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1 Chitosan in viscosupplementation: in vivo effect on rabbit subchondral bone

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14
15 **Abstract**

16 To investigate the effect of intra-articular injection of Chitosan (Cs) added to hyaluronic acid
17 (HA) on subchondral bone during osteoarthritis (OA), microarchitectural parameters and
18 mineral density were measured in a rabbit model of early OA. A novel hybrid hydrogel adding
19 reacylated Cs of fungal origin to HA was compared to high molecular weight HA commercial
20 formulation.

21 Eighteen rabbits underwent unilateral anterior cruciate ligament transection (ACLT) and were
22 divided into three groups (Saline-group, HA-group and Hybrid-group) depending on the intra-
23 articular injection compound. Eight contralateral knees were used as non-operated controls
24 (Contralateral-group). Micro-computed tomography was performed six weeks post-ACLT to
25 study subchondral bone microarchitectural parameters and mineral density at an early stage of
26 OA development.

27 Cartilage thickness mean value was reduced only in Saline-group compared to Contralateral-
28 group. When the Hybrid-group was compared to Saline-group, subchondral bone
29 microarchitectural parameters (trabecular thickness and trabecular bone volume fraction) were
30 significantly changed; subchondral bone plate and trabecular bone mineral densities (bone
31 mineral density and tissue mineral density) were reduced. When the Hybrid-group was

32 compared to HA-group, subchondral bone microarchitectural parameters (subchondral plate
33 thickness and trabecular thickness) and trabecular bone mineral densities (bone mineral density
34 and tissue mineral density) were significantly decreased.

35 Compared to HA alone, the novel hybrid hydrogel, constituted of Cs added to HA, enhanced
36 microarchitectural parameters and mineral density changes, leading to subchondral bone loss
37 in a rabbit model of early experimental OA.

38 *Keywords:* ACLT-induced osteoarthritis; Hyaluronic acid; Chitosan; Subchondral bone;
39 Microarchitecture; Mineral density.

40

41 **Background**

42 Osteoarthritis (OA) is a multi-causal disease that is difficult to mimic in complete *in*
43 *vitro* and *ex vivo* models. Animal models remain most common for new therapeutic strategies
44 development. Anterior Cruciate Ligament Transection (ACLT) in rabbits is a widely used
45 experimental model to study OA pathogenesis and drugs efficacy [1–7]. Though no therapy to
46 date has been approved as a disease modifying OA drug, several symptomatic slow acting OA
47 drugs have demonstrated clinically relevant effects. One of the drugs falling into this category
48 is viscosupplementation [8]. Viscosupplementation consists in the intra-articular injection of
49 hyaluronic acid (HA), a naturally occurring joint component, which contributes to viscoelastic
50 and mechanical properties of cartilage and synovial fluid (shock absorption, joint lubrication
51 and cartilage protection [9–11]). As the molecular weight and concentration of endogenous HA
52 are reduced during OA, viscosupplementation aims at restoring the metabolic and rheological
53 homeostasis of the joint [12].

54 Since the initial hypothesis regarding the role of bone in OA pathogenesis formulated
55 by Radin *et al.* [13], subchondral bone changes have been established as one of some possible
56 factors in disease onset and progression. The nature of subchondral bone remodeling changes
57 in OA [14] and the time frame in which they occur is not fully understood, particularly in the
58 early-stage of the disease [15]. High molecular weight HA is widely used for its
59 chondroprotective property [16], which preserves cartilage micro-structure but several studies
60 reported contradictory effects of HA on bone. Pilloni *et al.* [17] showed *in vitro* human
61 osteogenic potential, leading to osteoblast bone matrix protein expression. However, Prince *et*

62 *al.* [18] observed *in vivo* osteoclast activation, leading to bone resorption. To date no clear
63 action of HA has been depicted on bone and only few investigators have explored the effect of
64 intra-articular HA injection on key determinants of subchondral bone microarchitecture and
65 strength [19–21].

66 One currently used HA commercial product is Ostenil[®] which must be injected once a
67 week for three to five weeks to get an effect lasting around six months. In order to improve the
68 efficacy of HA within the articulation and reduce potential side-effects from the repetitive intra-
69 articular injections, one approach consists in associating HA with a second biopolymer:
70 chitosan. Chitosan (Cs) is a linear polysaccharide originated from a deacetylation of chitin, the
71 primary structural polymer of the exoskeleton of marine invertebrates, insects, and cell wall of
72 fungi [22]. Cs has been proven to be chondroprotective and to increase chondrocyte
73 proliferation when injected intra-articularly in rat [23] and rabbit [24,25] OA models. Cs was
74 also shown to promote bone formation in porous Cs sponge *in vitro* [26] and osteoinduction *in*
75 *vivo* [27,28]. However, as Hoemann *et al.* [29,30] pointed out, Cs increased initial resorption
76 of microdrilled subchondral bone in rabbit knees. While Cs is a promising compound for
77 viscosupplementation, as evidenced by its chondroprotection properties in preclinical studies,
78 no study in ACLT rabbit model of OA has described Cs effects on subchondral bone yet.

79 The aim of the study was to investigate the effect of intra-articular injection of a novel
80 hybrid hydrogel based on reacylated fungal Cs added to HA on subchondral bone. Several
81 microarchitectural parameters (subchondral plate thickness, trabecular thickness, trabecular
82 separation, bone volume fraction) as well as bone mineral density and tissue mineral density
83 were investigated. Final evaluations were performed six weeks after ACLT in rabbits (*i.e.*
84 rabbits in the early-stage of the disease). More precisely, the hypothesis that the novel hybrid
85 hydrogel compared to a high molecular weight HA commercial formulation would change
86 subchondral bone microarchitectural parameters and diminish bone mineral density was tested.
87 Such an early change on the subchondral bone might be symptomatic of an OA disease
88 modification.

89 **Material and Method**

90 *Animal model*

91 The experimental work on rabbits was performed under the authorization of the ethical
92 committee of VetAgro Sup, veterinary campus of Lyon (authorization number 1373) and in full

93 accordance with European legislation and the ARRIVE statement [31]. Eighteen healthy adult
94 male white New Zealand rabbits (5 months of age, 3.68 ± 0.18 kg) were provided by Centre Lago
95 (Vonnas, France). As described previously [32], after 2 weeks in acclimation and quarantine in
96 individual boxes, experimental OA was surgically induced by unilateral ACLT performed by a
97 trained veterinary surgeon. Before surgery, the animals received subcutaneous injections of 30
98 mg/kg Borgal[®] (sulfadoxine and trimethoprim) bid, 0.1 mg/kg morphine, and 0.4 mg/kg
99 Meloxidyl[®] (meloxicam). Deep anaesthesia was induced by intra-muscular injection of 40
100 mg/kg Ketamine 1000[®] and 80 ml/kg Domitor[®] (medetomidine) and then maintained by 1-
101 3.5% isoflurane administered *via* endotracheal intubation. After careful shaving and
102 disinfection with Vetedine[®] (povidone iodine) soap and solution, ACLT was performed on the
103 left leg with a lateral approach [7], while the right knee was not operated. The complete rupture
104 of the ACL was assessed by the anterior drawer sign (manual horizontal dislocation) before the
105 closure of the articular capsule. The operated leg was not immobilized, and rabbits were allowed
106 to move freely in their individual cages after the surgery.

107 *Postoperative care*

108 The rabbits received subcutaneous injection of 0.01 mg/kg buprenorphine bid for 4 days
109 to avoid pain, 0.5 mg/kg Emeprid[®] (metoclopramide) for 3 days, 15 mg/kg Borgal[®] bid for 9
110 days and 1 cp/day Feligastryl[®] (eserine) for 3 days to reduce the risk of obstipation. Cothivet[®]
111 spray was applied to the wound for 6 days after surgery. Veterinarians closely monitored the
112 recovery, and a careful clinical follow-up was performed every other day. This postoperative
113 care reduced pain and prevented lameness. All rabbits recovered fully from the surgery.

114 *Formulation administration*

115 The operators of the injections and evaluations were blinded to the formulations. The
116 18 rabbits were randomized into 3 groups of 6 rabbits. Each ACLT knee (left knee) was treated
117 by intra-articular injection of 0.2 ml of either Saline solution, HA commercial formulation
118 (Ostenil[®], TRB Chemedica, Switzerland), or novel hybrid hydrogel formulation (Cs added to
119 HA) [25,33,34], respectively called hereafter Saline-group, HA-group and Hybrid-group. Eight
120 non-operated right knees (randomly chosen within groups) were used as non-operated controls
121 (referred hereafter to as Contralateral-group, n=8). Intra-articular injections were performed at
122 weeks 1, 2, 3, 4, and 5 post-ACLT after a short time of anaesthesia (40 mg/kg Ketamine 1000[®]
123 and 80 µg/kg Domitor[®]) and careful disinfection (Vetedine[®] soap and solution).

124

125 *OA grading and micro-computed tomography imaging of subchondral bone*

126 After a 6-week observation period, the rabbits were sacrificed by intra-vascular
127 administration of 1 ml/kg Dolethal[®] (pentobarbital) after being chemically restrained by intra-
128 muscular injection of 40 mg/kg Ketamine 1000[®] and 80 mg/kg Domitor[®]. Careful dissection of
129 both knees and a saw section of the proximal part of the tibia was performed. Cartilage
130 degradation, as well as osteophyte production, were graded using a macroscopic grading system
131 developed by Lavery *et al.* [34]. To assess the stage of OA for each tissue, a mean value of OA
132 score was calculated as an average of femoral and tibial grades prior to micro-computed
133 tomography.

134 Full details on micro-computed tomography imaging technique on eXplore Locus
135 system (General Electric, Fairfield, USA) are provided in our previous study [33]. The
136 acquisition was performed with 45 μm^3 isotropic resolution at 80 kV and 450 μA with a FOV
137 80 mm in diameter and 35 mm in depth. After acquisition and reconstruction, 16-bit images
138 were calibrated with a phantom containing hydroxyapatite, water, and air and expressed in
139 Hounsfield Units (HU: air 1000 HU and calcified tissues >100 HU).

140 *Microarchitectural parameter measurements*

141 Image treatment and parameters measurements were performed by a single operator
142 blinded for the assessment using MicroView software ABA 2.2 (General Electric, Fairfield,
143 USA). An anisotropic filter was applied to the images. Individual semi-automatic segmentation
144 was performed based on Otsu method [35] to distinguish subchondral bone plate from
145 trabecular bone. Subsequently, based on Hounsfield numbers, the region of interest (ROI) was
146 defined manually using a contour-based tool on the weight-bearing area of the medial tibial
147 condyle, as follows and illustrated in Figure 1. Firstly, the X (medio-lateral) and Y (cranio-
148 caudal) axes were set from the intercondylar area to the lateral edge of the cortical bone and
149 from the intercondylar area to the caudal aspect to the medial condyle, respectively. Secondly,
150 the Z axis (proximo-distal axis) was adapted to each tissue: subchondral bone plate, from the
151 calcified cartilage to the end of the subchondral bone plate; and trabecular bone, from the
152 subchondral bone plate/trabecular bone junction to the end of the epiphyseal line.
153 Approximately 100 slices were delineated by interpolation for the subchondral cortical bone
154 and approximately 80 for the trabecular bone. The difference in slice number was attributed to

155 ROI of trabecular volume which became too small where the intercondylar area met the
156 epiphyseal line.

157 FIGURE 1

158 The microarchitectural parameters were then calculated according to classical 2D
159 histomorphometric method [36]: (i) subchondral bone plate: mean subchondral bone plate
160 thickness (Pt.Th [mm]); (ii) trabecular bone: mean trabecular thickness (Tb.Th [mm]), mean
161 trabecular separation (Tb.Sp [mm]) and mean trabecular bone volume fraction (Tb.BV/TV
162 [%]). Moreover, mean bone mineral density (BMD [mg of mineral content per cc]) and mean
163 bone tissue mineral density (TMD [mg of mineral content per cc]) were measured for both the
164 subchondral bone plate, denoted Pt, and the trabecular bone, denoted Tb. For each voxel inside
165 the ROI, 3D local thickness was obtained based on Hildebrand and Rüegsegger method [37].

166 *Microarchitectural and mineral density parameters statistical analysis*

167 Statistical analysis was performed with R (R Foundation for Statistical Computing,
168 Vienna, Austria version 3.1.2014-10-26) using the Kruskal-Wallis ANOVA test ($\alpha=0.05$)
169 followed by the Mann-Whitney-Wilcoxon rank test ($\alpha=0.05$) as post hoc analysis when
170 Kruskal-Wallis showed statistical significance. Firstly, Saline-group, HA-group and Hybrid-
171 group were compared to Contralateral-group to assess the effects of ACLT on subchondral bone
172 6-weeks post-surgery, hereafter statistical significance is denoted by the symbol #. Secondly
173 HA-group and Hybrid-group were compared to Saline-group to explore the
174 viscosupplementation effects on subchondral bone, hereafter statistical significance is denoted
175 by the symbol *. Finally, to test the combined effect of Cs and HA, Hybrid-group was compared
176 to HA-group, in this case statistical significance is denoted by the symbol Δ .

177 **Results**

178 Mean OA scores obtained for Contralateral, Saline, HA and Hybrid groups were 0.45 ± 0.11 ,
179 1.8 ± 0.72 , 2.14 ± 0.62 and 1.53 ± 0.3 , respectively. By consequence, all operated groups exhibited
180 early OA lesions, while contralateral knees showed no macroscopic sign of OA. Figure 2 shows
181 typical micro-tomography images of tibial plates from Hybrid-group and Contralateral-group.
182 Bone modifications were striking, in particular the subchondral bone plate thickness was
183 importantly reduced in Hybrid-group (Figure 2). To confirm and measure this observation,
184 subchondral bone microarchitectural parameters and mineral density were investigated
185 quantitatively for all groups (Table 1).

186 FIGURE 2

187 Firstly, cartilage thickness (C.Th), subchondral bone plate thickness (Pt.Th), trabecular bone
188 thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular bone volume fraction
189 (Tb.BV/TV) mean values were measured.

190 Secondly, bone mineral density (BMD) and tissue mineral density (TMD) mean values were
191 measured in both subchondral bone plate (Pt) and trabecular bone (Tb).

192 TABLE 1

193 *Comparison to Contralateral-group*

194 C.Th mean value (Figure 3) was significantly lower in Saline-group than in Contralateral-group
195 ($p=0.002$). No significant differences in C.Th mean values were observed in HA-group and
196 Hybrid-group compared to Contralateral-group.

197 FIGURE 3

198 Bone microarchitectural parameters are displayed in Figure 4.

199 In Saline-group compared to Contralateral-group, no significant differences were observed in
200 Pt.Th, Tb.Th, Tb.Sp and Tb.BV/TV mean values. In HA-group compared to Contralateral-
201 group, Tb.Sp mean value was significantly increased and Tb.BV/TV mean value was
202 significantly decreased ($p=0.043$ and $p=0.005$, respectively). In Hybrid-group compared to
203 Contralateral-group, bone parameter mean values were all significantly modified, with an
204 increase in Tb.Sp ($p=0.043$) and a decrease in Pt.Th, Tb.Th and Tb.BV/TV ($p=0.008$, $p=0.001$
205 and $p=0.003$, respectively).

206 FIGURE 4

207 Mineral densities (Pt.BMD, Tb.BMD, Pt.TMD, Tb.TMD) mean values are displayed in Figure
208 5.

209 Regarding bone mineral density, in Saline-group compared to Contralateral-group, Tb.BMD
210 and Tb.TMD mean values were significantly decreased ($p=0.29$ and $p=0.008$, respectively)
211 (Figure 5B-D). In both HA-group and Hybrid-group compared to Contralateral-group, Pt.BMD
212 mean values were significantly decreased ($p=0.001$ and $p=0.001$, respectively) (Figure 5A) and
213 Tb.BMD mean values were also significantly decreased ($p=0.003$ and $p=0.001$, respectively)
214 (Figure 5B).

215 Regarding tissue mineral density, in Saline-group compared to Contralateral-group Pt.TMD
216 and Tb.TMD mean values were significantly decreased ($p=0.029$ and $p=0.008$) (Figure 5B-D).
217 In both HA-group and Hybrid-group compared to Contralateral-group, Pt.TMD mean values
218 were significantly decreased ($p=0.001$ and $p=0.001$, respectively) (Figure 5C) and Tb.TMD
219 mean values were also significantly decreased ($p=0.003$ and $p=0.001$, respectively) (Figure 5C).

220

221 FIGURE 5

222

223 *Comparison to Saline-group*

224 No significant differences in C.Th mean value (Figure 3) were observed in HA-group and
225 Hybrid-group compared to Saline-group.

226 Concerning subchondral bone microarchitectural parameters, in HA-group compared to Saline-
227 group, no significant differences were observed in microarchitectural bone parameters (Pt.Th,
228 Tb.Th, Tp.Sp and Tb.BV/TV). In Hybrid-group compared to Saline-group, Tb.Th and
229 Tb.BV/TV mean values were significantly decreased ($p=0.009$ and $p=0.026$, respectively)
230 (Figure 4).

231 Regarding bone mineral density (Figure 5), in HA-group compared to Saline-group, Pt.BMD
232 and Pt.TMD mean values were significantly different ($p=0.026$ and $p=0.026$ respectively). In
233 Hybrid-group compared to Saline-group, Pt.BMD, Pt.TMD, Tb.BMD, and Tb.TMD mean
234 values were all significantly decreased ($p=0.009$, $p=0.026$, $p=0.004$ and $p=0.002$, respectively).

235 *Comparison between HA-group and Hybrid-group*

236 C.Th mean value showed no differences between HA and Hybrid groups (Figure 3).

237 Concerning subchondral bone microarchitectural parameters in Hybrid-group compared to HA-
238 group (Figure 4) Pt.Th and Tb.Th mean values were significantly decreased ($p=0.009$ and
239 $p=0.026$, respectively).

240 Regarding bone mineral density (Figure 5), Tb.BMD and Tb.TMD mean values were
241 significantly decreased in Hybrid-group compared to HA-group ($p=0.026$, $p=0.015$
242 respectively).

243 Finally, bone microarchitectural parameters and bone mineral density mean values on lateral
244 tibial condyle exhibited same significant changes as medial tibial condyle (Table 1).

245 **Discussion**

246 In this study, the effect of the addition of Cs to HA on OA subchondral bone during
247 viscosupplementation was investigated. The novel hybrid hydrogel constituted of reacylated
248 fungal Cs added to HA was compared to a high molecular weight HA commercial formulation.
249 Subchondral bone microarchitectural parameters and mineral density were measured 6 weeks
250 post-ACLT in rabbits. Our results support the hypothesis that Cs contributes to subchondral
251 bone changes and leads to increased subchondral bone loss in early OA in rabbits. The effect
252 on cartilage and especially cartilage histology has also been evaluated and published by Kaderli
253 *et al.*, [33].

254 A significant decrease of C.Th mean value confirming OA induction was observed when
255 the Saline-group was compared to the Contralateral-group, while no significant changes were
256 observed for all the bone parameters. Contrastingly, no significant decrease of C.Th mean value
257 was observed when the HA and Hybrid-groups were compared to the Contralateral-group
258 suggesting a preserved chondroprotective effect of HA when combined to Cs. Those results are
259 in accordance with other studies [19–21,39,40].

260 Notably, the present study highlights subchondral bone loss following HA-based
261 viscosupplement therapy in OA joints. On the one hand, literature show that during OA, intra-
262 articularly injected high-molecular weight HA penetrates into the OA affected subchondral
263 bone reaching the bone marrow space and osteocyte lacunae, where it can enhance intrinsic HA
264 synthesis [39]. On the other hand, intrinsic HA content of bone extracellular matrix is closely
265 linked to bone homeostasis in general and to osteoclast-mediated bone resorption in particular
266 [18]. In the present study, the decrease in subchondral bone microarchitectural parameters was
267 associated with demineralization, which suggests subchondral bone resorption, likely as a result
268 of the effect of HA on osteoclast activity in OA subchondral bone [39]. On the contrary, in a
269 rabbit model of OA, Permuy *et al.* [21] did not observe any significant subchondral bone loss
270 following HA intra-articular injection in a OA rabbit model [21]. This discrepancy might result
271 from the more severe induction technic (combining ACLT and meniscectomy *vs.* ACLT) and
272 the later endpoint used in their study (11 *vs.* 6 weeks) leading to a more advanced OA and thus
273 to the recovery of initial subchondral bone microarchitectural parameters and mineral density
274 [5,15].

275 The intra-articular injection of Cs added to HA (Hybrid-group) significantly decreased
276 several subchondral bone microarchitectural parameters as compared to intra-articular injection
277 of saline and HA . In particular, trabecular thickness, trabecular bone volume fraction, and bone
278 mineral densities mean values (Figure 4 and Figure 5) were reduced as compared to the Saline-
279 group, as well as subchondral bone plate thickness, trabecular thickness and trabecular bone
280 mineral density mean values compared to HA-group. Previous studies showed that, similarly to
281 HA, Cs mediates osteoclast cells adhesion to subchondral bone promoting resorption [40]. Our
282 results confirm the role of Cs on subchondral bone loss, as observed by Hoemann *et al.* [29,30]
283 on microdrilled rabbit knee defect filled with Cs-based implant. Interestingly, no loss in
284 mineralization was observed in the subchondral bone plate (*i.e.* cortical bone) in contrast with
285 the subchondral trabecular bone (Figure 5), which can be explained by the lower cortical bone
286 remodeling rate [41] (up to tenfold) compared to trabecular bone.

287 In the present study, OA induction was verified by X-ray osteophyte scoring on all
288 operated knees. The absence of such was also verified on all contralateral knees [33]. In order
289 to avoid group's size discrepancy, a "Contralateral-group" has been formed with randomly
290 chosen contralateral legs from each treated group. This approach has been used in numerous
291 studies [6,44–46]. Moreover, the same significant changes were found whether the medial or
292 lateral condyles were investigated. As well, when lateral condyles were compared to medial
293 condyles within each group, there were no significant differences within Contralateral-group,
294 Saline-group, HA-group and Hybrid-group. Thus, equilibrated contact-loads on tibial condyles
295 are suggested.

296 Viscosupplementation with high molecular weight HA significantly decreased
297 subchondral bone mineral density as compared to saline solution. Our study also shows that Cs
298 added to HA enhanced subchondral bone loss as compared to HA alone by decreasing
299 significantly subchondral bone parameters and bone mineral density. In order to go one step
300 further in the evaluation of the effect of Cs on subchondral bone, the impact of bone
301 microarchitectural parameters on the mechanical compliance as well as their relationship with
302 the cartilage integrity is going to be evaluated.

303 **Conclusion**

304 This study demonstrates that the commercial formulation of HA injected intra-articularly
305 induces subchondral bone demineralization in the early-stage of OA induced by ACLT in
306 rabbits. Moreover, Cs added to HA enhances this subchondral bone loss compared to HA alone..

307 To the authors' knowledge, this is the first study investigating the effects of Cs added to HA on
308 subchondral bone microarchitectural parameters and mineral density in experimental OA.

309

310 **Ethics approval and consent to participate**

311 The experimental work on rabbits was performed under the authorization of the ethical
312 committee of VetAgro Sup Lyon (authorization number 1373) and in full accordance with
313 European legislation and the ARRIVE statement.

314 **Consent for publication**

315 Not applicable

316 **Availability of data and material**

317 The datasets used and analyzed during the current study are available from the corresponding
318 author on reasonable request.

319 **Competing interests**

320 Not applicable

321 **Funding**

322 Not applicable

323 **Authors' contributions**

324 RR, CB, SK and TH have made substantial contributions to this study. RR and CB contributed
325 equally to the analysis and interpretation of the data, the drafting of the manuscript and the final
326 approval of the article. SK provided the study materials and contributed to the critical revision
327 of the article for important intellectual content and the final approval of the article. TH
328 contributed to the study design, critical revision of the article for important intellectual content
329 and final approval of the article.

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337

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457

458 **Legends**

459

460 Figure 1 : Medial tibial condyle ROIs at different locations in the Y (cranio-caudal) axis of subchondral bone
461 plate and trabecular bone from Contralateral-group.

462

463 Figure 2: Typical *ex vivo* microtomography images of tibial plate (6 weeks after ACLT). A/ Hybrid-group (left knee);
464 B/ Contralateral-group (right knee).

465

466 Table 1: Microarchitectural parameters of medial and lateral tibial condyles: cartilage (C), subchondral bone plate
467 (Pt) and trabecular bone (Tb) at 6 weeks post ACLT and their statistical significance compared to Contralateral-
468 group (#), Saline-group (*) or HA-group (Δ).

469

470 Figure 3: Cartilage thickness (mean \pm SD) for Contralateral, Saline, HA and Hybrid groups. Statistical significance
471 compared to Contralateral-group is indicated by # (*i.e.*, pairwise Wilcoxon Rank test, $\alpha=0.05$).

472

473 Figure 4: Microarchitectural parameters (mean \pm SD) in Contralateral, Saline, HA and Hybrid groups. A/
474 subchondral bone plate thickness (Pt.Th); B/ trabecular thickness (Tb.Th); C/ trabecular separation (Tb.Sp); D/
475 trabecular bone volume fraction (Tb.BV/TV). #, * and Δ indicate statistical significance under pairwise Mann-
476 Whitney-Wilcoxon Rank test ($\alpha=0.05$) compared to Contralateral-group, Saline-group and HA-group,
477 respectively, performed after significant Kruskal-Wallis ANOVA test ($\alpha=0.05$).

478

479 Figure 5 : Bone Mineral Density (mean \pm SD) in Contralateral, Saline, HA and Hybrid groups. A/ subchondral bone
480 plate BMD (Pt.BMD); B/ trabecular bone BMD (Tb.BMD), C/ subchondral bone plate TMD (Pt.TMD); D/ trabecular
481 bone TMD (Tb.TMD). #, * and Δ indicate statistical significance under pairwise Mann-Whitney-Wilcoxon Rank

482 test ($\alpha=0.05$) compared to Contralateral-group, Saline-group and HA-group, respectively, performed after
483 significant Kruskal-Wallis ANOVA test ($\alpha=0.05$).