



Research article

Prednisone prevents particle induced bone loss in the calvaria mouse model



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HIGHLIGHTS

- Particle-induced bone loss in mice can be utilized to investigate osteoclast activity.
- Glucocorticoids show an osteoprotective effect on particle induced local bone resorption.
- Short-term low-dose glucocorticoids did not have measurable systemic side effects.

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ABSTRACT

Introduction: Glucocorticoids are essential in the treatment of many chronic inflammatory and malignant diseases but are known to have detrimental effects on bone. This study aimed to investigate the effects of prednisone on osteoclast functioning *in vivo* in the calvaria particle-induced bone loss mouse model.

Methods: 12-week-old male C57BL6/J mice received subcutaneously implanted prednisone (2.5 mg/d, 60 day release (n = 14)) or placebo pellets (n = 10). Osteolysis of the calvaria bone was induced two weeks later by application of ultra-high-molecular-weight polyethylene- (UHMWPE) particles to the dome (vs sham operation). The extent of osteolysis was determined histologically and by micro-computer tomography.

Results: Prednisone significantly inhibited particle-induced osteolysis in the skull. No significant difference in osteoclast numbers was seen in mice with prednisone vs placebo treatment. Prednisone treatment alone without particle application did not reduce bone mineral density or deterioration in bone microarchitecture parameters.

Conclusions: The calvaria particle-induced bone loss mouse model can be adapted to investigate osteoclast activity *in vivo* and the effect of prednisone on osteoclasts. In this preventive experimental design, the application of short-term low-dose prednisone has osteoprotective effects without measurable systemic side effects on bone parameters.

1. Introduction

Inflammatory disorders such as rheumatoid arthritis (RA) [1] are associated with reduced bone quality and secondary osteoporosis [2] and often require long-term treatment with glucocorticoids. There is evidence that increased systemic inflammatory activity increases bone resorption and decreases new bone formation [3]. Osteoclast function plays a

significant role in the pathogenesis of osteoporosis in inflammatory diseases [4, 5]. Proinflammatory cytokines stimulate the recruitment of osteoclast precursors and regulate osteoclast formation and function. Accordingly, immunosuppressive treatments like TNF α inhibitors prevent bone loss [4, 6].

Glucocorticoid-induced osteoporosis (GIO) is one of the most common forms of secondary osteoporosis [7]. GIO is characterized by

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stimulation of bone resorption, followed by a persistent and profound suppression of bone formation. These changes result in a systemic loss of bone mass, microarchitecture, and increased fracture risk [7, 8]. Glucocorticoids (GCs) suppress bone formation via inhibition of osteoblast activity through the Wnt signalling pathway [8, 9] and by dysregulating osteoblastogenesis [10]. Additionally, GCs stimulate osteoclastogenesis and inhibit osteoclast apoptosis through stimulation of receptor activator of nuclear factor κ B ligand (RANKL) expression and inhibition of osteoprotegerin (OPG), respectively [11, 12]. Due to the immunosuppressive actions, GCs are widely used to treat inflammatory disorders [13].

Interestingly, in RA low dose GCs have been shown to reduce the extent of disease-related periarticular osteoporosis [14] and reduce the rate of radiographic disease progression [15, 16]. Thus, by controlling the underlying inflammatory disease, glucocorticoid therapy might alleviate its negative impact on bone [17, 18]. A critical mechanism for these effects is the ability of glucocorticoids to reduce the synthesis of proinflammatory cytokines. Glucocorticoids are, therefore, considered to break the link between inflammation and bone loss [14].

Based on the experimental and clinical data, glucocorticoids seem to have variable effects on osteoclast functioning, ranging from stimulatory to inhibitory effects [11, 12]. Experimental studies on the impact of GCs under inflammatory conditions are rare but could provide helpful insights to estimate the potential risks vs protective effects of GC treatments on bone.

To investigate the effect of low-dose GC therapy on osteoclasts (OCs) in a local inflammatory environment *in vivo*, we utilized the calvaria particle-induced bone loss mouse model. This model was initially established to investigate aseptic prosthesis loosening. Wear particles, such as ultra-high-molecular-weight polyethylene (UHMWPE) particles, cause periprosthetic osteolysis by inducing local inflammatory response, which results in osteoclast activation and subsequent prosthetic failure [19, 20].

Therefore, mice received low-dose prednisone or placebo and subsequently UHMWPE-particle application or sham operation. Osteolytic effects of the particles were investigated via micro-CT and histomorphometry.

2. Material and methods

2.1. Declaration of approval for animal experiments

Experiments were performed and registered in accordance with the local authorities (Animal Use protocol: LANUV, North Rhine-Westphalia, Germany, 84-02.04.2012.A370). All animal experiments have been carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2. Animals and induction of particle-induced bone loss

Male C57BL/6J were purchased at 6 weeks of age from Charles River Laboratories (Sulzfeld, Germany) and housed in controlled conditions with a 12-hour light/dark cycle at $22 \pm 2^\circ\text{C}$ and relative room humidity at $55 \pm 5\%$ in the Central Animal Facility of the University Hospital Essen. Diet and water were supplied *ad libitum*.

At age 12 weeks, slow-release pellets with prednisone ($n = 14$) or placebo ($n = 10$) were implanted subcutaneously (placebo/prednisone at 2.5 mg; 60-day release; Innovative Research of America, Inc., FL-USA). Prednisone was chosen since it is the most commonly used glucocorticoid in treating inflammatory diseases in humans. Two weeks later, at 14 weeks of age, 30 μl of UHMWPE particles (Ceridust VP 3610, Clariant, Gersthofen, Germany) were administered using a surgical spoon to the skull in 7 mice of the prednisolone group and 5 of the placebo group, the remaining animals of the groups ($n = 7$ and $n = 5$) underwent a sham operation. Surgery and anaesthesia were performed as described previously [21]. Mice were sacrificed 14 days later and stored in formalin in 50 ml sample tubes at 4°C until Micro CT investigations of the skulls

were performed. During the experiment, mice were treated with prednisone or placebo for a total of 4 weeks and exposed to UHMWPE particles during the last 2 weeks.

2.3. Micro CT

The skulls were examined in micro-computed tomography (CT) (X-Ray Microtomograph 1072, Skyscan, Aartselaar, Belgium). Reconstruction of the acquired images for subsequent analysis was performed with the program Cone Beam Reconstruction (Skyscan, Aartselaar, Belgium). The reconstructed images were analyzed using the CT Analysis program (Skyscan, Aartselaar, Belgium). The sections from the sutura coronaris along the sutura sagittalis toward the sutura lambdoidea were investigated. This corresponded to the surgical area, i.e., the area of particle-induced osteolysis. Here, a region of interest (ROI) measuring 2×2 mm was placed over the sagittal suture so that the latter was in the centre of the area. A 'build cube' was then created, composed of the selected section and 100 other sections located in the occipital direction, so that a cuboid of $2 \times 2 \times 1$ mm was available for calculation. In 1 mm depth, a total of 8 cuboids were built following the sutura sagittalis. Cuboids 4–8 were used to analyse and calculate the bone volume bone volume (BV) and the relative BV/TV (bone volume/tissue volume).

For visualization, the 3D reconstructions were made with the program CTvox (version 1.0, Skyscan, Aartselaar, Belgium).

The fifth lumbar vertebra was analyzed with a microCT 35 (Scanco Medical) using an X-ray energy of 70 keV, an isotropic voxel size of 12 μm and an integration time of 200 ms. The trabecular bone density, tissue mineral density, the trabecular and cortical bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) were calculated from 310 slices using the Scanco bone evaluation software (IPL). The images were filtered using a Gaussian filter ($\sigma = 0.7$, support = 1) and segmented using a global threshold value corresponding to 20% of the maximum grey value [22].

2.4. Histomorphometric analysis

Calvaria bones were processed as described previously [23]: The calvaria were decalcified, dissected into four cross-sections and embedded separately in paraffin blocks. Afterwards, the sections were cut into thin coronal slides using a microtome (Reichert-Jung, Model 2065, Heidelberg, Germany). To determine the bone resorption area in the midline suture, the slides underwent hematoxylin & eosin (HE).

After that, all sections were digitally photographed at a magnification of 10×10 with the midline suture in the centre using a standard high-quality light microscope.

For histomorphometric analysis of the eroded surface area, the operator encircled an ROI within the midline suture according to the principles of bone perimeter measurement proposed by Parfitt et al. [24] and described by Wedemeyer et al. [23]. Bone destruction was quantified automatically based on image analysis software calculation (UTHSCA Image Tool, IT version 3.0; University of Texas, San Antonio, TX). Bone thickness was measured at the centre, at four 0.5 mm steps from the midline suture to the left and at four equivalent steps to the right (see Figure 3 for further illustration). Two independent investigators counted osteoclast numbers in the area adjacent to and in continuity with the midline suture. Osteoclasts were identified as large multinucleated cells located within a resorption lacuna with a surrounding peripheral cytoplasm that lacks organelles, as Kukita and Kukita [25] reported.

The Osteomeasure software (Osteomeasure Version 3.2.1.5; Osteometrics Inc. Decatur, Georgia, USA) was used following international standards [22].

2.5. Statistical analysis

Data are presented as mean standard deviation of mean if not otherwise indicated. Data were tested for normal distribution using the

Kolmogorov-Smirnov test. Normal distribution was assumed when $\alpha > 0.1$. T-tests were performed to detect pairwise differences within and between groups. For all tests, after Bonferroni correction for multiple comparisons, statistical significance was presumed at $P < 0.05$. Statistical analysis was performed using PRISM 8 for MAC OS X (La Jolla, CA, USA).

3. Results

3.1. Prednisone protects against particle-induced calvaria bone loss

As anticipated, the application of particles to the skull resulted in osteolysis with reduction of mean bone volume (BV) from $0.5692 \text{ mm}^3 \pm 0.0378 \text{ mm}^3$ in sham operated animals (placebo + sham) to $0.4754 \text{ mm}^3 \pm 0.036 \text{ mm}^3$ after particle application (placebo + particle) ($p < 0.01$) (Figures 1 and 2A). In addition, particle application resulted in a decrease of the cortical trabecular thickness (Tb.Th.) to 0.11 ± 0.0045 in placebo + particle vs 0.13 ± 0.001 in placebo + sham ($p < 0.05$).

Treatment with prednisone protected against particle-induced reduction of cortical bone volume (BV): In mice treated with prednisone, particle application did not significantly reduce cortical BV compared to sham-operated mice. Prednisone treated mice that received particles showed significantly higher cortical BV as compared to placebo treated mice (0.54 ± 0.041 vs 0.48 ± 0.036 , $p < 0.05$). Further, in prednisone treated mice, particle application did not significantly reduce trabecular thickness (Tb.Th.) ($p = 0.07$). Thus, Tb.Th. is preserved in prednisone treated mice but not in placebo treated mice (Figures 1 and 2A, B).

Treatment with prednisone in sham operated mice (prednisone + sham) had no significant effect on cortical bone volume or trabecular thickness.

3.2. Prednisone does not preserve cortical surface

Histological examination of the skulls of the mice showed a pronounced granulomatous foreign body reaction in both groups of mice that received particle application. However, this reaction appeared to be less noticeable in mice that received prednisone before particle application (Figures 2 and 3).

Cortical surface did not differ significantly between sham operated animals with and without treatment with prednisone. Particle application induced local erosions with increased bone surface, both in placebo and in prednisone treated mice ($4.38 \text{ mm}^2 \pm 0.852$ vs $1.469 \text{ mm}^2 \pm 0.287$; $p < 0.01$ and $2.767 \text{ mm}^2 \pm 0.629$ vs $1.207 \text{ mm}^2 \pm 0.003$; $p < 0.05$). Cortical surface did not significantly differ between animals with prednisone-treatment and particle application and animals with placebo-treatment and particle application ($2.767 \text{ mm}^2 \pm 0.629$ vs $4.382 \text{ mm}^2 \pm 0.852$ mm; $p = 0.057$) (Figure 2C).

3.3. Prednisone did not significantly reduce osteoclast number

Application of UHMWPE particles resulted in increased osteoclast numbers at the application site, both in placebo and prednisone treated mice. (5.650 ± 2.007 per High power field (HPF) vs. 1.333 ± 0.306 per

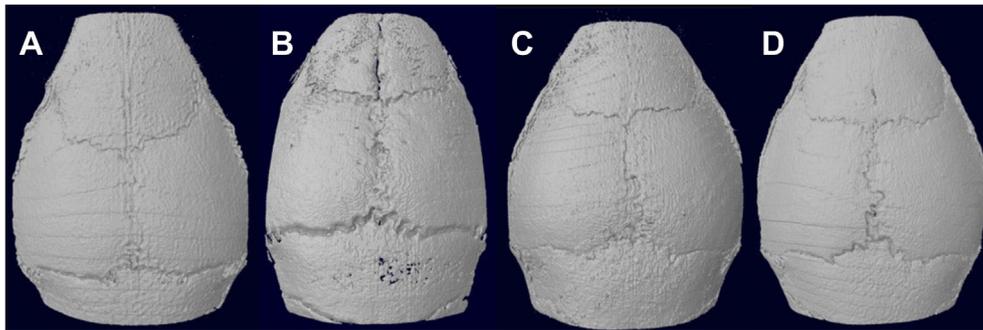


Figure 1. 3D-Reconstruction. 3D-Reconstruction of micro-CT analysis of the calvaria of (A) placebo treated sham operated mice (B) placebo treated mice with particle induced osteolysis, (C) sham operated mice, treated with prednisone and (D) lack of particle induced osteolysis in mice treated with prednisone.

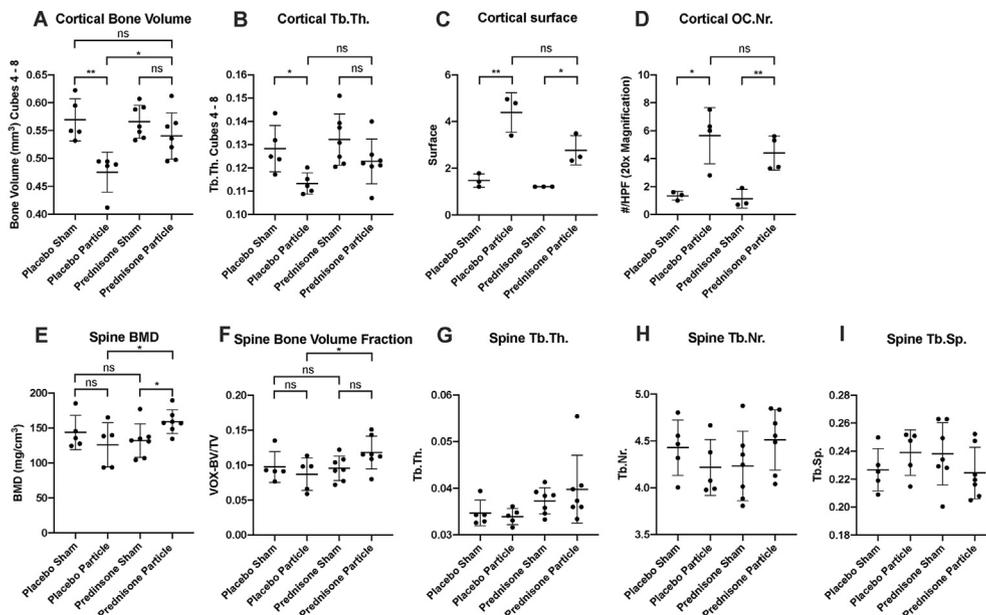


Figure 2. Skeletal findings in mice treated with/without prednisone, following particle implantation on the cranial dome vs Sham surgery: Cortical bone volume (BV) at the calvaria in mm^3 (A), cortical trabecular thickness (Tb.Th) (B), cortical surface (C) and number per HPF (high-power field) at the midline suture, and cortical osteoclast number (OC.Nr.) (D) in mice undergoing sham operation (Sham) or particle application (Particle) with (Prednisone) and without (Placebo) treatment with prednisone pellets are displayed (Mean \pm SD). Further, spinal bone mineral density (BMD) (E), spinal bone volume fraction (VOX-BV/TV) (F), spinal trabecular thickness (G), spinal trabecular number (Tb.Nr.) (H) and spinal trabecular separation (Tb.Sp.) (I) are shown. $20 \times = 20$ -fold magnification. * $p < 0.05$; ** $p < 0.01$.

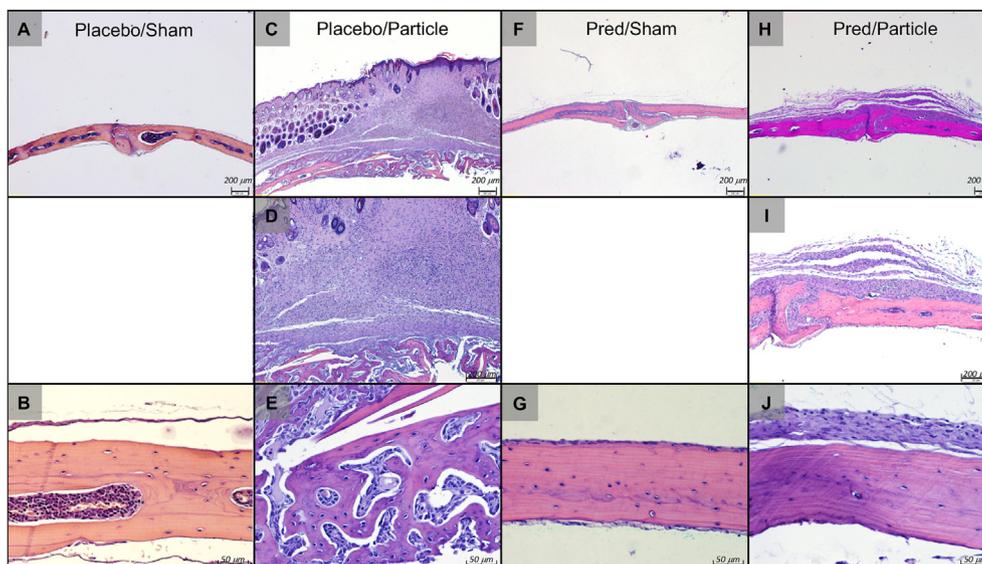


Figure 3. Histomorphometry. Histomorphometry (HE-staining) at the suture (calvaria) at 2.5x (upper row), 5.0x (middle row) and 20x (lower row) magnification displaying intact bone in placebo treated and Sham operated animal (A–B), eroded surface after particle application in a placebo treated animal (C–E), (H–I) intact bone in a prednisone treated and Sham operated animal (F–G) and eroded surface after particle application in a prednisone treated animal.

HPF; $p < 0.05$ and 4.400 ± 1.219 per HPF vs 1.133 ± 0.666 per HPF; $p < 0.01$).

Prednisone did not significantly reduce osteoclast numbers in animals with particle application (4.400 ± 1.219 per HPF) when compared to placebo-treated animals with particle application (5.650 ± 2.007 per HPF) (Figure 2D).

3.4. Low-dose Prednisone did not have side effects on the spine

Particle application at the skull did not significantly impact the spinal bone mineral density (BMD) in placebo treated mice. Further, in Sham operated mice, the 4-week course with prednisone did not have a significant impact on spinal BMD compared to placebo treated mice ($131.9 \text{ mg/cm}^3 \pm 24.08$ vs $143.6 \pm 24.62 \text{ mg/cm}^3$; $p = 0.428$).

Interestingly, animals treated with prednisone before particle application showed a significantly elevated BMD compared with animals treated with placebo before particle application ($159.1 \text{ mg/cm}^3 \pm 17.00$ vs $126.1 \text{ mg/cm}^3 \pm 31.44$; $p < 0.05$) or animals treated with prednisone before Sham operation ($159.1 \text{ mg/cm}^3 \pm 17.00$ vs $131.9 \text{ mg/cm}^3 \pm 24.08$; $p < 0.05$; Figure 2E). Consistently, animals treated with prednisone before particle application showed a significantly elevated spinal bone volume fraction (BV/TV) compared with animals treated with placebo before particle application (0.118 ± 0.023 vs 0.095 ± 0.018 ; $p < 0.05$). Akin to BMD, particle application alone and 4-week course with prednisone in Sham operated mice had no effect on spinal BV/TV (Figure 2F). When it comes to spinal microarchitecture parameters, including spinal trabecular thickness (Tb.Th.), number (Tb.N.) and separation (Tb.Sp.) no significant differences have been observed between the four groups (Figure 2G–I).

4. Discussion

We used the calvaria particle-induced bone loss mouse model to investigate effects of prednisone on the bone resorptive capabilities of osteoclasts *in vivo*. As expected, the application of particles resulted in osteolysis with significantly reduced bone volume, cortical trabecular thickness, increased cortical surface area, and a significant increase in osteoclast numbers. The local inflammatory response had no systemic effects on bone density and micro bone structure.

Prednisone inhibited particle-induced osteolysis of cortical bone volume, thereby displaying a bone-protective effect. However, this effect was not based on a significant reduction of the number of osteoclasts.

These results suggest that in mice the short-term application of prednisone predominantly suppresses osteoclasts function without affecting osteoclasts number. On the one hand, this observation is supported by the finding by *Jia et al.* that glucocorticoids lead to increased survival of osteoclasts *in vivo* [26] and the results of *Kim et al.*, that glucocorticoids reduce osteoclast functions [11, 27]. The reduced activity is most likely due to the immunosuppressive effect of GCs and subsequently reduced osteoclast stimulation, which is discussed below.

The 4-week long exposure to prednisone did not affect the BMD and BV/TV at the spine in this experiment. There was also no significant effect of prednisone on spinal microarchitecture parameters, such as Tb.Th., Tb.N. and Tb.Sp.. This seemingly stands in contrast to the pathophysiology of glucocorticoid-induced osteoporosis. However, *Chen et al.* studied the effect of glucocorticoid therapy in rats with 3.5 mg/kg/day prednisone for 7 and 21 days respectively and observed a decrease in the BMD of the femur metaphysis after 21 days but not after 7 days [28]. The mice in the present study were treated with 2.5 mg/d prednisone, a dose which corresponds to 2.0 mg/kg/d and is thus significantly less than that used by *Chen et al.* It is feasible that despite the more prolonged exposure of 4 weeks the lower dose prevented bone loss at the spine. In addition, the mouse strain choice might be partly responsible, since C57BL/6J mice require a higher amount of glucocorticoids as compared to FVB/N mice to induce osteoporosis [29, 30] and *Ersek et al.* could show, that osteoclasts from C57BL/6J mice were less responsive to GC treatment and tolerated higher doses than osteoclasts from CD1 mice [31]. Further, the mouse age might be a critical factor, since younger mice, as used here, show less decrease bone mineral density after glucocorticoid exposure [29, 31].

Interestingly, *Korczywska et al.* were able to show in humans that the anti-inflammatory effect of short-term GC therapy in patients with rheumatoid arthritis balances their direct negative impact on bone [32]. However, in high doses, even short term treatment with GCs can result in an increased risk of fractures [33] and long-term treatment with low dose GCs can result in a reduction of BMD [34, 35].

In summary, protective effect of GCs on local osteolysis in the calvaria particle-induced bone loss model, but no systemic side effects were

observed. These observations are consistent with osteoprotective effects of GCs in the context of inflammation [36].

The utilized mouse model was created initially to study sterile prosthesis loosening [21, 23]. Wear particles from the prosthetic material act to attract immune cells, which in turn, contribute to osteolysis by increasing tissue levels of cytokines that act to increase bone resorption (e.g., RANKL, TNF- α , IL-1 β , IL-17) [23,37,38]. These are the same pathways that are activated in inflammatory disease and likely responsible for bone loss in these disorders [2, 3].

Due to their anti-inflammatory properties, GCs can also have a protective effect on bone, by breaking the link between inflammation and bone loss. *Blavnsfeldt et al.* could not find any differences in BMD change in patients with early and active rheumatoid arthritis who were treated with prednisone/prednisolone versus placebo for 24 months [39]. This suggests that the suppression of inflammation by glucocorticoids may counterbalance their adverse effects on bone remodelling.

Limitations of the present work are the small number of mice per group. Possible limitations regarding the mouse strain and age are already discussed above. As with all mouse models, it should be noted that results may not transfer into pathophysiological processes in humans [40, 41]. Mice have a significantly higher metabolism and therefore metabolize drugs more quickly than humans [42]; thus effects of glucocorticoids might be lesser than expected given that the calculated dose was extrapolated from doses used for therapeutic reasons in humans. Of note, this model uses a preventive approach since the GCs are given prior to the inflammatory stimulus. In inflammatory diseases however, the therapy with GCs is initiated as a response to the inflammatory process. Thus, the study design does not match the 'natural history' of a patient with an inflammatory disease. Further, in the therapy of inflammatory diseases, the dosing of GCs is guided by the clinical effect and only to a lesser extent by potential side effects.

5. Conclusion

In summary, the calvaria particle-induced bone loss mouse model is suitable for the investigation of osteoclast function under inflammatory conditions *in vivo*. Our results underscore the heterogeneous effect of GCs on bone but point to beneficial impact of low-dose glucocorticoids in inflammatory conditions. The data deliver further experimental evidence for an osteoprotective effect of GCs on the bone as previously seen in clinical observations. Future studies should assess osteoclast function during short- and long-term glucocorticoid treatment in humans with inflammatory diseases e.g. via biochemical markers.

Declarations

Author contribution statement

Michael M Schündeln: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jakob Höppner: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Felix L Meyer; Wiebke Schmuck; Gero Hilken; Bodo Levkau; Martina Rauner: Performed the experiments; Wrote the paper.

Max D Kauther: Conceived and designed the experiments; Wrote the paper.

Corinna Grasmann: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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