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Title Page

Article Title: Surgical Technique and Comparison of Autologous Cancellous Bone Grafts from Various Donor Sites in Rats

Running Head: Cancellous bone graft in rats

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Abstract

Autologous cancellous bone graft is the gold standard in large bone defect repair. However, studies using autologous bone grafting in rats are rare. To determine the feasibility of autologous cancellous bone graft harvest from different anatomical donor sites (humerus, ilium, femur, tibia, tail vertebrae) in rats and compare their suitability as donor sites, a total of 13 freshly euthanized rats were used to describe the surgical technique, determine the cancellous bone volume and microstructure, and compare the cancellous bone collected quantitatively and qualitatively. It was feasible to harvest cancellous bone graft from all 5 anatomical sites with the humerus and tail being more surgically challenging. The microstructural analysis using µCT showed a significantly lower bone volume fraction, bone mineral density, and trabecular thickness of the humerus and iliac crest compared to the femur, tibia, and tail vertebrae. The harvested weight and volume did not differ between the donor sites. All donor sites apart from the femur yielded primary osteogenic cells confirmed by the presence of Alkaline phosphatase and Alizarin Red S stain. Bone samples from the iliac crest showed the most consistent outgrowth of osteoprogenitor cells. In conclusion, the tibia and iliac crest may be the most favourable donor sites considering the surgical approach. However, due to the differences in microstructure of the cancellous bone and the consistency of outgrowth of osteoprogenitor cells, the donor sites may have different healing properties, that need further investigation in an *in vivo* study.

Keywords: cancellous bone, autologous, rats, preclinical research, preclinical surgery

MANUSCRIPT TEXT

Introduction

Large bone defects and bone loss are considered one of the biggest surgical challenges for orthopedic surgeons.[1] Autologous bone grafting is the gold standard for bone substitution in bone defect repair,[2; 3] and involves using bone material harvested from the same individual receiving the graft.[4] The advantages of autologous bone result from the combined essential healing properties for new bone formation: osteogenesis, osteoinductivity, and osteoconductivity, as well as from its complete histocompatibility.[5] Despite the benefits of autologous bone graft, the additional invasive procedure required for harvesting, associated morbidity such as pain, infection and nerve injury, prolonged surgical time and the inherently limited availability in the patient[3] are motivating researchers to investigate new bone substitutes as a valid alternative to natural bone.

In vivo animal models are frequently used in preclinical research focusing on the bone regeneration properties of new biomaterials developed as bone substitutes which can be compared to the gold standard being autologous bone graft. Rodents such as rats are regarded as one of the first-choice preclinical models for *in vivo* tests for regeneration of bone[6] due to their small size and easy handling.[7] In rats, a bone graft can originate from a wide variety of bone donor sites[8-13] which makes interpretation and comparison of the results difficult, since the bone donor site is known to influence the bone regeneration potential in clinical applications.[14; 15] The small size of rodents' long bones and their thin and fragile cortices makes the use of allograft harvested from cadavers very attractive compared to autologous bone graft.[16] However, there is some disagreement whether autologous or allogenous bone grafts should serve as the positive control in preclinical studies,[11; 17] and the bone regeneration properties might differ between allograft and autograft. Consequently, the use of the correct bone graft as a gold standard in preclinical models is essential when comparing results for

clinical implementation and should not be dictated solely by the surgeon's preference nor ease of allograft harvesting compared to autograft.

The use of cancellous bone graft harvested from unreliable cancellous bone donor sites has the potential to significantly affect preclinical research results and decrease the reproducibility of the studies. Additional considerations must also be examined when planning an autologous bone graft procedure, including the reliability of the anatomical landmarks and availability of bone supply at each available donor site. Currently, there is no rationale on the most appropriate methodology to choose when harvesting autologous cancellous bone graft in rats, and most surgical harvesting techniques are anecdotal without being built upon well-established scientifically validated protocols.

Therefore, this study aimed to determine the feasibility of autologous cancellous bone graft harvest from different anatomical sites (humerus, ilium, femur, tibia, tail vertebrae) in rats and compare their relative suitability as donor sites. More specifically, the aims were 1) to describe the surgical feasibility, techniques, and anatomical landmarks of the cancellous bone graft suitable for autologous surgical harvesting, 2) to determine the cancellous bone volume and cancellous bone microstructure of the donor sites, and 3) to characterize and compare the cancellous bone collected quantitatively (weight) and qualitatively (*in vitro* osteogenic potential).

Materials and methods

Study Design

Thirteen female skeletally mature Sprague-Dawley rats were included in the study to identify and characterize suitable autologous cancellous bone harvesting sites believed to be applicable *in vivo* for preclinical research. The investigated harvesting

donor sites, based on cancellous bone donor sites described in rats, being the iliac crest,[8; 9] proximal tibia,[2] and tail vertebrae;[10] as well as donor sites commonly used in other small animals thought to also be suitable in rats, being the proximal humerus and distal femur (**Figure 1**).[18] Prior to euthanasia immediately before the start of the experiments, the rats were randomly assigned to one of the 3 study groups: 1) surgical feasibility (n=4 rats), 2) cancellous bone microstructure analysis (n=5 rats), and 3) *in vitro* osteogenic potential characterization (n=4 rats) (**Figure 1**). For all groups, autologous cancellous bone was harvested from each donor site and quantitatively assessed by weight measurement using a high precision scale. Surgical feasibility (group 1) included a description of the surgical technique and a description of the relevant anatomical landmarks. Cancellous bone microstructure analysis (group 2) was done quantitively using descriptive µCT studies of the potential harvesting donor sites before harvesting in conjunction with a post-harvesting scan to determine the total cancellous bone harvested volume. *In vitro* osteogenic potential (group 3) was qualitatively characterized from explant cell cultures of the aseptically harvested cancellous bone graft from each donor site.

Animals

The study was approved by relevant Swiss authorities (Cantonal authorities in Graubünden, Switzerland: Permission #21_2019). The 13 included female Sprague Dawley rats (21-28 weeks, 351±30g) were euthanized immediately prior to the start of the experiment. Following deep anesthesia using Sevoflurane gas, euthanasia was performed by intracardial puncture and administration of Pentobarbital (1 mL of 200mg/ml solution).

Cancellous Bone Harvesting and Surgical Feasibility

Surgical feasibility of autologous cancellous bone graft harvest was determined immediately following euthanasia in the 4 rats from group 1 (surgical feasibility) prior to the other experiments and was based on the anatomical structures surrounding the cortical bone access to reach the available cancellous bone, the required dissection of soft tissues from the bones, and the occurrence of intraoperative complications.

Cancellous bone graft was aseptically collected in the rats from group 2 (cancellous bone microstructure analysis) and group 3 (*in vitro* osteogenic potential characterization) immediately after euthanasia (group 3) or after preoperative CT image acquisition (group 2). Cancellous bone was harvested from the proximolateral humeral metaphysis, iliac crest, distolateral femoral metaphysis, proximomedial tibial metaphysis, and 3 of the most proximal tail vertebrae.

The rat was placed in lateral recumbency for all surgical approaches except for the tail vertebrae that required the rat to be placed in sternal recumbency. A detailed description of the surgical approaches used for autologous cancellous bone harvesting can be found in the supplementary file **Appendix S1, Surgical Approaches**. Briefly, the skin and underlying soft tissues were incised according to the anatomical landmarks identified in group 1. The bone marrow cavity of the humerus, tibia, femur, and tail vertebrae was exposed by creating a circular cortical bone defect using an 18G needle, and the cancellous bone was harvested in a standardized fashion by removing as much cancellous bone as possible using a combination of 18G needle and a small bone curette (size 0000) through the single cortical hole. The bone marrow cavity of the iliac crest was exposed after excision of the cranial half of the iliac wing and cancellous bone was collected from the excised bone using an 18G

needle. The surgical harvesting time from skin incision to end of each harvesting and occurrence of intraoperative complications was recorded for all rats (n=13).

Immediately following harvesting of each donor site, the obtained cancellous bone graft was placed in sterile microcentrifuge tubes filled with phosphate-buffered saline (PBS). The weight of the harvested material was recorded for all donor sites using a high precision scale by comparing the weight of the microcentrifuge tube filled with PBS before and after sample collection.

Cancellous Bone Microstructure of Donor Sites (µCT)

The cancellous bone volume and microstructure of the donor sites was determined in the 5 rats from group 2 (cancellous bone microstructure analysis) immediately after euthanasia and after cancellous bone harvesting using a µCT scanner (VivaCT40; Scanco Medical AG, Brüttisellen, Switzerland) at 19 μm voxel size (70 kV, 114 μA, 200ms integration time). For each donor site, the general region of interest (ROI) was identified using Amira software (Amira 2019.4, Thermo Fisher Scientific, Waltham, MA, USA) based on predefined anatomical landmarks and the number of CT slices to be included for analysis (**Table 1**).

For the µCT images obtained immediately after euthanasia, automated identification of the specific region of interest (ROI) of trabecular bone previously defined was performed using Matlab (R2020b, The Mathworks, Natick, MA, USA) and a previously in-house developed C++ algorithm.[19] Afterwards, the bone of the ROI was segmented and the parameters of interest were analyzed using Scanco's standard analysis software (Scanco Medical AG). Parameters of interest were the bone mineral density ([BMD], mg of calcium hydroxyapatite/cm^3), the volume of the region of interest (total volume [TV], mm^3), the volume of the region segmented as mineralized

bone (bone volume [BV], mm^3), the ratio of the segmented bone volume to the total volume of the region of interest (bone volume fraction [BV/TV], %), the mean thickness of trabeculae (trabecular thickness [Tb.Th], mm), the mean distance between trabeculae (trabecular separation [Tb.Sp], mm), and the average number of trabeculae per mm (trabecular number [Tb.N], 1/mm).

For the µCT images obtained after cancellous bone harvesting, the scans were registered to the pre-harvesting scans and segmented in Matlab. The volume of the cancellous bone empty defect created (harvested volume, mm^3) and the percentage of harvested volume to the TV before harvesting (percentage of available TV harvested, % of TV) were calculated for all donor sites except the iliac crest. The iliac crest could not be included in the post-harvesting analysis as the surgical approach involved the excision of the bone.

Osteogenic Potential

Under sterile conditions, the cancellous bone harvested under sterile conditions from the 4 rats in group 3 (*in vitro* osteogenic potential characterization) was processed for cell culture immediately after harvesting to determine the quality of viable osteoprogenitor cells based on previously published protocols of rat osteoblast cell cultures. [20; 21]

Explant Culture

The harvested material of each donor site from each rat was incubated in a T75 flask using standard culture medium (Dulbecco's modified eagle's medium (DMEM) 1g/l glucose, 10% fetal bovine serum, and 1% Penicillin-Streptomycin) at 37°C in a humidified 5% carbon dioxide and 95% air atmosphere and the medium was changed

twice weekly. The detailed protocol used for cell culture can be found in **Appendix S2, Cell Culture Protocol.**

After 25 days, the samples with outgrowth of primary osteogenic cells were subcultured. Four replicates per sample (2 replicates for osteogenesis and 2 as control) were then plated into 24-well plates on coverslips at a concentration of 28,500 cells/well in 0.5 ml standard culture medium and incubated as per explant culture above. When cell outgrowth was insufficient, only 2 wells (1 replicate for osteogenesis and 1 as control) or only 1 well (osteogenesis only) were subcultured. After allowing the cells to attach for 3 days, the medium of 1 (when only 1 or 2 wells were plated) or 2 replicates per sample was changed to an osteogenic medium (standard culture medium + 50 μg/ml l-ascorbic acid 2-phosphate, 5 mmol/l β-glycerophosphate, 10 nmol/l dexamethasone). The other replicates were kept in a standard culture medium as a control. The medium was changed 3 times per week and was collected and frozen at -20°C on days 7, 14, 21, and 28 for Alkaline phosphatase (ALP) analysis to assess the osteoprogenitor phenotype of the cell colonies.

Alkaline Phosphatase Assay

ALP activity in the culture medium collected on days 7, 14, 21, and 28 was measured using a colorimetric enzyme assay (Quanti-Blue, InvivoGen, Toulouse, France) according to the manufacturer's instructions. The optical density (OD) was measured at 620nm using a microplate reader and plate reader control software (TECAN, Männedorf, Switzerland).

Alizarin Red S Staining

On day 28, the cultures were stained using an Alizarin Red S Staining Quantification Assay (#8678, ScienCell Research Laboratories, Carlsbad, CA, USA) according to the

manufacturer's instructions to identify calcium deposits in the cultures. They were qualitatively analyzed using an inverted microscope. For quantitative assessment, the deposits were dissolved in acetic acid and the absorbance was read at 405nm as previously described for the ALP analysis. Alizarin Red S concentration (in mM) was determined using a standard curve, and the difference in concentration between each control and the osteogenic sample was calculated.

Statistical Analysis

Statistical analysis among the parameters of interest was performed with Prism software version 8 for Windows (GraphPad Software, San Diego, CA, USA). Descriptive data (mean ± SD or median and range) was provided.

The weight of the harvested material and harvesting times were averaged from both harvested sides (left and right) when applicable and could only be retrieved and included in the analysis for 11 rats. Cancellous bone microstructure and volume measurements (μCT) were averaged from the left and right side of each donor site resulting in one parameter measurement per donor site per rat. In 3 of the 5 rats in group 2, one donor site measurement contained only one donor site side (left or right) because of an inadequate scanning window which was then used for analysis instead of both side average.

Differences in weight of the harvested material and cancellous bone microstructure and volume measurements were assessed with nonparametric Friedman two-way analysis of variance for paired samples for harvesting site (humerus, iliac crest, femur, tibia, tail) with fixed effect of the rats. For all analyses, statistical significance was set at $p < 0.05$.

Results

Cancellous Bone Harvesting

Cancellous bone graft harvesting was successful from all 5 donor sites in all rats. The femur and tibia were the easiest donor sites to surgically approach as they were less covered by soft tissue structures. The created cortical bone access was too cranial to the predefined anatomical landmarks in 2 rats in one femoral and one humeral approach. Iatrogenic fracture of the humerus was detected during harvesting in 2 rats (n=2/26 humeri), and of an articular process of the tail vertebrae in 2 other rats (n=2/26 operated tail). The average duration of surgical harvesting ranged from 10 to 27 minutes and was shorter for the tibia and the tail (**Table 2**). The average weight of the harvested material was not statistically different between the donor sites (**Figure 2**).

Cancellous Bone Microstructure of Donor Sites (µCT)

The cancellous bone microstructure results for each donor site can be found in **Figure 3** and in the supplementary file in **Appendix S3, Cancellous Bone Microstructure Results**. The humerus and iliac crest both showed significantly lower BMD, BV, BV/TV, and Tb.Th compared to the femur, tibia, and tail vertebrae ($p < 0.05$; detailed p-values can be found in the supplementary file in **Appendix S3**). Additionally, the humerus showed significantly lower TV than the iliac crest, femur, tibia, and tail vertebrae ($p < 0.05$, see **Appendix S3**), and lower BV compared to the iliac crest ($p = 0.0388$). The trabecular number was significantly lower in the humerus compared to the femur and the tibia ($p = 0.0135$, 0.0331, respectively), but the trabecular separation was similar between all groups. While the harvested volume did not differ between the donor sites, the percentage of available TV harvested was significantly higher in the humerus compared to the femur, tibia, and tail ($p = 0.0486$, 0.0194, 0.0009, respectively; **Figure 4**).

Osteogenic Potential

The outgrowth of primary osteogenic cells from explants of harvested cancellous bone was inconsistent for each donor site and each individual rat. For instance, there was primary cell outgrowth from harvested femur samples in only one rat (n=1/4) with a cell number being only high enough for one subculture well. Therefore, the femur had to be excluded from the analysis of osteogenic potential. None of the 4 rats had positive cell outgrowth from all donor sites; 3 rats had outgrowth from 4 of the 5 donor sites and 1 rat for only 3 donor sites. The iliac crest was the only donor site that yielded viable osteogenic cells from all explants (100%) in large enough quantity to plate all 4 wells for the subculture. Moreover, its cells had to be passaged into a larger flask before subculturing them as their growth rate exceeded those of the other donor sites. As the number of cells growing in the explant cultures differed between the harvested bone samples, not all could be subcultured into 4 wells of the 24 well plate (**Table 3**).

For all donor site samples that yielded primary cell outgrowth, the osteoprogenitor phenotype could be confirmed by the presence of ALP within all subcultures and the ability of the cell colonies to form mineralized nodules and take up the Alizarin Red S stain. There was an increase in OD in all collected osteogenic cell culture medium samples between day 14 and day 21 (**Figure 5A**), equaling ALP concentration. The cells from all samples cultured in osteogenic medium showed a qualitatively stronger Alizarin Red S staining uptake compared to their control sample (**Figure 6**), which appeared more pronounced in the samples from the humerus and tibia than from the iliac crest and tail (**Figure 5B**). Some donor site samples still contained small bone pieces that could not be separated from the cells when transferring them from the

initial flasks to the well plates for subculture and prevented objective counting of nodules.

Discussion

This study's results suggest that autologous cancellous bone graft harvesting is surgically feasible in rats for preclinical studies from different donor sites and that there are substantial differences between the different sites. The tibia and iliac crest had reliable cancellous bone supply available with anatomical landmarks allowing straightforward surgical harvesting of autologous cancellous bone in rats. Those sites may be more favourable donor sites than the humerus and tail vertebrae which seem to be more prone to iatrogenic fracture and may be more reliable than the femur that yielded very inconsistent outgrowth of primary osteogenic cells from explants of harvested bone. However, the tibia and iliac crest were different in terms of their microstructure characteristics with the tibia showing significantly higher BMD, BV, BV/TV, and Tb.Th than the iliac crest, suggesting that both donor sites may have different healing properties for new bone formation and may not be used interchangeably.

Surgical Feasibility

Experience gained from the experiment indicated that the tibia and iliac crest are suitable sites for cancellous bone harvesting and are readily accessible with the rat in lateral recumbency. The main surgical advantage of the tibia as a donor site is minimal soft tissue dissection and retraction necessary to make the bone exposed for cancellous bone access. However, the weight-bearing function of the tibia is an important consideration for animal welfare if the preclinical model intended to be used involves a long bone defect creation to avoid bilateral limb impairment in the

operated rat. On the contrary, the iliac crest is a non-weight-bearing bone also surgically accessible which has the advantage of decreased consequences on ambulation.[22] The iliac crest also holds the advantage that it can be resected to perform a cancellous bone extraction outside the surgical site. The iliac crest was more deeply buried in soft tissue structures compared to the other donor sites and the ilium wing has multiple muscle attachments site making its surgical approach requiring more dissection. This may result in a longer intraoperative preparation time for dissection of soft tissues off the bones and hemostasis in the living rat and may predispose to incisional complications such as peri-incisional edema and seroma. The tail is another non-weight-bearing bone in the rat and has the advantage that it can be approached in ventral recumbency which is not the case for the other donor sites. This possibility can make this donor site more versatile for different preclinical surgical procedures compared to lateral recumbency. However, the small size of the vertebral structure made it more difficult to harvest cancellous bone and increased the risk of iatrogenic fracture and may require tail amputation. This could become a welfare concern since the tail is important for the rat's thermoregulation and thermosensation,[23] and its amputation is not recommended in a living rat. The humerus and femur, despite being surgically acceptable bones, may not be suitable donor sites for autologous cancellous bone graft. In fact, the humerus was the only long bone that fractured during harvesting. This finding aligns with a previous report describing an increased risk of fracture after bone graft harvesting in the humerus in other species.[24] Regardless of the site selected for autologous bone grafting, the researchers should consider the possible impacts of harvesting a substantial amount of cancellous bone from these sites to fill large bone defects, which could lead to pain or other problems such as abnormal ambulation.

Harvested Weight and Volume

The quantity of harvestable cancellous bone from the different donor sites is central since it needs to be established whether the described surgical approaches can provide enough cancellous bone for *in vivo* bone defect models knowing that the volume of bone graft placed in a defect affects its healing potential.[25] The weight of cancellous bone harvested was comparable between the different donor sites and was somehow similar to previously published harvested weight from the ilium in rats.[9; 26-28] The wider range obtained in the harvested volume from the tibia is suggesting that this site may be less reliable to retrieve reproducible amounts of cancellous bone for autologous grafting. Knowing whether the harvested cancellous bone quantity would be enough to fill *in vivo* bone defects can only be projected by indirect extrapolation based on previously published defect sizes in rats.[29; 30] Critical-sized bone defect models in the rat femur are frequently used with defect sizes ranging between 5 and 10 mm, depending on the model.[30] In this case, enough cancellous bone could be harvested in a volume sufficient for loosely filling a 5 mm femoral bone defect (25 mm^3; cylinder with a diameter of 2.5 mm and a height of 5 mm)[31] when harvested bilaterally, but the iliac crest may be the better choice with a TV around 40 mm^3 for each side.

Cancellous Bone Microstructure

The microstructural bone along with the biomaterial characteristics such as bone mineral density represents the mechanical strength of bone and is commonly evaluated as the main predictor of bone quality in bone grafting.[14; 15; 32; 33] Among all investigated donor sites, the humerus and iliac crest showed the lowest BV/TV, BMD, Tb.Th and Tb.N measurements. Similar findings in humans and primates previously reported lower BV/TV and Tb.Th in the humerus compared to the femur.[34; 35] In rats,

the total mineral content of the humerus was also previously reported to be lower than in the femur and tibia but the difference was not statistically different.[6] On the contrary, the iliac crest in humans was shown to have a higher BV/TV, Tb.Th and Tb.N than the vertebrae.[36] This difference could be explained by the different functions of the iliac crest and the tail vertebrae without significant weight-bearing in the rats compared to the vertebrae in humans having to support the body weight. Previous studies have found that bone graft volume maintenance and bone healing were enhanced by denser bone, dense trabecular connections, and a BV/TV ratio of 38.50%.[37-39] The femur, tibia, and tail vertebrae investigated in this study as potential cancellous bone donor sites showed BMD, BV/TV, and Tb.Th values exceeding those of the humerus and iliac crest, suggesting that their cancellous bone microstructure represents superior bone quality for grafting.

Cell culture

In this study, the osteogenic potential of the harvested cancellous bone from the different donor sites was compared based on their consistency in providing viable cells and specifically osteoprogenitor cells by measuring ALP concentration to assess the osteoblast phenotype[40] and by measuring their capacity to incorporate calcium using Alizarin Red S staining.[41]

The osteogenic cells from the bone graft need to be viable, before and remain viable after, transplantation, which is believed to be a critical factor in successful and early bone healing with bone graft.[42-44] The success of cell outgrowth in the explant cell culture was not consistent and varied between the harvested donor sites and rats. The samples from the tibia and femur showed cell outgrowth in only 50% and 25% of the rats, respectively. The poor consistency of cell outgrowth in the samples from the femur suggests that this donor site would be less reliable in providing a population of

viable osteogenic cells for autologous cancellous bone graft in rats. Similar experiences of primary cell culture outgrowth from different cancellous bone explants have been described in horses.[45]

Osteoblasts *in vitro* are recognized by their ability to produce mineralized (bone) nodules in culture and by increased ALP activity.[46] In this study, the measured ALP concentration was qualitatively higher in the samples obtained from the iliac crest compared to the other donor sites suggesting the presence of more osteoprogenitor cells. Similarly, the mean concentration of osteoblastic progenitor cells was shown to be higher in the iliac crest harvested from humans than from the tibia.[47]

The osteogenic potential from the cells cultured *in vitro* was also evaluated by staining calcium deposits using Alizarin Red S.[41] In the samples obtained from the iliac crest and tail, the difference in red staining between the cells cultured in osteogenic medium and their control was less pronounced suggesting lower calcium deposition compared to the humerus and tibia.

Limitations

Among the study's limitations is the fact that the impact of autologous bone graft harvest on donor site morbidity and postoperative complications could not be evaluated. While the sample size was sufficient to detect statistical differences between the donor sites in the microstructural analysis, the difficulties in establishing explant cultures for some of the donor sites limited the statistical power for the osteogenic potential. The remains of cancellous bone in the subcultured cell samples prohibited the direct quantification of Alizarin Red S stain. The study was only performed in female rats which may limit the generalization of the results to a male population as sex-specific differences may exist.

Conclusion

This study showed that autologous cancellous bone graft harvesting is surgically feasible in rats. The investigated donor sites were not equal in terms of cancellous bone microstructure and *in vitro* osteogenic potential. While all surgical approaches were able to retrieve similar amounts of cancellous bone graft, the tibia and iliac crest may be more favourable donor sites. While the microstructure of the cancellous bone from the tibia suggests superior bone quality for grafting, the iliac crest yielded a more consistent outgrowth of primary osteogenic cells from explants of the harvested bone. These findings warrant further study to compare the different healing potential of the cancellous bone from those donor sites in an *in vivo* bone defect healing model.

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Reference

1. Svedbom A, Hernlund E, Ivergård M, et al. 2013. Osteoporosis in the European Union: a compendium of country-specific reports. Arch Osteoporos 8:1-218.
2. Blokhuis TJ, Calori GM, Schmidmaier G. 2013. Autograft versus BMPs for the treatment of non-unions: what is the evidence? Injury 44 Suppl 1:S40-2.
3. Campana V, Milano G, Pagano E, et al. 2014. Bone substitutes in orthopaedic surgery: from basic science to clinical practice. J Mater Sci Mater Med 25:2445-2461.

4. Pape HC, Evans A, Kobbe P. 2010. Autologous bone graft: properties and techniques. J Orthop Trauma 24 Suppl 1:S36-40.

5. Myeroff C, Archdeacon M. 2011. Autogenous Bone Graft: Donor Sites and Techniques. JBJS 93:2227-2236.

6. Prodinger PM, Foehr P, Bürklein D, et al. 2018. Whole bone testing in small animals: systematic characterization of the mechanical properties of different rodent bones available for rat fracture models. Eur J Med Res 23:8.

7. Li Y, Chen S-K, Li L, et al. 2015. Bone defect animal models for testing efficacy of bone substitute biomaterials. J Orthop Transl 3:95-104.

8. Gill DR, Ireland DC, Hurley JV, et al. 1998. The prefabrication of a bone graft in a rat model. J Hand Surg 23:312-321.

9. Qiu Z, Wei L, Liu J, et al. 2013. Effect of intermittent PTH (1–34) on posterolateral spinal fusion with iliac crest bone graft in an ovariectomized rat model. Osteoporos Int 24:2693-2700.

10. Hamada T, Matsubara H, Hikichi T, et al. 2021. Rat model of an autologous cancellous bone graft. Sci Rep 11:18001.

11. Dreyer CH, Rasmussen M, Pedersen RH, et al. 2020. Comparisons of Efficacy between Autograft and Allograft on Defect Repair In Vivo in Normal and Osteoporotic Rats. Biomed Res Int 2020.

12. Shiga Y, Orita S, Kubota G, et al. 2016. Freeze-dried platelet-rich plasma accelerates bone union with adequate rigidity in posterolateral lumbar fusion surgery model in rats. Sci Rep 6:36715.

13. Lee Y-P, Jo M, Luna M, et al. 2005. The efficacy of different commercially available demineralized bone matrix substances in an athymic rat model. Clin Spine Surg 18:439-444.

14. Kon K, Shiota M, Ozeki M, et al. 2009. Bone augmentation ability of autogenous bone graft particles with different sizes: a histological and micro-computed tomography study. Clin Oral Implants Res 20:1240-6.

15. Kolk A, Handschel J, Drescher W, et al. 2012. Current trends and future perspectives of bone substitute materials - from space holders to innovative biomaterials. J Craniomaxillofac Surg 40:706-18.

16. An YH, Friedman RJ. 1999. Animal models in orthopaedic research. CRC press Boca Raton; p 241-261

17. Winge MI, Reikerås O, Røkkum M. 2011. Calcium phosphate bone cement: a possible alternative to autologous bone graft. A radiological and biomechanical comparison in rat tibial bone. Arch Orthop Trauma Surg 131:1035-41.

18. Fossum TW. 2018. Small Animal Surgery, 5th ed. Elsevier Health Sciences; p 991-993

19. Mys K, Stockmans F, Vereecke E, et al. 2018. Quantification of bone microstructure in the wrist using cone-beam computed tomography. Bone 114:206-214.

20. Declercq H, Van den Vreken N, De Maeyer E, et al. 2004. Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions: comparison of different isolation techniques and source. Biomaterials 25:757-768.

21. Ishida Y, Heersche J. 1997. Progesterone stimulates proliferation and differentiation of osteoprogenitor cells in bone cell populations derived from adult female but not from adult male rats. Bone 20:17-25.

22. Kraus K. 2012. Bone grafts and substitutes. Veterinary Surgery Small Animal Missouri: Elsevier Saunders:676-684.

23. Yehuda S. 1975. d-Amphetamine and the sensory role of a rat's tail in thermoregulation or what the rat's tail tells the rat's brain. Behav Biol 13:233-238.

24. Harriss FK, Galuppo LD, Decock HEV, et al. 2004. Evaluation of a Technique for Collection of Cancellous Bone Graft from the Proximal Humerus in Horses. Vet Surg 33:293-300.

25. DeVries WJ, Runyon CL, Martinez SA, et al. 1996. Effect of volume variations on osteogenic capabilities of autogenous cancellous bone graft in dogs. Am J Vet Res 57:1501-1505.

26. Abe Y, Takahata M, Ito M, et al. 2007. Enhancement of graft bone healing by intermittent administration of human parathyroid hormone (1–34) in a rat spinal arthrodesis model. Bone 41:775-785.

27. Yamaguchi A, Murakami T, Takahashi M, et al. 2011. Luminescence imaging of regenerating free bone graft in rats. Plast Reconstr Surg 127:78-87.

28. Sun Y-X, Sun C-L, Tian Y, et al. 2014. A comparison of osteocyte bioactivity in fine particulate bone powder grafts vs larger bone grafts in a rat bone repair model. Acta Histochem 116:1015-1021.

29. Kondo N, Ogose A, Tokunaga K, et al. 2005. Bone formation and resorption of highly purified β-tricalcium phosphate in the rat femoral condyle. Biomaterials 26:5600-5608.

30. Sato K, Watanabe Y, Harada N, et al. 2014. Establishment of reproducible, critical-sized, femoral segmental bone defects in rats. Tissue Engineering Part C: Methods 20:1037-1041.

31. Poser L, Matthys R, Schawalder P, et al. 2014. A standardized critical size defect model in normal and osteoporotic rats to evaluate bone tissue engineered constructs. Biomed Res Int 2014:348635

32. Fanuscu MI, Chang TL. 2004. Three-dimensional morphometric analysis of human cadaver bone: microstructural data from maxilla and mandible. Clin Oral Implants Res 15:213-218.

33. Pothuaud L, Van Rietbergen B, Mosekilde L, et al. 2002. Combination of topological parameters and bone volume fraction better predicts the mechanical properties of trabecular bone. J Biomech 35:1091-1099.

34. Doershuk LJ, Saers JP, Shaw CN, et al. 2019. Complex variation of trabecular bone structure in the proximal humerus and femur of five modern human populations. Am J Phys Anthropol 168:104-118.

35. Ryan TM, Shaw CN. 2013. Trabecular bone microstructure scales allometrically in the primate humerus and femur. Proc Biol Sci 280:20130172.

36. Dempster D, Ferguson-Pell M, Mellish R, et al. 1993. Relationships between bone structure in the iliac crest and bone structure and strength in the lumbar spine. Osteoporos Int 3:90-96.

37. Vinci R, Rebaudi A, Capparè P, et al. 2011. Microcomputed and histologic evaluation of calvarial bone grafts: a pilot study in humans. Int J Periodontics Restor Dent 31: e29-36

38. Ozaki W, Buchman SR. 1998. Volume maintenance of onlay bone grafts in the craniofacial skeleton: micro-architecture versus embryologic origin. Plast Reconstr Surg 102:291-299.

39. Kim YJ, Henkin J. 2015. Micro-computed tomography assessment of human alveolar bone: bone density and three-dimensional micro-architecture. Clin Implant Dent Relat Res 17:307-313.

40. Piattelli A, Scarano A, Corigliano M, et al. 1996. Effects of alkaline phosphatase on bone healing around plasma-sprayed titanium implants: a pilot study in rabbits. Biomaterials 17:1443-1449.

41. Orriss IR, Taylor SE, Arnett TR. 2012. Rat osteoblast cultures. In: Bone Research Protocols, Methods in Molecular Biology, vol 816: Springer; pp. 31-41.

42. Gray JC, Elves M. 1979. Early osteogenesis in compact bone isografts: A quantitative study of the contributions of the different graft cells. Calcif Tissue Int 29:225-237.

43. Bauer TW, Muschler GF. 2000. Bone graft materials: an overview of the basic science. Clin Orthop Relat Res 371:10-27.

44. Bassett CAL. 1972. Clinical implications of cell function in bone grafting. Clin Orthop Relat Res 87:49-59.

45. McDuffee LA, Anderson GI. 2003. In vitro comparison of equine cancellous bone graft donor sites and tibial periosteum as sources of viable osteoprogenitors. Vet Surg 32:455-463.

46. Sabokbar A, Millett P, Myer B, et al. 1994. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. Bone and mineral 27:57-67.

47. Hyer CF, Berlet GC, Bussewitz BW, et al. 2013. Quantitative assessment of the yield of osteoblastic connective tissue progenitors in bone marrow aspirate from the iliac crest, tibia, and calcaneus. JBJS 95:1312-1316.

Figure Legends

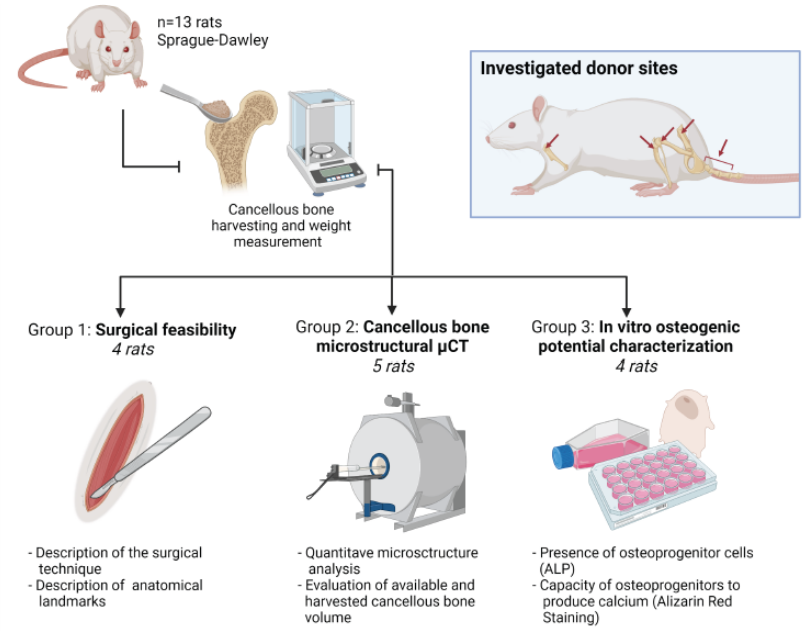

Figure 1 – Study Design.

Schematic representation of the study design used to identify and characterize suitable autologous cancellous bone harvesting sites in rats and representation of the investigated donor sites (red arrows; humerus, ilium, femur, tibia, tail vertebrae).

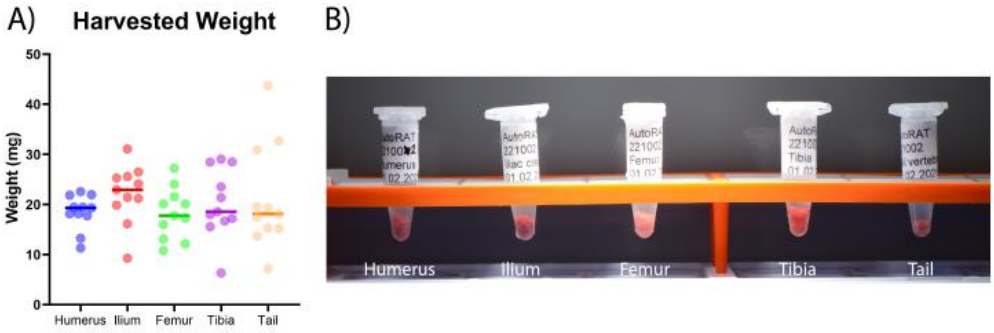

Figure 2 – **Cancellous Bone Harvesting.**

A) Scatter plots demonstrating the weight of harvested cancellous bone according to the donor sites. The line of each donor site represents the median. Note: the sampled weight for the tail was obtained from the 3 sampled vertebrae while the other donor sites' weight was obtained from only one bone.

B) Representative image of harvested cancellous bone from all 5 donor sites from one rat, stored in sterile PBS.

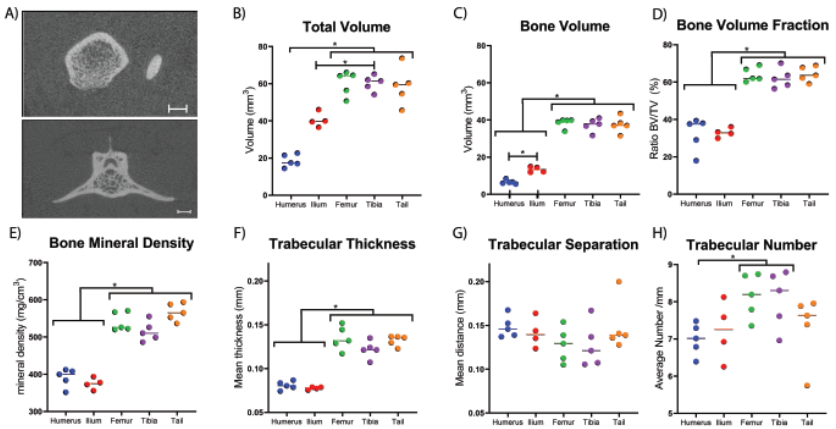

Figure 3 – Cancellous Bone Microstructure of Donor Sites (µCT).

A) Representative transverse cross-section of µCT images obtained for the humerus (top) and proximal tail vertebra (bottom) showing the differences in the cancellous bone microstructure; scale bar: 1 mm.

B) – H) Scatter plots demonstrating the cancellous bone microstructure results before harvesting according to the donor sites. The line of each donor site represents the median.

Asterisks indicate a significant difference between the donor sites ($p < 0.05$).

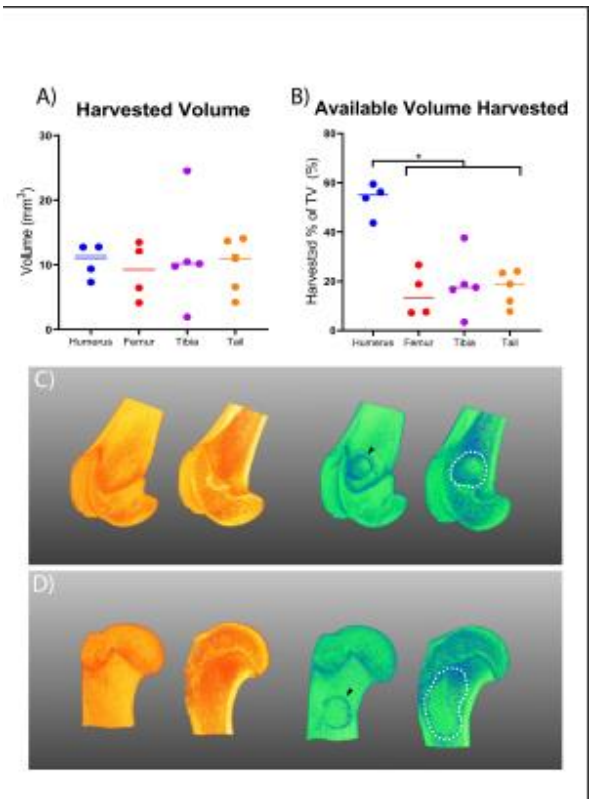

Figure 4 – Harvested Cancellous Bone Volume.

Scatter plots demonstrating the cancellous bone volume results obtained after harvesting according to the donor sites. The line of each donor site represents the median.

A) The volume of the cancellous bone empty defect created from cancellous bone harvesting corresponding to the harvested volume.

B) Percentage of harvested volume in relation to the total volume of bone available before harvesting corresponding to the available volume harvested.

C-D) Representative 3D rendered images of µCT scans of the femur © and humerus (D); top is proximal and left is cranial. From left to right, the images represent the cortical shell and bone cross-section showing the available cancellous bone before harvesting (yellow bones), followed by the cortical bone access (arrowhead) made through the cortical shell and the empty defect within the cancellous bone (dashed line) after harvesting (green bones).

Asterisks indicate a significant difference between the donor sites ($p < 0.05$).

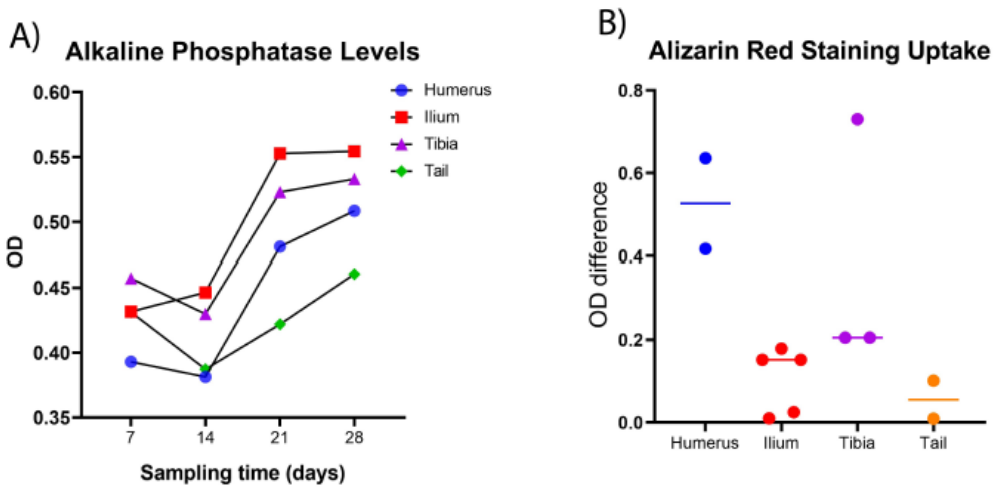

Figure 5 – Osteogenic Potential.

A) Variation of the mean optical density (OD) indicative of alkaline phosphatase levels over time obtained from the osteogenic cell culture medium used for bone graft explant cultures according to the donor sites.

B) Scatter plots demonstrating the difference in Alizarin Red S concentration (in mM) between the subcultured cells incubated in osteogenic medium and the control samples in a standard medium according to the donor sites. The line of each donor site represents the median.

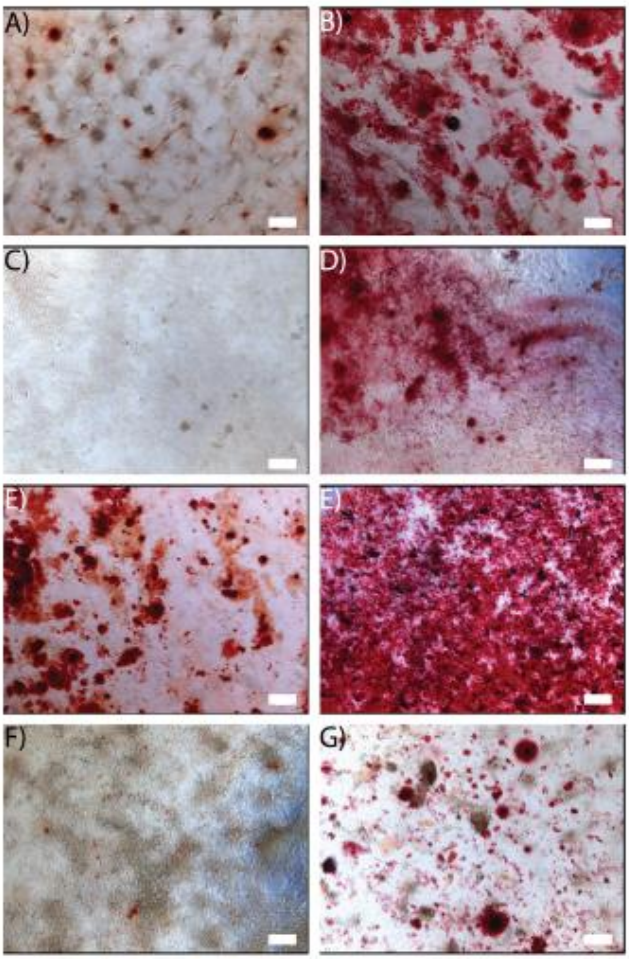

Figure 6 - Alizarin Red S staining.

Representative microscopical images observed during Alizarin Red S staining of the control samples kept in standard medium (left column) compared to samples placed in osteogenic medium (right column); scale bar: 200 µm.

A-B) humerus, C-D) iliac crest, E-F) tibia, G-H) tail. Note the positive staining (red) of the control samples due to residual pieces of bone in the cell subcultures.

Tables

Table 1 - Anatomical limits used to define the region of interest for cancellous bone microstructure evaluation

	Proximal or cranial limit	Distal or caudal limit
Proximal humerus	Epiphyseal cortical bone	6.65 mm distal from epiphyseal cortical bone
Iliac crest	Cranial cortex of iliac crest	6.65 mm caudal from cranial cortex of iliac crest
Distal femur	6.65 mm proximal from epiphyseal cortical bone	Epiphyseal cortical bone
Proximal tibia	Epiphyseal cortical bone	6.65 mm distal epiphyseal cortical bone
3 cranial tail vertebrae	Cortical bone of cranial endplate	Cortical bone of caudal endplate

Anatomical limits used to define the region of interest to be calculated in Matlab. Region of interest represents cancellous bone judged to be surgically accessible and is used to calculate cancellous bone microstructural parameters.

Table 2 – Surgical harvesting time

Donor site	Harvesting time (min) ± SD
Humerus	20.4 ± 3.9
Iliac crest	19.2 ± 3.3
Femur	19.1 ± 3.5
Tibia	14.6 ± 2.2
Tail	13.6 ± 2.9

Mean surgical time for autologous cancellous bone graft harvesting from skin incision to end of harvesting for the 5 different donor sites (n=12 rats).

Note: the harvesting time for the tail was obtained from the 3 sampled vertebrae while the other donor sites' harvesting time was obtained from bilateral harvesting.

Table 3 - Number of wells used to subculture cells for the analysis of osteogenic potential

	Humerus	Iliac crest	Femur	Tibia	Tail
Rat 1	4	4	1	0	4
Rat 2	1	4	0	4	4
Rat 3	2	4	0	0	1
Rat 4	1	4	0	4	4

The number of wells used per bone sample to subculture outgrown cells to analyze the osteogenic potential *in vitro*. Whenever possible, 4 wells were used. Differences derive from different amounts of cell outgrowth in explant culture.