrocyte, modifying its functional aspects. Another limitation of *in vitro* experimentation is the lack of effects linked to movement and joint load that are very important for the modality of afflux and outflow of anabolic and catabolic material in the cartilagineous matrix. It has also been demonstrated that mechanical factors can modify the metabolism and the morphology of chondrocytes. [15, 30, 35, 36] The results of *in vitro* studies are then clearly influenced by some characteristics relative to the "material" utilized for preparing the culture, such as the origin (animal or human) of the cartilage and the age of the patient or the animal from which it was taken and, above all, the conditions (normal or pathological) of the tissue. [34]

Furthermore, for the specific context of this review we have to consider the controversial and unsolved problems of the absorption and the concentrations reached by minerals dissolved in thermal waters to joint space *in vivo*. Unfortunately, no study has been conducted on this topic.

In conclusion, the presented data showed a chondroprotective activity of mineral waters or singular mineral element and elucidate their potential mechanism of action at the molecular level. These reports represent an important contribute to understand the clinical efficacy of balneotherapy in OA. However, it is impossible to ignore the existence of a complex series of problems and limits that make it important to take caution when extrapolating these *in vitro* results to *in vivo* situations.

Further studies are needed *in vitro* to confirm these preliminary findings and to further clarify the possible chondroprotective effects of mineral waters or mineral components *in vivo*.

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Table 1. Effect of thermal mineral sulphur waters on various mediators of cartilage degradation or inflammation

Author	H_2S donors	Proteases	Cytokines	Inflammation	Pathways involved
Li et al. [16]	GYY4137	-	↓ TNF-α, IL-6	↓ NO, PGE_2, NOS, COX-2	↓ NF-KB activation and activity
Kloesch et al. [18]	NaHS	-	↓ IL-6, IL-8	-	-
Burgera et al. [21]	NaHS, GYY4137	↓ MMP-13	↓ IL-6	↓ NO, PGE_2 synthase, NOS, COX-2	↓ NF-KB traslocation
Ha et al. [22]	H_2S	↓ MMP-13	-	↓ NO, PGE_2, NOS, COX-2	↓ NF-KB activation
Sieghart et al. [27]	NaHS	↓ MMP-2, MMP-14	↓ IL-6, IL-8	-	↓ MAPKs phosphorilation ↓ AKT 1/2

Legend:
H_2S = Hydrogen Sulfide; GYY4137 = slow-releasing water soluble H_2S donor; TNF-α = Tumor Necrosis Factor α; IL= Interleukin; NO = Nitric Oxide; PGE_2 = Prostaglandin E_2; NOS = Nitric Oxide Synthase; COX-2 = Ciclooxygenase 2; NF-KB = Nuclear Factor KB; NaHS = Sodium Hydrosulfide; MMP = Metalloproteinase

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