

Effects of platelet-rich fibrin and hyaluronic acid gel matrix on bone healing in a rat femoral fracture model

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Abstract

Background

Advances in the understanding of fracture healing biology have led to new methods aimed at accelerating healing and improving union rates. This study evaluated the effects of hyaluronic acid (HA) and platelet-rich fibrin (PRF) on bone healing in a rat femoral fracture model. Although previous studies have examined similar materials, this is the first to compare HA and PRF together in a long-bone fracture model.

Materials and methods

Forty-eight Sprague–Dawley rats were used. Four were sacrificed to obtain PRF, and the remaining forty-four were divided into four groups: control (C), hyaluronic acid (H), PRF (P), and combined (P + H). A standardized femoral fracture was created by open osteotomy and fixed with a Kirschner wire. HA and PRF were applied individually or together at the fracture site. Half of the animals were sacrificed on day 15 and the rest on day 30. All femora were analyzed by micro-CT for callus volume and bone mineral density (BMD), followed by histopathological evaluation using Huo et al.'s union criteria.

Results

When the treatment groups were compared with one another and with their respective control groups, a radiological increase in callus volume was observed in all groups; however, the mean callus volume of the P + H group showed a significant increase compared with the other groups. Bone density increased in every group; however, the mean BMD of the P group was higher than those of the control, H, and P + H groups, and no significant differences were observed among the remaining groups. Both materials stimulated histological union more than the control group, but the mean histological score of the H group was lower than that of the P + H group.

Conclusion

Based on these results, we conclude that the potential osteoinductive effect of HA, which we initially envisaged as a matrix to enhance osteoinduction, is limited, and that HA may be more useful as a carrier scaffold for growth factors rather than as a standalone treatment. Additionally, PRF, which is frequently used by dentists to accelerate soft- and hard-tissue healing, may also have beneficial effects in stimulating union in long-bone fractures.

1. Introduction

Following both surgical and conservative treatment, fracture healing proceeds without scar formation and with anatomical and functional characteristics as close as possible to the original tissue. Despite these treatment modalities, union problems are observed in approximately one-tenth of all fractures, leading to increased morbidity, loss of labor, and high treatment costs. Various treatment methods aiming to accelerate fracture healing and maximize union rates have been investigated, and the four basic materials used for the repair of bone defects are autografts, allografts, heterografts, and alloplastic materials.

Allografts and xenografts offer advantages such as availability in sufficient quantities, long-term storage, and avoidance of a second operative site; however, they may also result in complications, including adverse immunological reactions and transmissible infections. To avoid these complications, biomaterials have been developed as a result of advances in tissue engineering [1, 2].

Among these biomaterials, hyaluronic acid (HA) reduces granulation tissue formation and positively influences cell migration, differentiation, and proliferation at the operative site. By contributing minimally to extracellular matrix organization, it stimulates osteogenic activity and accelerates wound healing [3, 4].

Fibrin is the activated, insoluble form of the plasma protein fibrinogen and is found in high amounts in plasma and in the α -granules of platelets. Fibrin forms the initial scar matrix at the injury site, and this structure, acting as a biological adhesive, creates a protective wall around the initial platelet aggregates during coagulation [5].

The α -granules of platelets, which are abundantly represented in platelet-rich fibrin (PRF), contain many growth factors (such as platelet-derived growth factor [PDGF] and transforming growth factor- β [TGF- β]) and cytokines involved in inflammation [6, 7]. PRF is considered a potent stimulator of bone formation and regeneration by enhancing the proliferation of osteogenic cells [8, 9].

PRF has several advantages, including reduction of bleeding, acceleration of soft- and hard-tissue healing, promotion of vascularization, anti-inflammatory properties, absence of allergic reactions when used autologously, ease and rapidity of preparation, and lack of risk of disease transmission [8, 10].

Based on these findings, HA and PRF are considered to play an important role in bone regeneration. The aim of this study was to evaluate the effects of HA and PRF on bone healing in a rat femoral fracture model.

2. Materials and methods

2.1. Study Design

Local ethical approval for experimental animals was obtained from the Bağcılar Training and Research Hospital Experimental Research and Skill Development Center (BADABEM) on 29 August 2016 (approval

number: 2016/32). Forty-eight adult female Sprague–Dawley rats were used in this study. All experimental procedures were performed in accordance with the relevant institutional and national guidelines and regulations for the care and use of laboratory animals.

The mean age of the rats was 2.75 months (10–12 weeks), and the mean body weight was 228 g (193–263 g). Four rats were allocated as donors for obtaining PRF. The remaining 44 animals were randomly divided into four equal groups (Table 1). Rats were monitored in the laboratory for 48 hours preoperatively. Throughout the study, they were given tap water and standard rodent chow ad libitum. The animals were kept at 22°C with a 12-hour light/12-hour dark cycle. All surgeries were performed by the same surgeon. Each rat was housed individually for the first 24 hours after surgery for wound inspection, dressing, and analgesic monitoring. After this 24-hour postoperative period, the animals were regrouped and housed in cages containing 11 rats each. No wound-site infections were observed in any animal during follow-up.

Five randomly selected rats from each group were sacrificed at the end of week 2, and the remaining animals were sacrificed at the end of week 4 under general anesthesia by cervical dislocation. The mean age of the rats at baseline was 2.75 months (10–12 weeks), and the mean body weight was 228 g (193–263 g). Animals sacrificed at week 2 were approximately 12–14 weeks old, and those sacrificed at week 4 were approximately 14–

16 weeks old. Body weights at the time of sacrifice were not recorded separately.

After sacrifice, the left femora were disarticulated at the hip and knee joints and removed with their soft tissues intact to avoid damaging the callus. All left femora were wrapped in moist gauze and stored in 10% formalin solution until clinical, radiological, and histological examination. The specimens were scanned by micro-computed tomography at TETLAB, affiliated with Istanbul University Technology Transfer Center, to evaluate newly formed bone tissue (callus) volumetrically and in terms of bone mineral density (BMD). After imaging, samples were submitted to the Pathology Laboratory of Bağcılar Training and Research Hospital for histopathological evaluation. Histological classification of healing was performed according to the scale published by Huo et al.

2.2. Surgical Technique

Animals that had undergone the necessary preparation and preoperative monitoring were taken to the operating room. General anesthesia was induced by intraperitoneal administration of ketamine HCl 80 mg/kg and xylazine HCl 5 mg/kg. Preoperatively, a single dose of antibiotic prophylaxis was administered as subcutaneous gentamicin 8 mg/kg.

After shaving the left hip and thigh regions, the skin was prepared with povidone–iodine, and a 2-cm longitudinal lateral thigh incision was made to expose the femoral shaft while preserving the periosteum. An osteotomy was performed at the middle one-third of the femur using a microblade.

For stabilization, a 1-mm Kirschner wire was applied antegrade (Fig. 1). After fixation, a chamber directly associated with the fracture site was created at the osteotomy line, allowing placement of the agents to be evaluated.

In the P group, PRF obtained from the donor rats was placed into the chamber. PRF was prepared by collecting blood from sacrificed rats and centrifuging it (Fig. 2).

On the first day, surgeries for the P + H group were performed after completion of the P group procedures. After osteotomy and fixation, 0.5 mL PRF and 0.5 mL HA gel were applied to the chamber at the osteotomy line in the left thigh of 11 rats (Fig. 3).

On the second day, surgeries for the H and Control groups were performed. In the H group, after osteotomy and fixation, 0.5 mL HA was applied to the osteotomy line.

In the Control group, no additional substance was applied after osteotomy and fixation.

Rats were monitored in the laboratory in four groups with 11 animals per cage.

At the end of week 2, five animals from each group were randomly selected and sacrificed under general anesthesia by cervical dislocation. The left femora were removed by disarticulation at the hip and knee joints together with the surrounding soft tissues (Fig. 4).

Specimens were wrapped in moist gauze, placed in labeled containers, and stored in 10% formalin solution.

At the end of week 4, the remaining six animals in each group were sacrificed under general anesthesia by cervical dislocation, and their left femora were amputated and stored in labeled containers containing 10% formalin.

2.3. Radiological Examination

Radiological evaluation was performed at Istanbul University Technology Transfer Center (TETLAB). Femora were assessed using micro-computed tomography with a focus on two parameters:

1. Newly formed callus volume (mm^3)
2. Bone mineral density (BMD) of the newly formed bone (g/mm^3)

Scans were acquired using a Bruker Micro-CT SkyScan 1174 system, and analyses were performed with Bruker CTAn software. BMD calibration was performed using $0.25 \text{ g}/\text{mm}^3$ and $0.75 \text{ g}/\text{mm}^3$ CaHA phantoms. Three-dimensional reconstructions were obtained from the acquired images (Fig. 5).

2.4. Histological Evaluation

All femora were fixed in 10% buffered formalin for 2 days. After fixation, samples were decalcified in 10% hydrochloric acid for 2 hours and then placed into cassettes under macroscopic guidance. Samples were dehydrated in a closed-system tissue processor through alcohol, acetone, xylene, and paraffin stages, followed by paraffin embedding. Longitudinal 4- μ m sections were taken, and hematoxylin–eosin (H&E) staining was applied. Mounting medium was added, and coverslips were placed. Sections were examined using an Olympus BX51 light microscope. Healing was classified using the histological scale defined by Huo et al.

2.5. Statistical Analysis

Statistical analyses were performed using Number Cruncher Statistical System (NCSS) 2007 Statistical Software (Utah, USA). In addition to descriptive statistical methods (mean, standard deviation, median, interquartile range), one-way analysis of variance (ANOVA) was used for comparison of normally distributed variables across multiple groups, with subgroup comparisons performed using the Tukey multiple comparison test. For comparison of two independent groups, the independent t-test was used. For variables not normally distributed, the Kruskal–Wallis test was used for comparisons among multiple groups, and the Mann–Whitney U test was used for pairwise comparisons. Results were evaluated at a significance level of $p < 0.05$.

3. Results

The newly formed bone (callus) volume data obtained from micro-computed tomography were evaluated in cubic millimetres and recorded numerically (Table 2).

The mean callus volume on Day 30 in the P + H group was found to be statistically significantly higher than the mean on Day 15 ($p = 0.034$)(Table 3).

Bone density data obtained from micro-computed tomography were evaluated in g/mm^3 and recorded numerically(Table 4).

On Day 30, the mean BMD values of the P, H, and P + H groups were found to be statistically significantly higher than their Day 15 means ($p = 0.001$, $p = 0.002$, and $p = 0.0001$, respectively) (Table 5).

The mean BMD of the P group was statistically significantly higher than those of the Control, H, and P + H groups ($p = 0.014$, $p = 0.003$, and $p = 0.033$, respectively), and no statistically significant differences were observed among the remaining groups ($p > 0.05$). This finding indicates that the P group was superior to all other groups in terms of bone density at Day 30 (Table 6).

Four longitudinal sections were obtained from each femur and photographed after H&E staining. Each section was scored for union according to the criteria described by Huo et al(Table 7).

The Day 30 mean histological scores of the P, H, and P + H groups were statistically significantly higher than their Day 15 means ($p = 0.002$, $p = 0.0001$, and $p = 0.0001$, respectively).

A statistically significant difference was observed among the Day 15 mean histological scores of the Control, P, H, and P + H groups ($p = 0.0001$). This significant difference was primarily attributable to the early period (Table 8).

The mean histological scores of the Control group were statistically significantly lower than those of the P, H, and P + H groups ($p = 0.0001$, $p = 0.007$) (Table 9). Both agents, when applied separately or together at the osteotomy line, positively influenced histological union.

4. Discussion

Fractures heal uneventfully with conservative or surgical treatment in the vast majority of cases. Only 5–10% of fractures exhibit healing problems such as nonunion or delayed union, which require additional intervention. Due to repeated treatments, loss of labor, increased morbidity, and rising healthcare costs, many methods have been investigated to accelerate fracture healing and to return patients to normal function as quickly as possible by maximizing union rates, and some of these approaches have found clinical application [11–13].

In a study conducted by Lee et al. on rabbits, a calvarial defect was reconstructed with PRF combined with silk fibroin, while the contralateral defect was left unrepaired [14]. Although no statistically significant difference was observed in computed tomography or histomorphometric analyses performed 6 weeks postoperatively, evaluations performed at 1 week demonstrated that bone healing was significantly accelerated in the area reconstructed with PRF.

Huang et al. applied HA of different molecular weights and concentrations to mesenchymal cells derived from rat calvaria and evaluated its effects on osteogenic differentiation [15]. They reported that the stimulatory effect of HA on osteoblastic differentiation in mesenchymal cells is dependent on both dose and molecular weight, with high-molecular-weight HA demonstrating a more pronounced effect.

In light of these findings, our micro-CT evaluation of callus volume showed that, although all groups demonstrated a marked increase in callus volume between Day 15 and Day 30, only the P + H group exhibited a statistically significant increase in mean volume at Day 30 compared with Day 15 ($p = 0.034$).

According to the histological union scores, all groups showed a significant increase at Day 30 compared with Day 15. A statistically significant difference was observed among the early-period (Day 15) histological scores ($p = 0.0001$). When groups were evaluated individually, the Control group was found to lag behind all other groups. This result indicates that both bioactive materials used in the study exerted a positive effect on histological union during the early period. However, in the same comparison, the H group performed worse than the combined P + H group. We believe this may be explained by HA acting as a barrier between the perifracture soft tissues—known to be a major source of undifferentiated

mesenchymal cells that are highly active in healing and responsive to growth factors—and the fracture line.

Nevertheless, the superiority of HA over the Control group in accelerating fracture healing suggests that, in addition to serving as an effective mechanical barrier for soft tissues, HA is biocompatible and functions as an auxiliary extracellular matrix component.

Its significantly higher early-period histological score compared with the Control group supports the notion that HA can stimulate mesenchymal cell differentiation and osteoinduction, may serve as a carrier for growth factors, and can contribute to forming an effective matrix in the fracture healing environment. Consistent with the known positive effects of growth factors on fracture healing [16–18], the beneficial effects of PRF—which is rich in these factors—on bone healing, especially in terms of bone density and histological improvements, align with previously published findings.

Furthermore, when callus volumes, bone densities, and histological healing scores at Day 15 and Day 30 were compared within each of the four groups, Day 30 values were superior in all cases, a result that is consistent with the existing literature [16, 17, 19, 20].

5. Conclusions

This experimental study found that hyaluronic acid (HA) and platelet-rich fibrin (PRF) are effective bioactive materials for stimulating fracture healing.

We believe that HA, although an expensive material when considered as a matrix component, is not sufficiently suitable for fracture applications in terms of cost and standalone effectiveness when compared with its counterpart in this study. However, based on its biocompatibility and its lack of immunogenic and infectious risks as reported in the literature, we conclude that HA can be safely used as a carrier scaffold for growth factors and other bioactive materials.

We also conclude that PRF, owing to its ease of preparation and application, absence of required additives, low cost, high concentration of growth factors, and capacity for sustained release, may serve as an effective alternative material capable of accelerating union in long bone fractures. As stated in the literature, the fibrin network of PRF, which can function as a gel-form matrix or membrane and is widely used in dental practice, appears promising for broader clinical applications.

We recommend that this experimental study be regarded as a preliminary investigation. The results are encouraging for the design of larger studies in bigger animal models with increased sample sizes, in which additional parameters of fracture healing—such as dose-finding analyses and biomechanical assessments—can be evaluated in detail.

Ethical Approval and Consent to Participate

Ethical approval for the experimental study was obtained from the Local Animal Ethics Committee of Bağırcılar Training and Research Hospital Experimental Research and Skill Development Center (BADABEM) on August 29, 2016, with decision number 2016/32.

Declarations

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Acknowledgment/disclaimers/conflict of interest

The authors would like to thank all participants who contributed to this study. This study is derived from the specialty thesis of the corresponding author.

Ethical approval for this experimental study was granted by the Animal Experiments Local Ethics Committee of Bağırcılar Eğitim ve Araştırma Hastanesi Deneysel Araştırma ve Beceri Geliştirme Merkezi (BADABEM) (Approval No: 2016/32, dated August 29, 2016).

The authors declare that they have no conflict of interest.

Informed consent

Not applicable

Ethical Approval and Consent to Participate

Ethical approval for the experimental study was obtained from the Local Animal Ethics Committee of Bağırcılar Training and Research Hospital Experimental Research and Skill Development Center (BADABEM) on August 29, 2016, with decision number 2016/32.

Consent for Publication

Not applicable.

Data Availability

The datasets generated and/or analyzed during the current study are not publicly available due to institutional data protection policies but are available from the corresponding author on reasonable request.

Informed Consent

Due to the retrospective design of the study, the requirement for informed consent was waived by the ethics committee.

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Competing Interests

The authors declare that they have no competing interests.

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Tables

Table 1. Distribution of experimental animals

Group Name	Abbreviation	Number of Rats
Control	C	11
Platelet-Rich Fibrin (PRF)	P	11
Hyaluronic Acid	H	11
PRF + Hyaluronic Acid	P+H	11

Table 2. Micro-computed tomography volume data (mm³)

Volume (mm ³)				
n	Control	P	H	P+H
1	54.25924	60.64803	81.53661	60.16963
2	74.76154	84.54467	64.31888	60.46488
3	44.31275	98.93454	129.23210	82.12158
4	67.91095	68.29239	115.59709	50.43504
5	112.70590	53.15247	66.65630	67.94035
6	73.13485	69.04285	128.34392	74.23746
7	132.66686	51.39605	113.24466	80.92909
8	97.40117	127.81082	225.21639	128.90596
9	152.25835	78.58578	212.95761	154.64277
10	97.22224	103.42802	76.54909	128.23914
11	243.36045	75.37466	67.65721	70.00107

Table 3. Comparison of group callus volumes at Day 15 and Day 30

Group	15th Day (mm ³)	30th Day (mm ³)	p
Control Group	70.79 ± 26.25	132.67 ± 61.12	0.066
P	73.11 ± 18.54	84.27 ± 27.15	0.457
H	91.47 ± 29.41	137.33 ± 67.31	0.193
P+H	64.23 ± 11.78	106.16 ± 35.55	0.034
P	0.297	0.264	

Table 4. Micro-computed tomography bone mineral density (BMD) data (g/mm³)

n	BMD (g/mm³)			
	Control	P	H	P+H
1	0.59736	0.68835	0.65920	0.80785
2	0.60405	0.47168	0.79450	0.71451
3	0.67622	0.72620	0.42891	0.76133
4	0.72909	0.81133	0.75115	0.77777
5	0.73367	0.78352	0.58934	0.67128
6	0.92524	0.95767	1.01220	1.11538
7	1.13441	1.49837	0.83003	1.30530
8	1.17390	1.45748	0.98639	1.20935
9	1.06916	1.97154	0.88779	1.02393
10	1.25215	1.31084	1.12888	1.01241
11	0.94390	1.62442	1.12642	1.09439

Table 5. Comparison of group BMD values at Day 15 and Day 30

Group	15th Day (mm³)	30th Day (mm³)	p
Control Group	0.67 ± 0.07	1.08 ± 0.13	0.0001
P	0.70 ± 0.13	1.47 ± 0.34	0.001
H	0.64 ± 0.14	1.00 ± 0.12	0.002
P+H	0.75 ± 0.05	1.13 ± 0.11	0.0001
p	0.495	0.003	

Table 6. Tukey multiple comparison test results for Day 30 BMD values

Comparison	p value
Control Group vs P Group	0.014
Control Group vs H Group	0.868
Control Group vs P+H Group	0.981
P Group vs H Group	0.003
P Group vs P+H Group	0.033

Table 7. Histological scoring of fracture healing

Histopathological Examination				
n	Control	P	H	P+H
1	2	4	2	4
2	2	4	4	4
3	2	4	3	5
4	2	4	4	5
5	2	5	4	5
6	6	6	6	6
7	6	7	6	6
8	7	7	5	6
9	7	7	5	7
10	6	7	6	7
11	2	6	7	7

Table 8. Comparison of histological union scores at Day 15 and Day 30

Group	15th Day (mean $\pm$ SD)	30th Day (mean $\pm$ SD)	p
Control Group	2.00 $\pm$ 0.00	5.67 $\pm$ 1.86	0.002
P	4.20 $\pm$ 0.45	5.83 $\pm$ 0.75	0.002
H	3.40 $\pm$ 0.89	6.67 $\pm$ 0.52	0.0001
P+H	4.60 $\pm$ 0.55	6.50 $\pm$ 0.55	0.0001
p	0.0001	0.316	

Table 9. Tukey multiple comparison test for Day 15 histological union scores

Comparison	p value
Control Group vs P Group	0.0001
Control Group vs H Group	0.007
Control Group vs P+H Group	0.0001
P Group vs H Group	0.160
P Group vs P+H Group	0.689
Group H / Group P+H	0.02

Figures



Figure 1

Early postoperative radiograph of the femur.

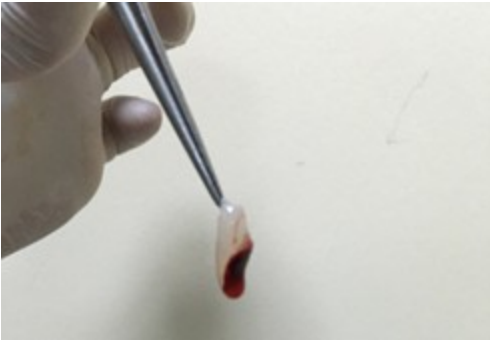


Figure 2

Ready-to-use PRF removed from the tube after centrifugation.



Figure 3

Application of hyaluronic acid to the osteotomy line.

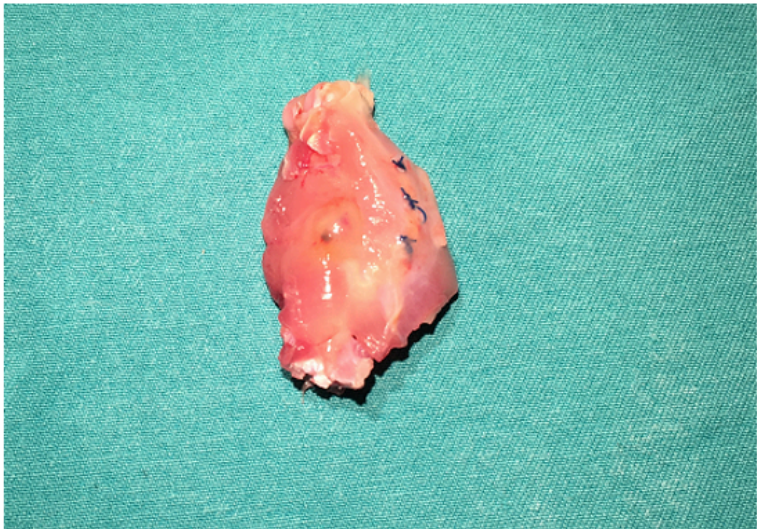


Figure 4

Amputation of the left femur after sacrifice, removed together with the surrounding soft tissues.

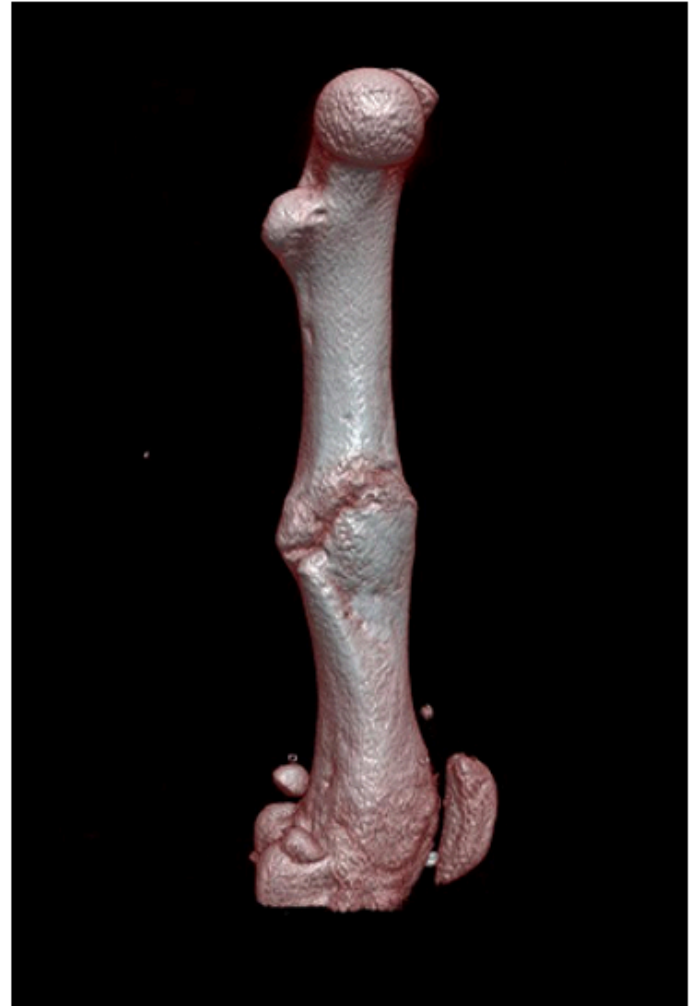
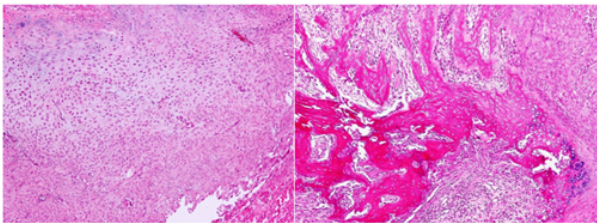
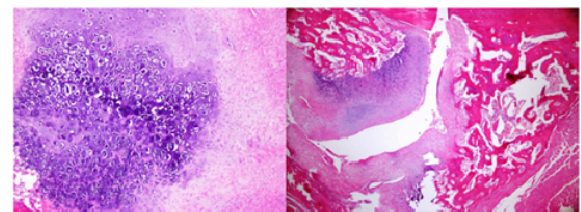


Figure 5

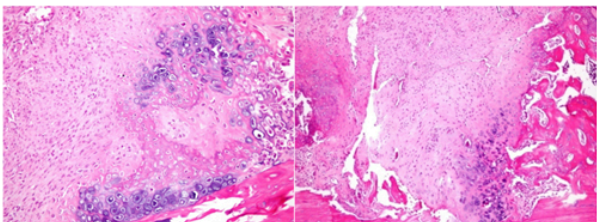
Example micro-CT image showing three-dimensional reconstruction.



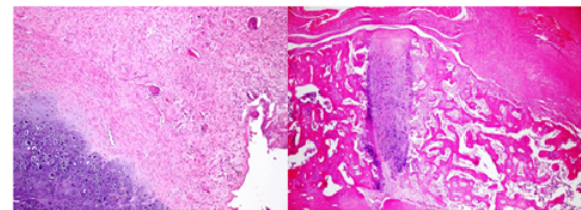
P Group: Sample 2 (Day 15) shows entirely cartilage tissue (H&E, $\times 100$); Sample 8 (Day 30) shows predominantly immature bone with a small amount of cartilage tissue (H&E, $\times 100$).



P+H Group: Sample 1 (Day 15) shows entirely cartilage tissue (H&E, $\times 100$); Sample 7 (Day 30) shows equal amounts of cartilage and immature bone (H&E, $\times 40$).



H Group: Sample 4 (Day 15) shows entirely cartilage tissue (H&E, $\times 200$); Sample 7 (Day 30) shows equal amounts of cartilage and immature bone (H&E, $\times 100$).



Control Group: Sample 1 (Day 15) shows predominantly fibrous tissue with a small amount of cartilage tissue (H&E, $\times 100$); Sample 9 (Day 30) shows predominantly immature bone with a small amount of cartilage tissue (H&E, $\times 40$).

Figure 6

Histopathological examination of all groups at Days 15 and 30.

