

## Alternative therapy of earth elements increases the chondroprotective effects of chondroitin sulfate in mice

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Accepted 5 June 2005

Abbreviations: CS, chondroitin sulfate; DAS, disruption of articular surface; DRT, duplication and rupture of the tidemark; FA, fibrillar appearance; GAG, glycosaminoglycans; HC, hypocellularity of chondrocytes; HE, haematoxylin; IGF-1, Insulin-like Growth Factor-1; LI, labelling index; MLSS, moderate loss of safranin-O staining; MMP, matrix metalloproteases; NO, nitric oxide; OA, osteoarthritis; PSR, picro sirius red; ROS, reactive oxygen radicals; SLSS, severe loss of safranin-O staining; TGF  $\beta$ , Transforming Growth Factor  $\beta$

### Abstract

The administration of mineral sulphur water is an alternative experimental approach for the treatment of rheumatic diseases, such as osteoarthritis (OA), that cause the degeneration of bone and cartilage and sufferance to the patients. Chondroitin sulfate (CS) is a symptomatic slow acting nutraceutical agent currently used in molecular therapy of OA. Therefore, we have studied the role and efficacy of the selective soil paste from the mineral sulphur enriched spring (mud)-therapy alone or in combination with CS in the treatment of OA. The study was performed on 40 C57 Black 6N mice, an experimental model which spontaneously develop an osteoarthritic process. The animals were divided in 4 groups and were treated with the single agents or with the combination. After

30 days of treatment all the mice were sacrificed and right knees and blood were collected. It was found that CS determined a reduction of radiological and histological features of chondrodegeneration and that mud-therapy increased the effects of CS in the animal group treated with the combination. However, the effects of thermal therapy alone were not statistically significant. Since OA is characterized by an increase of the production of nitric oxide (NO) by chondrocytes in extracellular matrix with its consequent elevation in serum and synovial fluid, we have evaluated the effects of the treatments on serum NO levels. CS alone induced a statistically significant reduction of NO serum levels ( $90 \pm 13 \mu\text{M}$  vs  $219 \pm 60 \mu\text{M}$  of control group,  $P < 0.05$ ) while mud-therapy alone induced a not statistically significant reduction of serum NO ( $170 \pm 62 \mu\text{M}$ ,  $P > 0.05$ ). However, the latter strongly potentiated the decrease of serum NO induced by CS ( $31 \pm 1.5 \mu\text{M}$ ) with a high statistical significance if compared to both the control group ( $P < 0.01$ ) and the CS-treated group ( $P < 0.05$ ). In conclusion, this study demonstrates that mud-therapy with sulphur mineral water could represent an important phase of the therapeutic strategy of OA. This experimental strategy could integrate and potentiate the standard pharmacological tools. Moreover, we have set a valid experimental *in vivo* model for the study of the thermal effects on the development of OA.

**Keywords:** chondroitin sulfate; mud therapy; nitric oxide; osteoarthritis; sulfur

### Introduction

In recent years it is becoming more evident that the demand of a scientific validation of thermal therapy according to the modern experimental medicine methods in order to give scientific validation to the so-called Thermal Medicine is increasing. Until today the mechanisms of action and the activity of thermal therapy (mineral waters, mud baths and caves) in the control of rheumatic diseases are only partially known.

Osteoarthritis (OA) is one of the diseases that can endow the therapeutic action of mud-therapy (Costantino *et al.*, 1999; Costantino *et al.*, 2001; Nappi *et al.*, 2001; Flusser *et al.*, 2002; Van Tubergen *et al.*, 2002). The latter could be an additional strategy able to obtain

a reduction of analgesic and/or non steroidal anti-inflammatory drugs consumption with a consequent amelioration of the quality of life and of the pharmaceutical costs.

On the basis of these considerations we have evaluated the chondroprotective action of a cycle of mud-therapy with sulphur mineral water alone or in combination with the chondroitin sulphate (CS), a currently used chondroprotective pharmacological agent. In addition, we have investigated if this therapy could induce adverse reactions and we have setted a useful *in vivo* experimental model for the study of the thermal therapy effects on the development of OA.

## Materials and Methods

### *In vivo* studies

The fine soil paste (mud) used for this study was the mineral sulphur water of Rosapepe Spa in Contursi (Salerno, Italy), derived from a mineral water that, for its chemical and physical characteristics, could be considered like bicarbonate alkaline sulphur mineral water (Table 1) (Marotta and Sica, 1929; Nappi *et al.*, 2001). The experiments were performed on 40 Black 6N Mice C57 of both sexes (not pregnant). In details, we have used 20 males, with mean weight of  $30 \pm 0.5$  g and 20 females with mean weight of  $28 \pm 0.5$  g. The mice C57B 6N are genetically altered in order to spontaneously develop a osteoarthritic process (Wilhelm *et al.*, 1976). All the animals were maintained in fit conditions of nutrition and surrounding ( $T = 21^\circ\text{C}$ ; humidity ratio = 55% 10). European Union guidelines for the care of animals used for experimentation and other scientific purposes (Guideline 86/609/CCC and R.D. 223/88 B.O.E.) were as follows. After 30 days of treatment each mouse, after anesthesia with intraperitoneal injection of ketamine (80 mg/Kg), has been sacrificed and blood samples were collected in order to determine the levels of serum nitric oxide (NO) and their right knee joints were immediately extracted.

### Radiology

Forty knee joints were radiographed with a micro-radiography unit (Faxitron; HewlettePackard, Rockville, MD) and high-detail film (Min-R; Eastman Kodak, Rochester, NY). Antero-posterior images from each specimen were obtained to further analyze them based on knee-joint OA criteria suggested by Shimizu *et al.* (1993).

### Tissue preparation

Forty tibial epiphyses were fixed in 4% neutral buffered formalin for one day, decalcified in 10% EDTA, sec-

**Table 1.** Chemical, physical and chemical-physical characteristics of the mineral water from Rosapepe terme.

|  |         |
|--|---------|
| <i>General organological characteristics:</i>                  |         |
| 1) Aspect = clear  |         |
| 2) Favour = sulphureous  |         |
| 3) Taste = slightly saline                                     |         |
| <i>Microbiological test:</i> bacteriologically pure            |         |
| <i>Chemical-physical characteristics:</i>                      |         |
| 1) temperature of water at the well = $+ 37.6^\circ\text{C}$ ; |         |
| 2) temperature of air outside = $+ 4^\circ\text{C}$ ;          |         |
| 3) Electric conductivity = 4.37 m/S;                           |         |
| 4) thickness = 1.002;  |         |
| 5) pH = 6.62   |         |
| <i>Chemical characteristics:</i>                               |         |
| 1) fixed residue at $110^\circ\text{C}$ = 2999 mg/l;           |         |
| 2) fixed residue at $+ 180^\circ\text{C}$ = 2957 mg/l;         |         |
| 3) tot. alkalinity (in HCl N/10/l) = 362 ml;                   |         |
| 4) total hardness in French degrees = 178.8;                   |         |
| 5) ammonia = traces;   |         |
| 6) nitrites = traces;  |         |
| 7) nitrates = present  |         |
| <i>Gas dissolved in 1 lt of water:</i>                         |         |
| 1) Carbonic anhydride at well = 1408.0 mg/l                    |         |
| 2) sulphydric degree = 15.25 mg/l                              |         |
| <i>Analysis of residue in mg/l:</i>                            |         |
| sodium   | 420     |
| potassium  | 65      |
| calcium  | 525.32  |
| magnesium  | 115.57  |
| lithium  | 1.00    |
| chlorine   | 567.25  |
| iron   | 0.04    |
| manganese  | 0.13    |
| fluorine   | 0.18    |
| barium   | absent  |
| hydrogen carbonate   | 2208.20 |
| sulfites   | 243     |
| silica   | 12.6    |
| bromine  | trace   |
| iodine   | trace   |

*Classification:* IPER-THERMAL SULPHUR SODIC-CHLORIDE BICARBONATE ALKALINE MINERAL WATER

tioned in frontal planes, and then paraffin-embedded for further routine histopathology analysis with light microscopy. Serial frontal 5 mm sections were cut and placed on poly-L-lysine treated slides or on non-treated slides.

### Histomorphology and histochemistry

From all samples haematoxylin (HE), alcian blue PAS,



and safranin stains were performed in order to evaluate matrix abundance, cellularity and the content of glycosaminoglycans. Two sections of each specimen were stained with picro sirius red (PSR) (Junqueira *et al.*, 1979) and analyzed by polarized light microscopy to evaluate the arrangement of collagen bundles. Articular cartilage was evaluated following Mankin *et al.*'s guidelines (Mankin *et al.*, 1971). To evaluate articular cartilage four zones are defined: superficial, transitional, radial and calcification (Hunziker *et al.*, 1992) within this tissue layer usually appear collapsed. The borderline between hyaline articular cartilage and the zone of calcified cartilage is called the tidemark.

### TUNEL

For *in situ* visualization of apoptotic cells (APOC cells), the TUNEL method was performed following the method described by other authors (Gavrieli *et al.*, 1992). In both immunohistochemistry and TUNEL stainings, positive cells were counted throughout three microscopic magnified fields (40x) of the central region of each articular cartilage. Only dark brown cells were considered as positive in these counts. The labeling index (LI) was calculated as the percentage of positive cells in each cartilage zone.

### Treatment schedule

At the beginning of the experimental work the animals were divided into 4 experimental groups (A, B, C, and D). Each group consists of 10 animals, all including 5 males and 5 females (Figure 1):

The A group has been assumed as control group and have not subjected to any treatment. Control animals received similar application of distilled water to animals receiving mud-therapy and were injected with equal volume of 0.8% NaCl solution to animals receiving CS. The B group has been treated with a mud-therapy cycle with mineral sulphur water including 12 applications, administered once a day and lasting 15 min at the temperature of 40°C; The C group was treated with orally administered CS at the dose of 0,3 mg/day for 12 consecutive days; The D group was treated with a combination of mud-therapy with mineral sulphur water + CS for 12 consecutive days, as described above.

### NO spectrophotometric determination

The determination of serum NO concentration, expressed in  $\mu\text{M}$ , has been performed with an indirect method through the measure of serum concentrations of nitrates and nitrites. For NO determination the spectrophotometric reaction of Griess was used (Tithera-dge, 1998).

### Statistical analysis

The mean and standard deviations of each variable were calculated. The comparison between groups was performed using the Mann-Whitney U-test, which tests differences in distribution of ranks.

## Results

### Radiographic examination

Knee-joint space narrowing with marked sclerosis of subchondral bone and irregularity of joint surface were seen in 100% of the control group and in 80% of mice treated with only mud-therapy ( $P > 0.05$ ). However, these alterations were observed only in 60% of mice treated with CS ( $P < 0.05$ ) and in 30% ( $P < 0.01$ ) of mice treated with CS plus mud therapy (Figure 1). Thereafter, the treatment with mud therapy alone did not seem to prevent chondrodegenerative processes, but it increases chondroprotective effects of CS.

### Histological observations

The control group showed progressive histopathological changes characteristic of developing OA. These included disruption of the articular surface (19.2%), cleft (63.5%), cloning (80.8%), hypocellularity of chondrocytes (16.2%), moderate (50.6%) to severe (40.4%) loss of safranin-O staining, and duplication and rupture of the tidemark (54.5%) (Table 2). In addition, a superficial layer with a fibrillar appearance was seen in 20% of the cases. Degenerative areas with decreased metachromasia and indicative of matrix breakdown were also observed. These morphological changes were not significantly reduced in the mice treated with mud ther-

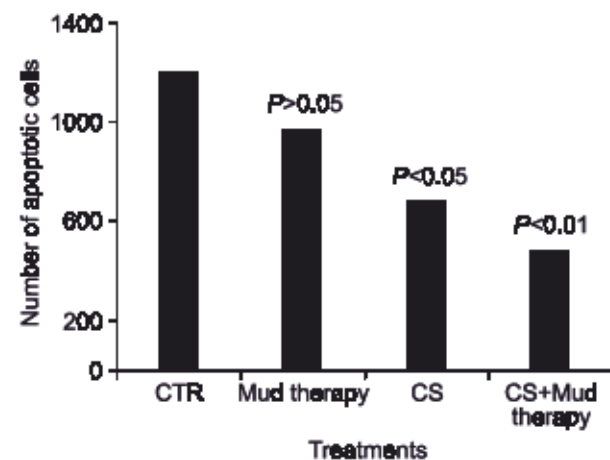


Figure 1. Effects on apoptosis after treatment with mud-sulphur therapy and/or pharmacological therapy with chondroitin sulfate in C57 black 6N mice.

**Table 2.** Histological modifications of the mice knee cartilage after the different treatments.

| Histological changes | Control | Mud-therapy | CS      | CS + Mud-therapy |
|----------------------|---------|-------------|---------|------------------|
| DAS                  | 19.2%   | 15.1%       | 10.1%*  | 7.6%**           |
| Cleft                | 63.5%   | 53.1%       | 40.1%*  | 30.2%*           |
| Cloning              | 80.8%   | 70.6%       | 35.3%** | 20.9%**          |
| HC                   | 16.2%   | 15.6%       | 10.1%*  | 5.6%**           |
| MLSS                 | 50.6%   | 40.6%       | 30.2%*  | 12.3%**          |
| SLSS                 | 40.4%   | 35.6%       | 30.8%*  | 20.1%**          |
| DRT                  | 54.5%   | 53.2%       | 30.9%*  | 20.1%**          |
| FA                   | 20.0%   | 18.0%       | 10.0%*  | 5.0%**           |

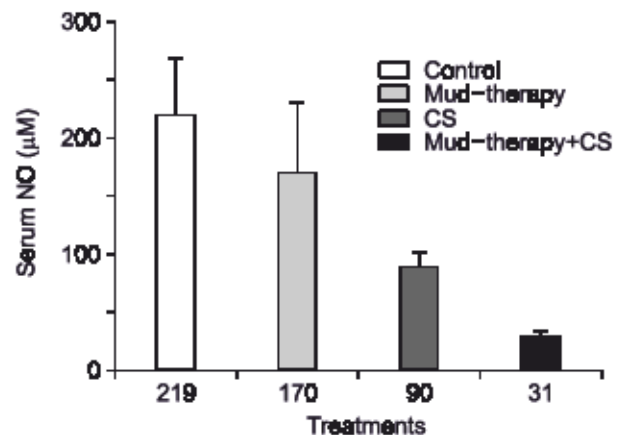
DAS, disruption of articular surface; HC, hypocellularity of chondrocytes; MLSS, moderate loss of safranin-O staining moderate; SLSS, severe loss of safranin-O staining; DRT, duplication and rupture of the tidemark; FA, fibrillar appearance.

\*Significant statistical difference ( $P < 0.05$ ); \*\*Highly significant statistical difference ( $P < 0.01$ ).

apy alone (see Table 2). The treatment with CS alone caused a statistically significant reduction of disruption of the articular surface (10.1%), cleft (40.1%), cloning (35.3%), hypocellularity of chondrocytes (10.1%), moderate (30.2%) to severe (30.8%) loss of safranin-O staining, and duplication and rupture of the tidemark (30.9%) (Table 2). Moreover, the appearance of fibrillar structures was observed only in 10% of the tissue. The differences were statistically significant ( $P < 0.05$ ) in almost all the cases and highly statistically significant in case of cloning ( $P < 0.01$ ). In the mice treated with mud therapy plus CS the chondrodegenerative histological features were furtherly reduced. In fact, it was observed disruption of 7.6% articular surface in, 30.2% cleft, 20.9% cloning, 5.6% hypocellularity of chondrocytes, 12.3% moderate to 20.1% severe loss of safranin-O staining, and 20.1% duplication and rupture of the tidemark (Table 2). The statistical differences between CS plus mud therapy group and control group were highly statistically significant ( $P < 0.01$ ). Therefore, mud therapy demonstrated to potentiate the chondroprotective effects of CS.

#### Effects of mud and CS treatment on apoptotic labelling index

In order to evaluate the effects of the treatments on the degeneration of cartilage of the mice we have detected the apoptotic index of chondrocytes of the cartilage of the sacrificed animals. In the cartilage of control group the apoptotic cells were  $1205 \pm 189$  while the treatment with mud therapy showed statistically non-significant reduction of the apoptotic chondrocytes ( $974 \pm 236$ ,  $P < 0.05$ ) (see Figure 1). On the other hand, CS induced a significant reduction of apoptotic chon-



**Figure 2.** Effects on serum concentration of NO after treatment with mud-sulphur therapy and/or pharmacological therapy with chondroitin sulfate in C57 black 6N mice.

drocytes ( $680 \pm 90$ ,  $P < 0.05$ ) while the combined treatment furtherly decreased the apoptotic cell number ( $480 \pm 70$ ,  $P < 0.01$ ) (see Figure 1). These results suggested that the combined treatment could be active in arresting the degenerative potential of arthrosic cartilage through the decrease of the apoptotic index of cartilage cell compartment.

#### Effects of mud and CS treatment on serum NO concentration

Since NO generation by pro-inflammatory cytokines has been involved in the pathogenesis of OA through the induction of chondrocyte apoptosis and the consequent decrease of extracellular matrix production we have evaluated the effects of the treatments on NO serum levels. The treatment with CS induced a significant ( $P < 0.05$ ) reduction of serum NO concentration (C group:  $90 \pm 13$ ) if compared with the control group (A group:  $219 \pm 60$ ) (Figure 2). The combination between thermal treatment with mud-sulphur therapy and pharmacological therapy with chondroitin sulfate enhanced the reduction of NO serum concentration (D group:  $31 \pm 1.5$ ) (Figure 2). This difference was again statistically significant as compared with both controls ( $P < 0.01$ ) and CS-treated mice ( $P < 0.05$ ).

Finally, in the group of animals treated with mud sulphur therapy alone (B group:  $170 \pm 62$ ) a reduction of the NO serum concentration was also recorded, as compared to the control group. However, this reduction was not statistically significant ( $P > 0.05$ ) (Figure 2). These data appear to confirm the chondroprotective effect of CS and of CS + mud therapy and suggest a role for the inflammatory processes in the regulation of osteoarthrotic mechanisms in this experimental model.



### Adverse effects

During the experiments no adverse reactions or weight reduction were observed in the three groups of animals subjected to pharmacological and/or thermal treatment.

### Discussion

OA is the most common degenerative osteoarticular disease characterized by the loss of the homeostasis between the anabolic and catabolic processes of bone tissue (Radine *et al.*, 1986; Pelletier 1999). This alteration induces the degradation of the articular cartilage, synovium inflammation and the bone inspissation. All these pathological changes produce pain during articular movements, functional difficulty and articular stiffness. Data from literature (Cecchetti *et al.*, 1995; Bellometti *et al.*, 1996; Nguyen *et al.*, 1997) seems to highlight the modulator role of mud-thermal treatment on the chondrocyte, a basic element of the cartilage, which reacts to the stimulus derived from the application of the mud thermal therapy through the activation of an anabolic response. In fact, it has been demonstrated that, during mud thermal therapy, a decrease of IL1 and TNF- $\alpha$ , both mediators of inflammation and of degenerative OA processes, is recorded. On the other hand, an increase of the serum concentration of ceruloplasmine and transferrin, both proteins involved in the circulating antioxidant defense system, is found. Other data (Nappi *et al.*, 2001; Flusser *et al.*, 2002) have demonstrated the protective role of mud thermal therapy on collagen and proteoglycans, thus avoiding the occurrence of degradative processes. It was also shown that mud-therapy has beneficial effects on several rheumatic diseases (Nguyen *et al.*, 1997; Costantino *et al.*, 1999; Costantino *et al.*, 2001; Nappi *et al.*, 2001; Flusser *et al.*, 2002; Van Tubergen *et al.*, 2002). Moreover, mud-therapy appeared to be able to prevent the progression of the cartilage degeneration through a chondroprotective effect in osteoarthritis (Chiodo *et al.*, 1993; Anderson *et al.*, 1999; Blanco 2000). Recently, it was found glycoso aminoglycans (GAG) have a chondroprotective effect through the modulation of the synthesis of cartilage. Among these molecules the CS, the most abundant cartilage GAG, is able to restore the metabolic homeostasis of the chondrocyte. In osteoarthritis, CS seems to ameliorate the clinic symptoms through its anabolic activity, through the increase of the synthesis of hyaluronic acid, glucosamine sulphate and type II collagen. Moreover, CS has an anti-catabolic action (block of the degradation of the extracellular matrix molecules), and a chondroprotective effect from the triggering of apoptotic processes induced by some toxic mediators like NO during OA. In fact, an increase of NO, tumour necrosis factor  $\alpha$ , interleukin 1, metalloproteases (MMP) and re-

active oxygen radicals (ROS) is observed in OA, demonstrating the participation of these factors to the genesis and maintainance of degenerative processes in OA (Osborn *et al.*, 1989; Moskowitz, 1993; Lotz *et al.*, 1995; Blanco *et al.*, 2000). NO is mainly produced by chondrocyte, induces apoptosis of the chondrocyte itself (Attisano *et al.*, 1994; Borderie *et al.*, 1999; Fukui *et al.*, 2001; Purple *et al.*, 2001), and inhibits the synthesis of components of cartilage matrix such as collagen type II and GAGs; NO, moreover, increases the activity of MMP, decreases the proliferative response to the Insulin-like Growth Factor-1 (IGF-1) and reduces the endogenous production of Transforming Growth Factor  $\beta$  (TGF  $\beta$ ), by chondrocyte, furtherly decreasing the synthesis of GAGs and of collagen fibers. Recent *in vitro* studies (Blanco *et al.*, 2000) have demonstrated that CS blocks the synthesis of NO at the articular level.

On the basis of these considerations we have evaluated the probable *in vivo* antioxidant effect of a cycle of mud-therapy with sulphur mineral water versus a chondroprotective pharmacological agent, such as CS or the combination between the two different treatment modalities. We have designed an *in vivo* experimental model in mice that spontaneously develop chondrodegenerative processes and we have evaluated the feasibility of the treatment with mud-therapy in animals.

Our results have confirmed the antioxidant and potentially chondroprotective action of the CS, as compared to the control group; however, the statistical difference was furtherly increased when mud-sulphur thermal therapy was combined to pharmacological therapy ( $P < 0.01$ ). In fact, we have found that these treatments induced a decrease of radiological and histological features of chondrodegeneration and these effects were likely mediated by a significant reduction of the production of endogenous oxidant agents such as NO, a product of pro-inflammatory processes. On the other hand, mud therapy alone appeared to reduce the serum levels of NO as compared to CS-treated mice, but the difference was not statistically significant.

In conclusion, our data suggest that alternative therapy of old earth elements could be an important therapeutic tool in the treatment of OA. In fact, it could potentiate the efficacy of the pharmacological therapy with CS, thus inducing both more potent therapeutic effects and decreasing the required dose of CS. Moreover, our experimental models and tools could be useful to perform further investigations on the biological, molecular and pharmacological effects of this kind of therapy *in vivo*.

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