ORIGINAL ARTICLE

Effect of Hydrogen Sulphide Containing Mineral Water on Experimental Osteoporosis in Rats

Tamara Popović,[1] Ljiljana Amidžić,[2, 3] Mile Čeko,[4] Siniša Marković,[4] Ranko Škrbić[5]

Abstract

Background/Aim: Sulphur mineral water is widely used in the treatment of musculoskeletal diseases. Hydrogen sulphide is an important regulator of bone metabolism and its application in the treatment of osteoporosis is intensively researched. The aim of this study was to analyse biochemical and histological effects of H_2S containing mineral water of "Mlječanica" spring on ovariectomy-induced experimental osteoporosis in rats.

Methods: In this experiment a 14-week-old Wistar female rats were used. The animals undergone bilateral ovariectomy (OVX groups) as an experimental model for oestrogen-deficient osteoporosis. After six weeks, animals were divided into control and the experimental group. Rats from the experimental group treated with H_2S (SW group) containing mineral water *ad libitum* during five weeks. Biochemical parameters for monitoring sulphur water effects were concentration in serum of osteocalcin, alkaline phosphatase, calcium and phosphorus. Histological analyses of the left tibia coloured with haematoxylin-eosin were carried out.

Results: Regarding the biochemical parameters, a statistically significant increase was observed in the OVX group for osteocalcin, alkaline phosphatase calcium and phosphorus compared to the sham-operated (CNT) group ($p < 0.01$). In SW + OVX, alkaline phosphatase was statistically significantly decreased ($p < 0.01$) and serum osteocalcin and phosphorus increased ($p < 0.01$). Calcium values were increased without significance. In the OVX + SW group, histological analyses showed numerous osteoblasts along the trabecular endosteum and the growth of young chondrocytes in the central bone zone and their migration to the peripheral parts.

Conclusion: Drinking the H_2S containing "Mlječanica" mineral water has led to decreased alkaline phosphatase, increased osteocalcin and phosphorus concentration in serum and stimulated the bone reparation in osteoporotic rats.

Key words: Osteoporosis; Hydrogen-sulphide; Sulphur water; Bone metabolism.

1. Institute for Physical Medicine and Rehabilitation "Dr Miroslav Zotović", Banja Luka, the Republic of Srpska, Bosnia and Herzegovina.
2. Centre for Biomedical Research, Faculty of Medicine, University of Banja Luka, the Republic of Srpska, Bosnia and Herzegovina.
3. Department for Human Genetics, Faculty of Medicine, University of Banja Luka, the Republic of Srpska, Bosnia and Herzegovina.
4. Hospital for Physical Medicine and Rehabilitation "Mlječanica" Kozarska Dubica, the Republic of Srpska, Bosnia and Herzegovina.
5. Department of Pharmacology, Toxicology and Clinical Pharmacology, Faculty of Medicine, University of Banja Luka, Banja Luka, the Republic of Srpska, Bosnia and Herzegovina.

Correspondence:
TAMARA POPOVIĆ
M: +387 65 650 230
E: tamara.popovic@med.unibl.org

ARTICLE INFO

Received: 29 November 2022
Revision received: 21 December 2022
Accepted: 21 December 2022

Introduction

With population aging there is an increase in the number of people with osteoporosis. It is estimated that 10 % of total population is suffering from osteoporosis. Decreased mineralisation and impaired bone microarchitecture in osteoporosis leads to fractures.[1] Forearm, hip and vertebral fractures are the most common osteoporosis consequences. Fractures cause chronic pain, disabilities and increased health care costs and mortality in elderly patients.[2] Treatment periods for osteoporosis are very long and last for years, even decades. The most common type is post-

This article should be cited as follows: Popović T, Amidžić LJ, Čeko M, Marković S, Škrbić R. Effect of hydrogen sulphide containing mineral water on experimental osteoporosis in rats. Scr Med 2022 Dec;53(4):291-7.

menopausal osteoporosis, while the secondary osteoporosis is caused by malignancy, endocrine and systemic diseases. Due to very serious comorbidities the growing number of patients is not able to be treated with pharmacological therapy.[3] This represents a major challenge for treatment safety and encourages investigation of new therapeutic modalities.

Hydrogen sulphide (H_2S) is endogenously produced gas in human cells and regulates many physiological and pathophysiological functions.[4] Within the last several years the studies related to effects of H_2S on bone metabolism have been intensified.[5] With aging, the concentration of endogenous H_2S levels in the blood decreases and becomes negatively associated with oxidative stress.[6] Its reduction is registered both in ovariectomised mice[7] and in a rat model with a methionine-reduced diet.[8] It is possible to apply H_2S exogenously using H_2S donors with therapeutic effects that have been intensively studied. It is known that H_2S stimulates osteoblastogenesis and H_2S donors are currently being tested on experimental oestrogen deficient model. Grassi et al showed that in ovariectomised mice treated with the H_2S-donor GYY4137 (GYY), the serum H_2S was normalised, trabecular bone loss was prevented and bone formation was increased.[9]

There are natural mineral waters that are rich with sulphur in the forms of sulphurous ions (SO_4^{2-}) and dissolved H_2S gas. Mineral waters with the S^{2-} level of at least 1 mg/L are characterised as "sulphurous waters". Our country is rich with H_2S containing mineral water springs.[10] The use of sulphur containing waters for bath, inhalation or drinking has been known in balneology since ancient times.[11] The positive effects of sulphur containing mineral waters on dermatological and cardiovascular disease are well explained in the literature.[12, 13]

The natural healing springs with sulphur containing water have been known in the area of Kozarska Dubica town, in north-west of the Republic of Srpska, Bosnia and Herzegovina, since ancient times and have been used for the treatment of mainly local residents in the form of bath. The first mineral water spring in "Mlječanica" was discovered by locals digging wells on their lands. Among folks, they were referred to by pejorative name "stinkies", because of their scent of rotten eggs. The well-known Austrian balneologist, E. Ludwig, was the first to mention the mineral water "Mljecanica" in his book back in 1888.[14] It has been shown that this mineral water had positive clinical effects on lower limb spasticity after stoke, as well as the positive effects on hypertension.[15, 16] Although, this mineral water has been widely used as balneotherapy for musculoskeletal disorders, the effects of H_2S containing mineral water "Mlječanica" has not been studied in osteoporosis.[17] So, the aim of this study was to analyse biochemical and histological effects of H_2S containing mineral water from "Mlječanica" spring on experimental oestrogen deficient osteoporosis in rats.

Methods

Experimental animals

The twenty 14-week-old female Wistar rats, weighing between 180-220 g were used in this study. The animals were housed in Plexiglas cages, with the room temperature regulated at 22 ± 2 °C, humidity of 55 % ± 15 % and a 12/12 h light/dark cycle. All procedures on animals were conducted in accordance with the Guidance for the Care of Animals in Experimental Researches and approved by the Ethics committee for the Protection and Welfare of Experimental Animals of the Faculty of Medicine, University of Banja Luka.

Experimental osteoporosis

Experimental osteoporosis was induced by bilateral ovariectomy of 14-week-old female rats as most often used experimental model for post-menopausal osteoporosis with some modifications.[18] Ovariectomy was performed in general anaesthesia induced by ketamine (ketamine hydrochloride injection USP 50 mg/mL Rotexmedica, Tritan, Germany) in doses of 0.15 mL/100 g and diazepam 0.1 mg/100 g body weight (Bensedin® amp 10 mg/2 mL, Galenika a.d. Belgrade, Serbia). The anaesthetics were given intramuscularly.

A surgery was performed by all principles of asepsis and antisepsis and ventral approach was used as a method for ovariectomy as described previously.[19] Shortly, the cut was made along the central abdominal line and after the left horn of uterus was exposed the ligature of left ovary with surrounding adipose tissue was done and ovary was removed. The same procedure was repeated for the right ovary. After the ovariectomy was successfully done, the abdominal wall and the skin were sewed separately and antibiotic (neomycin-bacitracin combination) powder (Bivacyn®, Lek, Ljubljana, Slovenia) was applied on the skin in thin layer. After the operations were completed, the animals were housed in individual cages and monitored for following seven days to be sure that the wounds were properly cicatrised. After the ovariectomies were successfully done, the animals were kept at the same standard laboratory conditions for following 6 weeks during which the oestrogen deficient osteoporosis was developed.[20]

Experimental design

Twenty rats were randomly divided into control and experimental group. The sham operated rats were assigned to the first control group (CNT, n = 6), while the ovariectomised rats were divided in two groups: group I (OVX group, n = 6) treated with tap water and group II (OVX + SW, n = 8) treated with H_2S containing mineral water from “Mlječanica” spring. All animals were offered to drink water *ad libitum* (an average of 25 mL per day) during five weeks. At the end of experimental protocol, all animals were anaesthetised and sacrificed by exsanguination.

The physicochemical parameters of the “Mlječanica” mineral water were previously determined by a certified laboratory at the Institute of Public Health of the Republic of Srpska (Table 1).

Test conditions for all parameters but conductivity, active reaction (H^+) and fixed residue were measured at temperature 21 ± 3 °C.

Table 3: Physicochemical parameters of “Mlječanica” spring mineral water

Parameters	Measurement	
	Units	Values
Temperature	*°C*	*12.6*
Concentration H^+	*pH*	*6.9- 7.1*
Conductivity at 20 °C	*mS cm^{-1} (20 °C)*	*4150*
Fixed residue at 105 °C	*mg/L*	*4069*
Turbidity	*NTU*	*91.6*
Alkalinity	*mmol/L*	*501.5*
Total hardness, $CaCO_3$	*mg/L*	*1995*
Dissolved total hydrogen sulphide	*mg/L*	*136*
(H_2S) and free gas		*71.3*
Ammonium ion NH_4^+	*mg/L*	*0.95*
Nitrites NO_2	*mg/L*	*0.10*
Calcium	*mg/L Ca^{++}*	*359.7*
Magnesium	*mg/L Mg^{++}*	*266.8*
Sulphates	*mg/L SO_4^{--}*	*2380*
Hydrogen-phosphate	*mg/L HPO_4^{--}*	*0.65*
Chlorides	*mg/L Cl^-*	*< 5.0*
Iron total	*mg/L Fe*	*< 0.05*
Sodium	*mg/L Na^+*	*433*
Potassium	*mg/L K^+*	*3.5*

“<…” – below the limit of detection

Biochemical analyses of blood samples

For biochemical analysis the blood samples were taken from periorbital veins. The sample was taken (3 mL) after 11 weeks at end of experiments. The serum samples were stored at -20 °C until the final biochemical analyses. The osteocalcin (OC) level was determined using immunochemical method by means of COBAS E411 (Roche Diagnostics GmbH, Mannheim, Germany), while alkaline phosphatase level (AP), serum calcium (Ca) and phosphorus (P) levels were measured using COBAS C311 (Roche Diagnostics GmbH, Mannheim, Germany).

Histological analysis of bone

For histological analysis the left tibia was prepared and fixed in 10 mL 10 % formalin. After 28 days of fixation, tibias were dehydrated, decalcified through classical histological procedure, embedded in paraffin and cut with microtome into 5 µm thick longitudinal sections. Sections of tibia were stained with haematoxylin and eosin (HE) and histological analysis were done on light microscope Reichert (model 310 professional compound, Vienna, Austria).

Statistical analysis

The numeric data were shown as mean values ± standard deviation. Significance of differences of mean values of tested biochemical parameters between control and experimental group was determined by using t-test. Significance level was set at $p < 0.05$.

Results

Biochemical analysis

The average values of serum alkaline phosphatase, calcium, osteocalcin and phosphorus were significantly increased in ovariectomised rats compared to controls. In rats treated with H_2S containing mineral water the alkaline phosphatase was decreased. At the same time this treatment did not affect the calcium level, while the levels of osteocalcin and phosphorus were increased significantly (Table 2).

Pathohistological analysis

No macroscopic changes in bones were observed of the tibia in control animals. Histology of its cross-section showed a normal cortical compact bone structure with an outer periosteum, a matrix containing blood vessels and osteocytes within their lacunae and a smooth endosteal sur-

Table 2: The average value of serum alkaline phosphatase, calcium, phosphorus, and osteocalcin (mean ± SD) in the sham-operated control, ovariectomised rats and ovariectomised rats treated with H_2S containg water

Parameters	CNT (mean ± SD)	OVX (mean ± SD)	t-value	OVX + SW (mean ± SD)	t-value
Alkaline phosphatase (U/L)	77.4 ± 6.54	152.7 ± 4.8[1]	-20.860	133.9 ± 13.06[1]	3.111
Calcium (mmol/L)	2.39 ± 0.02	2.54 ± 0.03[1]	-9.375	2.58 ± 0.07[NS]	-0.657
Osteocalcin (ng/L)	12.6 ± 1.46	28.2 ± 0.86[1]	-20.690	31.90 ± 2.00[1]	-3.889
Phosphorous (mmol/L)	2.15 ± 0.01	2.45 ± 0.01[1]	-4.769	2.66 ± 0.01[a]	-6.176

CNT - control, sham operated; OVX - ovariectomised; SW - sulphurous water; [1]p < 0.01; NS - not significant

face (Figure 1a). Particularly, at the periosteal side of the cortical bone, connective and muscle tissues were visible, along that at the endosteal side of the medullary canal the structure was filled with bone marrow. Blood vessels and osteocytes in their lacunae can be seen in the bone matrix (Figure 1b).

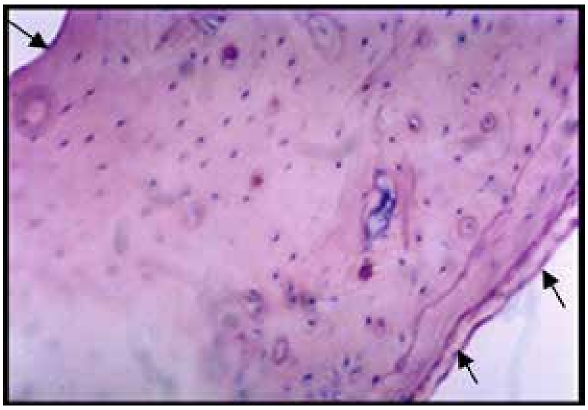

Figure 1a: A cross-sectional image in the cortical compact bone of the tibia of the control group of the sham-operated (CNT) rats (H & E x 250). It showed a normal cortical compact bone structure with an outer periosteum (one arrow), a matrix containing blood vessels and osteocytes within their lacunae and a smooth endosteal surface (arrows).

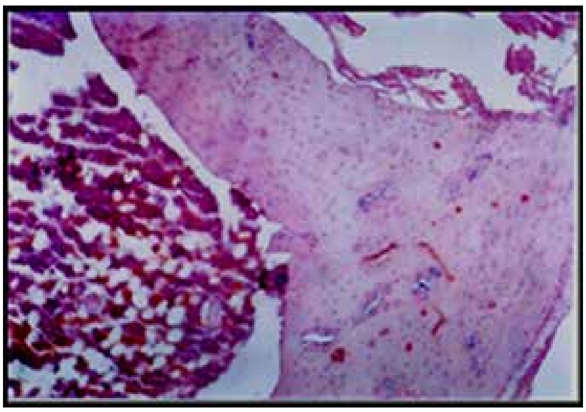

Figure 1b: A cross-sectional image of bone the sham-operated (CNT) group (H & E x 100). On the periosteal side of the cortical bone (CNT group), connective and muscle tissue is visible, and along the endosteal side of the medullary canal, the structure is filled with bone marrow. Blood vessels and osteocytes in their lacunae can be seen in the bone matrix.

Macroscopically, the tibias of ovariectomised rats did not deviate in shape and size from tibias of normal control animals. However, in the cross sections of tibia, the results revealed further progress of osteoporosis (Figures 2a, 2b). The medullary canal and area containing bone marrow were more extended. Focally, in the cortical bone, the whole osteon was decomposed and replaced by a cavity filled with bone marrow. Thus, the cortical bone showed histological features of trabecular bone (Figures 2a, 2b) indicating that

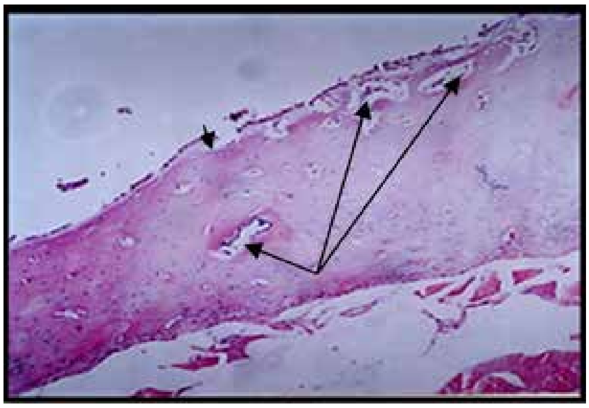

Figure 2a: On the cross-section of the tibia in the osteoporotic group of rats (OVX), multiple resorption cavities (long arrows) and eroded endosteal surfaces are visible (shot arrow) (H & E x 250)

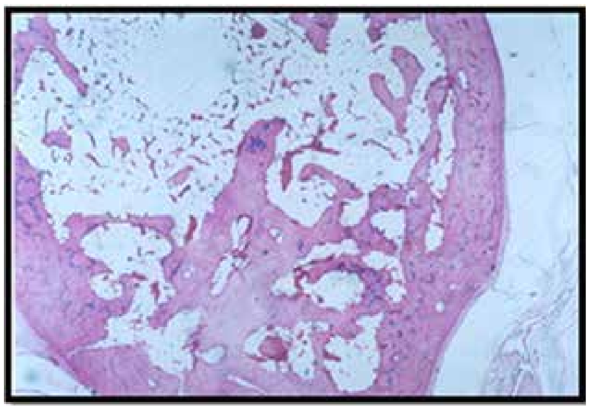

Figure 2b: On the cross-section of the tibia in the osteoporotic group of rats (OVX) shows a discontinuous bone network trabeculae with filling extensions bone marrow (H & E x 250)

the bone become more porous. There were multiple resorption cavities and eroded endosteal surfaces were visible. Centres of enchondral ossification were visible while trabeculae were perforated and very thinned. The trabeculae within cancellous bone were short, parallel and transversally connected, aligned towards the mechanical load distribution (Figure 2b).

In the experimental group treated with H_2S containing water, preserved bone architecture compared to the ovariectomised were observed. The cross-section of the tibia showed the bone trabecula with irregular bone lamellae and osteocytes located in their lacunae. The spaces between the trabeculae were filled with bone marrow. There were numerous osteoblast lines with trabecular endosteum and discrete osteoid deposits (Figure 3a). Along with osteocytes in the bone tissue, the growth of young chondrocytes (in the central zone) that migrate to the peripheral parts of the bone were seen (Figure 3b).

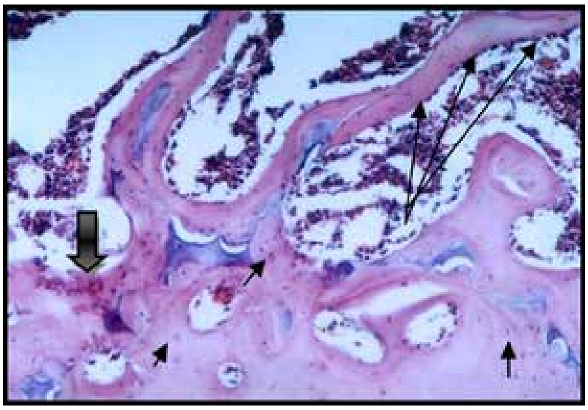

Figure 3a: On the cross section on the tibia in the osteoporotic group of rats treated with H_2S containing mineral water (OVX + SW group), showing preserved bone architecture compared to the ovariectomised (OVX) group (H & E x 100). Bone trabecula with irregular bone lamellae and osteocytes located in their lacunae (shot arrow). Bone trabecula with irregular bone lamellae and osteocytes located in their lacunae (shot arrow). The spaces between the trabeculae are filled with bone marrow. Numerous osteoblasts line the trabecular endosteum (long arrows). Discrete osteoid deposits can be seen (thick arrow).

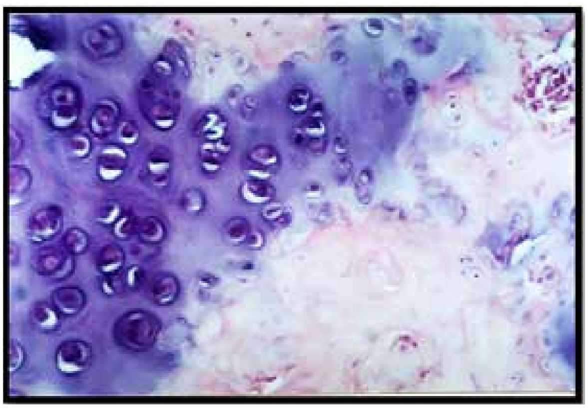

Figure 3b: On the cross section on the tibia in the osteoporotic group of rats treated with H_2S containing mineral water (OVX + SW group), showing preserved bone architecture compared to the ovariectomised (OVX) group (H & E x 100)

Discussion

In order to examine the effects of H_2S containing “Mlječanica" mineral water on oestrogen-deficient osteoporosis, the well-known model with ovariectomised rats was used. The values of alkaline phosphatase, phosphorus, calcium and osteocalcin in the control group after ovariectomy were elevated indicating the development of osteoporosis. In the experimental group that was treated with sulphur containing mineral water during five weeks, the significant decrease in alkaline phosphatase and significant increase in osteocalcin and phosphorus were noticed. These changes indicate increased osteoblast activity in the group treated with H_2S containing mineral water. Histological analysis confirmed the existence of bone reparation in the group that were treated with sulphur water.

It has been shown that H_2S is a key molecule in the maintenance of bone mass, by regulating the entry of calcium ions through Ca^{++} channels and osteogenic differentiation of stem cells. Its deficiency reduced the differentiation of stem cells and thus affects the bone formation.[21] *In vitro* experiments on human periodontal ligament cells treatment with H_2S increased protein expression levels of alkaline phosphatase, osteocalcin and collagen type I.[22] The metalloproteinase activity in bone matrix was reduced after exposure to H_2S which accelerates matrix mineralisation.[20] The study of Xu et al showed that H_2S protects osteoblast cells from the harmful effects of hydrogen peroxide (H_2O_2) and other free radicals.[23]

The natural mineral waters have a specific and stabile chemical composition with potential for different therapeutic effects. “Mlječanica” mineral water contains dissolved H_2S gas in a concentration of 71.3 mg/L and total gas concentration of 136 mg/L.[10] The pH values, as well as the water temperature could significantly affect the gas stability. This water has a pH of 6.9-7.1 and is considered as slightly acidic mineral water. Acidic waters have the highest gas stability, while in the physiological range of pH 7.2-7.4, only a third of the sulphur was presented as H_2S gas. Sulphur (S^{--}) is inert in alkaline waters and does not have a beneficial therapeutic effect.[24]

In vivo studies demonstrated the beneficial effect of drinking of natural sulphur containing water on animal models of diabetes and glucose metabolism, as well as gastroprotection, antioxidant effects in intestinal physiology and improvement of lipid metabolism and lipid lowering effect.[25-31] It can be concluded that there is no evidence of a minimum effective concentration of H_2S in drinking water. Different concentrations of H_2S in natural waters exhibit different biological effects regardless of H_2S level. The composition of mineral waters is specific and it is certain that H_2S in combination with other minerals like calcium, magnesium, or bicarbonates can lead to different biological responses of certain organs and tissues.[32]

In a clinical study that was conducted on patients with knee osteoarthritis, who used to drink "Mlječanica" mineral water, the lipids were significantly lowered in a short-term period.[33] Bulgarian authors performed a clinical trial with sulphated mineral water, but with much lower sulphate content, on the oxidative status and markers of inflammation on healthy volunteers. The intake of this mineral water improved the redox status of the body.[34] In clinical practice, the sulphurous mineral water is more often used in the form of baths and less often by drinking because of its unpleasant rotten egg smell. When the H_2S gas is used for bath treatment it penetrates through the skin 150 times more rapidly than if used in other forms. H_2S gas has a role in many biological processes depending on its concentrations. Certainly, a higher concentration of this gas in the blood is achieved if both modalities of gas intake are used, ie through the skin and through intestinal resorption. High levels of gas intake could be toxic, while the low levels are well tolerated and have antioxidants, anti-inflammatory and cytoprotective effects.[35] A recent study has proved that the physiological concentration of H_2S in the peripheral blood ranges from 30 to 100 μmol/L, but there was no evidence of its level in bone tissue.[36] In this study, only one modality of gas resorption was used and water was taken *ad libitum* without the possibility of dosing the H_2S concentration.

The limitation of this study is that the H_2S blood concentrations in ovariectomised rats before the beginning of experiment was not determined. Future experimental studies should clarify the mechanisms of action of H_2S containing mineral water on prevention of osteoporosis. A well-designed clinical study on patients with osteopenia and osteoporosis would contribute to the improvement of the clinical application of natural mineral water with H_2S and more precise dosing of both, the baths and oral use.

Conclusion

Drinking of H_2S containing mineral water led to decreased alkaline phosphatase, increased osteocalcin and phosphorus concentration in serum and stimulated the bone reparation in osteoporotic rats.

Acknowledgements

None.

Conflict of interest

None.

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