Ultrasound Expedites Decalcification of Mouse Tibia

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Introduction

Bone Composition [1]
- 30% organic matrix and cells
- 70% is hydroxyapatite (HA) ($Ca_{10}(PO_4)_6(OH)_2$) crystals

Decalcification
- HA is aligned along the collagen fibers to reinforce the matrix
- Removal of HA is favorable for paraffin histological preparation
- Many factors influence the duration of the decalcification (thickness, temperature, decalcifying agent, etc.)
- Decalcifying agents include Acids and Chelating agents
- Ethylenediaminetetraacetic (EDTA) is a chelating agent
- Ultrasound (US) has been seen to greatly decrease the duration of decalcification without influencing histology [2]
- This was not researched in depth with mouse bones which is one of the primary subjects for research

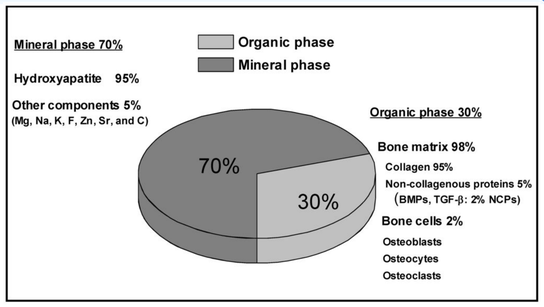

Fig. 1. Shows the composition of the bone [1]

Objectives

- To expedite the decalcification process with the least amount of materials
- Observe influence of US on the morphology of the mouse tibia

Hypothesis

- Ultrasound will significantly decrease the decalcification time and not influence the histology of the bone
- The time difference between containerized and containerless would be very similar

Methods

Bone samples
- Bones used for this experiment were mouse tibia from wild-type mice.
- Each tibia was extracted from the specimen and placed in formalin for 24 hours prior to decalcification

Decalcification processes
- First conditions (Control):
  - 2 tibia samples were decalcified in 10% EDTA for 2 weeks
  - The EDTA was changed every 3 days
- Second condition (Containerless):
  - 1 tibia sample was placed in the US decalcifier (DeCa DX1100; Pro-Cure Medical Technology Co. Ltd, Hong Kong, 50 W at a frequency of 40kHz) in 2 Liters of 0.5 M EDTA.
  - The EDTA solution was changed every 24 hours
- Third condition (Containerized):
  - 1 tibia sample was placed in a beaker with 200 mL of 0.5 M EDTA and then placed in a water bath within the US decalcifier
  - The EDTA solution was changed every 6 and 18 hours
- The decalcification conditions were kept at a constant temperature range of 35-40℃.

Decalcification Assessment
- The decalcification was assessed through the scout-view of the Scanco *vitaCT* 80 system at every 24 hour mark

Sectioning
- Once decalcification was confirmed the bones were were embedded in paraffin and sectioned at 5 µm along the cross-section of the tibia

Histology
- The sectioned bones were placed under two staining conditions: Hemotoxin and Eosin (H&E) and tartrate-resistant acid phosphatase (TRAP)
- H&E and TRAP staining were performed using standard staining procedure [3,4]
- The stained sections were then observed under a light microscope
- Cell morphology and the presence of artefacts or detrimental effects were observed

Results and Discussions

Decalcification Assessment
- The tibias were seen to have completely decalcified at times at 48 hours and 78 hours for the containerless and containerized solution of EDTA, respectively (Fig. 2). The time saved with these US conditions was 85% for the containerless and 76% for the containerized solution of EDTA.
- The bone was confirmed to be decalcified when the scout-view was seen to be similar to the 2 week.

Histology
- H&E staining showed no significant difference between the samples
- The staining of the cell nuclei in the bone showed no signs of deterioration in any of the decalcification conditions (Fig. 3).
- TRAP staining showed no significant difference between the samples.
- The staining of the osteoclasts in the bone showed no deterioration in any of the decalcification conditions (Fig. 4).
- There were also no artefacts of detrimental effects on the tissue for either of the stains performed.

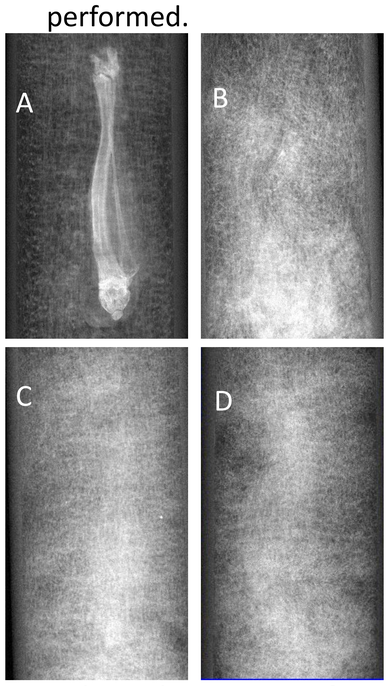

Fig. 2. Shows the scout-view for the (A) Fully calcified Tibia (B) Tibia decalcified in absence of US for 2 weeks (C) Tibia decalcified in Containerless US environment for 48 hours(D) Tibia decalcifies in Containered environment for 78 hours

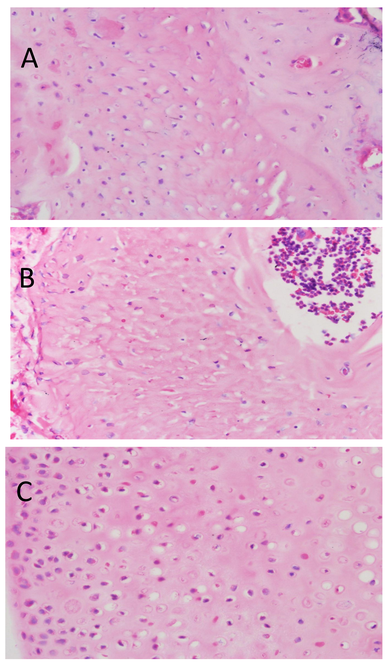

Fig. 3. H&E staining for
(A) 2 week Decalcification
(B) Containerless Decalcification
(C) Containerized Decalcification
The Blue/Purple stains represent the cell nuclei and the pink stain represents surrounding tissue and proteins

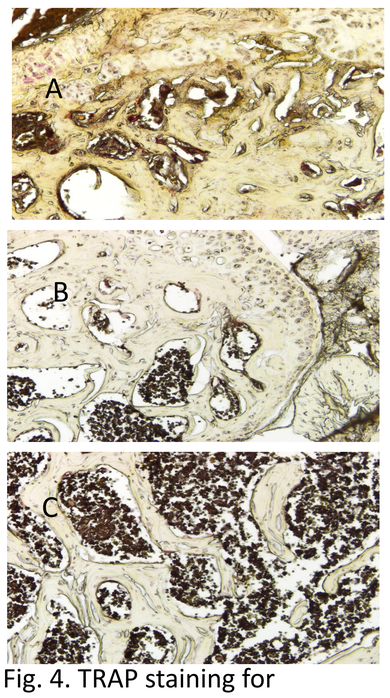

Fig. 4. TRAP staining for
(A) 2 week Decalcification
(B) Containerless Decalcification
(C) Containerized Decalcification
The red stains represent osteoclasts, the brown represents cell nuclei, and the yellow represents surrounding tissue

Conclusion

- We found that there was a significant difference in time that it takes to decalcify the bones but no significant difference between the quality of the staining
- The stain quality was high for all of the samples
- With these results it could be concluded that ultrasound can both expedite the decalcification process and the process could also use less materials making it more efficient.
- The time difference between the containerless and containered samples could be a result of the different volumes of EDTA in each condition [2]
- Future work would be to use bones from the same mouse in order to do a cell count and to perform immunohistochemistry to stain more specific proteins within the bone.

References

[1] Alvarez K, Et al. *Materials,* 2009
[2] Chow DH, Et al. *JOT,* 2019
[3] Sampias, C, Et al. *Leica,* 2022
[4] Sigma-Aldrich , 2014