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Osteoclasts are recruited to the subchondral bone in naturally occurring post-traumatic equine carpal osteoarthritis and may contribute to cartilage degradation

Authors: Andrea Bertuglia^*, Mathieu Lacourt^, Christiane Girard[§], Guy Beauchamp[§], Hélène Richard^, Sheila Laverty^

Affiliation: ^Comparative Orthopaedic Research Laboratory, Département de sciences cliniques, Faculté de Médecine Vétérinaire, Université de Montréal, 3200 Rue Sicotte, St-Hyacinthe (QC) J2S 2M2, Canada

§Département de Pathologie et Microbiologie Vétérinaires, Faculté de Médecine Vétérinaire, Université de Montréal, 3200 Rue Sicotte, St-Hyacinthe (QC) J2S 2M2, Canada

*Andrea Bertuglia's current address is: Dipartimento di Scienze Veterinarie, Università degli Studi di Torino, Largo P. Braccini 2, Grugliasco 10095 (To), Italy. E-mail: *andrea.bertuglia@unito.it*

Address correspondence and reprint requests to: S Laverty MVB, Dipl ACVS & ECVS, Département de sciences cliniques, Faculté de Médecine Vétérinaire, Université de Montréal, 3200 Rue Sicotte, St-Hyacinthe (QC) J2S 2M2, Canada. Tel1-450 7788100. Email: sheila.laverty@umontreal.ca

Running Title: Osteoclasts in equine post-traumatic osteoarthritis

Abstract

Background: The role of osteoclasts (Ocs) in osteochondral degeneration in osteoarthritis (OA) has rarely been investigated in spontaneous disease or animal models of OA.

Objective: The objectives of the current study were to investigate Oc density and location in post-traumatic OA (PTOA) and control (C) specimens from racehorses.

Methods: Cores were harvested from a site in the equine third carpal bone, that undergoes repetitive, high intensity loading. Histological and immunohistochemical (Cathepsin K and Receptor-antagonist of Nuclear Factor-kβ ligand (RANKL)) sections from the cores were scored and the Oc density calculated. A global score for each section and a subregional (ROI: 1-mm regions of interest) score were made. The cartilage histological scores were compared with Oc density and RANKL scores.

Results: There was a greater density of Ocs in ROIs in PTOA samples and they were preferentially located in the subchondral bone plate (SCB-P). RANKL scores positively correlated to the scores of cartilage degeneration and the Oc density. The relationship between hyaline articular cartilage RANKL score and Oc density was stronger than that of the SCB RANKL score suggesting that cartilage RANKL may have a role in recruiting Ocs. The RANKL score in the articular calcified cartilage (ACC) correlated with the number of microcracks also suggesting that Ocs recruited by RANKL may contribute to calcified cartilage degeneration in PTOA.

Conclusion: Our results support the hypothesis that Ocs are recruited during the progression of spontaneous equine carpal PTOA by cartilage RANKL, contributing to calcified cartilage microcracks and focal SCB loss.

Keywords

Osteoclast, Subchondral Bone, RANKL, Post-traumatic Osteoarthritis, Racehorses, Articular Cartilage, Osteoarthritis

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Introduction

Osteoarthritis (OA), a slowly progressive degenerative joint disease, is characterized histologically by fibrillation and erosions of the hyaline articular cartilage (HAC), remodelling of the subchondral bone (SCB) and periarticular osteophytes. The HAC and SCB are juxtaposed and coupled biomechanically and metabolically, but their complex interactions, particularly in the early stages of OA, are not entirely understood. Radin et al. proposed that an increase of the stiffness gradient in the SCB may initiate and promote progression of OA[1]. However an early reduction in SCB bone mineral density (BMD) has now been measured in many animal models of OA[2-9] suggesting that very early resorptive remodelling events arise as part of the disease process. Furthermore bone resorption is increased in patients with progressive knee OA[10] and SCB remodelling correlates with severity of overlying HAC pathology in human OA[11].

On the other hand, direct HAC injury in post-traumatic osteoarthritis (PTOA) initiates an immediate cascade of events within the HAC itself that include: chondrocyte death, matrix microcracks, release of matrix molecules, disruption of collagen structure and inflammation (reviewed by Lotz)[12]. Matrix breakdown and cracks, in an already permeable ACC, identified in human and animal joint disease [1,13-20], may facilitate bi-directional molecular diffusion of a variety of stimulatory molecules across these interfaces potentially regulating cartilage matrix degradation by chondrocytes, osteoblast signalling and osteoclastogenesis. It is likely that the mechanisms and chronology of events and prevailing direction of crosstalk will vary depending on the initial cause of the OA.

OA arises frequently in racehorse joints and it is the only spontaneous, naturally-occurring, model for investigation of the pathophysiological events of PTOA. Racehorse joints sustain repetitive periods of high intensity loads, particularly in the carpal joints, during athletic activity that initially induce adaptive (reviewed by Martig)[21], but later degradative events, in the HAC, ACC and SCB.

The physiological adaptive modelling of the SCB with exercise is observed as increased bone density on radiographs. However, the tipping point leading to pathological events in the osteochondral unit remains to be elucidated. The osteochondral degradative events are focal, with characteristic patterns for each joint due to extreme loading. Several investigations of the mineralized tissues in equine PTOA have revealed that microcracks arise in the ACC[19,22,23] and SCB[18,22,24], combined with bone remodelling with excessive resorption and porosity[17,19,22,23,25,26]. The complex inter-play between the initial structural damage and the biological events, orchestrated by cells in the HAC and underlying SCB is central to understanding PTOA pathophysiology, progression, prevention and therapy.

SCB remodelling is executed by basic multicellular units (BMUs), spearheaded by bone resorbing osteoclasts (Ocs) that excavate tunnels followed by bone forming osteoblasts that lay down osteoid in their wake. The Ocs are recruited from circulating peripheral blood monocytes in the bone marrow and undergo differentiation and activation. The activated Ocs attach to the bone matrix, release H+ ions to demineralize the matrix and produce enzymes, including cathepsin K and matrix metalloproteases, to digest principally type I collagen in bone (reviewed by Cappariello)[27]. Mature Ocs are large, multinucleated cells with a lifespan of 9-10 days, but BMUs can be active for up to 4 months. Oc function is controlled by the Receptor Activator of NF-kβ (RANK), the Receptor antagonist of Nuclear Factor-kβ ligand (RANKL) and the RANKL natural antagonist Osteoprotegerin (OPG). RANKL, a member of the Tumor Necrosis Factor cytokine ligand superfamily is essential for Oc differentiation and regulates their bone resorptive function[28] and prevents Oc death. RANKL gradients also steer BMU cutting cones[29]. RANKL is produced in greatest abundance by osteocytes[30] but also by osteoblasts[28], and chondrocytes embedded in the matrix[30-32]. RANKL expression is up-regulated in human OA cartilage specimens[33] and in rabbit models of OA. As RANKL is expressed by chondrocytes this raises the question as to whether HAC chondrocytes could modulate Oc recruitment, SCB remodelling and structure in OA by signalling to cells in the SCB.

Oc biology and its role in SCB turnover in OA and its progression is receiving more attention recently as it is now recognized that Oc activity may be an important target for therapy (reviewed by Karsdal)[34]. Although Ocs are the principal effectors of SCB resorption, there is only sparse data on their number in the SCB in human OA[35]. Resorption pits extending from the subchondral bone into hyaline cartilage have been observed in greater numbers in OA patient specimens and it was posited that osteoclastogenic factors were released from chondrocytes[36]. In addition, a new study has revealed that blood monocytes from OA patients have an enhanced capacity to generate Ocs[37] with increased resorptive activity and reduced Oc apoptosis. Ocs are also being increasingly identified in early OA in the subchondral plate in experimental animal models of OA[38-40]. These findings support the hypothesis that Ocs participate in the pathophysiology of OA by altering bone metabolism.

We hypothesize that there is a focal increase in Ocs in regions of overload in PTOA linked to cartilage and bone loss in these tissues and that cartilage RANKL expression may drive this process. The objective of this investigation was to measure Oc density in subregional areas in the SCB in both control and naturally occurring equine PTOA specimens and to study the association of Oc density with lesions in the HAC and ACC and RANKL expression.

Methods

Tissue source

The osteochondral sections examined in this study were from tissues banked from a previous study on equine carpal PTOA[19]. Briefly, cores (10 mm) were cut from the third carpal bone of racehorses (n=15), where the most severe cartilage PTOA lesions frequently arise (Figure 1). They were classified as healthy controls (n = 5) or affected with PTOA (n = 10), based on the articular cartilage changes observed on macroscopic examination of the articular surface as previously described[19].

Histological sections

The cores were fixed in paraformaldehyde solution (4% in Phosphate buffer solution (PBS)) for 24h at +4°C, stored in PBS at +4°C, decalcified in 10% EDTA and embedded in paraffin. Five-µm thick sections were cut and stained with hematoxylin, eosin, phloxine and saffron (HEPS) for cellular and morphologic evaluation, and Safranin-O-Fast-Green (SOFG) for cartilage assessment.

Immunohistochemical stained sections

Immunohistochemistry was performed to detect both cathepsin K and RANKL protein expression. For details please see immunohistochemistry protocol (Supplementary information online). All sections were stained in a single session to eliminate interassay variability. Negative controls consisted of phosphate buffer solution-bovine albumin with of omission of the primary

antibody (Supplementary information online, Figure 1,).

All stained sections were captured as digital images at both 2.5x and 20x magnification using a Leica DM 4000 B with a camera Prosilica GT workstation. The HAC and ACC were separated at the level of the osteochondral junction in each image to provide 2 images stored separately that permitted a blinded, independent evaluation of the HAC/ACC and SCB changes separately and by different readers.

Complete section and ROI assessment

All the sections were assessed in a dual fashion: first, as complete sections and subregionally within smaller regions of interest (ROI) as PTOA lesions are focally distributed. ROIs were created by digitally dividing all complete section images into 1-mm-width ROIs (Figure 1). This strategy permitted us to more accurately capture focal changes and make meaningful comparisons within these and also increased the number of data points.

HAC score & ACC evaluation

The images of complete sections and ROIS were scored blindly by 2 readers employing a modified Mankin score for cartilage degeneration[41] (Table 1, Supplementary information online). The numbers of microcracks in the ACC and cartilage pits were assessed as described previously[19].

SCB histomorphometry to assess bone structure

Digital images of the SCB from complete sections and ROIs, stained with HEPS and cathepsin K,

were employed for 2-D bone histomorphometry. Images were processed with image analysis software as described previously[42]. The total section area (TA), the bone area (BA) and the bone perimeter (BPm) were measured. Bone volume (BV%) and porosity (BP%) fraction were derived with the following formulae, BV%=BA/TA, BP%=1/BV.

Osteoclasts (Ocs): numbers, density & location

Oc numbers were calculated by 2 independent evaluators in the HEPS sections and the cathepsin K immunostained sections. Ocs were defined as multinucleated (≥ 3 nuclei) giant cells observed in bone lacunae. The Oc numbers were then normalized to TA, BA and BPm to provide surface and linear Oc densities.

In addition, to explore the depth location of Ocs in the mineralized tissues, three zones below the HAC were selected for Oc density assessment: the ACC, from the tidemark to the cement line; the SCB-plate (SCB-P), from the chondro-osseus junction to 3-mm-depth, and the SCB-TB (subchondral trabecular bone) below the 3-mm-line.

RANKL expression score

RANKL expression in all the tissues was assessed semi-quantitatively on digital images of the whole sections (SL and CG) and ROIs (HR and AB) by expanding a previously described score for human HAC[33] (Table 2, Supplementary information online).

Statistical analyses

Intraclass correlation coefficient (ICC) was employed to assess interobserver agreement for all semi-quantitative parameters (HAC and RANKL scores) and Oc numbers in complete sections and 20 randomly selected ROIs. When the ICC values were ≥ 0.8, scores of one observer were used for subsequent statistical analyses. Pearson's correlation was employed for comparisons between the different Oc normalization methods.

Wilcoxon's test were employed to detect differences between groups for ordinal scores (HAC total score, RANKL score, zonal location of Ocs) and t-tests for quantitative variables (ACC microcrack number, cartilage pit numbers in ACC, BV(%), BP(%), BA, TA, BPm, Oc density) in the complete sections and ROIs.

Spearman rank correlations were employed to compare Oc density with: total HAC scores and individual HAC parameter scores in the complete sections. A Pearson's correlation test was employed to assess the association between the Oc density and SCB histomorphometric parameters and RANKL score. A Cochran-Mantel-Haenszel test for ordinal variables was used to test the association between the RANKL score (RANKL Total score, RANKL HAC score, RANKL Tidemark score and RANKL SCB score) and the HAC score, the ACC score, the BV (%) and BP (%) in the SCB. For the subregional assessment a mixed linear model was also employed to test the association between the same parameters in the ROIs as described above for the complete sections to determine if the associations changed when assessed at a more focal level.

The coefficient of determination (%) was calculated for all the significantly associated variables. The horse ID was considered as a random effect to take into account the repeated measurements for each individual. Statistical analysis was performed using SAS v.9.3 (Cary, N.C.) and Graphpad Prism v6.0

(Graphpad Software Inc. USA). Data are presented as Mean+/-SD. The level of statistical significance was set at 0.05.

Results

Macroscopic & histologic HAC assessment

Complete sections from the cores were in the C group (n = 5) or PTOA (n = 10) based on macroscopic assessment. When separated into ROIs it yielded C = 42 regions or PTOA= 83 regions for analyses. Mean ± SD HAC histopathological score for control and PTOA complete sections and ROIs are provided in Table A. There was excellent agreement between the readers on HAC histopathological score (ICC:0.93) on complete sections and the total HAC histopathological scores were significantly different between C and PTOA groups (Table 3, Supplementary information online).

ACC assessment

Mean ± SD microcrack numbers and SCB pits for complete sections and ROIs in C and PTOA groups are provided in Table A. A significant difference in the microcrack density and the number of SCB pits were also detected between C and PTOA specimens (Table 3, Supplementary information online).

SCB histomorphometry

BP(%) and BV(%) data are in Table 3 (Supplementary information online) and no differences were detected between groups.

Oc numbers

Oc densities are provided in Table 3 (Supplementary information online). There was a high interobserver agreement for the Oc numbers on the HEPS and cathepsin K stained specimens (ICC: 0.90) and between two readers (ICC: 0.94). Ocs with ≥ 3 nuclei were frequently seen in PTOA specimens and were usually localized in resorbing bays, often in apposition to bone tissue. Giant, hypernucleated, cells with more than 20 nuclei were occasionally observed (Figure 2. C, F, I). Cathepsin K staining revealed the enzyme within the cytoplasm (Figure 2. D, E, F).

Oc density

There was a strong association ($p<0.001$, $r = 0.99$ for Oc/BA and Oc/TA; $p = 0.002$, $r = 0.74$ for Oc/TA and Oc/BPm and $p = 0.003$, $r = 0.71$ for Oc/BA and Oc/BPm) between the Oc density normalization methods. Oc/TA was employed in all the subsequent comparisons. Mean ± SD Oc densities for complete sections and ROIs in control and PTOA cores are provided in Table 3 (Supplementary information online). The Oc density was significantly higher in the PTOA group compared with the C group (Figure 3).

Oc location

Oc density in the mineralized tissue zones (ACC, SCB-P and SCB-TB) are presented in Table 3 (Supplementary information online)**.** The Oc density was significantly increased in the SCB-P, compared to ACC and SCB-TB in the PTOA but not in the C group (Figure 3).

RANKL expression

RANKL scores are provided in Table 3 (Supplementary information on line). There was high interobserver agreement for RANKL expression in both complete sections (ICC: 0.87) and ROIs (ICC: 0.89). RANKL expression in the HAC and ACC was pericellular and in the matrix. RANKL expression was mostly located in the middle and deeper layers of the HAC, with a distribution that paralleled the pattern of the glycosaminoglycan loss in the matrix, detectable on the contiguous SOFG stained specimens (Figure 4). A patchy RANKL expression was also observed in the SCB, preferentially around the bone lacunae staining both osteoblasts and blood vessels. There was a highly significant difference in total RANKL score between C and PTOA in ROIs, but this was a trend ($p = 0.05$) when assessed in the complete sections (Figure 5).

Correlations between Oc density and HAC scores, ACC scores, SCB parameters and RANKL score

There was a significant positive correlation between the Oc density and the total HAC score, the HAC structure score, the HAC staining score (Figure 6), the HAC clusters, the ACC microcracks (Figure 6) and the cartilage pits in both the complete section and ROI analyses (Table 1). A significant positive correlation was also detected between the Oc density and the RANKL HAC score on both the complete and ROI analyses (Figure 7). On the complete section analyses there was also a positive correlation between the Oc density and the RANKL SCB scores, but the significance was lower than that observed with the HAC cartilage RANKL score. A significant association with a weak correlation was found between the Oc density and the BP(%) in the SCB.

Correlation of RANKL score with HAC scores, ACC scores and SCB parameters.

There was a significant positive correlation between the total RANKL score and the total HAC score and HAC structure score in the complete section (Table 1). A positive correlation was also detected between the RANKL HAC score and Total HAC score, HAC structure score, HAC staining score and ACC microcrack density, in the complete sections. The SCB RANKL was also correlated to the ACC microcrack and cartilage pits in the complete sections. Interestingly only the ACC RANKL score was correlated with the Total HAC score, the HAC staining, the HAC structure and the ACC microcrack density in the ROIs (Figure 2, Supplementary information on line).

Discussion

The findings of this study provide a number of novel insights into PTOA pathology that are clinically relevant as the samples are from specimens with naturally occurring disease. First, there was a greater density of Ocs in focal subregions in OA samples when compared to control sites and, importantly, they were preferentially located in the subchondral plate, directly under the articular cartilage. Second, RANKL, an essential and potent osteoclastogenic molecule, was localized in the middle and deep layers of the HAC and was correlated both to the degenerative processes in cartilage and the Oc density in the mineralized tissues signifying that it could be a key signalling molecule in crosstalk between cartilage and bone tissues. The relationship between the HAC RANKL score and Oc density was stronger than that of the SCB RANKL score suggesting that HAC RANKL may have a role in recruiting OCs. Finally, the RANKL score in the ACC correlated with the number of microcracks also suggesting that, combined with the Oc sites in the subchondral bone plate, these powerful cells may also contribute to ACC degeneration in PTOA.

This is the first study to relate cartilage RANKL expression to Oc density in the SCB in PTOA tissues. The observation of increased density of Ocs in the SCB in PTOA tissues agrees with the findings of others as Ocs have been observed in human[35,36] and equine[19,43] naturally occurring OA and several experimental animal models of OA[38-40]. This observation provides a biological explanation for our previous findings of SCB hypomineralization in these tissues[19]. The assumption in the past has been that Ocs are recruited in the advanced stages of OA alone. However recent observations from animal models of OA challenge this dogma. Oc numbers were increased in the subchondral bone plate in animal models of early OA[38-40]. As Ocs have a short lifespan, their increased detection in the subchondral bone plate in OA suggests that an active osteoclastogenic process or molecule is recruiting them to this site.

The subregional assessment approach, by examining ROIs in the tissues, emphasized the very focal nature of the changes, at least in these PTOA tissues, and supports the observations of others about the very focal nature of cartilage lesions in early human and animal OA[19]. The OC density increased significantly, up to 9 fold, in the subregional SCB regions, below the degenerated HAC and there was a positive and significant correlation between the increased OC density and the bone porosity in the same regions.

These findings raise 2 questions: The first question is why are Ocs recruited to the SCB plate in PTOA? and second: what are their effects on the osteochondral unit? Although it is logical to investigate the crucial osteoclastogenic molecule RANKL in respect to Oc recruitment, other factors such as inflammation and microdamage could also play a role. With respect to RANKL, its expression was upregulated in the middle and deep layers of the HAC and correlated with the cartilage degradation score and the Oc density in the mineralized tissues suggesting a link between these events and that it could be a key signalling molecule in crosstalk between cartilage and bone tissues in PTOA. Several recent investigations have suggested that soluble cartilage RANKL may

modulate subchondral bone remodelling in a paracrine fashion in OA[31,32]. Furthermore, it is tempting to speculate that microcracks and degeneration in the ACC would facilitate diffusion and signalling of cartilage RANKL to SCB marrow cells to recruit Ocs in disease. The RANKL score in the articular calcified cartilage correlated with the number of microcracks in this tissue also raising the possiblity that Ocs localized in the juxtaposed subchondral bone plate may have a role in ACC degeneration, and overlying HAC degeneration as Ocs have the capacity to digest articular cartilage[44].

Although we provide evidence for increased expression of RANKL in PTOA cartilage with increased Oc density in the SCB, it is recognized that there may be alternative pathways to modulate osteoclastogenesis in PTOA. These pathways could be either independent or synergistic with the cartilage RANKL pathway we and others[31,33] propose. It is well recognized that inflammation is induced by trauma or overload in PTOA and that a proinflammatory cytokine milieu is a major driving force in cartilage degradation in OA (reviewed by Lotz)[12]. Therefore, alternative potential osteoclastogenic pathways in PTOA include the influence of inflammatory cells and their cytokines such as 1L -1, 6, 23, 17 & 34 (reviewed by Adamapoulos)[45]. An interesting link between repeated biomechanical overload and inflammation has also been established in animal models. Repeated biomechanical overload increased Ocs, their activity, cortical thinning and reduced bone mineral density in the radial metaphyseal trabeculae in rats[46]. There was a concomitant increase in the inflammatory cytokines IL-1beta and TNF-alpha, both locally in the bone and systemically and the changes were prevented by the administration of Ibuprofen. This suggests repetitive bone overload induces local inflammatory cascades and could be a potential pathway for Oc recruitment in equine PTOA. In an experimental rat overload model of the temporomandibular joint Ocs also significantly increased in the SCB at 20 days[39]. The proximal articular surface of the equine 3rd carpal bone studied here, sustains cyclic loading of high forces in the radial facet during both training and racing. The normal SCB's response to loading is anabolic or catabolic depending on the magnitude,

345 frequency and duration of the load and determines the bone's density, structure, matrix composition
346 and strength (reviewed by Robling)[47]. Characteristic features of the SCB in equine repetitive PTOA in
347 racehorses include an initial adaptive sclerosis but later, microcracks in the sclerotic bone (reviewed
348 by Martig)[21]. Oc lacunae and sites of bone resorption have been identified[18,19,26]. The role of Ocs in
349 the process, the only bone resorbing cell, has never been examined. Repair of microcracks induced by
350 repetitive overload is important for the maintenance of the bone's mechanical properties and
351 microdamage activates BMUs[15]. However, precisely how Ocs are recruited to the microcracks
352 remains to be elucidated. Osteocytes are mechano-sensors transducing mechanical stimuli into
353 biological signals and are the main producers of bone RANKL for osteoclastogenesis[48,49]. Specifically,
354 membrane bound RANKL, located on osteocyte processes is now believed to orchestrate bone
355 remodelling[49]. Soluble RANKL is cleaved by matrix metalloproteinases from the membrane bound
356 form. Recent studies suggest that membrane bound RANKL and cell to cell contact is necessary for
357 osteoclastogenesis, pointing to a greater role of osteocytes in the process[48,49]. In these specimens
358 where pathology is induced by repetitive trauma, microcracks in the bone matrix may injure
359 osteocytes leading to osteocyte apoptosis and may contribute to target remodelling of the bone by
360 BMUs[47]. However osteocytes are reduced in regions where microcracks are detectable in the equine
361 SCB[50].

362 The findings of this study lend support for the strategy of targetting osteochondral tissue
363 remodelling in OA therapy, either to prevent or arrest the progression of disease. This strategy could
364 be either a downstream decrease in the number Ocs as with bisphosphonate therapy, specifically
365 inhibiting the powerful collagenases cathepsin K produced by osteoclasts or alternatively an upstream
366 approach targetting RANKL and other pro-osteoclastogenic molecules[34]. However the downside of
367 this approach is that BMUs are required for normal bone remodelling and repair and potential long

term adverse effects of their shutdown on the skeletal tissue health, particularly in athletes with bone microcracks requires additional elucidation.

Conclusion

Taken together these results support the hypothesis that Ocs are recruited in the SCB during the progression of spontaneous equine carpal PTOA by the classic RANK/RANKL pathway and RANKL expression by chondrocytes may be an important chemo-attractant for Ocs to the SCB-P, contributing to focal SCB bone loss, ACC microcracks, cartilage collapse into the SCB-P and potentially osteochondral fractures. Ocs, by releasing cathepsin K a powerful collagenase capable of degrading bone type I and cartilage type II collagens focally, may participate and, precipitate, degradation of articular cartilage during progression of natural OA. A better understanding of Ocs attraction and migration to the SCB-P in naturally occurring OA pathologic tissues, could help identify additional drug targets to inhibit excessive bone resorption and preserve bone integrity structure and function during OA.

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Author contributions

SL conceived and designed the study and evaluated the histological specimens and wrote the manuscript. AB participated in study design, evaluated the histological specimens and wrote the manuscript. ML collected the specimens. HR performed the histological and immunohistochemical analyses. CG evaluated the histological specimens and revised the manuscript. GB performed the statistical analysis and revised the manuscript.

Conflict of interest

None of the authors had any conflict of interest.

Index of Abbreviations

OA= Osteoarthritis

ROI=Region of interest

HAC= Hyaline articular cartilage

ACC= Articular calcified cartilage

SCB= Subchondral bone

SCB-P= Subchondral bone plate

TB= Trabecular bone

TA= Total Area

BA= Bone Area

BPm= Bone perimeter

BV(%)= Bone Volume fraction

BP(%)= Bone Porosity fraction

BMD= Bone mineral density

Ocs= Osteoclasts

RANKL= Receptor-antagonist of Nuclear Factor-kβ ligand

RANK= Receptor-antagonist of Nuclear Factor-kβ

OPG= Osteoprotegerin

SOFG= Safranin-O-Fast Green

HEPS= Hematoxylin-Eosin-Phloxine-Saffron

BMU= Basic Multicellular Unit

PTOA= Post-Traumatic Osteoarthritis

C= control

ICC= Interclass Coefficient Correlation

PBS= Phosphate buffer solution

PBS-BSA= Phosphate buffer solution-bovine albumin

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561

Legend

Figure 1. **Study design**

The intercarpal surface of equine third carpal bones (C3) was scored for articular cartilage macroscopic changes and classified as post-traumatic osteoarthritis affected surface (PTOA) or control (C). A cylindrical osteochondral core was cut from the radial facet of C3 close to the dorsal border of the bone and notched for orientation purposes and decalcified. Sections were stained with Hematoxylin Eosin Phloxin and Safran (HEPS) and Safranin O fast green (SOFG). Immunohistochemistry was performed with Cathepsin K and receptor activated nuclear kappa α ligand (RANKL). All the sections were digitalized and the hyaline articular cartilage (HAC) and articular calcified cartilage (ACC) were digitally separated from the subchondral bone (SCB) and blindly evaluated. A computer analysis was employed to generate a bone mask of the SCB. The SCB mask and the corresponding cartilage were subdivided in 1-mm-width regions of interest (ROI) for a subregional histomorphometric assessment. Parameters assessed included HAC modified Mankin score, ACC microcracks and cartilage pits, Osteoclast density, histomorphometry and RANKL expression.

Key: C3 = Third carpal bone; PTOA=post traumatic osteoarthritis; C=Control; HEPS=Hematoxylin Eosin Phloxin and Safran ; SOFG=Safranin O fast green; RANKL=receptor activated nuclear kappa α ligand; HAC=hyaline articular cartilage; ACC=articular calcified cartilage; SCB=subchondral bone; ROI=region of interest.

Figure 2. HEPS, SOFG and Cathepsin K immunohistochemical stained sections revealing osteoclasts.

A. A BMU with arrow pointing to 3 osteoclasts in a cutting cone .

B & C. Numerous osteoclasts visible in this section. Dotted square magnified in C. Arrow reveals large osteoclasts.

D, E and F. Cathepsin K stained sections with intracellular Cathepsin K in osteoclasts. Insert from E is magnified (F) to reveal morphology.

G, H and I. Additional examples of osteoclast morphology observed in the subchondral bone.

Key: BMU=bone remodelling unit.

Bar=100μm.

Figure 3. Comparisons between Control and PTOA specimens: Hyaline articular cartilage score, Oc density and Oc location in bone.

A. Total HAC scores in either complete section or subregional ROI analyses in C and PTOA groups.

B. Oc density (Oc/TA cells/$mm^{2)}$ scores in either complete section or subregional ROI analyses in C and PTOA groups.

C. Oc location in bone in subregional ROI analyses

Key: HAC=hyaline articular cartilage; ROI=region of interest; C= Control; PTOA=post-traumatic osteoarthritis; Oc=osteoclast; TA=Total area.

Figure 4. SOFG and RANKL immunohistochemical stained osteochondral sections.

A. SOFG stained section revealing intact HAC structure with loss of staining in the superficial zone of cartilage. Microcracks visible in the ACC.

B. RANKL staining in the superficial zone of the HAC and patchy staining in the subchondral bone.

C. Close up of dotted square in B revealing superficial diffuse staining. Patchy artefactual staining within the HAC.

D. Loss of staining in all the HAC. Coalescence of microcracks in the SCB.

E. RANKL staining (intracellular and pericellular) in deep zone of the HAC and ACC. Marked staining in the SCB matrix.

F. Close up of dotted square in E

G. Loss of staining principally in the HAC. Collapse of the ACC into the SCB on the left of the image.

H. RANKL staining in the intercellular zone in the matrix of the HAC and in the SCB matrix.

I. Close up of dotted square in H

J. Loss of up to 50% of HAC matrix and loss of SOFG staining.

K. & L. Intense focal uptake of RANKL stain in the ACC.

M. Focal loss of HAC structure and SOFG staining in lesion top the left of the image.

N. & O. RANKL staining inversely proportional to loss of SOFG staining.

P. Focal loss of HAC structure and SOFG staining in lesion top the left of the image.

Q. & R. RANKL staining both intracellularly and matrix.

Key: HAC=hyaline articular cartllage ; ACC=articular calcified cartilage ; SOFG=safranin O fast green; RANKL=receptor activated nuclear kappa α ligand.

Bar =1 mm:500μm in C, F, I, L, O, R

Bar=100μm. A, B, D, E, G, H, J, K, M, N, P, Q.

Figure 5. RANKL expression in control and PTOA osteochondral sections

Total RANKL score in the complete sections and subregional ROI analyses in both C and PTOA specimens

Key: RANKL=receptor activated nuclear kappa α ligand; ROI=region of interest; C=Control. PTOA=post traumatic osteoarthritis.

Figure 6. Correlations between Osteoclast density and total HAC score, HAC structural & Safranin O stain scores and ACC microcracks in complete sections and on subregional analysis.

A. Correlation of Oc density and total HAC score in both complete sections and ROIs

B. Correlation of Oc density and HAC structure score in both complete sections and ROIs

C. Correlation of Oc density and HAC Safranin O stain score in both complete sections and ROIs

D. Correlation of Oc density and ACC microcracks in both complete sections and ROIs

Key: Oc=Osteoclast; TA=total area; HAC=hyaline articular cartilage; ACC=articular calcified cartilage.

Figure 7. Osteoclast density correlated with the hyaline articular cartilage RANKL expression

Correlations between osteoclast density and RANKL HAC score in complete sections and on subregional analysis.

Key: Oc=Osteoclast; TA=total area; RANKL=receptor activated nuclear kappa α ligand. HAC = hyaline articular cartilage.

On line

Figure 1, Supplementary information on line. Sections stained immunohistochemically with either RANKL or Cathepsin K antibodies and controls.

A. Osteochondral section stained with RANKL. Increased staining can be observed at the surface of the cartilage and also in the subchondral bone matrix.

B. Negative control: Adjacent section to A stained with PBS-BSA.

C. Cathepsin K stained section with increased staining at the surface of HAC and also visible in the bone matrix.

D. Negative control: Adjacent section to A stained with rabbit antiserum.

Key: RANKL=receptor activated nuclear kappa α ligand. HAC=hyaline articular cartllage.

Bar=500 μm

Figure 2, Supplementary information on line. Articular calcified cartilage RANKL score correlated with hyaline articular cartilage histological scores and microcracks

A. Total HAC score correlated with the ACC RANKL score

A.1. HAC structure score correlated with the ACC RANKL score

A.2. HAC safranin O score correlated with the ACC RANKL score

D. Microcracks in ACC correlated with ACC RANKL score

Key: HAC=hyaline articular cartilage. ACC=articular calcified cartilage; RANKL=receptor activated nuclear kappa α ligand.