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Local RANKL delivery improves socket healing in bisphosphonate treated rats

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Abstract

Medication related osteonecrosis of the Jaws (MRONJ) is a severe complication of antiresorptive and antiangiogenic medications. Osteoclast inhibition is central in MRONJ pathogenesis. Here, we investigated if local application of RANKL (a key molecule in osteoclast activation) could enhance osteoclast generation and improve extraction socket healing in the presence of bisphosphonates. Thirty Wistar-Han rats received one saline or 66 μg/kg zoledronate (ZA) i.p. dose before surgery. A week later, mandibular molars were extracted bilaterally. Collagen tapes infused with water or RANKL were placed in the extraction sockets of 60 hemimandibles of veh (veh/RANKL−, veh/RANKL+) or ZA treated rats (ZA/RANKL−, ZA/RANKL+). Rats were euthanized 3 or 12 days after surgery. Animals euthanized at 12 days received two additional veh or ZA injections. Clinical, radiographic and histologic assessments were performed.

Visually, at the 3-day timepoint, no sockets demonstrated complete healing. At the 12-day timepoint, sockets of veh/RANKL− and veh/RANKL+ rats showed intact mucosa, while mucosal defects were noted in ZA/RANKL− rats. Importantly, ZA/RANKL+ sockets showed absence of bone exposure. RANKL delivery increased bone healing in the ZA/RANKL+ sites 12 days after extraction compared to the ZA/RANKL− sites. Histologically, at the 3-day timepoint, ZA/RANKL− sockets demonstrated extensive bone exposure and osteonecrosis. In contrast, ZA/

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CRediT authorship contribution statement

Akrivoula Soundia: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Danny Hadaya:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Yee Chau:** Data curation, Methodology, Writing – review & editing. **Ioannis Gkouveris:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Olga Bezouglaia:** Data curation, Methodology, Writing – review & editing. **Sarah Dry:** Data curation, Methodology, Writing – review & editing. **Flavia Pirih:** Data curation, Investigation, Writing – review & editing. **Tara Aghaloo:** Data curation, Investigation, Writing – review & editing. **Sotirios Tetradis:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing.

RANKL+ rats showed granulation tissue coverage and significantly reduced osteonecrosis, similar to the veh groups. Importantly, in the ZA/RANKL+ group, osteoclasts attached to the bone surface and osteoclast numbers were higher compared to ZA/RANKL− sites. At the 12-day timepoint, persistent osteonecrosis and bone exposure were detected in the sockets of ZA/RANKL− animals. Contrary, ZA/RANKL+ rats demonstrated socket epithelialization and reduced osteonecrosis. Significantly more total and bony attached osteoclasts persisted in the ZA/RANKL+ vs the ZA/RANKL− group. We present a novel approach towards improving socket healing, in the presence of ZA, by enhancing osteoclastic numbers and attachment through local RANKL application. Our approach is clinically applicable and could improve treatment outcomes of patients on high-dose ZA therapy.

Keywords

Bisphosphonates; ONJ; RANKL; Osteonecrosis; Rats; Zoledronate

1. Introduction

Medication related osteonecrosis of the Jaw (MRONJ) is a rare but serious side effect of antiresorptive medications and can cause significant morbidity to patients. MRONJ is characterized by areas of exposed bone in the oral cavity for a period of more than 8 weeks [1–3]. The most common instigating local factor of MRONJ is tooth extraction or other dental surgical procedures, such as implant placement. A classic presentation of the disease is when a tooth is extracted and the extraction socket does not heal. However, tooth extraction in patients undergoing antiresorptive treatment often is unavoidable [3–5].

The main class of medications associated with MRONJ are antiresorptive agents that function as inhibitors of osteoclastic function or differentiation [1–3]. Antiresorptive medications are mainly used for the management of osteoporosis, and less frequently but at higher doses in patients with bone malignancy [6–9]. Indeed, the fear of MRONJ is a main contributor for osteoporotic patients to not be compliant with their antiresorptive medications [10]. Osteoporosis is the most common metabolic bone disease with 43.4 million people affected by osteoporosis or osteopenia in the USA, representing 44% of the people aged 50 and older [11]. Reports in the scientific and public literature express serious concern about not treating osteoporosis more aggressively [10,12–14]. A successful intervention to minimize the MRONJ risk would be beneficial for millions of osteoporotic patients that need to undergo a dental surgical procedure, such as a tooth extraction.

Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) is a key molecule for the production of osteoclasts [15–17]. RANKL binds to the RANK receptor of pre-osteoclastic cells as a homotrimer and mediates the fusion of neighboring osteoclast precursors causing them to become multinucleated under the influence of other genes, such as DC-STAMP. RANKL also mediates the transcription of several osteoclast-specific genes such as TRAP, cathepsin K and calcitonin receptor committing the cells to an osteoclastic phenotype. Additionally, RANKL promotes osteoclast survival by upregulation of small G-proteins while anti-apoptotic proteins are inhibited in its absence [18]. Certain antiresorptive

treatments target RANKL as a way to inhibit osteoclast formation, and thus bone resorption [19,20].

Given the central role of RANKL in osteoclastic differentiation, function and survival, we hypothesized that RANKL could be delivered locally within the extraction socket in rats undergoing bisphosphonate (BP) treatment to enhance the local generation of osteoclasts, mitigate the local BP effect on resorption and improve socket healing without discontinuing the systemic antiresorptive treatment.

2. Materials and methods

2.1. Animal care

Thirty Wistar-Hun 7-week-old rats were randomly assigned to receive saline or 66 μg/kg of zoledronic acid (ZA), (LKT laboratories, St. Paul, MN). Rats were injected intraperitoneally once a week in morning hours. Animals were kept and treated according to guidelines of the UCLA Chancellor's Animal Research Committee [21–24].

One saline or ZA dose was administered to rats before surgery. A week later, the first and second mandibular molars of both sides were extracted in all rats. After extraction, resorbable collagen tapes (ACE Surgical Supply, Brockton, MA) containing water or recombinant rat RANKL (Peprotech, Rocky Hill, NJ) were placed in the extraction sockets. 4 μl of water or aqueous RANKL solution (0.1 mg/ml) were placed in each collagen tape. Water or RANKL loaded collagen tapes were placed in the extraction sockets of 30 hemi-mandibles of veh-treated rats and 30 hemimandibles of ZA-treated rats (a total of 60 hemimandibles were used). Each rat received a veh infused tape on one side of the mandible and a RANKL infused tape on the other. Sides were alternated to account for possible discrepancies in surgical technique.

ZA treatment continued and rats were euthanized 3 or 12 days after tooth extraction utilizing CO_2. Twelve rats were euthanized 3 days after surgery (6 veh treated and 6 ZA treated) and 18 rats were euthanized 12 days after surgery (9 veh treated and 9 ZA treated). Rats that were sacrificed 3 days after surgery did not receive another dose of veh or ZA. Rats that were sacrificed on the 12-day timepoint received one dose of veh or ZA 3 days after surgery and one dose 10 days after surgery.

2.2. Mucosal healing

Mandibles were dissected and photographs of the specimens were obtained utilizing a digital optical microscope (Keyence VHX-1000, Osaka, Japan). Specimens were photographed at 6.3× magnification and images were imported into Image J (NIH, imagej.nih.gov). Size of mucosal defects was measured utilizing the area measuring tool.

2.3. Micro CT

Micro CT scanning was performed, as described [24]. Trabecular bone volume-total volume ratio measurements were performed in the distal root of the first molar starting from the apex and including 50 axial slices towards the coronal third, as we have previously described

[24]. Examples of the region of interest for each of the groups are provided in Supplemental Fig. 1.

2.4. Histology

Mandibles were fixed for 48 h in 4% paraformaldehyde and then decalcified in 14% EDTA for 4 weeks. Samples were paraffin embedded, and 5 μm-thick cross sections were made perpendicular to the long axis of the alveolar ridge at the area of the mucosal defect. If the mucosa was completely healed, sections were made at the area between the first and second molars, approximately 2 mm mesial to the mesial cusp of the third molar. H&E stained slides were digitally scanned. Image analysis was performed using the Aperio Image Scope software (Aperio Technologies, Inc., Vista, CA, USA). The osteonecrotic area(s) and empty osteocytes over total bone area(s) were quantified [25,26].

2.5. TRAP assay, Picrosirius red staining, immunohistochemistry

For enumeration of osteoclasts, tartrate-resistant acid phosphatase (TRAP) staining was performed (387A-IKT Sigma Aldrich, St. Louis, MO, USA). Osteoclast numbers were normalized over bone length. Acid phosphatase assay (ab83370, Abcam) was used to measure serum TRAP levels 3 days before and 3 days after surgery on all rats. Picrosirius red (Pc red) staining was used to study collagen organization (four samples per group) [22]. Anti-RANKL (sc-7628, Santa Cruz) and Anti-Cytokeratin 14 (ab51054, Abcam) were used for immunohistochemistry (four samples per group) [27].

2.6. Statistics

Sample size was calculated anticipating a 75% decrease in bone exposure incidence with a Type I error probability of 0.05 and a power level of 0.80. Raw data were analyzed using the GraphPad Prism Software (GraphPad Software, Inc. La Jolla, CA). Descriptive statistics were used to calculate the mean and the standard error of the mean (SEM). Data were analyzed by a two-way ANOVA and post-hoc Tukey's test for multiple comparisons among the various groups and *t*-test for a single comparison with a statistical significance of $p < 0.05$.

3. Results

3.1. Specimen photographs 3 and 12 days after surgery

Specimen photographs 3 days after tooth extraction showed mucosal defects in all groups and ongoing mucosal healing (Fig. 1A–E, red circles). Specimen photographs 12 days after tooth extraction revealed an intact alveolar mucosa in the mandibles of veh/RANKL− and veh/RANKL+ rats (Fig. 1F, G, blue circles). Alveolar mucosal defects, granulation tissue and exposed bone were noted in the alveolus of ZA/RANKL− (Fig. 1H, red circle). Interestingly, intact mucosa was noted in most of the ZA/RANKL+ hemi-mandibles (Fig. 1I, blue circle). Areas of unhealed mucosa were significantly smaller in ZA/RANKL+ rats compared to ZA/RANKL− rats (Fig. 1J).

3.2. Radiographic assessment 3 and 12 days after surgery

BV/TV values were less than 10% in all specimens from all groups 3 days after surgery (data not shown). Significant healing with woven bone was observed in the veh/RANKL− and veh/RANKL+ sites 12 days after surgery (Fig. 2A, A1, B, B1). Significantly decreased BV/TV was seen in the extraction sockets of the ZA/RANKL− and ZA/RANKL+ groups compared to the veh treated groups (Fig. 2C, C1). Interestingly, ZA/RANKL+ sites showed increased bone healing compared to the ZA/RANKL− sites 12 days after surgery (Fig. 2D, D1, E, yellow arrows).

3.3. RANKL immunohistochemistry

Three days after surgery, RANKL immunohistochemistry revealed increased signal in the sockets of veh/RANKL+ and ZA/RANKL+ rats compared to the non RANKL treated groups. RANKL signal was noted in the soft tissue of the sockets (Fig. 3A, B, C, D, E) and on multinucleated cells in the RANKL treated groups (Fig. 3B, D, insets, yellow arrows) 3 days after surgery. No significant differences were seen in the RANKL signal in the soft tissue of the mandibles 12 days after surgery (data not shown).

3.4. Serum TRAP assay

Serum TRAP assay was performed to ensure absence of off-target effects in rats. TRAP levels 3 days before and 3 days after surgery were compared in veh and ZA treated animals. TRAP levels before and after extraction and local RANKL application did not show a statistically significant increase neither in the veh nor in the ZA treated groups (Fig. 3F).

3.5. Histologic assessment of extraction sockets 3 days after surgery

Histologic evaluation 3 days after surgery showed granulation tissue (Fig. 4A, B, white arrows) and sparse collagen fibers (green arrows) overlying the sockets of veh/RANKL− or veh/RANKL+ rats. Absence of complete epithelialization of the wound was noted in both groups. In the ZA/RANKL− group, there was absence of soft tissue overlying the alveolar bone and prominent areas of bone exposure were revealed (Fig. 4C, black arrows). Approximately 20% of bone in the extraction sockets of this group was necrotic (Fig. 4C, C1, E, cyan arrow). Contrary, ZA/RANKL+ sockets showed thin epithelium (Fig. 4D, yellow arrow) and granulation tissue covering the sockets (Fig. 4D, white arrow). Importantly, osteonecrosis and empty osteocytes over bone area were significantly reduced compared to the ZA/RANKL− group (Fig. 4D, D1, E, F).

3.6. TRAP staining in extraction sockets 3 days after surgery

TRAP staining revealed higher numbers of osteoclasts in the sockets of veh/RANKL+ vs veh/RANKL− groups (Fig. 4A2, B2, G). Of note, statistically significantly more osteoclasts were also seen in the extraction sockets of ZA/RANKL+ compared to the ZA/RANKL− group (Fig. 4C2, D2, G). As we have previously described [21,25], many osteoclasts in ZA treated rats showed an altered, round morphology with pyknotic nuclei and were not in contact with the bone surface (Fig. 4C2, orange arrows). Interestingly, higher numbers of osteoclasts attached to the bone surface were seen in the extraction sockets of ZA/RANKL+ vs ZA/RANKL− sites (Fig. 4D2, H, blue arrows).

3.7. Histologic assessment of extraction sockets 12 days after surgery

Intact epithelium with rete peg formation was detected in the alveoli of veh/RANKL− and veh/RANKL+ rats. Connective tissue showed interval resolution of the inflammatory infiltrate observed in the 3 days timepoint (Fig. 5A, B, orange arrows). Significant amount of woven bone was seen occupying the extraction sockets in both groups of veh-treated animals. Osteonecrosis and empty osteocytes were minimal in the mandibles of both veh treated groups (Fig. 5A, A1, B, B1, E, F, white arrows). Histologic evaluation of ZA/RANKL− sockets showed areas of epithelial disruption, bone exposure (Fig. 5C, black arrow) and bony sequestration (Fig. 5C1, blue arrow). Significant amount of persistent osteonecrosis and empty osteocytes were also detected (Fig. 5C, C1, cyan arrows, E). In contrast, the extraction sockets of ZA/RANKL+ treatment demonstrated continuous keratinized epithelium with no evidence of bone exposure (Fig. 5D, yellow arrow). Statistically significantly less osteonecrosis and empty osteocytes were seen in the alveoli of these animals compared to the ZA/RANKL− group (Fig. 5D, D1, E, F).

3.8. TRAP staining in extraction sockets 12 days after surgery

Twelve days after tooth extraction, comparable numbers of osteoclasts were seen in the sockets of veh/RANKL− and veh/RANKL+ groups (Fig. 5A2, B2, G). Importantly, abundance of osteoclasts was noted in the alveoli of ZA/RANKL+ rats (Fig. 5D2). Statistically significant increase in total osteoclast numbers was detected in this group compared to the ZA/RANKL− group. Significantly higher numbers of osteoclasts attached to the bone surface were seen in the alveoli of ZA-treated rats with RANKL vs ZA treated rats without RANKL treatment (Fig. 5C2, D2, G, H).

3.9. Picrosirius red and cytokeratin 14

Pc red staining revealed an organized collagen network in both veh treated groups with strongly birefringent collagen fibers extending from the submucosal soft tissue and inserting within the vital bone (Fig 6A, B, A1, B1, blue arrows). Contrary, in ZA/RANKL− rats disruption of the bone-soft tissue interface was noted with absence of collagen fiber insertion in the alveolar bone (Fig. 6C, C1, white arrow). In the ZA/RANKL+ group, however, intact collagen network with strong collagen birefringence and collagen fiber insertion in the bone were noted (Fig. 6D, D1, blue arrows).

Cytokeratin 14 showed intact epithelium in veh groups (Fig. 6E, F). In contrast, epithelial disruption (Fig 6G, black arrow) adjacent to the necrotic bone (Fig. 6G, red arrow) was noted in ZA/RANKL− mandibles. The ZA/RANKL+ sockets showed intact, continuous epithelium covering the extraction socket, akin the veh treated rats (Fig. 6H).

4. Discussion

Medication related osteonecrosis of the jaws is a severe complication of antiresorptive and antiangiogenic medications that can considerably deteriorate the quality of life of already compromised patients. Recent reports have addressed a significant decrease in compliance with antiresorptive medication among osteoporotic patients, particularly post-menopausal women. A major reason for this change is patient concern about possible side-effects of

these pharmacologic agents, mainly MRONJ and atypical femoral fractures (AFF) [13,14]. Osteoporosis affects approximately 200 million people worldwide and osteoporotic fractures are responsible for 65,000 deaths in the United States every year [28,29]. A 50% decline in bisphosphonate use was reported in the osteoporotic population between the years of 2008 and 2012, a few years after MRONJ was introduced in the literature and patient and physician awareness increased [10]. Identifying a clinically feasible intervention which can prevent MRONJ development, ameliorate socket healing and help alleviate the fear among osteoporotic patients is of vital significance.

Inhibition of bone resorption is central in the pathophysiology of MRONJ [3,30]. Thus, we elected to enhance alveolar bone resorption by local application of RANKL, due to its fundamental importance in osteoclastic differentiation, function and survival [15]. In our study, woven bone formation was increased after RANKL delivery in ZA treated rats. This may be counter-intuitive given that RANKL facilitates bone resorption. This discrepancy resides on the fact that socket healing is distinctly different than osseous healing in a fracture model. While fracture healing occurs in an inflammatory but sterile environment, the extraction socket communicates with the oral cavity. The ZA+/RANKL− group showed delayed mucosal healing (Fig. 1) which compromised underlying osseous socket healing and resulted in decreased BV/TV (Fig. 2). Of note, BV/TV in both ZA groups was lower than BV/TV of either the veh groups, which highlights that inhibition of bone resorption is associated with delayed osseous socket healing (Fig. 2).

Osteoclasts are essential in socket remodeling after tooth extraction. Two months after tooth extraction the alveolar crest demonstrates an abundance of osteoclasts in human biopsy specimens [31]. A longer duration of bisphosphonate treatment is associated with a prolonged wound healing period after tooth extraction [32]. This is likely due to the disruption of socket healing, a process that initiates with clot formation, and progresses through formation of granulated tissue, establishment of connective tissue and pre-osseous tissue, filling of the extraction socket with trabecular woven bone, and remodeling of the socket to produce lamellar bone [33]. Additionally, intermittent PTH that increases bone turnover improves healing of MRONJ lesions in patients and animals, possibly due to increased osteoclast activity [34–37].

Even if socket healing was incomplete at the end of the experiment, ZA/RANKL− rats demonstrated interval mucosal and osseous healing when comparing the 3-day and 12-day timepoints. From this study, it is not clear whether ZA/RANKL− would eventually reach complete healing if the animals were sacrificed at a later timepoint. Our data showed that enhancing osteoclast numbers by RANKL delivery in ZA treated rats accelerated the healing process. From a clinical standpoint this is significant, as it would minimize chances of socket re-infection, a confounding factor for MRONJ development [30,38].

In our experimental design, it was important that administration of antiresorptives was not discontinued. This would be crucial in the clinical setting, both for patients receiving antiresorptives for the control of metastatic disease, and for patients with osteoporosis [1,39–41]. We continued to administer ZA at 66 μg/kg throughout the duration of the experiment to parallel the dose prescribed to oncologic patients. In patients with bone malignancy that

receive monthly doses of antiresorptives, any extraction would be within two weeks of an antiresorptive dose either prior to or after the tooth extraction. Considering that extraction socket healing of multirooted teeth can last 3 months or longer [42], this antiresorptive dosing is relevant to the socket healing process. Furthermore, teeth in these patients are extracted as the last treatment option when they are non-restorable and are associated with significant periapical or periodontal inflammation [4,43]. In such teeth, bisphosphonates would be concentrated at higher levels in these areas of dental disease with localized high alveolar bone turnover [44–46] The therapeutic approach we describe should also be efficient in osteoporotic patients, given that the cumulative bisphosphonate dose they receive is lower compared to cancer patients due to the utilization of less severe regimens [47–49]. Achieving improved socket healing without discontinuing bisphosphonate treatment is crucial because ‘a drug holiday’ may potentially compromise the patient’s skeletal health [1,39].

A second parameter that we assessed was that our approach did not compromise the systemic inhibition of osteoclastic function, since increased RANKL production is key in osteoporosis and cancer metastasis [17,50–52]. RANKL application was local and a one-time intervention. Importantly, no difference in TRAP serum levels before vs after surgery in veh or ZA treated groups was observed, suggesting that RANKL effects were confined and osteoclastogenesis was not stimulated systemically.

Our study included an early timepoint at three days to capture the initial events of wound healing (inflammatory phase) and a later timepoint at 12 days to investigate a more mature socket healing stage (proliferative phase) [53,54]. Three days after extraction, a considerable amount of osteonecrosis was noted in the mandibles of veh- and ZA-treated rats. This is likely due to the significant trauma to the soft and bony tissues and disruption of the vasculature caused by tooth extraction. RANKL application significantly increased osteoclast numbers in both veh- and ZA-treated animals, but statistically attenuated the extent of osteonecrotic area only in the ZA-treated animals. This ZA-specific effect is likely due to the already enhanced bone resorption in the veh group, such that the additional RANKL application had only a modest effect to the removal of the osteonecrotic area. Twelve days after extraction, the osteonecrotic area significantly decreased, and in parallel osteoclast numbers increased in the veh animals irrelevant of RANKL application. However, in ZA-animals, osteonecrotic area was persistent, while RANKL application decreased the osteonecrosis, supporting the usefulness of our intervention in enhancing local osteoclastic numbers and bony attachment.

These observations point also to an additional significant conclusion. The presence of osteonecrosis at three days and subsequent decline at 12 days after extraction in veh animals suggests that osteonecrosis was not primarily induced by bisphosphonates, but that the inhibition of osteoclasts in bisphosphonate treated animals hinders the removal of necrotic bone originally caused by surgical trauma. This is further supported by our and other investigator’s observations that high levels of bisphosphonates in the absence of extraction or experimental dental disease are not associated with increased alveolar bone osteonecrosis [24,26,55].

In both veh groups and in ZA/RANKL+ rats, we were able to appreciate collagen fiber insertion within the alveolar bone, while in ZA/RANKL− animals the collagen network was discontinuous around the necrotic bone areas. This disruption in the interface between soft tissue and bone may play an important role in the initiation of epithelial migration which can result in sequestration and bone exposure. In a previous study, we have described altered socket healing after extraction of periodontally compromised teeth in rats treated with bisphosphonates. In particular, we noticed a similar disorganized collagen network with weak collagen bundle birefringence and lack of insertion of collagen fibers in the necrotic bone [56]. Epithelial migration approaching the osteonecrotic areas had also been observed and was rimmed by immune cells expressing MMP-9 and MMP-13. It is possible that the restoration of bone resorption and the ongoing remodeling of vital bone seen in ZA/RANKL+ sites might have prevented epithelial migration and helped accommodate the insertion of the collagen fibers after tooth extraction.

MRONJ has been reported with a similar prevalence and severity in patients with a history of bisphosphonates and denosumab (a monoclonal antibody to RANKL) [57–59]. Even though these two groups of drugs act by entirely distinct pharmacologic mechanisms, they both target osteoclasts and result in reduced bone resorption and suppressed bone turnover [60]. Our data indicate that increase of osteoclast numbers in a setting of BP treatment can reduce histologic osteonecrosis and accelerate mucosal healing after tooth extraction. We hypothesize that, in patients on denosumab, a high-enough level of local RANKL delivery could overcome the systemic inhibition and thus improve socket healing. Thus, it would be important to extend the present study and investigate RANKL application in the presence of systemic administration of RANKL inhibitors.

We recognize some limitations to our study. First, we elected RANKL application through a collagen substrate due to its wide clinical application. Although the collagen tape provides a short substrate release, within the experimental parameters of this study design, RANKL delivery was effective. In future studies, we aim to improve delivery methods utilizing engineered hydrogels that allow a slower molecule release [61]. Another notable limitation is the relatively short experimental duration. Although these timepoints allowed us to study the effects of RANKL delivery in socket healing in an antiresorptive environment, the experimental timeline should include longer timepoints to investigate MRONJ according to its definition. We intent to expand our study to include longer timepoints (6–8 weeks) to replicate the clinical setting of MRONJ. Additionally, we selected the use of young animals in this study to better facilitate surgery procedures and post-op survival rate. However, it is anticipated that healing of extraction sockets would be less robust in older animals. Finally, as mentioned earlier, we need to expand our future studies to include bisphosphonate types and doses that are more common in the management of osteoporosis, as well as other types of antiresorptive, such as RANKL inhibitors.

Here, we present a clinically relevant application of local RANKL delivery, as it is plausible that a collagen sponge (a very commonly used substrate during extractions) could be infused with RANKL and inserted within the extraction socket of patients on antiresorptives, in an effort to accelerate wound healing and minimize the incidence of MRONJ. The intervention we describe would have beneficial effects, not only for minimizing MRONJ occurrence,

but also to alleviate the fear of osteoporotic patients and thus improve compliance with antiresorptive medications. Additionally, an approach that could effectively accelerate socket healing would allow clinicians to more easily elect extraction of hopeless teeth in patients at risk of MRONJ and therefore, minimize complications from persistent dental infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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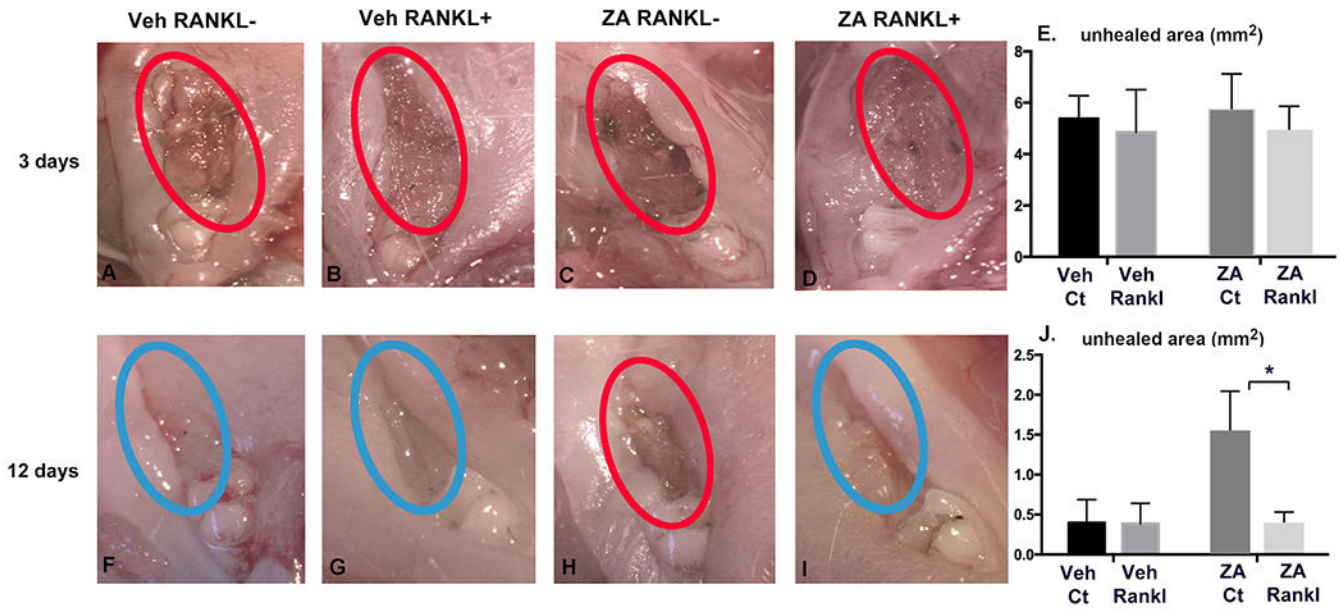

Fig. 1.

Visual images of mandibles 3 and 12 days after surgery. Mucosal defects and incomplete mucosal healing are seen 3 days after surgery (1A-D, red circles). Quantification of unhealed mucosal area over total mucosal area 3 days after surgery (1E). $n = 6$, two-way ANOVA, post-hoc Tukey's. Images of the mandibular mucosa of veh or ZA treated rats with or without RANKL treatment 12 days after surgery (1F-I). Complete mucosal healing is shown by blue circle, mucosal defect by red circle. Quantification of unhealed mucosal area over total mucosal area 12 days after surgery (1J). $n = 9$, two-way ANOVA, post-hoc Tukey's * = statistically significant with a p value<0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

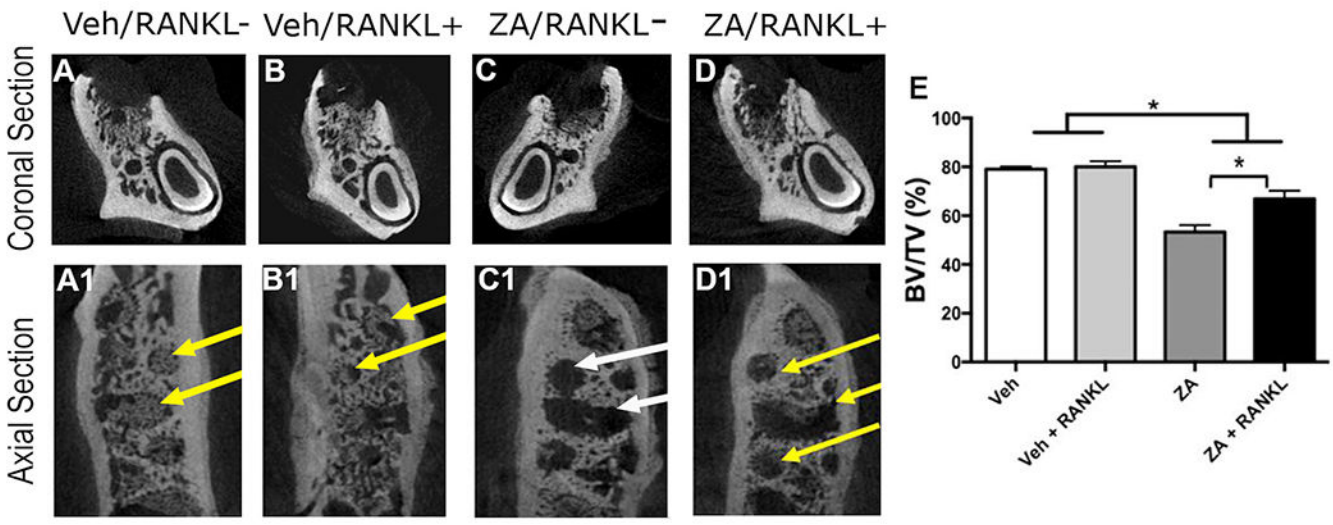

Fig. 2.

Coronal (2A-D) and axial micro-CT views (2A1, D1) of veh or ZA treated sites with or without RANKL treatment 12 days after surgery. Yellow arrows point to woven bone formation, white arrows point to lack of bone formation. Quantification of bone volume over tissue volume (2E). n = 9, two-way ANOVA, post-hoc Tukey's * = statistically significant with a p value <0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

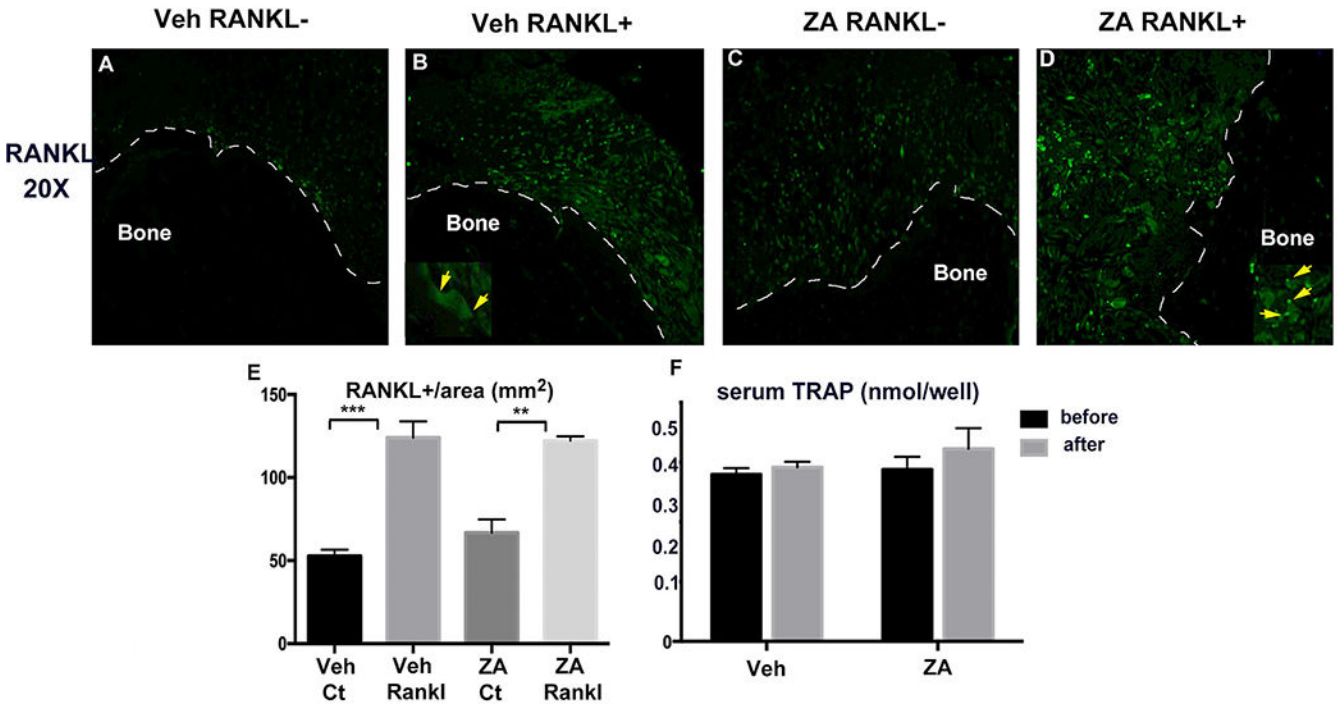

Fig. 3.

RANKL immunohistochemistry of veh or ZA treated rats with or without RANKL treatment 20× magnification 3 days after surgery (3A-D). Yellow arrows in onsets point to RANKL stain on multi-nucleated cells. Quantification of RANKL positive cells over soft tissue area (3E). $n = 4$, two-way ANOVA, post-hoc Tukey's ** = statistically significant with a p value <0.01, *** = statistically significant with a p value <0.001. Serum TRAP levels 3 days before and 3 days after surgery in veh and ZA treated rats (3F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

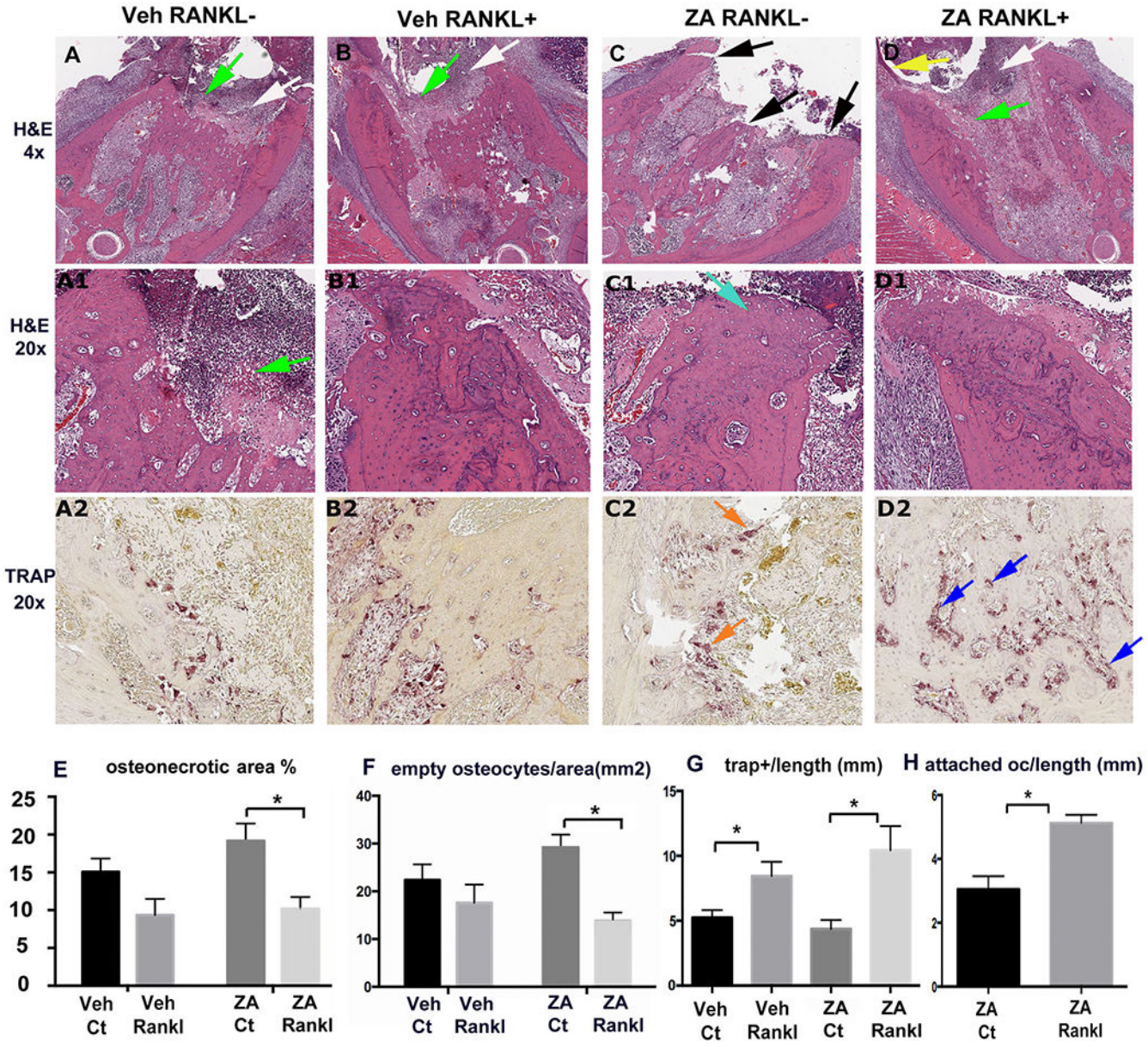

Fig. 4.

Histologic sections of extraction sockets of veh or ZA treated rats with or without local RANKL treatment 3 days after surgery. H&E 4× magnification (4A-D). White arrows point to granulation tissue, green arrows point to collagen fibers, black arrows point to bone exposure, yellow arrows to epithelium. H&E 20× magnification (4A1-D1). Cyan arrow points to bone necrosis. TRAP staining 10× magnification (4A2-D2). Orange arrows point to detached osteoclasts, blue arrows to osteoclasts attached to the bone surface. Quantification of osteonecrotic area over total bone area (4E) and empty osteocytes over bone area (4F) n = 6, two-way ANOVA, post-hoc Tukey's. TRAP positive cells over bone length (4G) and attached osteoclasts over bone length (4H) n = 6, Student's *t*-test * = statistically significant

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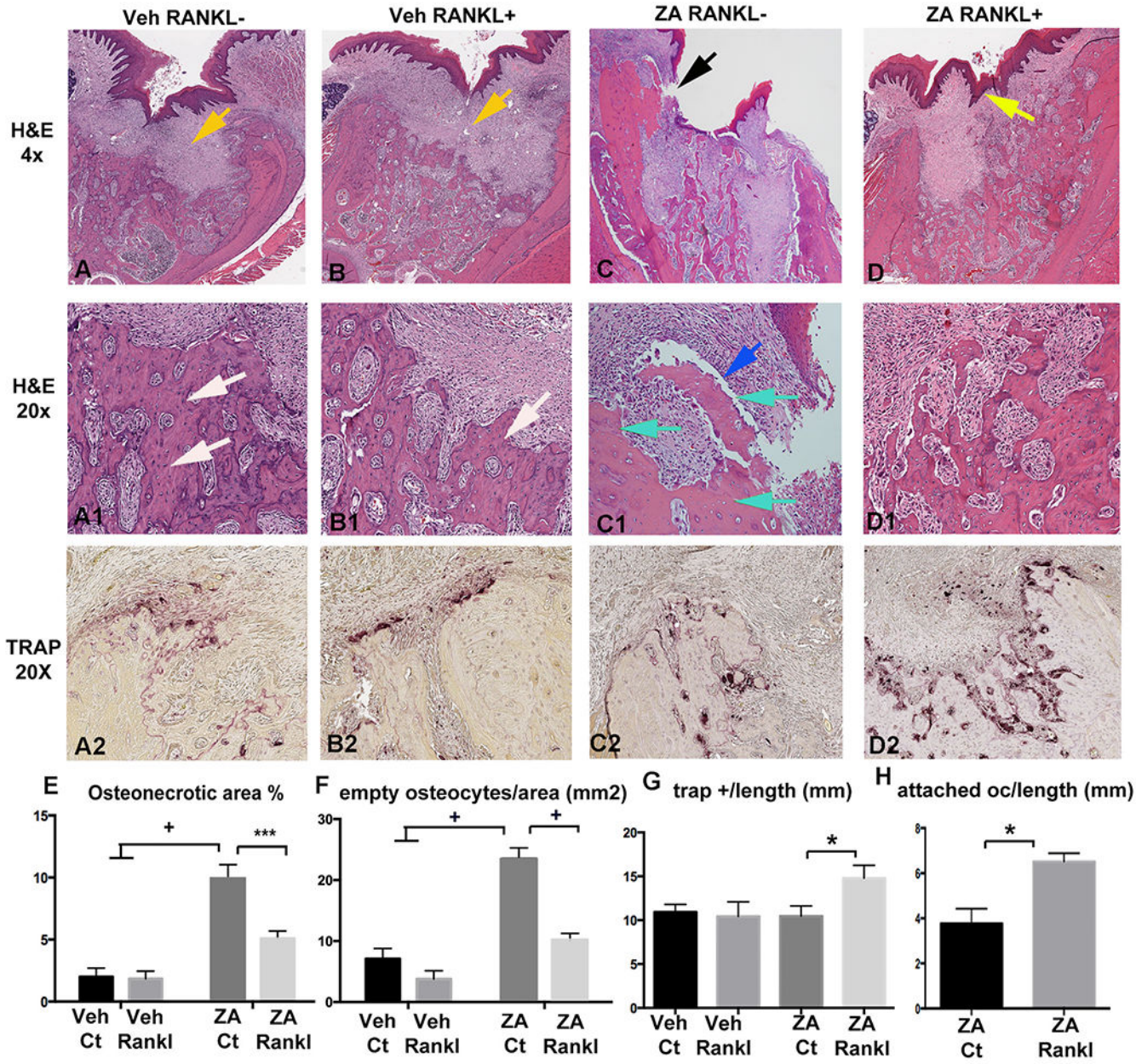

Fig. 5.

Histologic sections of extraction sockets of veh or ZA treated rats with or without local RANKL treatment 12 days after surgery. H&E 4× magnification (5A-D). Orange arrows point to interval resolution of inflammation, black arrows point to epithelial disruption/bone exposure, yellow arrow points to intact epithelium. H&E 20× magnification (5A1-D1). White arrows point to vital bone, blue arrow points to sequestration, and cyan arrow to bone necrosis. TRAP staining of veh or ZA treated rats with or without RANKL treatment (5A2-D2). Quantification of osteonecrotic area over total bone area (5E), empty osteocytes over bone area (5F) n = 9, two-way ANOVA, post-hoc Tukey's. TRAP positive cells over bone length (5G) and attached osteoclasts over bone length (5H) n = 9, Student's *t*-test *** = statistically significant with a p value <0.001, + = statistically significant with a p value

<0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

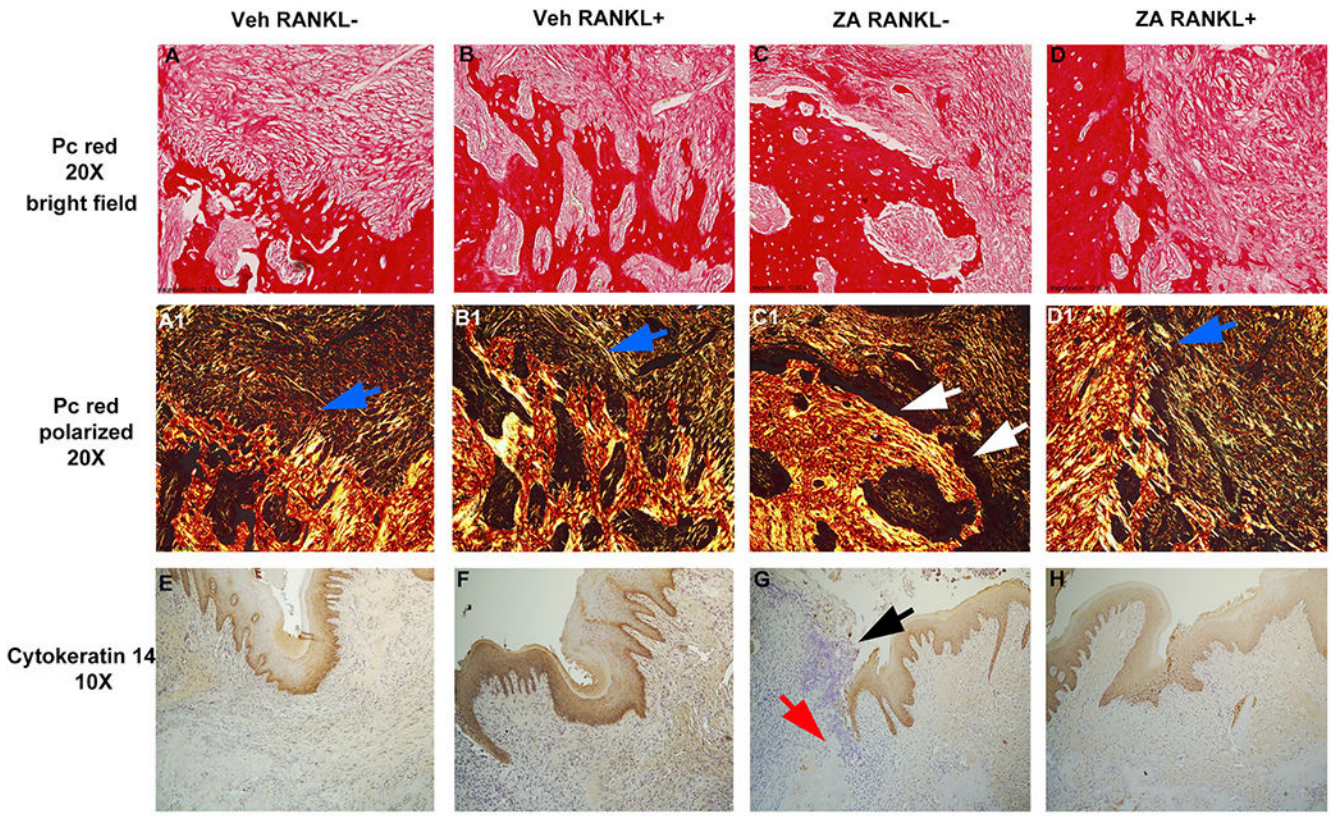

Fig. 6.

Picrosirius red stain of veh or ZA treated sites with or without RANKL treatment (bright field-5A-D, polarized light-5A1-D1). Blue arrows point to insertion of collagen fibers in the bone. White arrows point to lack of collagen fiber insertion in the bone. Cytokeratin 14 stain of veh or ZA treated sites with or without RANKL treatment (5E-H). Black arrows point to epithelial disruption, red arrow points to necrotic bone. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)