Protective effect of methyl gallate on murine antigen-induced arthritis by inhibiting inflammatory process and bone erosion

Luana Barbosa Correa¹,² · Tatiana Almeida Pádua¹,² · Paulo Vinicius Gil Alabarse³ · Elvira Maria Saraiva⁴ · Esdras Barbosa Garcia⁵ · Fabio Coelho Amendoeira³ · Fausto Klubund Ferraris⁵ · Sandra Yasuyo Fukada³ · Elaine Cruz Rosas¹,² · Maria G. Henriques¹,²

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Abstract
Methyl gallate (MG) is a plant-derived phenolic compound known to present remarkable anti-inflammatory effect in different experimental models, such as paw oedema, pleurisy, zymosan-induced arthritis and colitis. Herein we investigated the effect of MG in the mice model of antigen-induced arthritis (AIA), a model with complex inflammatory response, driven primally by immune process and that cause bone and cartilage erosion similarly found in rheumatoid arthritis. Arthritis was induced by intra-articular injection of albumin methylated from bovine serum (mBSA) in C57BL/6 male mice previously immunized. The dose–response analysis of MG (0.7–70 mg/kg; p.o) showed that maximum inhibition was reached with the dose of 7 mg/kg on paw oedema and cell infiltration induced by AIA at 7 h. Treatment with MG (7 mg/kg; p.o) or with the positive control, dexamethasone (Dexa, 10 mg/kg; ip) reduced AIA oedema formation, leukocyte infiltration, release of extracellular DNA and cytokine production 7 and 24 h (acute response). Mice treated daily with MG for 7 days showed no significant weight loss or liver and kidney toxicity contrary to dexamethasone that induced some degree of toxicity. Prolonged treatment with MG inhibited the late inflammatory response (28 days) reducing oedema formation, cell infiltration, synovial hyperplasia, pannus formation and cartilage degradation as observed in histopathological analyses. Ultimately, MG reduced bone resorption as evidenced by a decrease in tartrate-resistant acid phosphate (TRAP)-positive cells number in femur histology. Altogether, we demonstrate that MG ameliorates the inflammatory reaction driven primarily by the immune process, suggesting a potential therapeutic application in arthritis treatment.

Keywords Methyl gallate · Inflammation · Antigen-induced arthritis · Osteoclastogenesis

Introduction
Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial inflammation, which can promote progressive cartilage and bone damage (Smolen et al. 2018; Giannini et al. 2020), ultimately leading to substantial disability in RA patients (Fardellone et al. 2020). Pro-inflammatory mediators are released in the synovial compartment by resident and infiltrated cells, playing a crucial role in arthritis pathogenesis. Cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-17, and IL-23, increase the recruitment of neutrophils into the joint and trigger chemokines release and degradative enzymes production (Brennan and McInnes 2008; Cecchi et al. 2018). Moreover, this pro-inflammatory milieu leads to an increase of macrophage colony-stimulating factor (M-CSF) levels and receptor activator of nuclear factor-κB
ligand (RANKL) expression, which stimulates the differentiation and activity of osteoclasts, promoting cartilage destruction and bone erosion (Karmakar et al. 2010; Llorrente et al. 2020).

The current therapy for RA includes non-steroidal anti-inflammatory drugs, disease-modifying anti-rheumatic drugs (DMARDs) of synthetic and of biological origin (Burmester and Pope 2017). However, traditional DMARDs such as glucocorticoids and methotrexate frequently present side-effects such as cytopenia, transaminase elevation, and poor tolerability. There are biological DMARDs that target tumour necrosis factor, IL-6 or IL-6 receptor, co-stimulatory molecules CD80 and CD86 or the molecule CD20, expressed in B cells. These drugs are usually administered in association with MTX but still, there is a high rate of unresponsive patients (Smolen et al. 2018). More recently, medications that suppress the Janus kinase (JAK) pathways have shown noticeable effects as RA treatments. Nevertheless, the JAK inhibitors, also often cause gastrointestinal side-effects, lymphopenia, neutropenia, elevated cholesterol, and more infections (Burmester and Pope 2017). Therefore, the investigation of alternatives to treat RA is still relevant. In the last decade studies have been trying to identify complementary therapies for RA from natural products with low toxicity (Sung et al. 2019). Polyphenols is one of the major classes of natural products that have been studied in this context. They are plant secondary metabolites and numerous studies have shown that polyphenol-rich diets exert cardioprotective, anti-cancer, anti-diabetic anti-inflammatory and anti-aging effects (Pandey and Rizvi 2009).

Methyl gallate (MG; Fig. 1) is a phenolic compound found in medicinal and food plants, such as Schinus terebinthifolius, Galla Rhois, Rosa rugosa e Givotia rotteriformis Griff. (Cho et al. 2004; Kang et al. 2009; Kamatham et al. 2015; Rosas et al. 2015), which presents pharmacological properties, such as antioxidant (Huang et al. 2021), antitumor (Lee et al. 2013), antimicrobial (Acharyya et al. 2015) and anti-inflammatory (Chae et al. 2010). MG attenuates osteoclastic differentiation in vitro and reduces the bone loss induced by lipopolysaccharide in mice (Baek et al. 2017). MG presented a potent anti-inflammatory effect inhibiting paw oedema, hyperalgesia and articular inflammation induced by the zymosan by decreasing cell accumulation and inflammatory mediators (Correa et al. 2016, 2020). This effect was associated with the modulation of NF-κB signaling and MAPK pathway triggered by TLRs (Correa et al. 2020). However, these studies have several limitations and are less representative of RA processes.

This study aimed to investigate a potential therapeutic effect of MG in mice under antigen-induced arthritis (AIA), a model with a complex inflammatory response, driven primarily by immune process and that cause extensive bone and cartilage erosion similarly to the reaction found in rheumatoid arthritis.

### Materials and methods

#### Animals

The experiments were performed in male C57BL/6 mice weighing between 20 and 25 g from the Science and Technology in Biomodel Institute (Fiocruz, Rio de Janeiro, Brazil). Mice were kept with free access to food and fresh water in a room with temperatures ranging from 22 to 24 °C and a 12-h light/dark cycle. All animal care and experimental procedures performed were approved by the institution's Committee of Ethics in Animal Care and Use (CEUA) (registered under the number CEUA LW-43/14).

#### Treatments

Animals were fasted overnight and then received oral administration (p.o.) of MG (98% purity; purchased from Fluka-Sigma-Aldrich) in doses ranging from 0.7 to 70 mg/kg diluted in filtered water in a final volume of 200 μL, 1 h before mBSA (albumin methylated from bovine serum; Sigma-Aldrich, St. Louis, MO, USA). The dose range was based on previous results on paw and articular oedema induced by zymosan published before (Correa et al. 2016, 2020). Dexamethasone (10 mg/kg, 100 μL) was administered intraperitoneally (i.p.) 1 h before stimulation and used as a positive control as described before (Correa et al. 2016). The same volume of water (200 μL) was administered orally to the control groups.

In another set of experiments, mice were treated daily with MG (7 mg/kg; p.o.) or dexamethasone (10 mg/kg; i.p.) for seven consecutive days. Treatment started on day 21, 1 h before mBSA challenge and was administered daily until day 28 when animals were euthanized.

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*Fig. 1* Chemical structure of methyl gallate

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Induction of experimental arthritis—antigen-induced arthritis

Mice were immunized as described previously (Oliveira et al. 2011; Kanashiro et al. 2016). C57BL/6 male mice were immunized with 500 µg of mBSA in 0.2 mL of an emulsion containing 0.1 mL phosphate-buffered saline (PBS) and 0.1 mL complete Freund's adjuvant (CFA; Sigma-Aldrich) by subcutaneous (s.c.) route at day 0. Booster injection of mBSA was given 7 days after the first immunization. On days 21 and 25, mice were challenged by intra-articular (i.a.) injection of mBSA (30 µg/cavity) or sterile saline (immunized saline group; [sal]) through the patellar ligament into the knee joint with an insulin syringe (BD Ultra-Fine). This protocol was used to prolong knee inflammation and to induce proteoglycan degradation. Non-immunized (NI) mice were given similar injections but without the antigen (mBSA) during the immunization process and challenged in the knee joint with mBSA (Fig. 2).

Immunoglobulins (IgG) specific for the antigen mBSA were determined in serum, obtained at 24 h of mBSA challenge (day 21) by ELISA. The serum levels of anti-mBSA specific total IgG antibodies were significantly higher in immunized mice than in non-immunized control mice (Supplemental Fig. 1).

Knee joint oedema evaluation

Articular oedema of the tibiofemoral joint was evaluated by measuring the transverse diameters of each knee joint with a digital caliper (Digmatic Caliper, Mitsutoyo Corporation, Japan) at different timepoints after challenge with mBSA. Values of knee joint thickness are expressed as the difference (Δ) between the diameters measured before (basal) and after the induction of articular inflammation in millimeters (mm).

Determination of joint leukocyte infiltration

Knee synovial cavities were washed twice with 150 µL of PBS containing EDTA (10 mM) by inserting a 21 G needle into mouse knee joints, and the synovial washes were recovered by aspiration. Total leukocyte counts were performed in an automatic particle counter Z2 (Coulter Z2, Beckman Coulter Inc., Brea, CA, USA). Differential counts of leukocytes were performed using May–Grunwald–Giemsa-stained cytospin smears (Cytospin 3, Shandon Inc., Pittsburgh, PA, USA), and the values are reported as the number of cells per cavity (×10⁵). After cellular counts, the synovial washes were centrifuged at 400×g for 10 min at 4 °C, and the supernatant was stored at −80 °C for further analysis.

Histological analysis

Whole knee joints obtained from C57BL/6 mice 7 days after i.a. administration of mBSA or saline were removed, dissected, and fixed in 10% formalin for 48 h. After that, the knees were kept in 10% EDTA in PBS solution for 2–3 weeks for decalcification. Tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) or safranin O/fast green, mounted on permanent glass slides, and analyzed under an optical microscope (Olympus BX41, Olympus, Japan). To assess pathological changes in the synovial cavity, images were taken at 100× and 200× magnification to allow the visualization of the entire area of the synovial

Fig. 2 Experimental protocol. In vivo experimental protocol of antigen-induced arthritis and methyl gallate treatment in mice.
cavity. The following parameters were subjectively graded: synovial hyperplasia (pannus formation; from 0 = no hyperplasia to 3 = most severe hyperplasia); synovial infiltrate (from 0 = no inflammation to 3 = most severe inflammation), bleeding (from 0 = no bleeding to 3 = most severe bleeding). The safranin-O/fast green staining was used to assess cartilage protein loss, suggesting cartilage degradation, and was also included in the assessment (from 0 = no cartilage degradation to 3 = severe cartilage degradation). Scores for all parameters were subsequently summed to give an arthritis index (AI; expressed as the mean ± SEM).

Cytokine and chemokine measurements (ELISA)
Levels of IL-17, CCL-2, and CCL-3 were quantified in the knee joint washes, and TNF-α concentration was quantified in the tissue joint at 7 h after injection of mBSA. Briefly, joints were dissected out, frozen with liquid nitrogen, crushed in a mortar and pestle, then were homogenized in 500 µL of the appropriate buffer containing a cocktail of protease inhibitors (Sigma-Aldrich), centrifuged and the supernatants were used to determine TNF-α levels. According to the manufacturer's instructions, cytokines were measured using standard sandwich ELISA using matched antibody pairs (Quantikine, R&D Systems, Minneapolis, MN, USA). The results are expressed as picograms of each cytokine or chemokine per milliliter (pg/mL) or per mg of tissue.

Measurement of extracellular dsDNA
Synovial fluids from animals subjected to AIA were collected 7 and 24 h after i.a. challenge with mBSA. The washings were centrifuged to remove cells and 50 µL of the supernatant was added to 50 µL of Quanti-it™ PicoGreen® dsDNA Assay kit (Thermo Fisher Scientific, Waltham, USA), and the reading determined at 485/538 nm emission/excitation according to (Gardinassi et al. 2017). The results were expressed in arbitrary units (AU).

Assess of acute oral toxicity of MG
To assess the potential toxic of MG, healthy mice were treated for 14 consecutive days with different concentrations of MG (5, 50, 300 and 1000 mg/kg) accordingly to OECD guideline 420. After oral administration, the animals were followed for 14 days to assess the following aspects: food intake, clinical signs (assessed daily) and assessment of body weight. The organs evaluated were: kidneys, liver, heart and spleen. The absolute weight was recorded from the direct weighing of each organ, and the relative weight was obtained by calculating the absolute weight of the organ divided by the animal weight, to obtain the proportion of organ weight (g) per gram of body weight. For the hematological and biochemical evaluation, abdominal aortic puncture was performed and samples were processed according to the manufacturer’s instructions. The tests were performed using the dry chemistry methodology using the Vitros 250 equipment (Ortho clinical; Jonhson & Jonhson) and pocH-100iV Diff equipment (Sysmex Corporation) for hematological evaluation.

Evaluation of plasma enzyme activities after prolonged treatment with MG
Mice were treated daily with MG (7 mg/kg; p.o.) or dexamethasone (10 mg/kg; i.p.) for seven consecutive days. Treatment started on day 21, 1 h before mBSA challenge and was administered daily until day 28. Blood samples were collected by cardiac puncture (day 28) and added into microtubes containing anticoagulant (Heparin; Sigma-Aldrich) to determine enzymatic activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as indicators of hepatotoxicity and urea and creatinine kinase levels as indicators of nephrotoxicity. Plasma was separated by centrifugation (200× g, 10 min, 4 °C), samples processed according to the manufacturer's instructions and the tests were performed using the Vitros 250 equipment. Results were presented as U/mL (AST and ALT) and mg/dL (urea and creatinine kinase) of plasma. Bodyweight was monitored throughout the experiment from day 0 to 28.

Histochemical staining for TRAP
Sections of knee joints obtained from C57BL/6 mice at 7 days after i.a. mBSA administration were stained according to tartrate-resistant acid phosphate (TRAP) kit 387A (Sigma-Aldrich, St. Louis, MO, USA). TRAP was used as a histochemical marker of osteoclasts in 5 µm sections (Ballanti et al. 1997) and TRAP-positive cells appeared as dark purple. Osteoclasts were identified as TRAP-positive multinucleated cells and counted in the joint growth area (femur). The result was expressed as the number of TRAP-positive cells.

Osteoclast culture and in vitro TRAP analysis
Bone marrow cells (BMCs) from 8-week-old male C57BL/6 mice were obtained by flushing tibias and femurs with α-MEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 µg/mL). Cells were transferred to 10 cm Petri dishes and then cultured in the presence of M-CSF (30 ng/mL; R&D Systems, Minneapolis, MN, USA) for 3 days. After that, floating cells were discarded, and the adhering cells were classified as BMMs (bone marrow macrophages). To generate osteoclasts, BMMs were cultured in
96-well plates (2 × 10^4 cells/well) at 37 °C/5% CO₂ in the presence of osteoclastic media, which consists of M-CSF (30 ng/mL) and RANKL (10 ng/mL), with different concentrations of methyl gallate (0, 3, 10, 30 µM). After 3 days, the cells were stained according to TRAP kit 387A (Sigma-Aldrich, St. Louis, MO, USA). TRAP-positive multinucleated cells with more than three nuclei were identified as osteoclasts. Mature osteoclasts were counted and represented as multinucleated TRAP^+ cells/well. To determine the osteoclast area, we analyzed the images using ImageJ software.

**Cell viability**

BMMs were seeded in 96-well plates (2 × 10^4 cells/well) and cultured with osteoclastic media and different concentrations of MG (0, 3, 10, 30 µM) as described above. On the third day, 5 mg/mL MTT solution (22.5 µL) was added to each well. After 3 h, the plate was centrifuged (200 × g/5 min), the medium was removed, and DMSO (150 µL) was added to dissolve the formazan dye. The optical density (OD) was measured at 570 nm using a microplate reader. The mean OD of the control group (RANKL) was set as 100%, and the experimental groups were compared to RANKL.

**RNA extraction and qRT-PCR**

BMMs were seeded in 24-well plates (2 × 10^5 cells/well) and cultured with osteoclastic media and MG (30 µM) for 48 h. Total RNA was isolated from cell culture using the SV Total Isolation System kit (Promega) and quantified using NanoDrop with an A260/280 ratio of 1.9–2.1. Complementary DNA was synthesized using the High-Capacity synthesis kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). Quantitative PCR (qPCR) was performed on a StepOnePlus system (Applied Biosystems, Carlsbad, CA, USA) using a predesigned TaqMan Universal master mix II (Applied Biosystems). The relative levels of mRNA expression were determined by the 2^−ΔΔCt method using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control (reference gene). Specific primers for nuclear factor of activated T cells, calcineurin-dependent (Nfatc1; Mm00479445_m1), tartrate-resistant acid phosphatase (TrACP5; Mm00475698_m1), integrin-β 3 (Itgb3; Mm00443980_m1), calcitonin receptor (CTR; Mm00432282_m1) and GAPDH (Mm99999915_g1) genes were employed (TaqMan® Gene Expression Assay). The graphs show the relative expression of the gene of interest normalized to the control population (RANKL).

**Statistical analyses**

In all experiments, values are expressed as means ± SEM. Differences between groups were tested for significance by one-way ANOVA followed by Tukey multiple comparison tests using GraphPad Prism 5 software. A p value of ≤ 0.05 was considered significant.

**Results**

**Dose–response analysis of methyl gallate on oedema and cellular influx in the antigen-induced arthritis model**

We investigated the anti-inflammatory effects of methyl gallate (MG) in a well-established model of antigen-induced arthritis (AIA) in mice, which involves adaptive immunity to generate the acute inflammatory response (Asquith et al. 2009; Benson et al. 2018).

Pretreatment with MG inhibit antigen-induced oedema formation at doses of 7 and 70 mg/kg, whereas no effect was observed for pretreatment with MG at 0.7 mg/kg (Fig. 3A). As shown in Fig. 3B–D, only the dose of 7 mg/kg inhibited significantly total leukocyte recruitment from 1.065 ± 0.093 to 0.628 ± 0.095 × 10^5 cells/cavity. Considering these results, further analysis was performed using a dose of 7 mg/kg.

**Methyl gallate inhibits acute articular inflammatory response in AIA model**

The antigen injection in the knee joints of immunized C57BL/6 mice induced significant oedema formation and increased total leukocyte, mononuclear cells, and neutrophil count in its joint exudate 7 and 24 h after the challenge (Fig. 4). Treatment with dexamethasone (10 mg/kg; i.p.) or MG (7 mg/kg) was performed 1 h before the challenge, and the mice were euthanized 7 or 24 h after the challenge. Prior oral administration of MG significantly reduced the increase in joint diameter at both times evaluated to the same extent as the positive control, dexamethasone (Fig. 4A, B). Treatment with MG also decreased the number of total leukocytes accumulated in the joint at 7 h (56% of inhibition) and 24 h (62% of inhibition), compared with immunized and challenged mice (Fig. 4C, D). Treatment with MG also significantly reduced mononuclear cells and neutrophils accumulation into the synovial cavity (Fig. 4E–H). Inhibition in all parameters evaluated was statistically similar to that of the positive control, dexamethasone.

An excessive formation of neutrophil extracellular traps (NETs) is one of the primary mechanisms by which neutrophils cause tissue damage and promote autoimmunity in RA (Apel et al. 2018). We evaluated the release of extracellular...
DNA in the synovial wash to infer MG’s effect on NET formation. The i.a. injection of mBSA on joint of immunized mice induced an increase of DNA release on synovial wash 7 and 24 h after the challenge (Fig. 4I, J), suggesting NET formation in this model. MG treatment reduced the release of extracellular DNA similarly as dexamethasone at both times evaluated (Fig. 4I, J). These results collectively indicate that MG is efficient in modulating the inflammatory process involved in AIA.

**Methyl gallate inhibited the production of pro-inflammatory cytokines in the AIA model**

Chemokines and cytokines play a pivotal role in the recruitment of inflammatory cells and the development of arthritis. The levels of CCL-2, CCL-3, IL-17 and TNF-α were evaluated in the synovial wash and periarticular tissue by ELISA (Fig. 5). The chemokines CCL-2 and CCL-3, related to the recruitment of monocytes and polymorphonuclear leukocytes, were increased in the synovial wash of AIA mice compared to the control groups (non-immunized mice and immunized-saline group). Treatment with MG reduced CCL-2 and CCL-3 production (Fig. 5A, B). It was also observed an increase in cytokines IL-17 and TNF-α 7 h after the challenge with mBSA. Pretreatment with MG significantly reduced IL-17 and TNF-α production at 7 h, to a similar extent to the dexamethasone pretreatment effect (Fig. 5C, D). In this way, inhibition of pro-inflammatory chemokines and cytokines by MG correlates with reducing the inflammatory reaction in the AIA model.
Fig. 4  Effect of methyl gallate on oedema, leukocyte influx and DNA released in the synovial wash. Mice were treated with MG (7 mg/kg, po,—1 h) or dexamethasone (10 mg/kg, ip,—1 h) before i.a. injection of mBSA (30 µg/25 µL). Control group was injected with the same volume of sterile saline. Non-immunized mice also were challenged i.a. with mBSA. Knee joint diameter was evaluated with a digital caliper 7 and 24 h after mBSA challenge (A, B). The numbers of total leukocytes (C, D), mononuclear cells (E, F), and neutrophils (G, H) are plotted as the number of cells × 10^5. Knee synovial cells were recovered 7 and 24 h after mBSA challenge. Levels of DNA released in synovial wash induced by injection of antigen (I, J). The results are presented as the means ± SEM of seven mice per group per experiment and are representative of three separate experiments (*p ≤ 0.05 compared to the vehicle group; +p ≤ 0.05 compared to the immunized mBSA group [one-way ANOVA followed by Tukey test]). NI not-immunized; IM immunized.
Effect of daily treatment with methyl gallate on liver and kidney enzymes

An important issue to be addressed is whether prolonged treatment with MG present toxicity effects. Accordingly, the acute oral toxicity assay showed no changes in hematological and biochemical patterns or in the size or morphology of vital organs, such as heart, liver, spleen and kidneys even after treatment with the highest MG dose (1000 mg/kg) (Table 1). Next, we evaluated a potential toxicity effect of prolonged treatment with MG in animals affected by AIA. Mice were treated daily with MG or dexamethasone for seven consecutive days after i.a. challenge with mBSA. During the disease’s progression, the mice weight was monitored, and it was not observed a significant difference between non-immunized and immunized mice groups (Fig. 6A). However, daily treatment with dexamethasone induced a significant weight loss. MG treatment did not cause weight loss, but the animals did not gain weight in the same proportion as the control groups (Fig. 6A). Next, plasma samples were collected to determine the concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea BUN and creatinine kinase (CK). Dexamethasone induced an increase in AST and ALT levels (Fig. 6B, C). Significantly, the treatment with MG did not alter the plasma concentration of AST (Fig. 6B), ALT (Fig. 6C), urea BUN (Fig. 6D) and CK (Fig. 6E) compared to control groups. These results suggest that prolonged treatment with MG does not interfere with mice liver or kidney function.

Daily treatment with methyl gallate inhibits late articular inflammatory response in the AIA model

AIA model presents several parameters of a chronic inflammatory process at day 28, after two challenges with mBSA. The daily treatment with MG for 7 days after the first injection of mBSA reduced significantly the antigen-induced oedema formation (Fig. 7A). The treatment was also able to reduce the arthritis index calculated with histopathological parameters: severity of synovial hyperplasia (pannus formation), intensity of the inflammatory infiltrate, bleeding and cartilage degradation (Fig. 7B). Figure 7C shows representative images of histological sections of mice femorotibial joints obtained 7 days after the i.a. challenge with mBSA stained with H&E or Safranin-O. Immunized-saline group exhibited a typical architecture of the femorotibial joint, with a normal appearance of the cartilage lining, without any inflammatory infiltrates in the synovial tissue (Fig. 7C). Non-immunized histological
sections show moderate inflammatory infiltrate in the tissue. Histological sections from immunized-mBSA mice presented an abnormal histologic appearance of the joint, with pronounced synovial inflammation (black arrows) and severe decrease in fat cells, synovial hyperplasia and pannus formation (*) (Fig. 7C). The analysis of immunized group showed an increase in the overall arthritis score in comparison to control mice (Fig. 7B).

In contrast, the animals treated with dexamethasone showed significant inhibition in cell infiltration compared with vehicle-treated mice, leading to a reduction in the arthritis index (Fig. 7B). MG treatment was also effective in reducing all scores of histologic features analyzed and the arthritis index was decreased in a similar proportion to the observed with dexamethasone treatment (Fig. 7B, C). Safranin-O stain was used to assess the loss of cartilage protein. Histological sections from mBSA-immunized mice, suggest cartilage degradation as evidenced by loss of safranin staining (Fig. 7D).

Table 1 Biochemical data, hematological data and relative organ weight of C57BL/6 mice, 14 days after administration of methyl gallate (MG)

| Parameters                          | Reference values | CTRL     | MG (5 mg/kg) | MG (50 mg/kg) | MG (300 mg/kg) | MG (1000 mg/kg) |
|-------------------------------------|------------------|----------|--------------|---------------|---------------|-----------------|
| Glucose (mg dL⁻¹)                   | 90–192           | 157 ± 28 | 183 ± 33     | 133 ± 21      | 131± 44       | 171 ±62         |
| Urea (mg dL⁻¹)                      | 36–96            | 38.7 ± 16.9 | 28.2g± 4.1   | 31 ±4.0       | 30.4 ± 7.9    | 38.5 ± 9.0      |
| Creatinine kinase, CK (U/L)         | 68–1070          | 46 ± 14  | 58 ± 12      | 46 ± 8        | 45 ± 24       | 70 ± 56         |
| Alkaline phosphatase, ALP (U/L)     | 30–200           | 185 ± 47 | 141±24       | 129±44        | 139±39        | 141±29          |
| Uric acid (mg dL⁻¹)                 | 1.7–5.4          | 1.9 ± 0.8 | 1.7 ± 0.7    | 1.0 ± 0.2     | 1.0 ± 0.2     | 1.4 ± 0.5       |
| AST (U/L)                           | 59–247           | 51 ± 11  | 39 ± 8       | 61 ± 31       | 52 ± 18       | 46 ± 14         |
| ALT (U/L)                           | 38–106           | 52 ± 16  | 54 ± 5       | 68 ± 18       | 47 ± 6        | 46 ± 8          |
| Total proteins (g dL⁻¹)             | 3.6–6.6          | 4 ± 1.0  | 3 ± 0.2      | 3 ± 0.1       | 3 ± 0.8       | 3 ± 0.7         |
| Albumin (g dL⁻¹)                    | 2.5–4.8          | 1.9 ± 0.6 | 1.4 ± 0.1    | 1.4 ± 0.1     | 1.4 ± 0.4     | 1.5 ± 0.4       |

Table 1: Biochemical data, hematological data and relative organ weight of C57BL/6 mice, 14 days after administration of methyl gallate (MG).

| Parameters                          | CTRL     | MG (5 mg/kg) | MG (50 mg/kg) | MG (300 mg/kg) | MG (1000 mg/kg) |
|-------------------------------------|----------|--------------|---------------|---------------|-----------------|
| Red blood cell count (RBC) (milhões/mm³) | 7.72 ± 1.40 | 8.89± 0.47   | 9.26 ± 0.42   | 8.59 ± 1.87   | 9.11 ± 0.98     |
| Hemoglobin measurement (HGB) (g/dL) | 12.2 ± 1.3 | 13.3 ± 0.6   | 13.7 ± 0.5    | 12.8 ± 2.4    | 13.56± 1.32     |
| Hematocrit assessment (Hct) (%)     | 43.3 ± 25.7 | 47.6 ± 2.1   | 49.8 ± 2.6    | 46.2 ± 10.3   | 49.30± 5.51     |
| Mean corpuscular volume (MCV) (fm³) | 57± 8.3   | 53.5 ± 0.5   | 53.8 ± 0.8    | 53.7 ± 0.6    | 54.6± 0.74      |
| Mean corpuscular hemoglobin (MCH) (pg) | 16.1 ± 2.2 | 15.0 ± 0.1   | 14.8 ± 0.2    | 15.1 ± 0.8    | 14.90± 0.22     |
| Mean corpuscular hemoglobin concentration (MCHC) (g/Dl) | 28.2± 0.8 | 27.9 ± 0.2   | 27.5 ± 0.6    | 28.1 ± 1.8    | 27.56± 0.53     |
| White blood cells (WBC) (mil/mm³)   | 6.9± 4.6 | 3.9 ± 1.1    | 4.3 ± 1.8     | 4.6 ± 1.1     | 4.40± 2.09      |
| Platelet count (mil/mm³)            | 760 ± 220 | 866 ± 91     | 785 ± 171     | 727± 202     | 860.20± 206.99  |

| Parameters                          | CTRL     | MG (5 mg/kg) | MG (50 mg/kg) | MG (300 mg/kg) | MG (1000 mg/kg) |
|-------------------------------------|----------|--------------|---------------|---------------|-----------------|
| Heart                               | 4.6 ± 1.3 | 3.4 ± 0.2    | 3.7 ± 0.5     | 3.5 ± 0.4     | 4.1 ± 0.9       |
| Liver                               | 43.9 ± 4.9 | 44.4 ± 6.6   | 49.1 ± 1.9    | 51.1 ± 2.8    | 49.3 ± 2.0      |
| Spleen                              | 2.5 ± 0.2 | 2.0 ± 0.4    | 2.3 ± 0.8     | 1.8 ± 0.2     | 2.4 ± 1.1       |
| Kidneys                             | 10.9 ± 1.4 | 8.8 ± 0.6    | 9.6 ± 1.0     | 9.2 ± 1.2     | 9.9 ± 1.3       |

Values expressed as mean ± SEM. The difference between the groups was assessed through analysis of variance (ANOVA) followed by the Dunnett test. The symbol (*) indicates the statistical significance at the level of (p<0.05) between the treated group vs. group control (CTRL).

Table 1: Biochemical data, hematological data and relative organ weight of C57BL/6 mice, 14 days after administration of methyl gallate (MG).

Methyl gallate attenuates osteoclastogenesis in vitro and bone resorption in the knee joint in the AIA model

Bone erosion during arthritis is primarily the result of activated osteoclasts that express bone-resorbing enzymes. The expression of TRAP enzyme is a marker of osteoclast function and bone resorption intensity. Methyl gallate was previously described to reduce in vitro RANKL-dependent osteoclastic differentiation (Baek et al. 2017). We confirmed this finding by evaluating the number and area of multinucleated TRAP-positive cells as well as the expression of osteoclastogenic marker genes. Bone marrow macrophages (BMMs) were cultured with M-CSF, RANKL and different concentrations of MG (3, 10, 30 or 100 µM). Figure 8A shows the cell viability using MTT assay. The concentration...
of 100 µM presents a cytotoxic effect; however, the other concentrations tested were not cytotoxic. Numerous large TRAP-positive cells were formed after 3 days of culture of BMMs stimulated with M-CSF and RANKL (Fig. 8B, C and Supplemental Fig. 2A). The number of multinucleated TRAP-positive cells decreased in the presence of MG at 30 µM (Fig. 8B). The area of multinucleated TRAP-positive cells decreased in the presence of MG in a concentration-dependent manner (Fig. 8C), reaching a maximum effect at the concentration of 30 µM. Gene expression analysis showed that stimulus with M-CSF and RANKL upregulated the expression of integrin-β3, calcitonin receptor (CTR) and NFATc1 (Supplemental Fig. 2B–D). MG downregulated the expression of integrin-β3 and CRT (Supplemental Fig. 2B, C) and also decreased the expression of NFATc1 which is a key regulator of osteoclast differentiation (Supplemental Fig. 2D).

Next, we investigated the effect of daily treatment with MG in the knee joint in AIA model. TRAP-positive cells were determined by histochemical staining in knee joint tissue samples. AIA-induced a significant TRAP-positive cells detection compared to the control groups (non-immunized

![Image](image1.png)

NI: non-immunized; IM: immunized; *p ≤ 0.05 compared to the vehicle group; p ≤ 0.05 compared to the immunized mBSA group (one-way ANOVA followed by Tukey test).
mice and immunized mice challenge with saline). In turn, MG and dexamethasone treatment inhibit AIA-induced TRAP-positive cells (Fig. 8D). Quantitative analysis (Fig. 8E) of TRAP-positive cells confirmed that MG inhibited osteoclast activation. This result suggests that MG reduces in vivo osteoclast formation, and consequently, decreases the articular damage of murine antigen-induced arthritis model.

Discussion

Methyl gallate is a prevalent phenolic acid in the plant kingdom and is often found in herbs used in folk medicine to treat inflammatory conditions. In previous works, we already described that MG (0.7–70 mg/kg p.o.) inhibited in vivo inflammatory pain, edema, and cell migration in an acute inflammatory reaction induced by zymosan (Correa et al. 2016, 2020). This effect is related to the inhibition of neutrophil accumulation and inflammatory mediators, such as IL-1β, IL-6, TNF-α, CXCL-1, LTB4, and PGE2 which are relevant in the pathogenesis of rheumatoid arthritis (RA). Herein, we demonstrated the MG activity on the murine model of antigen-induced arthritis (AIA). This model is trigger primarily by immune processes and presents several characteristics of RA. Among others, a marked synovial-lining hyperplasia, proliferation of subliming cells, infiltration of inflammatory cells, local chemokine and cytokine production, pannus formation, hypernociception and articular cartilage destruction concomitant with prominent osteoclastogenesis (van Den Berg et al. 1981; Ferraccioli et al. 2010; Sachs et al. 2011).

Our data demonstrate that MG pretreatment significantly attenuates the development of AIA. This was evidenced by the reduction of clinical scores and synovial inflammation in treated animals. MG treatment also reduced the neutrophil influx into the joint cavity, oedema formation, articular cartilage damage, cytokines production, and osteoclast number in the synovial tissue. The inhibition achieved with MG was equivalent to the obtained with dexamethasone, except for the number of osteoclasts in synovial tissue. Dexmethasone is a synthetic glucocorticoid with anti-inflammatory and immunosuppressant properties commonly used to manage RA (Fraenkel et al. 2021). Dexmethasone binds to coactivators to directly inhibit histone acetyltransferase (HAT) activity and recruits histone deacetylase (HDAC)-2, which reverses histone acetylation, leading to suppression of activated inflammatory genes (Dinarello 2010). Our studies focused on the evaluation of the main signs of the inflammatory process and the production of inflammatory mediators, that is the reason we have chosen a positive control that acts directly on these pathways, such as dexamethasone.

Neutrophils have been recognized as critical participants in the onset and progression of rheumatoid arthritis. The pathogenic role of neutrophils includes increased migratory capacity, increased ROS and cytokines production and apoptosis delay (Martelli-Palomino et al. 2017; Marchi et al. 2018). Moreover, an exacerbated release of neutrophil extracellular traps is also observed (Khandpur et al. 2013; Wright et al. 2021). Many studies have confirmed a high degree of spontaneous NETs formation in RA neutrophils, showing a strong correlation between free circulating DNA levels and inflammatory markers, such as C-reactive protein (CRP), erythrocyte sedimentation rate, ACPAs titers, and levels of TNF-α and IL-17 (Khandpur et al. 2013; Sur Chowdhury et al. 2014; Pérez-Sánchez et al. 2017). Accordingly, we demonstrated that MG pretreatment reduced the release of extracellular DNA in the AIA model at both times studied (36% and 39%, of inhibition, respectively). It was recently shown that NETs are released in the joints in the AIA model and mediate articular pain through activation of TLRs (Schneider et al. 2020). It is interesting to note that MG can also inhibit inflammatory pain and cell signaling induced by Toll-like receptor ligands (Correa et al. 2020). However, we did not evaluate the effect of MG directly on NET release and we cannot rule out whether this reduction of circulating DNA on synovial wash is due to a direct decrease in the neutrophils number in the inflamed joint.

Several cytokines, including TNF-α and IL-17, play a fundamental role in the events that cause inflammation, joint destruction, and various comorbidities in RA (Tarner et al. 2007). MG inhibited AIA-induced pro-inflammatory cytokines production (TNF-α, IL-17, CCL-2 and CCL-3). We have recently demonstrated that MG (1–100 µM) is able to inhibit cytokines production induced by Toll-like receptor ligands by inhibiting MAPK and NF-Kb signaling impairing the ERK1/2, JNK and p38 MAPK phosphorylation as well as the 1κB-α degradation (Correa et al. 2020). IL-17 and TNF-α are increased in RA serum and synovial fluid, and their elevated level predicts joint damage progression. IL-17 and TNF-α are also described to induce NETosis in neutrophils from RA patients (Khandpur et al. 2013). IL-17 has a widespread inflammatory effect on the joint, orchestrating bone and cartilage damage progression, and causes pro-inflammatory mediators’ augmentation to the synovium (Maddur et al. 2012). Many chemokines are also increased in RA synovial fluid, including CCL-3 and CCL-2. These chemokines regulate the joint inflammatory response by recruiting and activating both innate and adaptive immune cells (Szekanecz and Koch 2016). Therefore, MG inhibition of mBSA-induced cytokine production may lead to reduced leukocyte recruitment reduction, oedema formation, and bone and cartilage damage.

RA patients commonly take corticosteroids; however, the induction of many side effects limits this therapy (Yasir and...
Sonthalia 2019). In this work, we investigated the action of MG and dexamethasone when administered in a prolonged treatment regimen, regarding weight gain and plasma enzymes. We observed a loss of weight and an increase in plasma levels of AST and ALT in mice treated daily with dexamethasone. On the other hand, treatment with MG did not cause any changes in plasma enzymes related to liver and kidney function. Our results showed that prolonged treatment with MG did not cause liver and kidney damage.

The histopathological study confirmed the effectiveness of the prolonged treatment with MG. We observed a reduction in cell infiltration, synovial inflammation, synovial hyperplasia and pannus formation at the same proportion as the prolonged treatment with dexamethasone. Treatment with MG or dexamethasone also protected cartilage degradation as observed with safranin-O stain.

Systemic bone loss in RA is a multifactorial and complex alteration in which chronic inflammation plays an important role. Osteoclast plays a critical role in bone loss and its differentiation and activation are mainly dependent on the presence of M-CSF and RANKL (Boyle et al. 2003). Pro-inflammatory cytokines such as TNF, IL-1 and IL-6 can enhance osteoclastogenesis by amplifying osteoclast function, inhibiting osteoblast function and perpetuating an inflammatory response that can lead to tissue degradation (Redlich and Smolen 2012).

It was previously described that MG inhibits osteoclastogenesis in vitro, by diminishing mRNA expression of OSCAR (osteoclast-associated receptor) and TRAP through downregulation of c-Fos and NFATc1 genes (Baek et al. 2017). Macrophages differentiate into bone-resorbing osteoclasts under stimulation of two necessary factors, M-CSF and RANKL. These factors induce the expression of genes that characterize the osteoclast lineage, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor (CRT) and the integrin-β3, leading to the development of mature osteoclasts (Han et al. 1998; Udagawa et al. 2021). NFATc1 is a pivotal regulator of osteoclastogenesis, modulating the expression of several osteoclast-specific molecules involved in fusion, differentiation, maturation and bone remodeling (Matsuoi et al. 2004; Huang et al. 2006; Park et al. 2017). We confirmed this effect, showing that MG can directly modulate osteoclastogenesis in vitro, diminishing the number and area of multinucleated TRAP-positive cells, as well as the expression of osteoclastogenic marker genes integrin-β3 and CRT and the expression of NFATc.

Importantly, we demonstrated that oral treatment with MG was able to reduce, the bone loss observed in the AIA model by quantifying the number of TRAP-positive cells. The number of TRAP-positive cells was decreased after prolonged treatment with dexamethasone or MG, reinforcing the in vivo effect on osteoclast differentiation.

**Conclusions**

The present data demonstrate that MG ameliorates antigen-induced arthritis. The reduction in the acute influx of neutrophils and cytokines and chemokines may be leading to cartilage degradation prevention. In addition, MG protected against bone erosion, possibly by its direct effect on osteoclast differentiation. These findings collectively demonstrate that MG exerts therapeutic effects on chronic inflammatory reactions driven primarily by an immune process characterized by extensive bone and cartilage erosion, such as in rheumatoid arthritis.

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Fig. 8 Effect of MG on osteoclast differentiation in vitro and osteoclasts number in synovial tissue. A–E Mouse bone marrow macrophage (BMMs) were cultured with osteoclastic media in the presence of the indicated concentration of MG (3, 10, 30 or 100 µM). A Cell viability was assessed by MTT assay. B After 3 days, cells were fixed and stained for TRAP. C Multinucleated TRAP-positive cells containing more than three nuclei were considered mature osteoclast and were counted (osteoclasts per well). D Longitudinal histological sections of the femorotibial joint were obtained 7 days after the challenge with the antigen, a representative image of at least six different animals per group (100x; bar = 100 µm). E Quantitative analysis of TRAP positive cells. The results are presented as the means ± SEM and are representative of two separate experiments [A–C, *p ≤ 0.05 compared to untreated and treated mice; #p ≤ 0.05 compared different doses of MG; E, *p ≤ 0.05 compared to the vehicle group; +p ≤ 0.05 compared to the immunized mBSA group (one-way ANOVA followed by Tukey test)]
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Data availability All the data of this study are transparent.

Code availability No applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethics approval All animal care and experimental procedures performed were approved by the institution’s Committee of Ethics in Animal Care and Use (CEUA) (registered under the number CEUA LW–43/14).

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