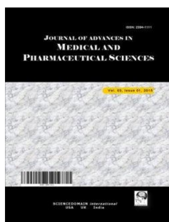

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Berberine Prevents Bone and Cartilage Destruction and Influences Cell Senescence in Experimental Arthritis

Petya Ganova[1], Lyudmila Belenska-Todorova[2], Tsvetelina Doncheva[3] and Nina Ivanovska[1*]

[1]*Institute of Microbiology, Department of Immunology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria.*
[2]*Medical Faculty, Sofia University, Bulgaria.*
[3]*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113, Sofia, Bulgaria.*

Authors' contributions

This work was carried out in collaboration between all authors. Authors PG and LB fulfilled the study and performed the statistical analysis, author NI designed the study and prepared the manuscript. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Background: Berberine (BB) is a protoberberine alkaloid with a wide spectrum of pharmacological activities considered to be a selective Janus kinase (Jak)3 inhibitor. Nevertheless, the mechanisms of berberine's actions on joint inflammation have not been well clarified.
Aim: This study evaluated the effect of berberine on cartilage and bone destruction, and on generation of senescent cells.
Methodology: The experiments were conducted in a murine model of erosive arthritis induced by intra-articular (i.a.) injection of zymosan (zymosan-induced arthritis, ZIA). Histopathologic changes were evaluated by haematoxylin and eosin (H&E), toluidine blue and Sudan Black B (SBB) staining.

*Corresponding author: E-mail: nina@microbio.bas.bg;

Bone marrow (BM) cells were shifted to osteoblast differentiation and alkaline phosphatase (ALP) positive cells were determined together with ALP activity in the cell lysates. Calvarial osteoblast generation was determined by von Cossa staining.
Results: Berberine decreased cartilage and bone erosion. The loss of glycosaminoglycans (GAGs) was lower in BB-treated group (*72.0 +/- 2.9% in ZIA mice vs 25.7 +/- 2.2 in BB group).* BB inhibited the generation of senescent cells and decreased the ALP activity of these cells (*0.550 +/- 0.006 for non-arthritic, 1.725.8 +/- 0.110 for ZIA and 0.750 +/- 0.0.064 for ZIA+BB*).
Conclusion: Berberine showed potential anti-arthritic properties in a model of erosive RA perspective for administration in joint disorders.

Keywords: Berberine; zymosan-induced arthritis; synovitis; osteoblasts.

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized with inflammation of the synovial lining of joints which leads to progressive erosion of bone in most cases irreversible, loss of function, and disability [1]. Onset of RA can begin at any age but increases with age. Although the prospects for most patients are now optimistic, many still do not respond to current therapies. Accordingly, new therapies are strongly required. Despite the central role of the adaptive immune system in RA pathogenesis, the innate immune system has a key role in this scenario. The underlying disease mechanisms remain unclear but are generally triggered by infections and inflammatory mediators [2]. At the articular borders, in a result of ongoing inflammation the lining layer forms a pannus that invades the adjacent articular cartilage and subchondral bone. Synovial T cells are attracted by chemokines and receive survival signals such as IL-7 and IL-15 [3]. The pannus formation is composed of infiltrating cells, such as monocytes/macrophages, as well as RA synovial fibroblasts. They secrete proinflammatory cytokines and chemokines that perpetuate inflammation. In addition, they release receptor activator of nuclear factor-kappa B ligand (RANKL) and promote osteoclast differentiation, leading to bone destruction [4]. Cartilage undergoes damage by catabolic effects in chrondrocytes after their stimulation by cytokines. Proteins from the synovial tissue of RA patients are extensively phosphorylated by intracellular tyrosine kinases, proving the importance of tyrosine kinases in the pathogenesis of RA [5]. Post-transcriptional and post-translational events further regulate mediator production. The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway has received less attention then other signaling pathways. However, several cytokines implicated in RA pathogenesis, including IL-6, IL-1, and interferons (IFNs), activate the JAK/STAT pathway, moreover STAT3 is constitutively activated in RA [6]. Many drugs are in development to target tyrosine kinases for the treatment of RA. The administration of anti–IL-6 antibody in RA that is predicted to block JAK/STAT activation has shown promising results. Kinase inhibitors might be considered as new therapeutic strategies for RA.

Berberine is a protoberberine alkaloid, isolated from root, rhizome and stem bark of various *Berberis* species. Its beneficial effect is established after application in metabolic disorders [7] related to lowering blood cholesterol, triglycerides and aterogenic apolipoproteins [8]. Berberine is reported to express antiarrhythmic, vasodilator and hypertensive properties [9-10]. The therapeutic dosage is most often 200 mg orally two to four times daily. Side effects are documented at high dosages, including gastrointestinal discomfort, lowered blood pressure or flu-like symptoms. Berberine is considered to be a predominantly selective Jak3 antagonist. Data from kinase assays and protein-compound docking simulation show that berberine blocked Jak3 catalytic activity after binding to the kinase domain of Jak3 [11]. Recent investigations prove that the alkaloid also activates Jak2/STAT3 pathway [12]. In previous investigations we have shown the beneficial effect of berberine in adjuvant-induced arthritis and in autoimmune tubulointerstitial nephritis administered under different schemes [13-14]. We have found that the alkaloid delayed the development of adjuvant arthritis when applied after its onset at a dose of 10 mg/kg. Also, we have established berberine's anti-osteoclastogenic effect *in vitro* [15]. The aim of the present study was to evaluate the effect of berberine on bone and cartilage erosion, and on synovial cell and osteoblast senescence in a mouse model of erosive arthritis induced by intraarticular injection of zymosan.

2. MATERIALS AND METHODS

2.1 Substance and Treatment

Berberine was isolated from the alkaloid fraction obtained from roots of *Berberis vulgaris* L. by chromatographic procedures based on column chromatography. The structure of berberine was confirmed by comparison of the R_f value, and IR and ^{1}H NMR spectral data with these of authentic sample. The alkaloid was used as a sulphate [(C20H18NO4)2-SO43H2O] dissolved in distilled H_2O and injected intraperitoneally i.p. in a dose of 10 mg/kg every other day from day 0 to day 12 of ZIA. The dose and time schedule of berberine were referenced by previous studies in adjuvant-induced arthritis [13].

2.2 Mice

Balb/c mice (Charles River Laboratories, Wilmington, MA, USA) were maintained at 12/12 light dark cycle in pathogen free conditions and were fed with pelleted food and tab water ad libitum. All experiments were conducted in accordance with the International and National Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee at the Institute of Microbiology (Guidelines №352 06.01.2012), Sofia.

2.3 Zymosan-Induced Arthritis (ZIA)

Mice received an intra-articular (i.a.) injection of 180 µg zymosan A from Saccharomyces cerevisiae (Sigma-Aldrich, Germany) under brief anaesthesia (sodium pentobarbital 50 mg/kg, i.p) (day 0). Control animals received an i.a. injection of an equal volume of sterile phosphate buffered saline (PBS).

2.4 Histopathologic Examination

Knee joints were dissected at the active phase of ZIA (day 18) and at the late chronic phase (day 56), fixed for 4 days in 10% formalin, decalcified by incubation in 10% EDTA/0.2% PFA in PBS at 4°C for 20 days and embedded in paraffin. Tissue samples 5 μm thick were dewaxed with xylene, dehydrated until 70% ethanol and stained with haematoxylin and eosin (H&E) for routine histological evaluation (n = 7 per group in three separate experiments). For Sudan Black B (SBB) staining the slides were mounted in saturated 70% ethanol solution for 8 min and then embedded into 50% ethanol, transferred and washed in distilled water (n = 7 per group in three separate experiments). Lipofuscin staining was considered positive when perinuclear and cytoplasmic aggregates of blue-black granules were evident inside the cells. For detection of proteoglycans joint sections were stained with 1% toluidine blue. Toluidine blue-stained sections were examined to measure the areas of total and negatively stained cartilage to show glycosaminoglycan (GAG) distribution and loss (n = 5 per group in three separate experiments). The proportion of damaged cartilage was calculated by dividing the area of negatively stained cartilage by the area of total cartilage. All histologic assessments were performed in a blinded protocol. Images were captured with a coupled device camera and exported to Adobe Photoshop CS6.

The degree of injury was graded for infiltration (score 0, normal; score 1, mild infiltration; score 2, moderate infiltration; score 3, marked infiltration; score 4, severe infiltration). Bone erosion was graded as score 0, no abnormality; score 1, small areas of resorption; score 2, more numerous areas of resorption, not readily apparent on low magnification; 3, obvious resorption in trabecular and cortical bone, and lesions apparent on low magnification; score 4.

2.5 Isolation of Bone Marrow Cells and Osteoblast Differentiation

Bone marrow was isolated aseptically from long bones of 8-10 weeks healthy mice or from mice at day 7 of ZIA. The suspension was gently aspirated to disrupt cell aggregates and after centrifugation cells were resuspended at a concentration of $1x10^6$/ml in 24 well plate in minimum essential medium (α-MEM, Sigma Aldrich, Germany), supplemented with 10% heat-inactivated FCS, 2% gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml L-ascorbic acid and 5 mM β-glycerophosphate for 21 days with media change every 3 days in the presence or absence of 20 μM BB from day 0 to day 5.

2.6 Alkaline Phosphatase (ALP) Staining and ALP Activity Assays

After cell culture, the fixed cells were incubated in 50 mM MgCl2 and 0.1 M Tris–HCl (pH 7.4) for 30 min. Cells were then stained for alkaline phosphatase with a mixture of 0.1 mg/ml Naphthol ASMX (Sigma-Aldrich) phosphate and

0.6 mg/ml. Fast blue RR salt (Sigma-Aldrich) in 0.2 M Tris–HCl (pH 8.5). An aliquot of cell lysate was added to ALP substrate buffer, containing 2 mg/ml p-nitrophenyl phosphate (Sigma), 0.1 M diethanolamine, 1 mM $MgCl_2$, 0.1% Triton X-100 (pH 9.8), and the mixture was incubated at 37°C for 30 min. The enzymatic reaction was stopped by the addition of 0.5 M NaOH, and the absorbance was read at 405 nm. A calibration line was constructed from different concentrations of p-nitrophenol.

2.7 Von Kossa Staining of Cultured Calvarial Osteoblasts

Calvaria were removed from 3-day-old neonatal mice. Cells were liberated by five sequential 15-min incubations of calvaria with bacterial collagenase (Sigma-Aldrich), 0.1% trypsin, and 0.8 mM Na_2EDTA in Ca^{2-}, Mg^{2-} free PBS. Cells at a concentration of $1x10^6$/ml were cultivated in α-MEM supplemented with 10% FCS (Sigma-Aldrich), gentamicin 50 µg/ml, penicillin G 100 µg/ml (Sigma_Aldrich), L-ascorbic acid 50 µg/ml (Sigma-Aldrich) and 5 mM β-glycerophosphate (Sigma-Aldrich). From day 0 of culture, 20 µM berberine solution was added. The cultured cells were fixed, incubated with 5% silver nitrate solution, exposed to light, washed and un-reacted silver was rinsed with 5% sodium thiosulfate.

2.8 Statistical Analysis

Data represent mean ± SEM. Statistical significance was assessed using one-way ANOVA or *Student's t-test*, as indicated in the figure legends, considering a *P* value = .05 as significant. Statistical analyses were performed using InStat3.0 and GraphicPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

3. RESULTS AND DISCUSSION

We aimed to study the effect of berberine on cartilage and bone destruction at different stages of arthritis. The onset of ZIA was attended with strong erythema and joint swelling well exerted at day 7 ameliorated in BB treated mice (Fig. 1A). Further at the late phase the swelling was lower (data not shown). At the active phase of ZIA we observed deformation of cartilage surface and degradation with a presence of osteoclasts (Fig. 1B arrow). These data confirmed previously established presence of osteoclasts in subhondral bone shown by specific TRAP staining [15]. In the late chronic phase the synovial lesion invaded the adjacent bone and strongly supported osteophyte formation. The administration of berberine had a beneficial effect on the development and progression of ZIA as demonstrated by inhibited cartilage and bone damages and lack of osteophyte and pannus formation although, signs of cartilage hyperplasia were observed at day 56 (Fig. 1B). Berberine lowered the total histological score determined by the semi-quantitative grading and staging system (Fig. 1C).

Histopathological data showed significant loss of GAGs already at early phase of ZIA (day 7) while in BB treated mice cartilage was to a great extent preserved (Fig. 2) that corresponded to bone erosion and cell infiltration seen in Fig. 1B.

The detection of lipofuscin is highly specific for the visualization of senescent cells which are hyper-metabolic and full of lipids in the cytoplasm. The progressive chondrocyte senescence associated with alkaline phosphatase and beta-galactosidase enzyme expression leads to the age related loss of chondrocyte function [16]. By applying the SBB staining we observed that the development of ZIA is attended with the presence of senescent cells in synovium at the acute and chronic phases. While the senescent process was weakly inhibited by berberine treatment in the acute phase, SBB positive cells were almost absent in the chronic phase (Fig. 3).

Bone remodeling is characterized by a sequence of events occurring at bone surfaces, mediated by multicellular units comprised of a specialized group of cells, namely bone-resorbing osteoclasts, bone-forming osteoblasts and their precursors. Normally, the amount of bone removed by osteoclasts is equal to the amount of bone formed by osteoblasts, thus a stable bone mass is maintained. Osteoblasts are mononuclear, not terminally differentiated cells derived from mesenchymal cells. Mineralization of the extracellular matrix is one of the key bone formation markers and is influenced by cytokines. The inhibition of osteoblast differentiation contributes to the development of arthritic bone loss in RA as well as to the markedly diminished capacity of erosions to be restored [17]. Chronic inflammation is associated with aging and is often attributed to the progressive activation of immune cells over time [18-19]. No single marker may define a cell as senescent since none of these markers are exclusive to cellular senescence. Owing to the

absence of specific markers, bone marrow mesenchymal cells are usually identified by their ability to form colonies that stain positive for alkaline phosphatase when cultured *in vitro*. In present experiments, BM cells from non-arthritic mice and cells from mice with ZIA were cultivated in a specific medium containing β-glycerophosphate and ascorbic acid thus supporting osteoblast differentiation. After day 14 we registered the start of osteoblast generation and until day 21 we observed a presence of osteoblast nodules. Notably, in cultures of arthritic cells a lot of giant, diffuse, ALP positive cells were noticed (Fig. 4A, red arrows). These cells expressed high ALP activity pointing on their activated state (Fig. 4B). It is known that senescent cells exhibit a

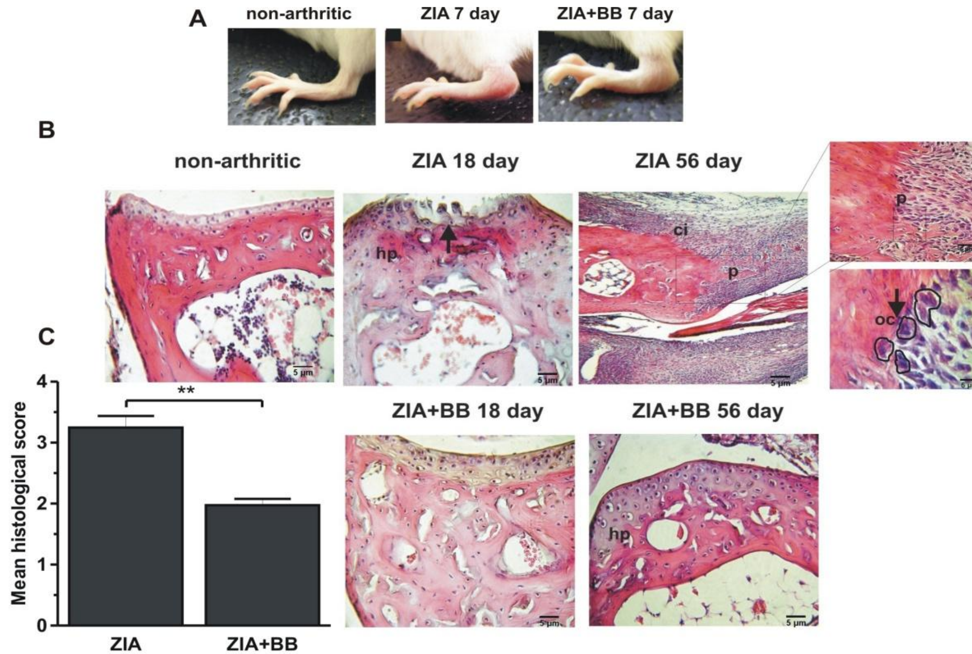

Fig. 1. Clinical and histological evaluation of healthy mice, mice with ZIA and mice with ZIA treated with berberine (BB) at different stages of arthritis

*(A) Erythema and joint swelling was observed in ZIA mice at day 7 less exerted in BB treated group. (B) Deformation of cartilage surface and degradation at day 18 and day 56 of arthritis with a presence of osteoclasts (oc) in arthritic mice (arrows), cell infiltration (ci), synovial hyperplasia (hp) and panus (p) all inhibited in BB treated mice. (C) Mean histological sore at day 56 of ZIA. n = 7 per group in three separate experiments. Data are expressed as the mean ± SD from the evaluation of 7 joints/group; Student's t-test; ** P =.01.*

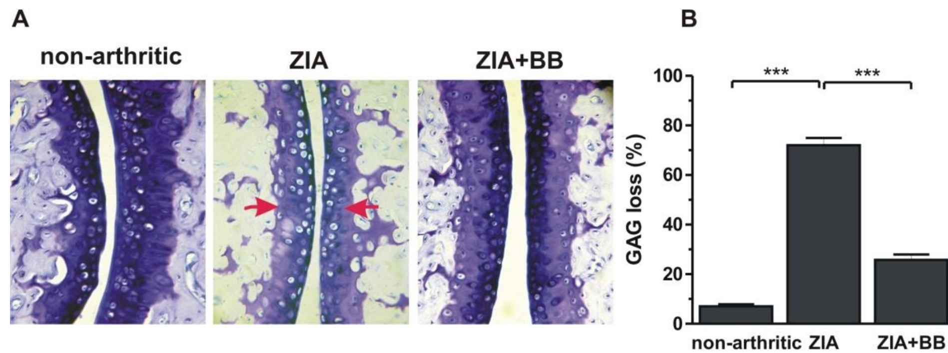

Fig. 2. Berberine (BB) reduced GAG thinning (toluidine blue staining)

*(A) Red arrows indicate destained articular cartilage. (B) Percentage of distained areas showing cartilage loss. n = 5 per group in three separate experiments. *** P =.001 from the evaluation of 5 joints/group.*

characteristic secretory phenotype involving inflammatory cytokines, growth factors, and matrix-degrading proteases that modulate and alter their local microenvironment. It is of importance that arthritic cells obtained at the active phase of ZIA are predisposed to accelerated senescence. As a consequence, the process of senescence probably leads to apoptosis of osteoblasts and lower capacity of bone remodeling.

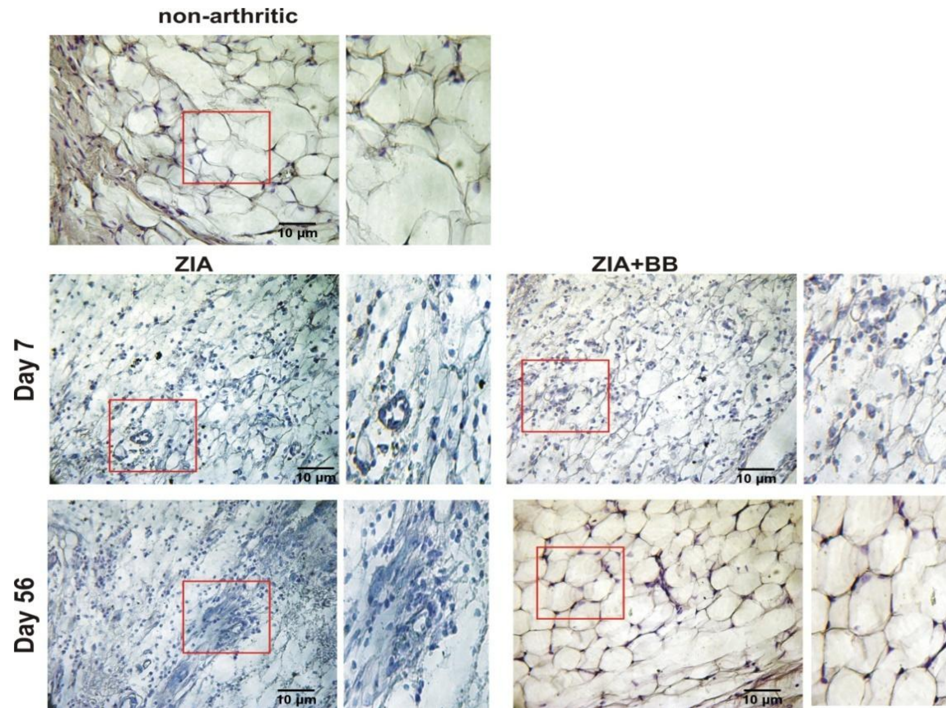

Fig. 3. Effect of berberine (BB) treatment on the accumulation of senescent cells in the synovium at day 7 and day 56 of ZIA

Representative pictures of cells stained with Sudan Black B (SBB). n = 7 per group in three separate experiments, from the evaluation of 7 joints/group.

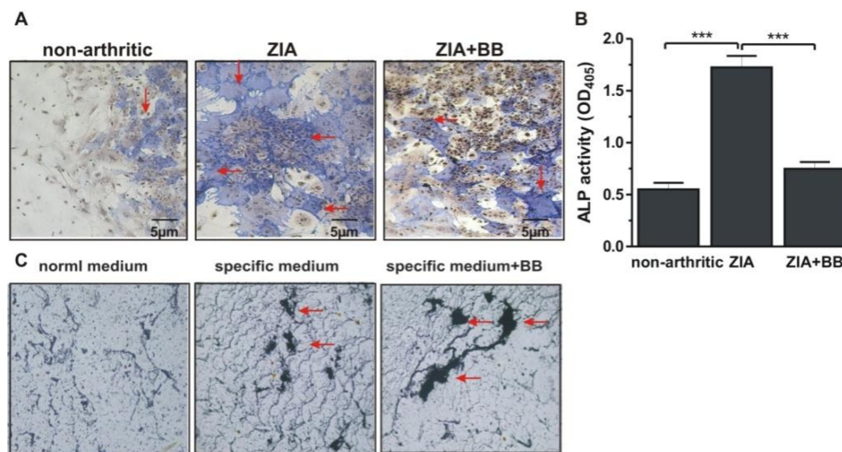

Fig. 4. Effect of berberine (BB) treatment on osteoblast ageing and calcium release

*Bone marrow cells isolated from healthy and arthritic mice at day 18 of ZIA were cultivated in specific medium for 21 days in the presence or absence of 20 μM BB from day 0 to day 5, and then stained with fast blue. Red arrows indicate senescent ALP positive cells (A). ALP activity is presented in (B). n = 5 per group in three separate experiments. *** P =.001. Calcium deposition in calvarial osteoblast cultures (C) stained with silver nitrate at day 21.*

Additionally, the influence of berberine on osteoblast activity was determined in calvarial cell cultures. After 14 days we observed signs of mineralization and further formation of mineralised nodules widespread at day 21. Berberine stimulated this process (Fig. 4C, red arrows) thus indicating that it can support bone remodeling in vivo.

The experiments included in this study were not pointed on the elucidation of the direct relation between berberine's effects and Jak pathway. Further investigations are required to define the role of Jak2/3 inhibition in bone remodeling. There are 3 general conditions for which we should consider berberine: metabolic syndrome, inflammation, and cancer. In the clinical trials the drug was applied in diabetes 2 patients at a dose of 500 mg 3 times daily [20,21] for 3 months. It supposed non-toxic long duration of treatment and supposed positive effect in chronic conditions such as particular types of arthritis.

4. CONCLUSION

The present study demonstrated that berberine reduced cartilage and bone destruction through decreased cell infiltration and prevention of GAG loss. The substance inhibited the generation of senescent cells in the synovium thus leading to ameliorated synovitis. Concomitantly, berberine diminished the appearance of senescent cells during osteoblast differentiation in vitro and enhanced the processes of mineralization. The effect of the substance can be defined as a long-time since the assessment was done 6 weeks after the last treatment. These findings provide new mechanistic insight into the anti-arthritic effects of berberine and are promising basis for its use in arthritic disorders.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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