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Bone Cysts After Osteochondral Allograft Repair of Cartilage Defects in Goats Suggest Abnormal Interaction Between Subchondral Bone and Overlying Synovial Joint Tissues

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Abstract

The efficacy of osteochondral allografts (OCA) may be affected by osseous support of the articular cartilage, and thus affected by bone healing and remodeling in the OCA and surrounding host. Bone cysts, and their communication pathways, may be present in various locations after OCA insertion and reflect distinct pathogenic mechanisms. Previously, we analyzed the effect of OCA storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on cartilage quality in fifteen adult goats after 12 months *in vivo*. The objectives of this study were to further analyze OCA and contralateral non-operated (Non-Op) CONTROLS from the medial femoral condyle to (1) determine the effect of OCA storage on local subchondral (ScB) and trabecular (TB) bone structure, (2) characterize the location and structure of bone cysts and channels, and (3) assess the relationship between cartilage and bone properties. (1) Overall bone structure after OCA was altered compared to Non-Op, with OCA samples displaying bone cysts, ScB channels, and ScB roughening. ScB BV/TV in FROZEN OCA was lower than Non-Op and other OCA. TB BV/TV in FRESH, 4°C/14d, and 4°C/28d OCA did not vary compared to Non-Op, but BS/TV was lower. (2) OCA contained “basal” cysts, localized to deeper regions, some “subchondral” cysts, localized near the bone-cartilage interface, and some ScB channels. TB surrounding basal cysts exhibited higher BV/TV than Non-Op. (3) Basal cysts occurred (a) in isolation, (b) with subchondral cysts and ScB channels, (c) with ScB channels, or (d) with subchondral cysts, ScB channels, and ScB erosion. Deterioration of cartilage gross morphology was strongly associated with abnormal μ CT bone structure. Evidence of cartilage-bone communication following OCA repair may favor *fluid intrusion* as a mechanism for subchondral cyst formation, while bone resorption at the graft-host

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interface without affecting overall bone and cartilage structure may favor *bony contusion* mechanism for basal cyst formation. These findings suggest that cysts occurring after OCA may result from aberrant mechanobiology due to (1) altered compartmentalization that normally separates overlying cartilage and subchondral bone, either from distinct ScB channels or more general ScB plate deterioration, and (2) bone resorption at the basal graft-host interface.

Keywords

osteocondral allografts; bone cysts; micro-computed tomography; animal model

1.1. Introduction

The efficacy of osteochondral allografts (OCA) for repairing defects of articular cartilage may be affected by the healing of subchondral bone, as determined by remodeling of both the graft and surrounding host bone. Clinically, graft failure is defined by progressive increase in patient symptoms including new-onset pain, swelling, catching or locking, feeling of instability, decreased range of motion, and interference with activities of daily living, attributable to the graft [1–6]. The diagnosis of graft failure is often supported by radiographic evidence of bony non-union, late fragmentation, and collapse, all indicative of insufficient osseous support [1–6]. In particular, patients may present with persistent bone sclerosis, bone resorption, and subchondral bone cysts, the latter being identified as well-demarcated and often rounded areas of translucency in bone. In addition, articular cartilage often deteriorates (decreased volume, histopathological index of degeneration, decreased stiffness) near bone lesions [7–11]. In animal models, subchondral bone cysts also occur following osteochondral grafting [12–15], and other cartilage repair treatments [16–19]. The presence of bone cysts following cartilage repair treatments suggests abnormal biological remodeling, and bone cysts following osteochondral autograft transplantation and autologous chondrocyte implantation are often associated with inferior clinical results [20, 21]. However, the etiology of such subchondral bone pathology and its relationship to overlying articular cartilage is unclear, particularly in the setting of OCA.

In OCA repair, subchondral bone cysts may arise from mechanisms proposed to underlie the formation of such cysts after injury and in osteoarthritis, as well as from mechanisms specific to OCA implantation. The *Synovial Fluid Intrusion* hypothesis suggests that subchondral bone cysts form as degenerative changes of the overlying cartilage lead to aberrant communications between the joint space and subchondral bone, forcing pressurized synovial fluid to exude into the subchondral bone; such fluid intrusion alters mechanical and biological homeostasis in the bone [22, 23]. This mechanism is plausible in the setting of OCA due to resurfacing by the implant. Alternatively, the *Bony Contusion* hypothesis suggests that subchondral bone cysts form as a biological reaction of the subchondral bone in response to excessive, concentrated loads on the bone; such bony contusion leads to decoupled bone metabolism favoring resorption over formation [24]. This mechanism may be relevant to OCA, either by excessive loading of bone, during or after OCA insertion. Finally, bone unloading due to gaps between implant and host may lead to abnormal bone mechanobiology and metabolism. Delineation of the structural properties of subchondral bone, and relationships between cartilage and underlying bone following OCA repair may support the above mechanisms, and clarify the etiology of OCA-associated bone cysts and their significance.

Some methods have been established for assessing the structure of subchondral bone cysts and the microarchitecture of surrounding bone, and for mapping nearby bone channels and regions of subchondral bone deterioration. Cysts are well-demarcated as substantial regions,

devoid of trabecular bone and on (micro)computed-tomography (μ CT) or histology [18], and exhibit signal intensity similar to that of joint cavity fluid on MRI [8]. Cyst size is typically determined in the image slice with the greatest lesion size [9], via semi-quantitative grading [9, 18, 25–27], or quantitative 2D measurements of maximum diameter [7], or by quantitative 3D volume estimates from three orthogonal slices [8]. Subchondral bone discontinuities, allowing direct communication between the joint space and trabecular bone have been identified [7, 22, 28–30]; however, the extent of such discontinuities and their relationship to bone cysts are unclear. Bone microarchitecture around cysts is sometimes dense at the walls, and then more porous and branched with a relatively normal overall bone volume fraction in surrounding regions, and accompanied by increased porosity of the subchondral bone [19, 31]; however, application of methods developed for regional evaluation of bone [32] to bone cysts have been limited [33].

Thus, the objectives of the study were to (1) determine the effect of OCA storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on subchondral and trabecular bone structure in the graft region, (2) characterize the structure and location of bone cysts, and (3) assess the relationship between cartilage and bone properties following OCA repair at 12 months in the goat. The results of the analysis suggest mechanisms contributing to the development of bone cysts following cartilage defect repair by osteochondral allografts.

2.0. Materials and Methods

The tissues analyzed were from a previously described study in an adult goat model, with IACUC approval [34]. Osteochondral cores (d=15mm, h~8–10mm) at the medial femoral condyle (MFC), which encompassed the experimental graft site and portions of the surrounding host tissue, from FRESH, 4°C/14d, 4°C/28d, and FROZEN OCA (each, n=3–4), along with site-matched regions of contralateral non-operated control joints (Non-Op, n=15) were analyzed. Prior to implantation in donor OCA, chondrocyte viability varied with OCA storage, but subchondral bone viability did not, as subchondral bone contained only dead cells and no viable cells in all donor OCA, irrespective of OCA storage.

2.1. Experimental Design

2.1.1—The effect of OCA storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on subchondral bone plate (ScB) and trabecular bone (TB) structure after 12 months *in vivo* was determined by analysis of micro-computed tomography (μ CT) and histology data. First, a (semi-quantitative) general index of bone structure as visualized on μ CT was determined, identifying bone cysts and cyst size, ScB channels and channel size, and bone-cartilage interface roughness (see §2.2.2.). Then, histological sections were analyzed for bone healing, examining bone graft-host integration, calcified cartilage structure, and degree of bone marrow fibrosis (Fig. 1D, §2.3.2.). Finally, morphometric measures of bone structure from μ CT were analyzed with standard nomenclature [35] in ScB (Fig. 1B, §2.2.3.) and TB (Fig. 1C, §2.2.4.).

2.1.2—The structure and location of bone cysts and channels was assessed in more detail by further analysis of μ CT and histology data. Since bone cysts were present regardless of OCA storage group, the following analysis was performed by grouping all OCA samples together. Bone cysts were characterized based on location (§2.2.5.), analyzed quantitatively for cyst volume (V, Fig. 1E, §2.2.6.), and examined for histologic features differentiating different types of bone marrow lesions (§2.3.3.). In addition, the TB immediately surrounding some cysts was also analyzed using morphometric measures of bone structure from μ CT (Fig. 1F, §2.2.7.).

2.1.3—The relationships between cartilage and bone properties were analyzed qualitatively and statistically. Principal component analysis was used to assess the relationship between cartilage properties reported previously [34] (including gross morphological score, superficial zone cellularity, hexabrix gray value [inverse of matrix fixed charge], and material stiffness) and the OCA graft bone properties determined here (including overall structural μ CT score and morphometric measures of bone structure in graft ScB [BV/TV, BS/BV], graft TB [BV/TV, BS/TV, Tb.Th, Tb.Sp, Tb.N], and peri-(basal) cyst TB [BV/TV, BS/TV, Tb.Th, Tb.Sp, Tb.N]). Using some of the metrics identified from the principal component analysis, the possible relationships between bone cysts, ScB channels, and ScB erosion were assessed qualitatively from μ CT and histology images, tabulating the individual and combined occurrences of these features.

2.2. Micro-Computed Tomography (μ CT) and Bone Morphometry

2.2.1. Sample Set-Up—Osteochondral samples were fully submerged in tissue culture medium (low-glucose Dulbecco's modified Eagle's medium), oriented so that the proximal-distal axis of the condyle was approximately aligned with the longitudinal axis of the μ CT scanner (Skyscan 1076, Kontich, Belgium), and scanned at $(18\text{ }\mu\text{m})^3$ applying an electric potential of 70kVp and a current of 141 μ A, with a 1.0mm aluminum filter. A beam hardening correction algorithm was applied during image reconstruction of each scan and all samples were reconstructed using a dynamic range of 0 to 0.2827mm^{-1} . Selection of the threshold used for quantitative analysis was based on the histogram of pixel intensities for the full dataset, where three main components, corresponding to air, media, and bone in increasing linear attenuation coefficient, were identified. Sample volumes were oriented using DataViewer software (Skyscan) such that the transverse axis was parallel to the proximal-distal and medial-lateral interfaces between the graft and host bone in both the sagittal and coronal imaging planes (Fig. 1A), cropped to a $(10.44\text{mm})^3$ region, and saved as a new data set.

2.2.2. Structural Bone Score—A general index of bone structure as visualized on μ CT was evaluated semi-quantitatively for bone cysts and cyst size, ScB channel and channel size, and bone-cartilage interface roughness on a 0–9 scale, with high scores corresponding to deterioration and/or poor repair. The overall bone score identified structural features, such as bone cysts and channels, that quantitative μ CT morphometric measures may have not been sensitive to detect, and also provided a single measurement representative of both ScB and TB components. Bone cysts were identified as large void regions by μ CT using complete 3D data sets, with a maximum diameter greater than 1.0mm in a 2D slice, and found only in OCA samples. Bone cysts and cyst volume, calculated in 3D (§2.2.6.), were scored as 0, 1, 2, or 3, for total volume of 0, $<5\text{mm}^3$, $5\text{--}15\text{mm}^3$, or $>15\text{mm}^3$, respectively. ScB channels were identified as visible gaps (ranging in width from 0.2–3mm) at the bone-cartilage interface, and were scored for the degree of disconnect across the subchondral bone plate across both the graft and surrounding host bone, as 0, 1, 2, or 3, for ScB plate that was intact, had one gap $<0.5\text{mm}$, one large gap $0.5\text{--}2\text{mm}$, or large/multiple gaps $>2\text{mm}$, respectively. To visualize the superficial opening of such channels, and to help localize the channel in gross cartilage images, 3D renders of the bone-cartilage interface, viewed *en face*, were created in Mimics (Materialise, Belgium). ScB roughness visualized at the bone-cartilage interface was scored as 0, 1, 2, or 3 as smooth, containing either a step-off $>0.5\text{mm}$ at the graft-host interface or mild roughening, containing both a step-off $>0.5\text{mm}$ at the graft-host interface and mild roughening, or as severe roughening with or without a step-off. Scores were averaged from three observers.

2.2.3. Morphometric Measures of Graft Subchondral Bone (ScB)—ScB architecture was assessed near the bone-cartilage interface with standard morphometric μ CT

parameters (Fig. 1B). Graft ScB volume of interest (VOI) was defined as a cylinder ($d=5\text{mm}$) within the central graft region, beginning at the bone-cartilage interface to a depth of 0.54mm . The bone-cartilage interface was segmented from the sagittal plane in Matlab by identifying the bone contours using methods previously described [15, 16] and the VOI was imported into CTAnalyser. ScB within this VOI was segmented using a global threshold of 0.136 mm^{-1} to 0.283 mm^{-1} , eroded by one pixel to account for partial volume effects, and analyzed for bone volume fraction (BV/TV, %) and specific bone surface (BS/BV, mm^{-1}).

2.2.4. Morphometric Measures of Graft Trabecular Bone (TB)—TB architecture was assessed in bulk graft bone with standard morphometric μCT parameters (Fig. 1C). The transverse slice that corresponded to the proximal interface between the graft and host bone ($Z_{\text{HOST, Proximal}}$) was identified in DataViewer. Graft TB VOI was defined as a cylinder ($d=6\text{mm}$) within the central graft region, beginning 0.36mm from $Z_{\text{HOST, Proximal}}$ and extending 2.4mm deep (from $Z_{\text{TRAB, Surface}}$ to $Z_{\text{TRAB, Deep}}$). TB within this VOI was segmented adaptively with the Otsu method using CTAnalyser software, and analyzed for bone volume fraction (BV/TV, %), bone surface density (BS/TV, mm^{-1}), trabecular thickness (Tb.Th, mm), trabecular spacing (Tb.Sp, mm), and trabecular number (Tb.N, mm^{-1}) using 3D distance transform and sphere fitting methods [36].

2.2.5. Cyst Location—Bone cysts were characterized by location, as either “subchondral”, and localized near the bone-cartilage interface and within the initial implant region ($\sim 3\text{--}4\text{mm}$ depth, based on implant dimensions), or as “basal”, and localized in deeper regions of the sample. ScB channels were examined for connections to neighboring subchondral cysts.

2.2.6. Cyst Volume—Both subchondral and basal bone cysts were analyzed quantitatively for cyst volume (V) by fitting an ellipsoid to large void regions within μCT data sets (Fig. 1E). In Matlab (Mathworks, Natick, MA), data sets were segmented using a global threshold of 0.0032 mm^{-1} to 0.123 mm^{-1} , and processed in 3D to close trabecular gaps smaller than $200\text{ }\mu\text{m}$ (based on Tb.Sp in control joints) to isolate the void regions classified as cysts. Isosurface data was then extracted from the 3D void volume, and fit to an ellipsoid using least squares ellipsoid specific fitting [37]. Cyst volume was calculated as $4/3 * \pi * a * b * c$, where a, b, and c are the radii of the ellipsoid.

2.2.7. Morphometric Measures of Trabecular Bone (TB) Surrounding Basal Cysts—TB architecture was also assessed surrounding basal cysts with standard morphometric μCT parameters (Fig. 1F). Peri-(basal) cyst VOI, in a 1mm thick shell surrounding the localized 3D bone cyst region, was segmented adaptively with the Otsu method using CTAnalyser software, and analyzed for BV/TV, BS/TV, Tb.Th, Tb.Sp, and Tb.N (as described in §2.2.4). Although Non-Op samples did not contain cysts, ellipsoids (fit to the basal cyst of the corresponding OP joint) were site-matched in Non-Op samples (relative to $Z_{\text{HOST, Proximal}}$) as pseudo-cyst regions, and TB was analyzed analogously. For some Non-Op samples, pseudo-cyst regions were placed at different locations (up to 5mm medial, lateral, and distal to original location) and TB architecture parameters were within 5% at each location.

2.3. Bone & Cyst Structure in Histological Sections

2.3.1. Histological Processing—Sections along the sagittal plane were analyzed by histology. Osteochondral slabs were isolated from each sample site to include the central region, 2mm wide. Samples were fixed in 4% paraformaldehyde, decalcified in 30% formic acid, embedded in paraffin, sectioned at $7\text{ }\mu\text{m}$, deparaffinized, stained with Hematoxylin and Eosin (H&E), and imaged digitally at 20X (Leica SCN400). Histological sections were

scored from a single slice through a central proximal-distal path within a $5.0 \times 2.5 \text{ mm}^2$ region of interest (ROI) centered within the graft and directly below the bone-cartilage interface (Fig. 1D).

2.3.2. Histological Analysis of Graft Bone—Histological sections of OCA repair and contralateral control regions were analyzed for specific features of bone healing. Graft-host integration was assessed in a binary fashion (complete or absent); bony union was noted if trabeculae crossed the circumferential interface between graft and host bone. Calcified cartilage was also assessed in a binary fashion, as present/intact (+) or as thinning/absent (−); the presence of multiple tidemarks were also noted. Bone marrow was scored semi-quantitatively on a 0–3 scale for the degree of fibrosis within the graft ROI from 0% (normal with typical presence of fat cells, score=0), <33% (score=1), 33–66% (score=2), to >66% (score=3).

2.3.3. Histological Analysis of Bone Cysts—Bone cysts, when visible, were also examined for histologic features differentiating different types of bone marrow lesions [38]. Edema was noted as the presence of swollen fat cells surrounded by eosinophilic staining. Fibrosis was noted as replacement of fatty marrow with collagenous fibers and with spindle-shaped cells. TB surrounding the cyst was noted as intact, necrotic with loss of nuclei and sclerosis, or repeatedly remodeled with presence of reversal lines.

2.4. Statistics

Statistical analyses were performed using Systat 10.2 (Systat Software Inc., Chicago, IL). Data are presented as mean±SEM, with $p < 0.05$ considered significant. Statistical analysis was performed as a paired design on the difference between matched OP and Non-Op samples, since the variation between right and left knees of one animal was much less than the variation among animals (CV=2–5% vs. 11–16%, data not shown). For non-parametric data (i.e. structural μCT bone scores), the effect of OCA storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on overall bone score, and the individual components (bone cyst size, bone channel size, and ScB roughness) were determined by Kruskal-Wallis and Dunn's *post hoc* tests because structural μCT bone scores were ordinal rather than continuous variables. For quantitative μCT data, the effect of OCA storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on subchondral (ScB BV/TV, ScB BS/BV) and trabecular (BV/TV, BS/TV, Tb.Th, Tb.Sp, Tb.N) bone structure in the graft and peri-cyst wall (trabecular structure only) regions were determined by ANOVA with Tukey *post hoc* tests. In addition, planned comparisons were performed between each OCA storage group and Non-Op (i.e. FRESH vs. Non-Op, 4°C/14d vs. Non-Op, 4°C/28d vs. Non-Op, and FROZEN vs. Non-Op) with a one-sample *t*-test, comparing the mean of each OCA storage group to the Non-Op population mean=0 to determine which experimental group means differed from Non-Op.

Finally, to determine which cartilage and bone parameters were related to each other, a factor analysis by the method of principal components was performed. The principal components matrix was computed, and the loading coefficients (describing how each parameter contributed to each factor) were obtained. For each factor, the dominant contributors were identified as those whose coefficients (ranging from −1 to +1) had an absolute value greater than or equal to 0.33. The Cattell scree test was used to determine the number of meaningful factors, which indicated 2 factors prior to the first 'elbow' or break in the curve of proportion of variance vs. factor number (data not shown), and the contribution of each additional factor was 10%.

3.0. Results

3.1. Effect of OCA Storage (FRESH, 4°C/14d, 4°C/28d, FROZEN) on ScB and TB structure after 12 months *in vivo*

Overall structural bone score was altered by OCA compared to Non-Op joints ($p < 0.05$). All Non-Op joints were devoid of cysts, ScB channels, and ScB roughening, and received a total score of 0, while OCA samples displayed bone cysts, and ScB channels, with or without ScB roughening (Table 1, Fig. 2A–E). Overall structural bone score for 4°C/14d was similar to that for 4°C/28d and FRESH ($p > 0.2$), and lower than FROZEN ($p < 0.01$). Bone cyst size did not vary among OCA treatments ($p > 0.3$). ScB channel size in 4°C/14d, which was small (< 0.5 mm), tended to be larger than 4°C/28d ($p = 0.09$), but was smaller than FRESH and FROZEN ($p < 0.05$) OCA, in which channel sizes were larger (> 0.5 mm) (Fig. S1). The bone-cartilage interface was smooth in Non-Op, slightly rougher in FRESH, 4°C/14d, and 4°C/28d ($p > 0.05$), and rougher in FROZEN ($p < 0.05$, Fig. S1). Thus, all OCA repairs had some abnormal bone features compared to normal subchondral bone in Non-Op joints, and overall bone structure scores provided a general and useful index of the overall state of the repair bone.

Histological features of bone structure, including bone graft-host integration, calcified cartilage integrity, and bone marrow fibrosis, provided a general index of the overall quality of bone in the cartilage repair region which was indicative of the extent of bone healing following OCA. Histologically, all OCA exhibited bony union at the circumferential graft-host junction (Fig. 2F–T). The calcified cartilage was typically intact in Non-Op joints (15/15), as well as FRESH OCA (3/3), but absent in all FROZEN (4/4) and some 4°C stored OCA (3/4 for 4°C/14d, 2/4 for 4°C/28d, Fig. 2K–O); although some Non-Op and FRESH OCA had multiple tidemarks (8/15 for Non-Op, 1/3 for FRESH). Bone marrow in Non-Op exhibited typical presence of fat cells (11/15) or minimal fibrosis (i.e. $< 33\%$, 4/15); that minimal fibrosis was seen in most FRESH (3/3), 4°C/14d (4/4), and 4°C/28d (3/4) OCA, but predominant fibrosis was evident in FROZEN (3/4) OCA (Fig. 2P–T). Thus, all OCA repairs were variably accompanied by histological structural abnormalities within graft bone.

Standard μ CT morphometric analysis of ScB architecture in the graft region varied with OCA storage (each, $p < 0.001$). ScB architecture was altered in Non-Op versus FROZEN OCA, but not versus all other OCA. ScB BV/TV and ScB BS/BV in Non-Op was similar to FRESH, 4°C/14d, and 4°C/28d OCA ($p > 0.6$, Fig. 3). ScB BS/BV in FROZEN was ~30–40% lower than Non-Op, FRESH, 4°C/14d, and 4°C/28d OCA (each, $p < 0.001$, Fig. 3A). Concomitantly, ScB BS/BV in FROZEN was ~2–3x times higher than Non-Op, FRESH, 4°C/14d, and 4°C/28d OCA (each, $p < 0.001$, Fig. 3B).

Standard μ CT morphometric analysis of TB architecture in the graft region of OCA indicated that overall bone volume fraction was similar among Non-Op and all OCA storage groups ($p > 0.7$), but the distribution of bone trabeculae was altered in FRESH, 4°C/14d and 4°C/28d OCA. Non-Op and FROZEN OCA had similar BV/TV, BS/TV, Tb.Th, Tb.Sp, and Tb.N values ($p > 0.3$, Table 2). In Non-Op, BS/TV and Tb.N were higher than FRESH, 4°C/14d, and 4°C/28d OCA ($p < 0.05$, Table 2.), whereas Tb.Th and Tb.Sp were lower than 4°C/14d and 4°C/28d ($p < 0.05$, Table 2). Only two TB architecture parameters, BS/TV and Tb.N, varied with OCA storage; BS/TV and Tb.N were higher in FROZEN versus FRESH (Tb.N only), 4°C/14d, and 4°C/28d ($p < 0.05$, Table 2). Thus, standard μ CT bone morphometry measures were similar among FRESH, 4°C/14d, and 4°C/28d OCA, with altered TB distribution and similar ScB integrity versus Non-Op, but different than FROZEN OCA, which had similar TB organization versus Non-Op bone, but deteriorated ScB.

3.2. Structure and Location of Bone Cysts and Channels

Bone cysts were widespread after defect repair by OCA at 12 months, with variable location and cyst volume, and communication to overlying cartilage. Twenty-two bone cysts (volume= $8.5 \pm 1.3 \text{ mm}^3$, range= $0.8\text{--}23.3 \text{ mm}^3$), were identified by μCT in 15 OP joints (Fig. 4). All 15/15 OP joints contained basal cysts (15 basal cysts). In addition, 6/15 OP joints contained subchondral cysts (7 subchondral cysts). Basal cysts were typically larger in volume than subchondral cysts ($11.2 \pm 1.5 \text{ mm}^3$ vs. $2.7 \pm 0.6 \text{ mm}^3$, respectively). Also, 10/15 OP joints had ScB channels (11 ScB channels), which ranged in size from 0.4–3mm wide (Fig. S1). Of these ScB channels, 6/11 connected the nearby subchondral cyst volume to the overlying cartilage, while 5/11 penetrated into the TB, but did not directly connect to a cyst.

Qualitative histological features associated with bone marrow lesions, including edema, fibrosis, and TB necrosis, sclerosis, or remodeling indicated that the structure of and contents within bone cysts appeared to vary with the location of the cyst (subchondral vs. basal cysts). Non-Op control bone exhibited normal TB and marrow and no histological evidence of bone cysts. Some subchondral cysts, which were associated with a ScB channel, contained fibrous tissue in marrow spaces (Fig. S2A–D). Other basal cysts contained normal bone marrow, displaying primarily fat cells (Fig. S2E–H); not all cysts could be evaluated histologically, as not all sections were in a plane that transected the cyst. Other histologic features typically associated with bone marrow lesions were evident and included swollen fat cells and bone marrow edema (Fig. S2B), as well as abnormal trabecular remodeling with necrosis (Fig. S2E), reversal lines (Fig. S2E), and bone sclerosis (Fig. S2H) present.

Standard μCT morphometric analysis of TB architecture surrounding basal cysts indicated that bone structure was different than Non-Op bone, but quite variable within all OCA repairs. Peri-(basal)cyst wall μCT architecture was different than Non-Op bone, but did not vary with OCA storage ($p > 0.2$). Peri-(basal)cyst wall architecture in OCA generally had higher BV/TV, BS/TV, and Tb.N, lower Tb.Sp, and similar Tb.Th compared to Non-Op (Table 3). However, peri-(basal)cyst wall BV/TV in OCA ranged from 0–15% higher than Non-Op; compared to Non-Op, 7/15 OCA had similar BV/TV (each, 5%) and 8/15 OCA had much higher BV/TV (each, 10%). In the 8/15 OCA with increased peri-(basal)cyst wall BV/TV, Tb.Th was ~30% higher and Tb.Sp was ~15% lower than Non-Op samples.

3.3. Relationships between Cartilage and Bone Properties

Principal component analysis demonstrated that structural appearance of cartilage, indicated by macroscopic gross score, and bone, indicated by general μCT score of overall bone quality, were strongly and positively related (Table 4). Deterioration of cartilage gross morphology was strongly associated with decreased μCT bone structure. In addition, ScB integrity (ScB BV/TV and ScB BS/BV) was related to bone volume fraction of TB in the graft region and surrounding basal cysts. The factors in the principal component analysis (including components within the structural bone score, ScB integrity, and TB surrounding basal cysts) contributed substantially to the identification of the following repair patterns with OCA samples.

Considering all OP joints, four main patterns of subchondral cyst, basal cyst, ScB channel, and ScB erosion combinations were identified (Fig. 5). Some OCA contained basal cysts only (Group A, Fig. 5A); the peri-(basal)cyst wall architecture was similar to Non-Op. Some OCA contained both basal and subchondral cysts, with ScB channels (Group B, Fig. 5B); the peri-(basal)cyst wall bone architecture had higher BV/TV, BS/TV, and Tb.N, and lower Tb.Sp compared to Non-Op. Some OCA contained basal cysts and ScB channels, but without subchondral cysts (Group C, Fig. 5C), in which the peri-(basal)cyst architecture tended to have higher BV/TV and Tb.Th compared to Non-Op. Other OCA with both basal

and subchondral cysts and ScB channels were also accompanied by ScB erosion (Group D, Fig. 5D); the peri-(basal)cyst wall architecture was similar to that in Group B. When the ScB was intact, ScB channels were typically at the periphery of the OCA near the graft-host interface (Fig. 5B,C); ScB channels were typically at the center of the OCA when the ScB was eroded (Fig. 5D). Groups A and D were related to component 1 above and represented the best and worst cases relating overall cartilage and bone scores; in Group A, both cartilage and bone structure in these samples were relatively unaffected, whereas in Group D, both cartilage and bone structure were severely deteriorated. Furthermore, the characteristics of the bone in Group D were related to component 2, as deterioration of ScB was accompanied by altered TB in the graft and peri-(basal)cyst wall.

4.0. Discussion

These results demonstrate that altered bone structure in OCA versus Non-Op bone was similar among FRESH, 4°C/14d, and 4°C/28d OCA, and worse in FROZEN OCA, and suggest that certain cartilage repair patterns are related to channels and cysts in the subchondral bone. In Aim 1, FRESH, 4°C/14d, and 4°C/28d OCA displayed graft bone with an intact ScB and minimal marrow fibrosis, but altered architectural and histopathological abnormalities in TB versus Non-Op (Figs. 2–4, Tables 1–3), whereas FROZEN OCA exhibited eroded ScB and predominant marrow fibrosis, but TB architecture that was similar to Non-Op (Figs. 2–4, Tables 1–3). In Aim 2, all OCA samples contained basal cysts, some in combination with subchondral cysts and ScB channels and others in isolation. In Aim 3, the association of cartilage gross morphology and overall μ CT bone structure suggests that subchondral bone pathology is related to the overlying articular cartilage integrity (Table 4). Characterization of OCA based on cyst location, ScB channel size, and ScB erosion identified patterns of altered bone architecture in the peri-(basal)cyst wall (Fig. 5), associations of structural and histological abnormalities, and variations of bone failure within OCA storage groups, which may help to elucidate factors that are critical to the prevention of bone cysts following cartilage repair.

Examining bone and cyst structure after cartilage restoration by OCA involved the consideration of a number of issues. Since samples retrieved 12 months after OCA were used, the ability to directly observe an integrative view of bone and cartilage structure and biology was counter-balanced by an inability to analyze the time progression of individual lesions. It would be interesting to determine if longitudinal progression can be predicted from cross-sectional data. While the implantation of OCA stored at various conditions mimicked clinically relevant repair treatments, empty defect control does not provide a similar environment to this repair scenario and was not analyzed here; however, further controlled manipulation of this OCA repair model is needed to test potential mechanisms of cyst formation proposed in this study. Despite the variability between animals and limited sample numbers for each storage condition, the 15 operated knees provided a reasonable spectrum of bone lesions at different stages of progression, and clear differences in overall bone structure and ScB integrity between storage conditions were evident.

ScB deterioration in FROZEN allografts, in association with degradation of the articular cartilage [34], suggests that cartilage and bone coupling may affect biological and structural remodeling during osteochondral repair. Such FROZEN OCA exhibited severe cartilage degeneration at 12 months with softening, loss of chondrocytes, reduced proteoglycan content, and visible structural deterioration of the articular surface and bulk tissue, indicating cartilage failure [34], whereas other OCA did not fail and the cartilage was relatively intact at 12 months *in vivo*. These FROZEN OCA were characterized by the pattern described in Group D (Fig. 5), and may represent samples with the inability to maintain fluid pressurization due to eroded cartilage and permeable ScB (Fig. 6D), and

result in transmission of excessive loads and exudation of pressurized fluid into the underlying bone, altering the typical mechanical and biological bone environment (Figs. 2,3,5, Tables 1–3). Such abnormalities may be similar to those that occur with cartilage erosion in end-stage osteoarthritis (OA) and in experimentally induced OA models [39, 40]. Results from the principal component analysis were generally similar when conducted without qualitative cartilage and bone scores; however, the relationship between cartilage (superficial cellularity and material stiffness, instead of gross score) and bone (ScB BV/TV and ScB BS/BV, instead of structural score) properties were present in Factor 2 instead of Factor 1, and structural properties of graft bone and peri-(cyst) wall bone were present in Factor 1 instead of Factor 2 (data not shown). The strong association between macroscopic cartilage and bone structure (Table 4), together with the above observations, suggests that end-stage failure (i.e. softening and erosion) of articular cartilage may lead to subsequent bone failure.

Presence of bone cysts in all OCA repair samples, regardless of storage group, suggests that cysts may develop from mechanisms specific to OCA implantation. The lack of lateral cartilage integration, indicated by the clear separation between graft and host cartilage on histological sections [34], may act as a (soft tissue) channel and allow for cartilage-bone communication at implantation, described by patterns containing peripheral ScB channels (Fig. 6B,C). Different storage groups were investigated to examine the effect of chondrocyte viability on repair outcomes, as such viability is compromised during prolonged 4°C storage [41–44], but is the current commercial standard in the US to allow for OCA screening and processing [45–47]. While long-term *in vivo* cartilage performance appears to be related to chondrocyte viability at the cartilage surface, *in vivo* bone performance appears to be relatively unrelated to the state of OCA storage. Thus, while the end result (i.e. presence of cysts) may be similar among OCA, the mechanism by which these repairs formed cysts may be different (Fig. 6). Further mechanistic studies are needed to identify the mode(s) of cyst formation following OCA repair and potential treatment options specific to each one.

Regional bone evaluation of peri-(basal)cyst wall architecture allowed for quantification of structural abnormalities in OCA bone near visible bone cysts. Using standard geometric μ CT VOIs, measures of TB architecture in samples with visible bone cysts, especially bone volume fraction, were often unaffected, as voids and dense regions balance out, and cysts are simply identified qualitatively or scored semi-quantitatively [7, 18, 31]. Standardized methods to create VOIs that account for joint curvature have been used to more precisely analyze bone architecture [32], and have identified that bone structure is more porous and branched in regions containing bone cysts [19]; however, analysis of the architecture directly surrounding the bone lesions has been limited [33]. For subchondral cysts, peri-cyst wall architecture was not analyzed since the 1mm thick shell would have fallen outside of the bone region (above the cartilage interface), and there did not appear to be wall thickening in such regions. In this study, the peri-(basal)cyst wall was similar to non-operated bone in some samples, while other samples had higher bone volume fraction (Fig. 5). The variability in cyst wall architecture may distinguish two distinct pathologies of basal bone cysts (Fig. 6A vs. 6B,C,D), which may require unique treatments specific to each of the cyst pathologies.

The association of structural and histological abnormalities in bone healing within OCA suggests that altered biological bone homeostasis can contribute to the observed structural pathologies. ScB deterioration, especially in frozen OCA (Fig. 6D), and the presence of ScB channels (Fig. 6B,C) were associated with increased fibrosis in the marrow of underlying bone. The contents of subchondral cysts, often connected to the cartilage by ScB channels, were accompanied by edema and fibrous marrow, while basal cysts contained primarily normal marrow with fat cells surrounded by necrotic and sclerotic bone (Fig. S2). These

alterations in bone marrow suggest an osteoimmunological reaction, which may have potentially deleterious biological consequences on bone incorporation as the bone marrow cavity is important for the proper development and maintenance of the immune system [48]. Additionally, such non-characteristic histological abnormalities have also been associated with bone marrow edema-like (BMEL) signal in MRI [38]; cysts can develop from BMEL lesions, and commonly co-exist with severe lesions [8, 9]. In addition, bone healing may be influenced, in part, by the immunogenicity of allogenic bone, as reducing surface antigens (by freezing) or matching leukocyte-antigens, has improved osseous integration and clinical outcomes [49, 50]. While antigen matching was not attempted in this study, in accordance with current clinical practice, it may be a confounding factor. While osteoclasts were not visualized histologically at 12 months, the ScB deterioration and presence of cysts likely resulted from an increase in bone resorption (at earlier times) and abnormal regulation of bone turnover. Thus, future studies investigating bone metabolism and its relationship to cyst structure during cyst development may further elucidate potential biological mechanisms of cyst formation.

Categorization of OCA repair based on cyst structure may help to identify potential biophysical and biological mechanisms of bone cyst formation following cartilage repair (Fig. 6). Unrelated basal cysts may result from inadequate bone remodeling (Fig. 6A); new bone formation may not be able to fill voids created during surgery due to issues with graft fit and surgical tools (i.e. guide wires), but overall bone and cartilage structure is relatively unaffected. ScB channels allow for cartilage-bone communication and may induce subchondral cysts (Fig. 6B), introducing pressurized fluid into the bone and leading to a resorptive cavity near the bone-cartilage interface; overall cartilage structure is relatively unaffected, but bone marrow fibrosis and peri-(basal)cyst wall thickening may result. Bone cysts may also result from cartilage-bone communication due to reduced cartilage pressurization through either ScB channels, typically at the peripheral graft-host interface, (Fig. 6C) and/or cartilage degeneration (Fig. 6C,D) and increased ScB permeability (Fig. 6D); softened and eroded cartilage may be “leaky”, altering both biological and mechanical homeostasis in the osteochondral unit, and resulting in ScB degeneration and remodeling of peri-(basal)cyst bone architecture. Such evidence of cartilage-bone communication may favor the synovial fluid intrusion mechanism of cyst formation; however, the results reported here are limited to a particular snapshot of repair and do not provide a time course for such communication. Bone cysts may also form as the result of multiple mechanisms and not the action of a single mode. While specific pathological states of bone cysts after osteochondral allografting are identified here and may be useful to identify potential interventions tailored to each state, the progression of such lesions remains to be elucidated.

5.0. Conclusions

These findings suggest that cysts occurring after OCA may result from aberrant mechanobiology due to (1) altered compartmentalization that normally separates overlying cartilage and subchondral bone, either from distinct ScB channels or more general ScB plate deterioration, and (2) bone resorption at the basal graft-host interface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- OCA transplantation often led to a variety of abnormal bone structures.
- Cysts were widespread, and found in 2 main locations (subchondral or deep).
- Structure and contents of cysts appeared to vary with location.
- Cartilage-bone communication was increased via ScB channels and/or eroded cartilage.
- Certain aberrant bone structures were coupled to cartilage deterioration.

Figure 1. Sample orientation and definition of selected regions/volumes of interest (R/VOI)
 (A) Schematic diagram of sagittal, coronal, and transverse planes for samples at medial femoral condyle (MFC). Dotted lines indicate OCA region in surrounding host bone. R/VOI locations for graft (B) ScB and (C,D) TB, in addition to (E) bone cysts, fit to an ellipsoid to estimate volume (V), and (F) the peri-(basal)cyst wall defined on (B,C,E,F) typical μ CT or (D) histology slices. P=proximal, D=distal, M=medial, L=lateral.

Figure 2. Effect of OCA storage on bone structure after 12 months *in vivo*

Representative (A–E) 2D μ CT and (F–T) H&E histology images for (A,F,K,P) Non-Op, (B,G,L,Q) FRESH, (C,H,M,R) 4°C/14d, (D,I,N,S) 4°C/28d, and (E,J,O,T) FROZEN OCA. Bone cysts indicated by arrows, ScB channels indicated by arrowheads. In F–J, solid box indicates zoomed region in K–O, dotted box indicates zoomed region in P–T. Values in the lower right corner correspond to the overall bone score, calcified cartilage (CC) or bone marrow score of the representative image.

Figure 3. Effect of OCA storage on graft subchondral bone architecture after 12 months *in vivo* ScB (A) BV/TV and (B) BS/BV. *** $p < 0.001$ based on one-sample t-test of each OCA group vs. Non-Op. Letters (a and b) designate significantly different OCA groups ($p < 0.01$), with a shared letter indicating no difference based on one-way ANOVA and Tukey post-hoc tests.

Figure 4. Volume of bone cysts within OCA after 12 months *in vivo*

Data points correspond to individual cysts for FRESH (triangles), 4°C/14d (diamonds), 4°C/28d (circles), and FROZEN (squares) OCA. Open symbols indicate cysts near the bone-cartilage interface (i.e. “subchondral”), and filled symbols indicate cysts in deeper regions of the sample (i.e. “basal”). Dotted line indicates the average cyst volume (n=22). * indicates channel connects that cyst volume to the cartilage. x indicates channel is present, but does not directly connect to a cyst.

Figure 5. Characterization of bone cysts within OCA after 12 months *in vivo*

OCA had (A) basal cysts only, n=6, (B) both basal and subchondral cysts with ScB channels, n=3, (C) basal cysts with ScB channels, n=2, and (D) both basal and subchondral cysts with ScB channels and ScB, n=4. Representative images of (1) gross cartilage, (2) 3D renders of bone-cartilage interface, and (3) 2D coronal μ CT slices. (4) Peri-(basal)cyst wall architecture relative to corresponding Non-Op, represented as mean and range. Plus signs and thick arrows indicate ScB channels. Thin arrows indicate basal cysts. Arrowheads indicate subchondral cysts. Dotted lines indicate ~boundary of graft and host bone estimated from implant height. P=proximal, D=distal, M=medial, L=lateral.

Figure 6. Schematic diagram hypothesizing development of bone cysts following OCA repair
Cyst structure may result from abnormal interactions between synovial fluid and underlying bone or be (A) unrelated. Such aberrant bone mechanobiology may result from communication through (B) ScB channels, (C) softened cartilage + ScB channels, or (D) eroded cartilage and ScB.

Table 1

Effect of OCA storage on μ CT bone structure after 12months *in vivo*.

	Non-Op	FRESH	4°C/14d	4°C/28d	FROZEN
N	15	3	4	4	4
Structural Bone Score ^c	0	6.1±0.4 ^{**} ab	3.6±0.6 [*] a	4.8±0.6 ^{**} ab	7.3±0.9 ^{***} b
Bone Cyst Size	0	2.7±0.3 ^{**} a	2.0±0.3 ^{**} a	1.8±0.5 ^{**} a	2.3±0.5 ^{**} a
ScB Channel Size	0	2.3±0.3 ^{**} a	0.4±0.3 [*] b	1.8±0.5 [*] ab	2.4±0.5 ^{**} a
Interface Roughness	0	1.1±0.1 [*] a	1.1±0.5 [*] a	1.2±0.4 [*] a	3.0±0.0 ^{***} b

^c Structural bone score (0-9) was determined by assigning a score of 0-3 (with 0 representing normal, and 3 representing severe degeneration) for the following bone characteristics: bone cyst size, ScB channel size, and bone-cartilage interface roughness.

Mean±SEM.

* p<0.05,

** p<0.05,

p<0.001 based on one-sample t-test of each OCA group vs. Non-Op (mean population=0).

Letters (a and b) designate significantly different OCA groups (p<0.05), with a shared letter indicating no difference based on **Kruskal-Wallis** and **Dunn's** post-hoc tests.

Table 2

Effect of OCA storage on graft TB μ CT architecture after 12months *in vivo*.

	Non-Op	Difference = OP – Non-Op		
		FRESH	4°C/14d	FROZEN
n	15	3	4	4
BV/TV [%]	53±2	0±4 a	2±1 a	1±4 a
BS/TV [mm ⁻¹]	6.5±0.1	-0.7±0.2* ab	-0.9±0.2* a	0.1±0.4 b
Tb.Th [mm]	0.25±0.01	0.03±0.02 a	0.05±0.01* a	0.01±0.02 a
Tb.Sp [mm]	0.35±0.01	0.06±0.04 a	0.04±0.01* a	0.02±0.02 a
Tb.N [mm ⁻¹]	2.17±0.05	-0.25±0.05* a	-0.33±0.08* a	-0.06±0.12 b

Mean±SEM. For each OCA sample, the morphometric measurement (BV/TV, BS/TV, Tb.Th, Tb.Sp, and Tb.N) was calculated as the difference between the value of the OCA and the value of the contralateral Non-Op control knee of the same animal. The difference reported above is the Mean±SEM for each OCA group (FRESH, 4°C/14d, 4°C/28d, and FROZEN).

* p<0.05 based on one-sample t-test of each OCA group vs. Non-Op (mean population=0). Absolute morphometric measurement of Non-Op is reported above for reference. Letters (a and b) designate significantly different OCA groups (p<0.05), with a shared letter indicating no difference based on one-way ANOVA and Tukey post-hoc tests.

Table 3

Effect of OCA storage on peri-(basal)cyst wall TB μ CT architecture after 12months *in vivo*.

	Non-Op	Difference = OP – Non-Op		
		FRESH	4°C/14d	FROZEN
N	15	3	4	4
BV/TV [%]	39±2	10±1 *** a	6±3 * a	7±2 * a
BS/TV [mm ⁻¹]	6.0±0.2	0.2±0.6 a	0.6±0.1 * a	1.3±0.3 * a
Tb.Th [mm]	0.21±0.01	0.06±0.05 a	0.01±0.01 a	0.03±0.020 a
Tb.Sp [mm]	0.41±0.02	-0.05±0.2 * a	-0.05±0.01 * a	-0.02±0.01 a
Tb.N [mm ⁻¹]	1.83±0.02	0.06±0.31 ** a	0.19±0.05 *** a	0.05±0.11 *** a

Mean±SEM. For each OCA sample, the morphometric measurement (BV/TV, BS/TV, Tb.Th, Tb.Sp, and Tb.N) was calculated as the difference between the value of the OCA and the value of the contralateral Non-Op control knee of the same animal. The difference reported above is the Mean±SEM for each OCA group (FRESH, 4°C/14d, 4°C/28d, and FROZEN).

* p<0.05,

** p<0.01,

*** p<0.001

based on one-sample t-test of each OCA group vs. Non-Op (mean population=0). Absolute morphometric measurement of Non-Op is reported above for reference. Letters (a and b) designate significantly different OCA groups (p<0.05), with a shared letter indicating no difference based on one-way ANOVA and Tukey post-hoc tests..

Table 4

Principal component analysis to determine which of the many cartilage and bone parameters were closely related to each other. Dominant factors are boldface.

	Metric	Factor 1	Factor 2
Cartilage Properties	Gross Score	0.34	−0.26
	Superficial Cellularity	−0.24	0.26
	Hexabrix Gray Value	0.23	0.00
	Material Stiffness	−0.23	0.22
OCA Graft Bone Properties	Structural Score	0.35	−0.10
	ScB BV/TV	−0.24	0.33
	ScB BS/BV	0.20	−0.33
	BV/TV	0.05	0.40
	BS/TV	−0.27	−0.13
	Tb.Th	0.24	0.30
	Tb.Sp	0.24	−0.15
	Tb.N	−0.31	−0.03
Peri-(Base) Cyst Wall Bone Properties	BV/TV	0.23	0.35
	BS/TV	0.25	0.19
	Tb.Th	0.13	0.27
	Tb.Sp	−0.22	−0.30
	Tb.N	0.18	0.17
% variance explained		35.7	24.9