Structural and Cellular Responses of Supraspinatus Tendon Enthesis and Subchondral Bone to Pregnancy, Lactation, and Post-Weaning Recovery

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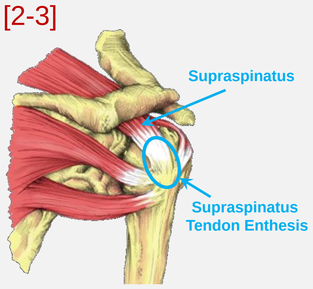
Introduction

- Reproduction-induced musculoskeletal changes:
  - Dramatic bone loss during pregnancy & lactation [1]
  - Fluctuating hormone levels during pregnancy and lactation increase the risk of musculoskeletal joint disorders, such as shoulder pain [2-3]
- Supraspinatus tendon enthesis:
  - Transfer force from the muscle to the humerus
  - Enable soft and hard tissues to function in unison
  - Minimizes stress concentrations
  - Complex structure in tissue organization with varying cellular compositions and mechanical properties



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- Unknown cellular mechanisms behind transient changes in mechanical properties of the supraspinatus tendon during pregnancy [4]

Objective: To track the structural and cellular changes in the supraspinatus tendon enthesis and underlying humeral bone

Hypothesis: Pregnancy, lactation, and weaning have differential effects on cellular activities, altering the dynamic change of microstructure in the supraspinatus tendon enthesis and its underlying subchondral bone.

Materials & Methods

In vivo longitudinal µCT study

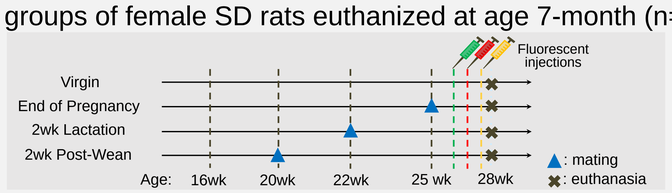
- 4-month-old female Sprague Dawley (SD) rats
  - Virgin (n=7) and Reproductive (n=6) female SD rats
- *In vivo* µCT scans on the right proximal humerus (Scanco vivaCT 80, voxel size: 21µm)
  - Mating
  - End of 3-week pregnancy
  - End of 3-week lactation
  - End of 6-week post-weaning recovery

- 3 ROIs analyzed in the humerus:
  - Epiphysis (red)
  - Adjacent to supraspinatus tendon enthesis (yellow)
  - Metaphysis (blue)

- Statistics: 2-way repeated-measures ANOVA with baseline adjustment and Bonferroni corrections for post hoc tests

Cryo-histomorphometry Study

- 5 groups of female SD rats euthanized at age 7-month (n=6)



- Calcein green, alizarin red, and tetracycline injected at 16, 9, and 2 days before euthanasia
- Multi-round cryo-histology imaging on left humeri head
  - Fluorochrome mineralization labels
  - Tartrate-resistant acid phosphatase (TRAP) staining to visualize catabolic enzyme activities
  - Toluidine blue staining for identifying cell population and different zones in the tendon enthesis
- Statistics: 1-way ANOVA and Tukey's HSD for post hoc tests

Results

Longitudinal µCT tracking showing bone dynamic changes during a reproductive cycle

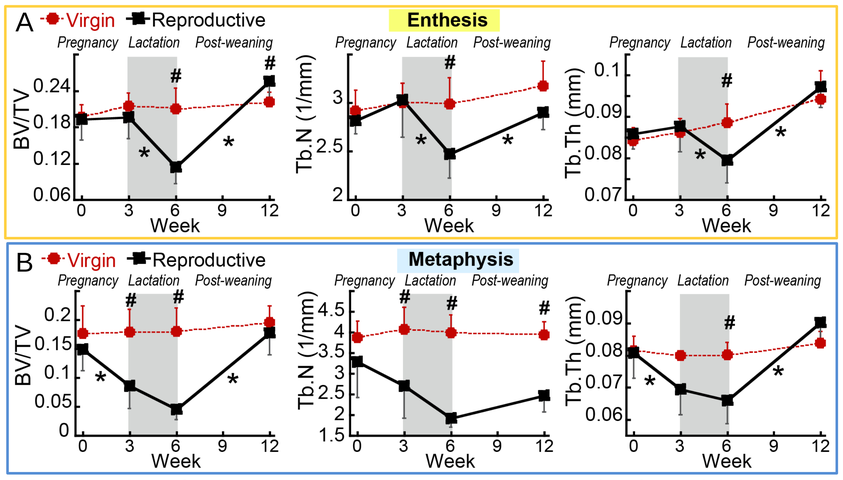


Fig 1. Trabecular bone microstructure at the (A) enthesis and (B) metaphysis. (p<0.05: # Virgin ≠ Reproductive; * within reproductive group) (C) Bone dynamic changes during 3wk lactation and 6wk post-weaning recovery. Red: bone resorption; Green: bone formation.

At the enthesis region

- No obvious changes in bone microstructure during pregnancy (Fig 1A)
- Significant reductions in BV/TV, Tb.N, and Tb.Th during lactation (Fig 1A)
- A substantial anabolic response triggered by weaning (Fig 1C), resulting in complete recovery in Tb.N and Tb.Th, and greater BV/TV in post-weaning rats than virgins (Fig 1A)

At the metaphysis region

- Significant reductions in BV/TV, Tb.N, and Tb.Th during pregnancy & lactation (Fig 1B)
- Lower Tb.N than baseline even after 6-week post-weaning recovery (Fig 1B)

Epiphysis *vs.* Enthesis

- The changes in trabecular bone microstructure: Enthesis > Epiphysis

Cryo-histomorphometry showing the dynamics of mineralization & cellular activities

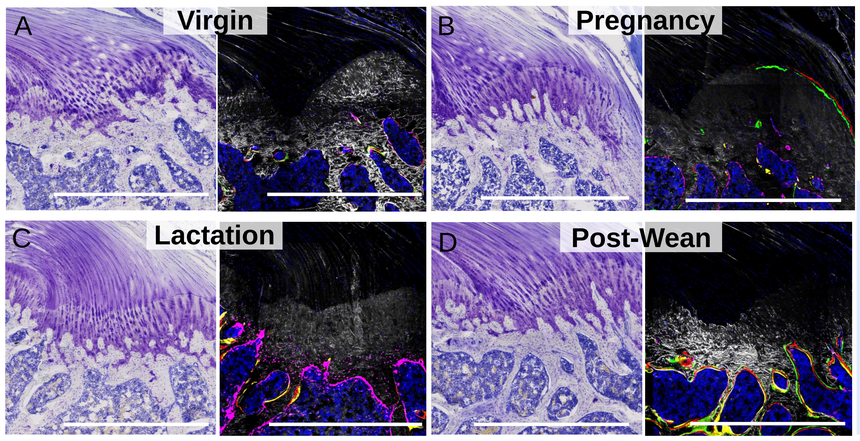


Fig 2. Representative cryo-images of (A) Virgin, (B) Pregnancy, (C) 2-week Lactation, and (D) 2-week Post-wean groups. White bar =1mm. Left: toluidine blue staining; Right: fluorescent image. (Green, Red, Yellow: fluorochrome mineralization labels; Blue: nucleus; Pink: TRAP staining)

Multicolor mineralization activities (Green, Red, Yellow)

- Found in the subchondral bone near the enthesis in all groups
- Strongest activities observed in the post-wean group (Fig 2D)
- Consistent with µCT data (Fig 1) showing substantial trabecular bone recovery after weaning

Osteocyte catabolic enzyme activities (TRAP: Pink)

- TRAP detected on bone surface in all groups
- TRAP: Highest expression level during lactation (Fig 2C)

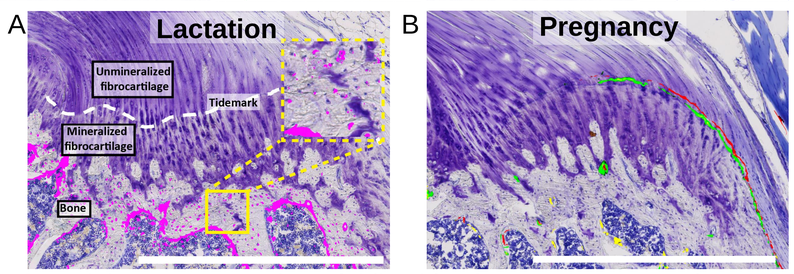


Fig 3. (A) A representative toluidine blue image of supra tendon enthesis overlapped with TRAP staining (pink) from lactation group. Tendon enthesis zones are divided into: *unmineralized fibrocartilage*; *tidemark*; *mineralized fibrocartilage*; *bone*. (B) A representative toluidine blue image overlapped with mineralization labels (Green, Red, Yellow) from pregnancy group.

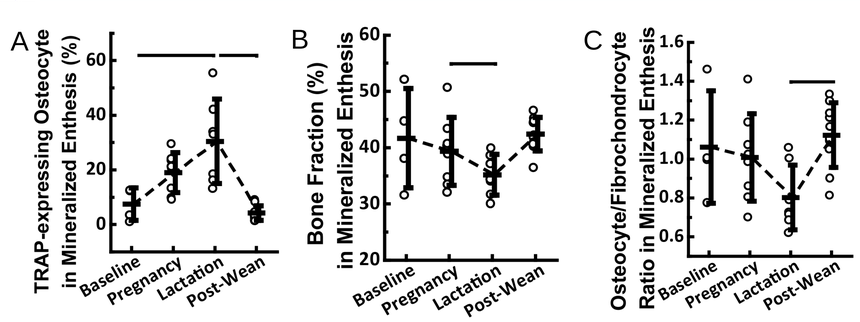


Fig 4. Quantifications of cellular activities within mineralized enthesis: (A) TRAP+ osteocyte fraction (%), (B) bone area fraction, (C) number ratio of osteocyte vs. fibrochondrocyte. (p<0.05: solid line indicates difference between two groups).

- **TRAP-expressing fraction of osteocytes** in bone (adjacent to the enthesis):
  - Rapidly increased from 8% (baseline) to 30% (lactation), peaking at 2-weeks after lactation
- **Fluorochrome mineralization labels** at the enthesis tidemark only found in the pregnancy group (Fig 3B)
- Within the enthesis, **bone area fraction** (Fig 4B) and **osteocyte/fibrochondrocyte ratio** (Fig 4C): 2wk post-wean group > 2wk lactation group

Conclusions & Discussion

Reproductive cycle causes striking changes in trabecular bone microstructure and cellular activities

- Substantial bone loss in the humerus during a reproductive cycle
  - At the epiphysis: only during lactation
  - At the metaphysis: as early as pregnancy
- Recovery after weaning:
  - At the epiphysis: complete recovery
  - At the metaphysis: deficits remain
- Striking changes observed at the location adjacent to enthesis
  - Mineral deposition at the enthesis tidemark during pregnancy, possibly activated by elevated progesterone [5]
  - Upregulated TRAP expression in osteocytes demonstrating increased osteocyte peri-lacunar remodeling (PLR) [6] at the subchondral bone during lactation

Acknowledgements

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References

[1] Kovacs, *Physiol Rev.*, 2015; [2] Watt+, *Post Reprod Health*, 2018; [3] Cucchi+, *Joints*, 2017; [4] Fung+, *SB3C*, 2019; [5] Bowman+, *JBMR*, 1996; [6] Qing+, *JBMR*, 2012.