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Modulating Wnt Signaling Pathway to Enhance Allograft Integration
in Orthopedic Trauma Treatment

PRINCIPAL INVESTIGATOR:

Amarjit S, Virdi, PhD

CONTRACTING ORGANIZATION:

Rush University Medical Center

Chicago, IL 60612-3839

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14. ABSTRACT The research project was designed to test a novel approach of modulating Wnt signaling pathway in the bone tissue repair by using monoclonal antibodies against sclerostin (Sost) and DKK-1 (donated by Amgen Inc., Thousand Oaks, CA under MTA). Since the previous annual report, the project has progressed at a rapid pace. We resolved all initial technical difficulties and successfully completed all the surgical procedures, harvested samples at prescribe time points and evaluated new bone formation at the allograft site using µCT scans and partially completed mechanical testing. Data presented in report reveals statistically that use of anti-Sost or anti-Dkk-1 antibodies enhances new bone formation around the allograft over all time points. Anti-Dkk-1 antibody treatment also seems to be superior to anti-Sost treatment. Mechanical testing of all available samples show increasing strength over time with Dkk-1-Ab being most effective. Freeze dried allografts performed better than fresh frozen allografts. Data obtained from this study supports our hypothesis.				
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Introduction

The scope of this project is to evaluate if the use of a novel anabolic treatment that targets the specific signaling pathways during osteogenesis that promotes bone healing will enhance the integration of allografts to the host bone in an animal model that simulates severe bone loss due to local trauma. In general, it is known that several different growth factors aid bone regeneration. In previous studies we have reported enhanced bone regeneration when growth factors, such as bone morphogenetic protein (BMP), are applied directly at the site of injury (1-10). It is also known that mechanical stimuli at the regenerate also accelerate the healing process. We and others have demonstrated that pulses of low intensity ultrasound, delivering mechanical stimulus, accelerates fracture healing (11-14). However, the focus of the proposed application is to employ a novel approach of modulating the LRP5/Wnt cell signaling pathway which is known to be critically involved in osteogenesis in order to repair large bone defects such as those experienced by soldiers in the battlefield due to ballistics related trauma to the extremities. Monoclonal antibodies raised against sclerostin and dickkopf-1 (Dkk-1) were proposed to be the test reagents employed to modulate the Wnt signaling pathway. An agreement with Amgen Inc. (Thousand Oaks, CA) was established for them to donate the reagents.

In order to carry out this research, we had proposed an animal model of segmental bone defect in the rat femur. In previous research projects in our laboratory we have employed this model to study the efficacy of combining BMP-2 and low intensity pulsed ultrasound to improve new bone regeneration in the gap. In the current study we had proposed to place an allograft in the created gap and to then treat the animals with systemic delivery of anti-sclerostin or anti-Dkk-1 antibodies for the prescribed period of time. The endpoints proposed were x-ray and μ CT imaging, mechanical testing and histology.

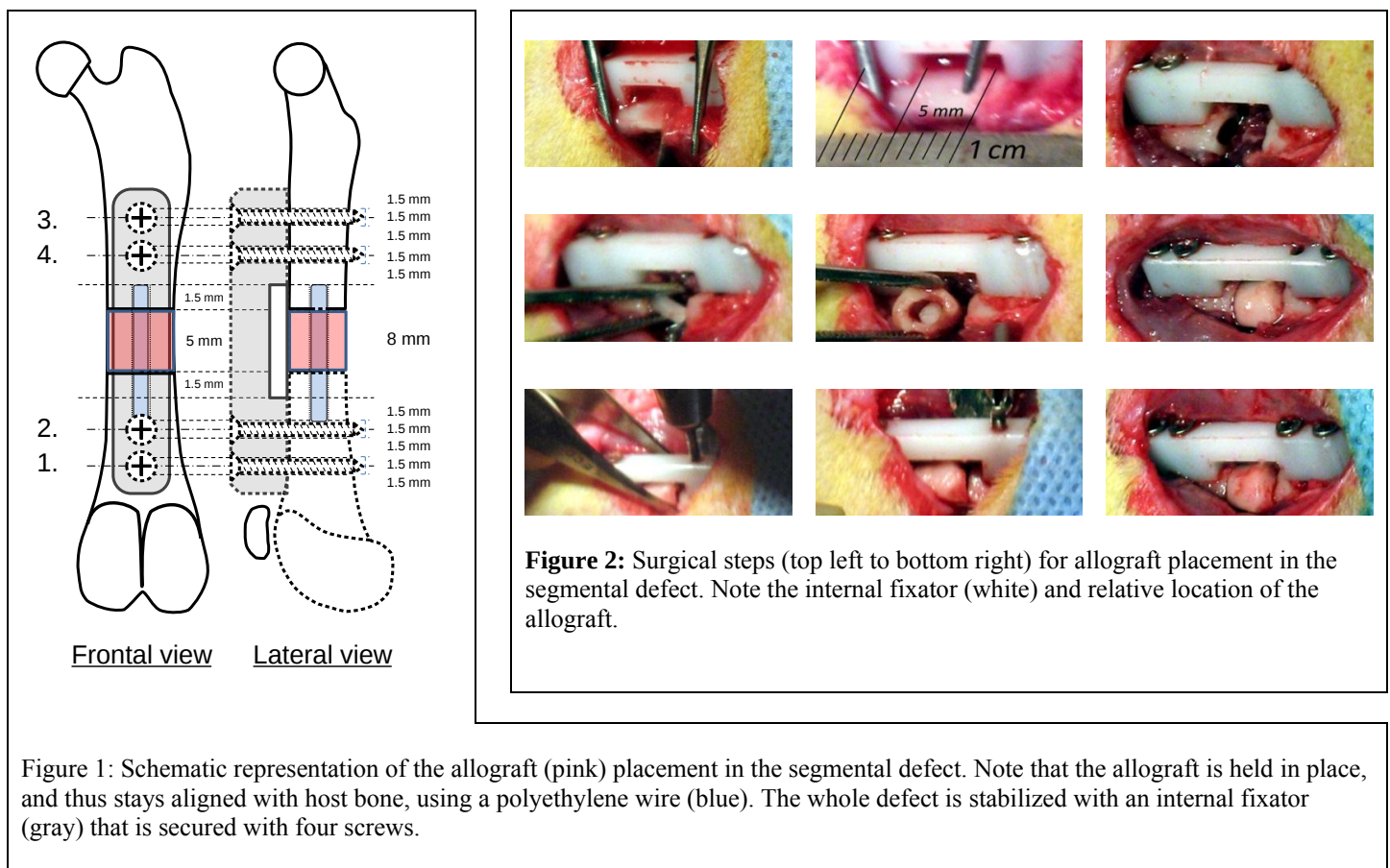
In addition to the text above (similar to last annual report), we have now completed the most time consuming portion of the project. All the surgeries, systemic treatments, sample harvesting, μ CT scanning and evaluation has been completed. Mechanical testing of the samples has also been completed. Histological evaluation has encountered technical issues but the results obtained from μ CT and mechanical testing provide support to our hypothesis.

We hypothesized that **neutralizing the LRP5/Wnt pathway inhibitors Sost or DKK1 with monoclonal antibodies will enhance allograft integration to the host bone**. The proposed work in this project was designed to test this hypothesis by addressing two specific aims.

- Aim 1:** Determine the effect of modulating the LRP-5/Wnt pathway with anti-Sost monoclonal antibody on allograft incorporation in a rat segmental repair model using radiographical, morphological and mechanical endpoints.
- Aim 2:** Determine the effect of modulating the LRP-5/Wnt pathway with anti-Dkk1 monoclonal antibody on allograft incorporation in a rat segmental repair model using radiographical, morphological and mechanical endpoints.

Within each these aims we proposed to use fresh frozen and freeze-dried allografts to emulate clinical scenarios where banked tissue available for use in patients is processed by these procedures.

Figures 1 and 2 depict our modified and working surgical model to ensure perfect placement of allograft that not only stays in place but also stays aligned with the host bone. This approach was critical in making sure that results from the study would be consistent and reliable.



Using this approach, we have completed all proposed surgical procedures. All groups have been treated with respective treatments for the stated duration. In vivo radiographs as well ex-vivo radiographs at the time of harvesting have also been completed for all samples.

We have analyzed all harvested bones from Fresh Frozen and Freeze-Dried allografts at 4, 8 and 12 week time points for saline, anti-Sost and anti-Dkk1 using μ CT scanning. Data is presented below. Quantitative output provides an extensive set of data but we have chosen to present the most relevant parameters that are reflected in the following outcomes.

- Total Volume (TV)** – this indicates the overall hard callus volume around the allograft and is suggestive of earlier healing events.
- Bone volume (BV)** – this indicates the amount of new bone formed around the allograft and represents the overall quantity and rate of bone regeneration. In general, higher BV correlates with better mechanical competence.
- Bone Volume over Total Volume (BV/TV)** – this outcome indicates the porosity of the new bone around the allograft.
- Bone Mineral Content** – this outcome indicates how much mineral is present in the healing area and correlates with bone density.

Pictures below (Figures 3, 4 and 5) show radiograph and μ CT images for representative samples from each group (Same as last annual report).

Figure 3: In vivo radiographs, ex vivo radiographs and μ CT scans for 4 week time point

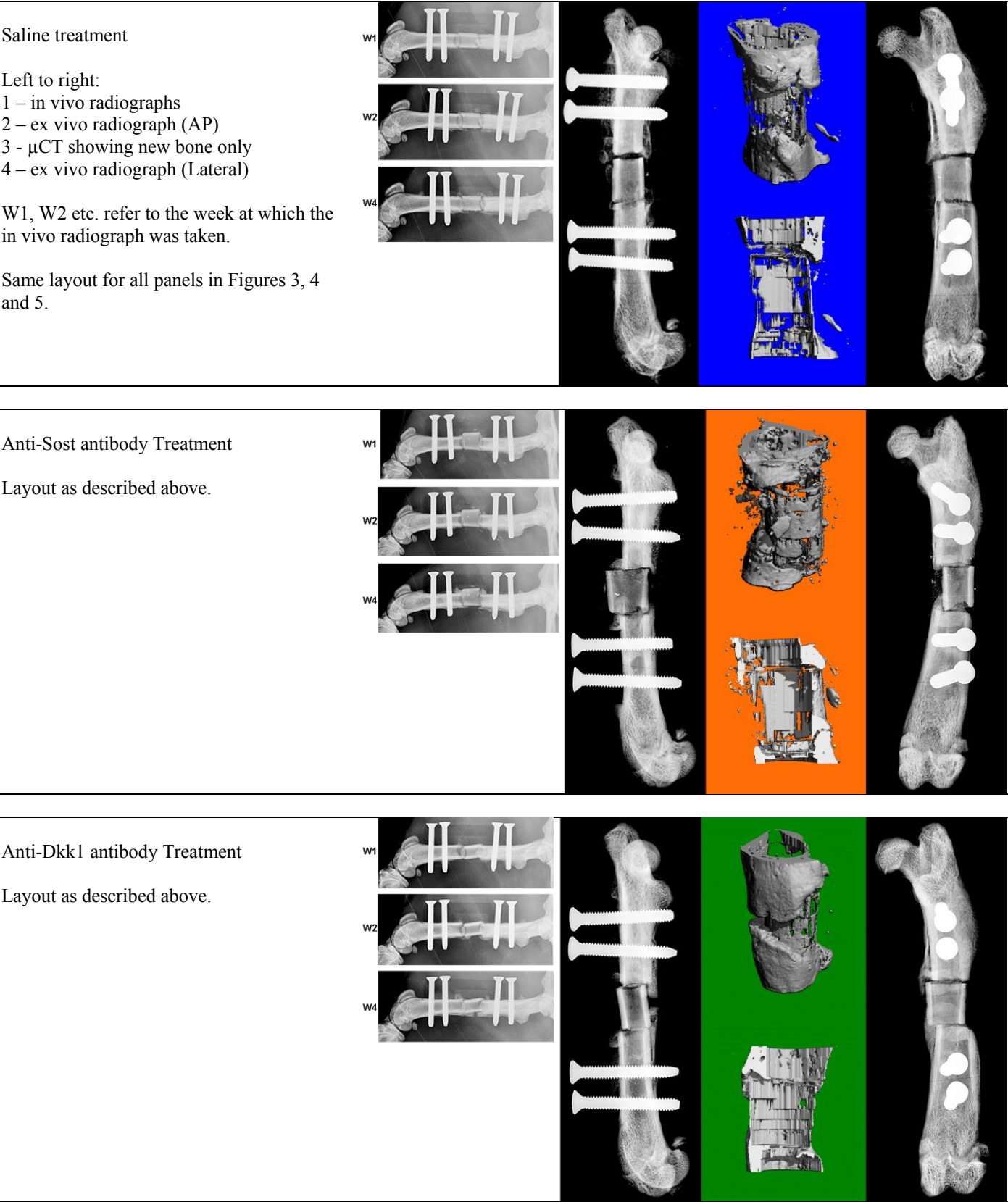


Figure 4: In vivo radiographs, ex vivo radiographs and μ CT scans for 8 week time point

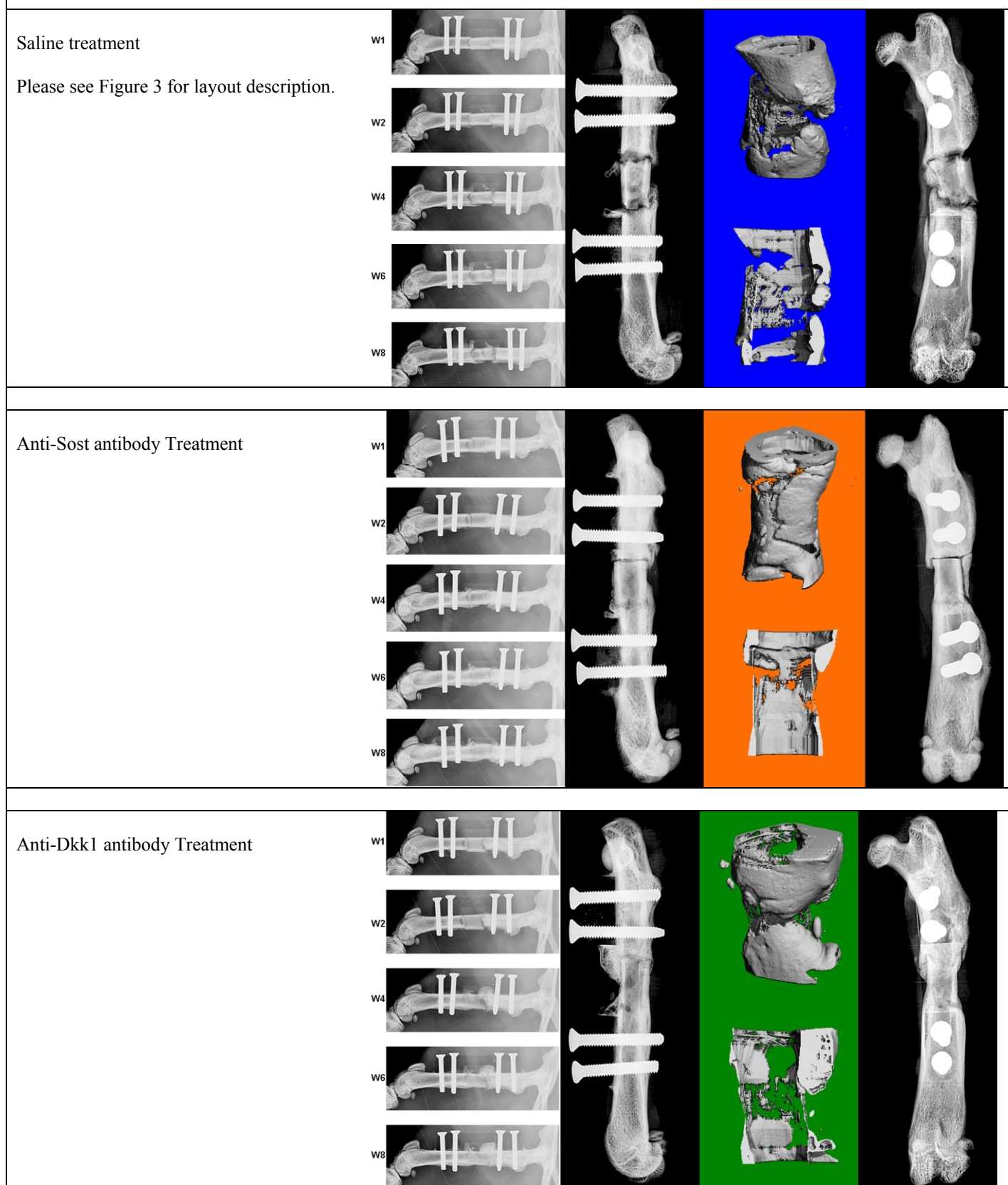
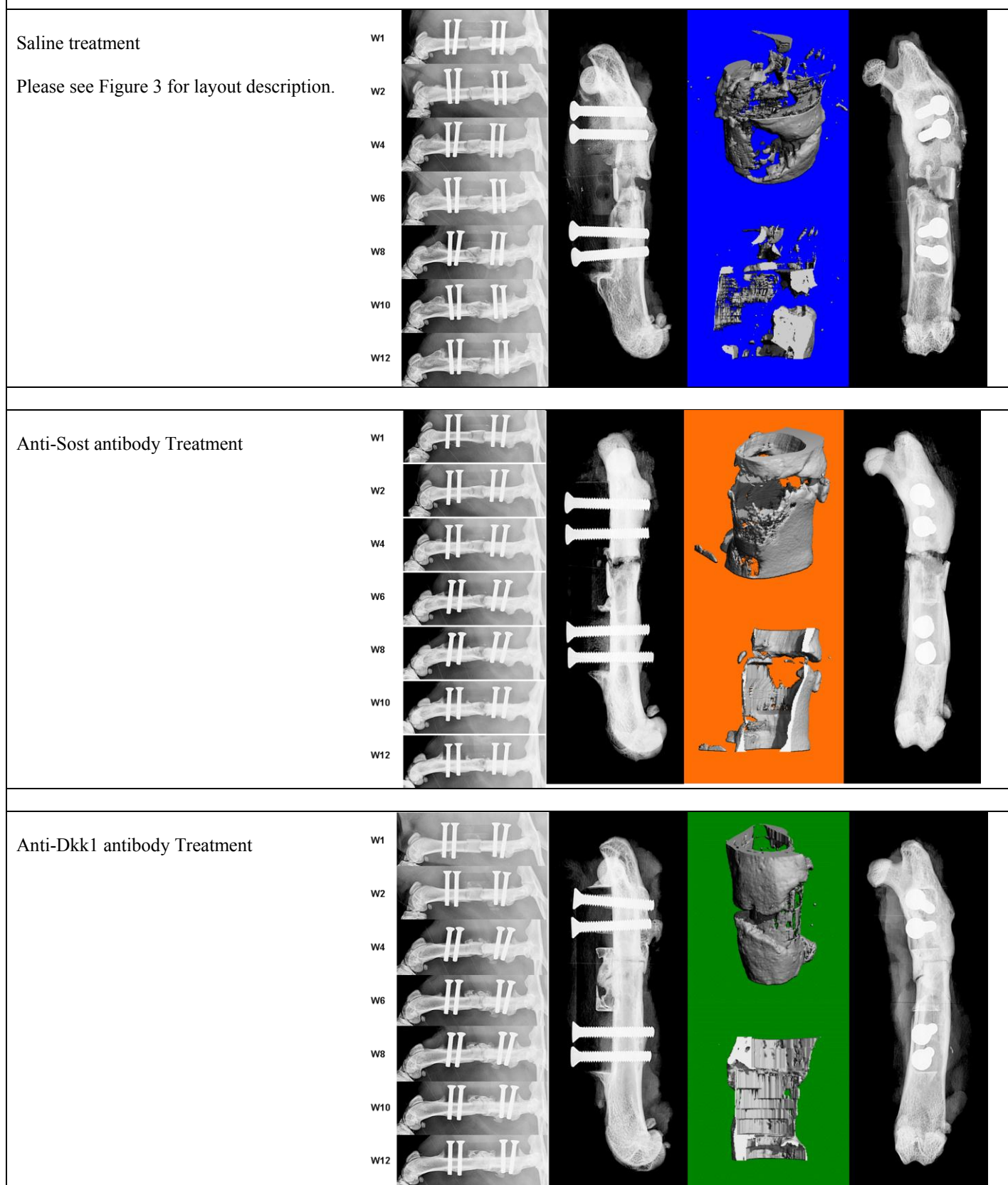


Figure 5: In vivo radiographs, ex vivo radiographs and μ CT scans for 12 week time point



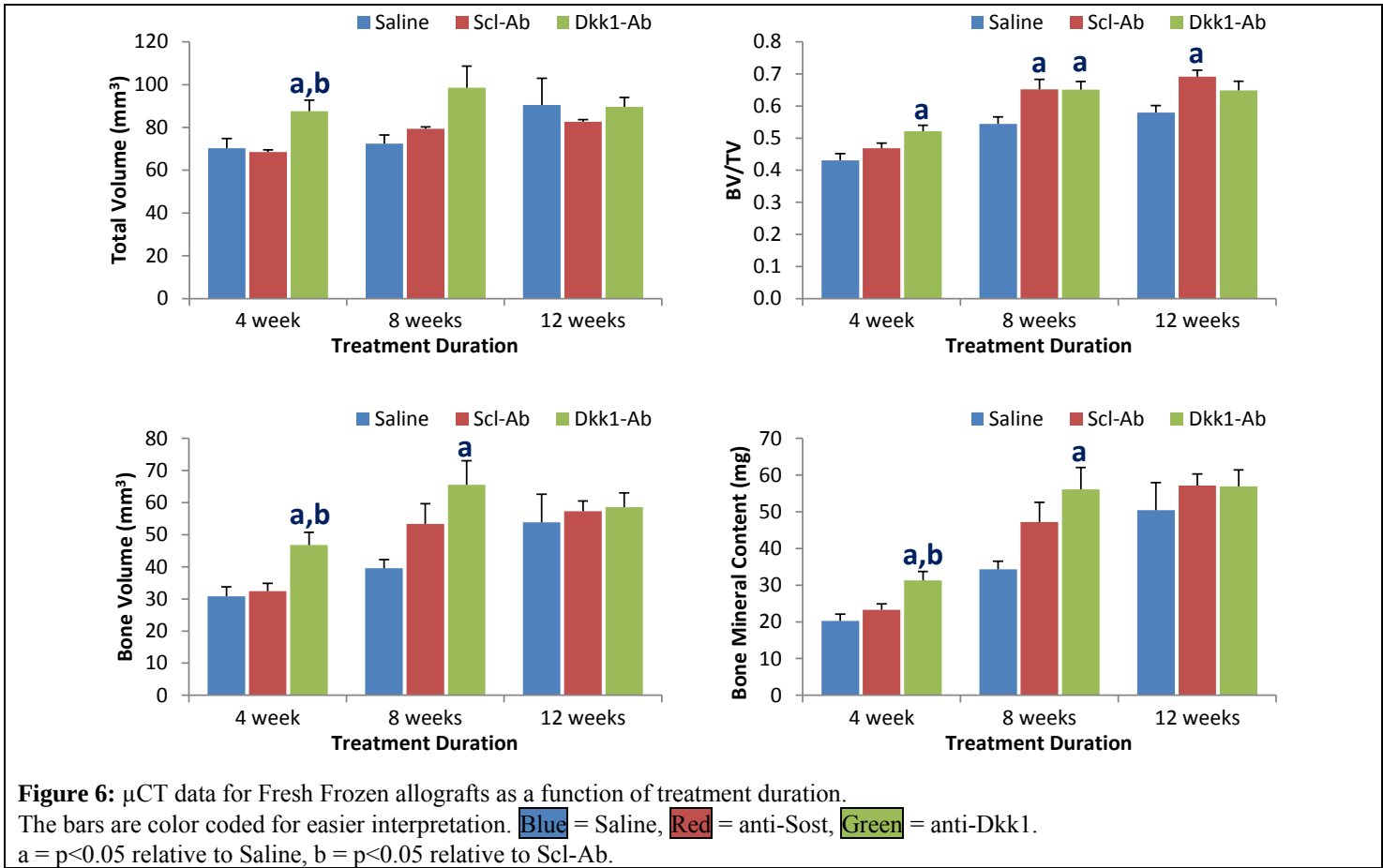
μCT evaluation data was analyzed for four most relevant outcomes as stated above. The data from Fresh Frozen and Freeze-Dried allografts are presented separately. Table 1 shows the number of samples analyzed for each graft, time point and treatment.

Fresh Frozen	Saline Treatment	Anti-Sost Treatment	Anti-Dkk1 Treatment
4 week	16	16	16
8 weeks	16	16	14
12 weeks	16	11	15
Freeze Dried	Saline Treatment	Anti-Sost Treatment	Anti-Dkk1 Treatment
4 week	15	14	15
8 weeks	13	14	16
12 weeks	14	14	15

Figures 6 and 7 depict graphs of μCT quantitative data for all treatments and time points from Fresh Frozen allografts. The data is presented as a function of time (Figure 6; 4 weeks, 8 weeks, 12 weeks) as well as function of treatment (Figure 7; Saline, Scl-Ab, Dkk1-Ab). Statistical analysis was performed on all groups and comparisons showing significance at p<0.05 is shown on the graphs.

In general, the data reveals that both anti-Sost and anti-Dkk1 antibody treatments enhanced bone formation (indicated by increase in BV, BV/TV and BMC) when compared with saline treatment. The mechanical testing has not been completed for all the samples but we expect that it will reflect the findings from the μCT data. If proven true, this would represent a practical means of enhancing repair of large bone defects in orthopedic trauma and can be translated into clinical practice in the near future.

Figures 8 and 9 depict graphs of μCT quantitative data for all treatments and time points from Freeze Dried allografts. The data is presented as a function of time (Figure 6; 4 weeks, 8 weeks, 12 weeks) as well as function of treatment (Figure 7; Saline, Scl-Ab, Dkk1-Ab). Statistical analysis was performed on all groups and comparisons showing significance at p<0.05 is shown on the graphs. Overall findings were similar to the Fresh Frozen allografts.



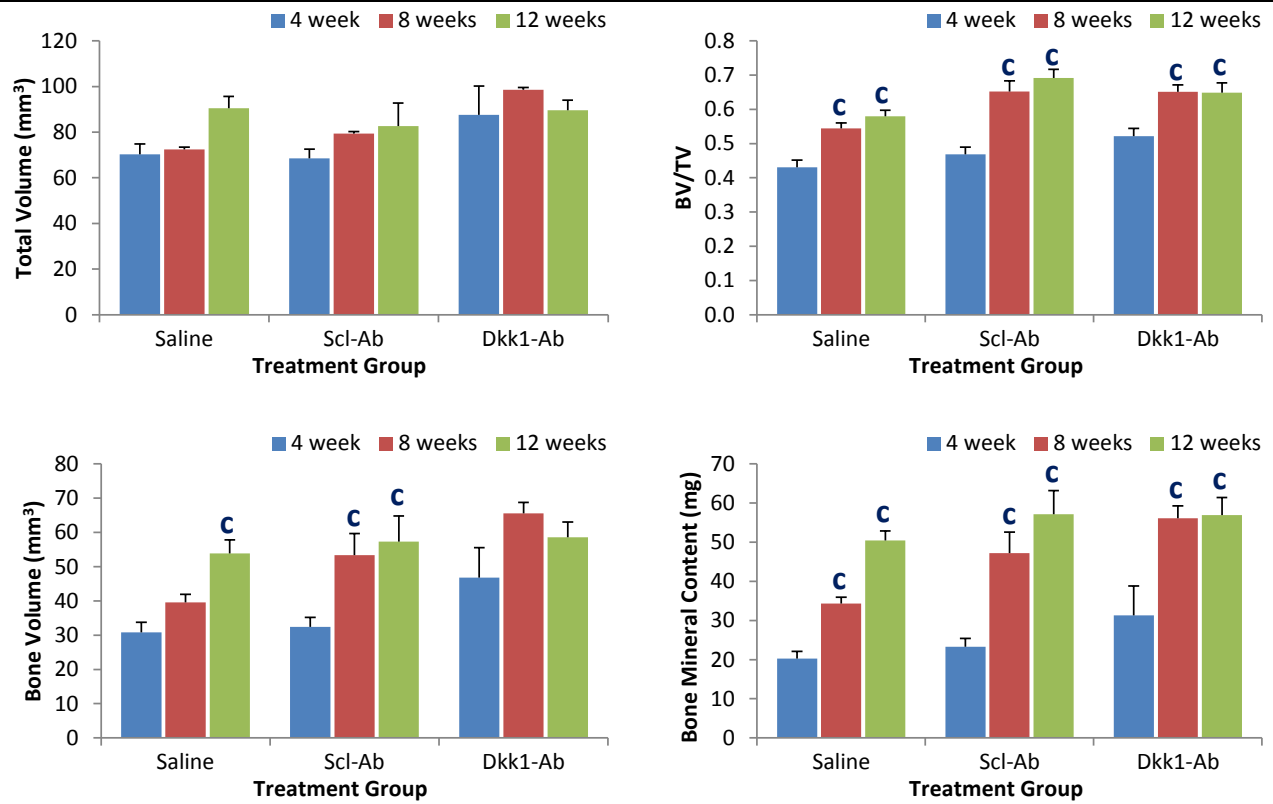


Figure 7: μCT data for Fresh Frozen allografts as a function of systemic treatment. The bars are color coded for easier interpretation. Blue = 4 weeks, Red = 8 weeks, Green = 12 weeks. c = p<0.05 relative to 4 Weeks, d = p<0.05 relative to 8 weeks.

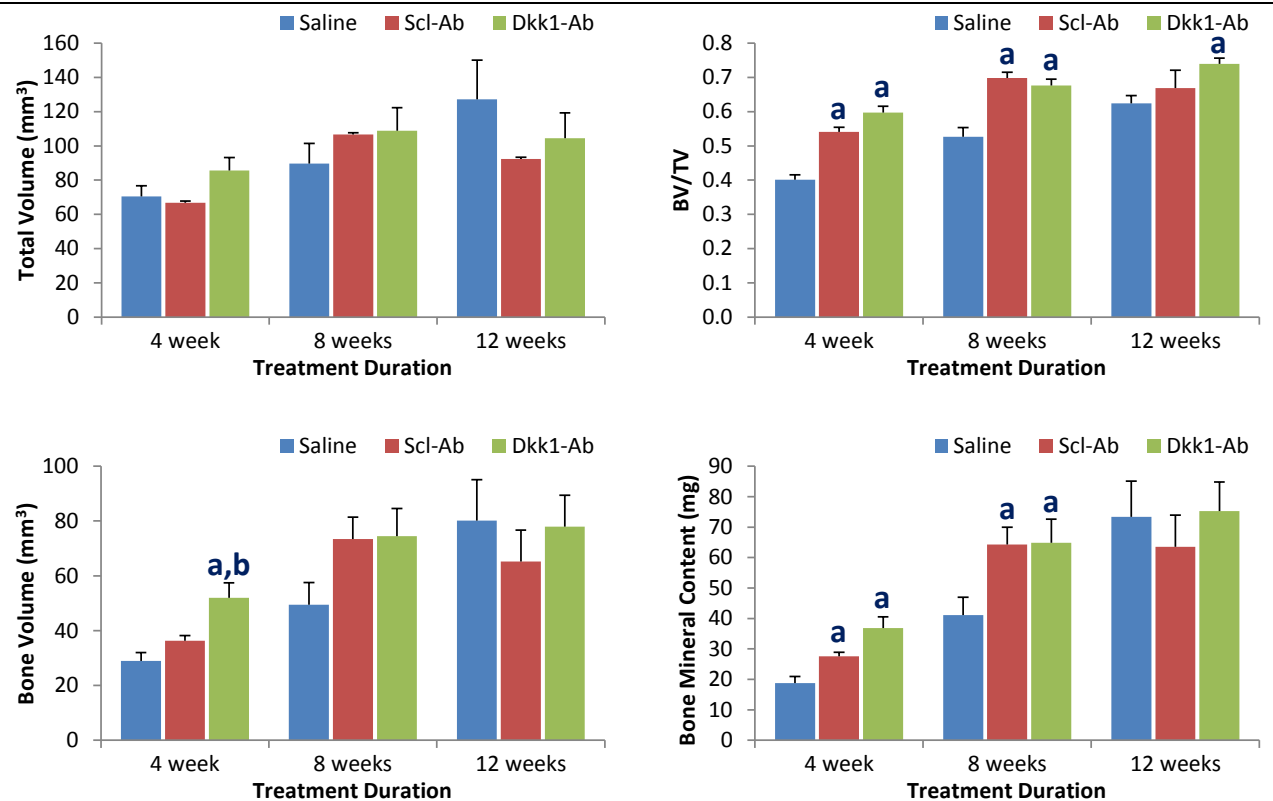


Figure 8: μCT data for Freeze Dried allografts as a function of treatment duration. The bars are color coded for easier interpretation. Blue = Saline, Red = anti-Sost, Green = anti-Dkk1. a = p<0.05 relative to Saline, b = p<0.05 relative to Scl-Ab.

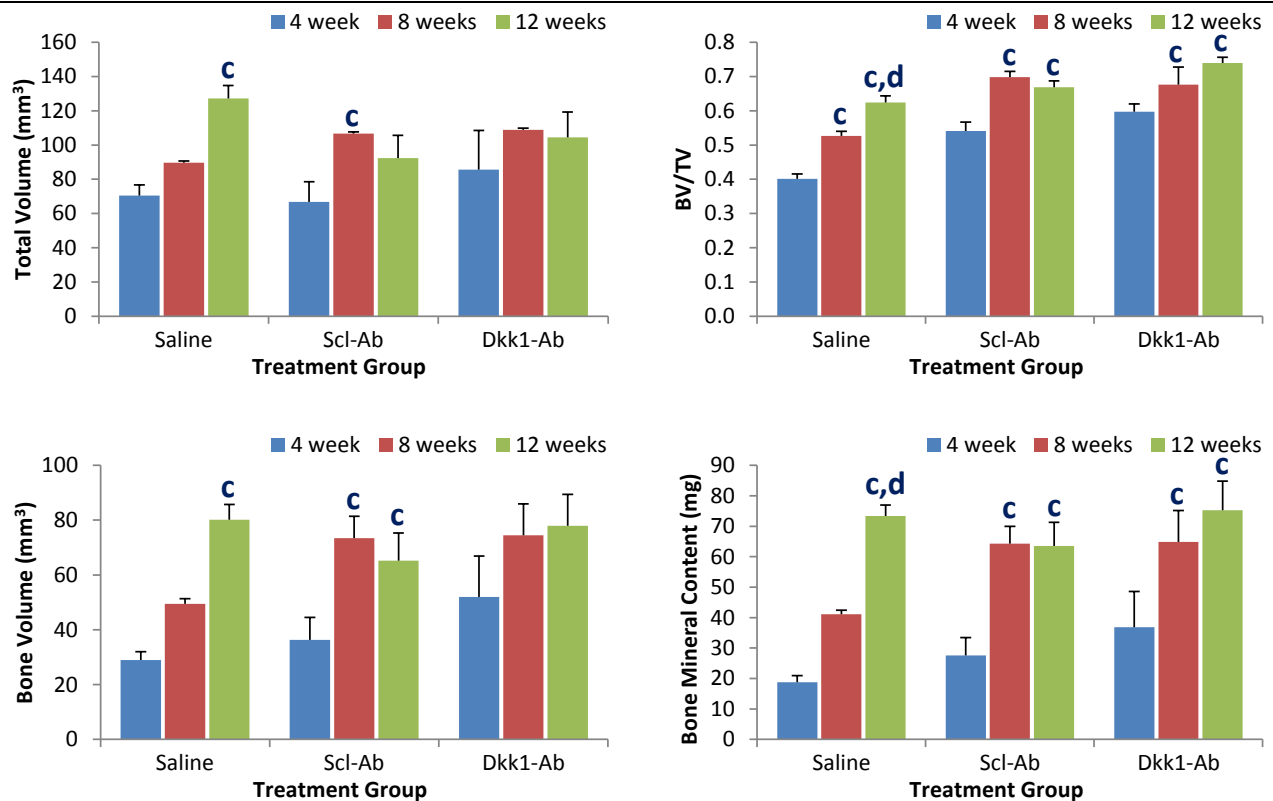


Figure 9: μCT data for Freeze Dried allografts as a function of systemic treatment. The bars are color coded for easier interpretation. Blue = 4 weeks, Red = 8 weeks, Green = 12 weeks. c = p < 0.05 relative to 4 Weeks, d = p < 0.05 relative to 8 weeks.

Figure 10 depicts the mechanical torsional test data in Fresh Frozen allografts. It is interesting to see that all treatments (including Saline) show time dependent increase in torsional strength (right hand panel) but Dkk1 treatment is exhibiting the greatest response. We had observed this with lesser samples in the previous report but now confirm the findings here. This observation is unexpected and is worth following in the future to investigate the true applicability of Dkk-1-Ab in restoring function.

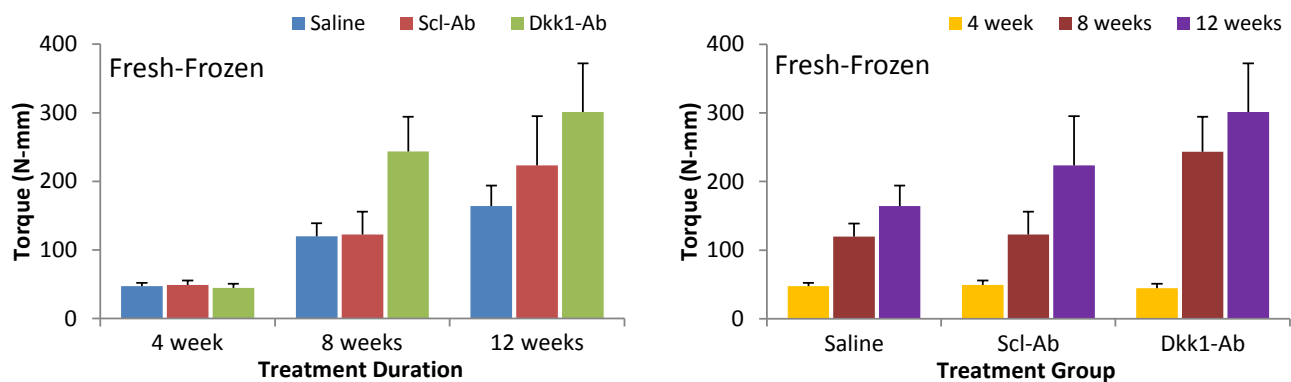
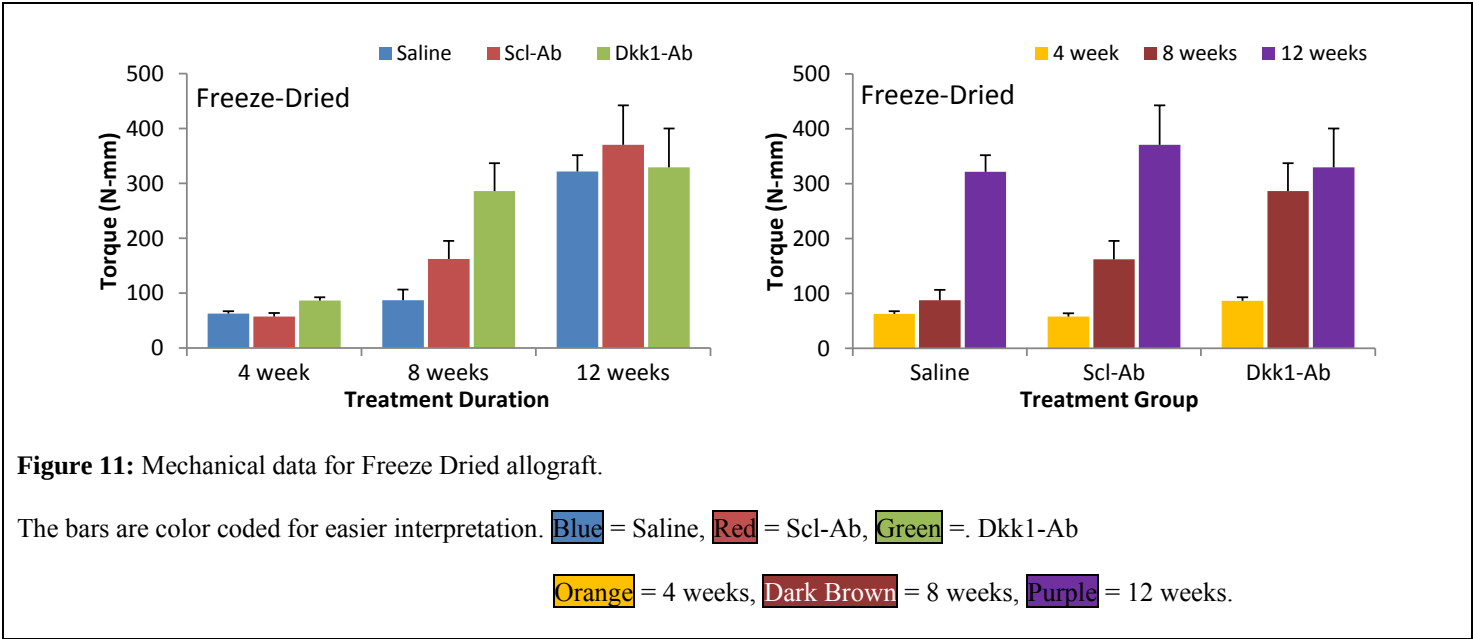


Figure 10: Mechanical data for Fresh Frozen allograft.

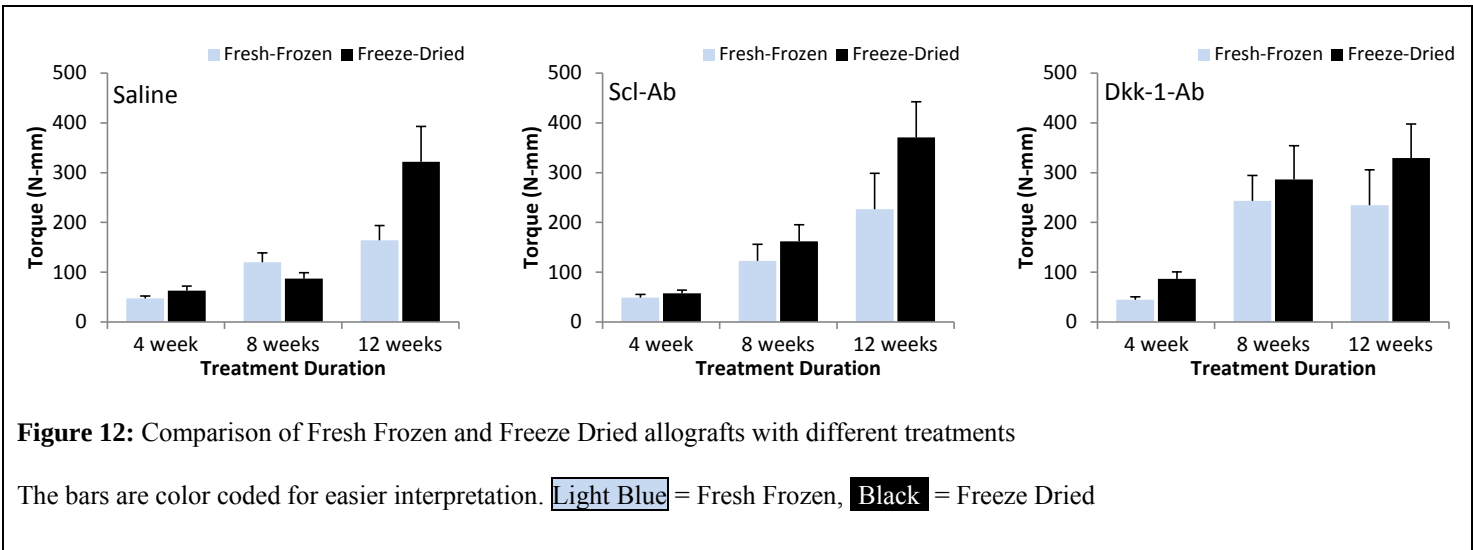
The bars are color coded for easier interpretation. Blue = Saline, Red = Scl-Ab, Green = Dkk1-Ab. Orange = 4 weeks, Dark Brown = 8 weeks, Purple = 12 weeks.

Figure 11 depicts the mechanical torsional test data in Freeze Dried allografts. As for Fresh Frozen allografts, there is a time dependent increase in mechanical strength for all treatments tested. Again, Dkk1 treatment is exhibiting the best response early on (8 weeks) but that advantage is not maintained at 12 week time point. This observation points to interaction between allograft material and the anabolic signal that in turn determines the cellular response at the repair site. In clinical terms, early enhanced bone repair and

return to function is desirable as it predicts the overall outcome in the long term. These novel findings will form the base of our future research direction.



We further analyzed the effect of allograft treatment as a co-variance. **Figure 12** shows the data comparison for each of the treatment for both allograft handling protocols. In general, the Freeze-Dried allograft performed better than the Fresh Frozen allograft. This difference was more pronounced at later time point and in Saline and Scl-Ab treatment. However, it is worth pointing out that although Dkk-1-Ab treatment did not show this effect clearly, the effect of Dkk-1-Ab treatment was earlier and rapid further supporting the observations in **Figures 10 and 11**.



Key Research Accomplishments

- All surgical procedures have been completed.
- All systemic treatments have been completed.
- All in vivo and ex vivo radiographs have been completed.
- All treated bones have been harvested.
- All harvested samples have been scanned by μ CT and evaluated for multiple parameters.
- All samples have been mechanically tested and data analyzed to reveal functional outcome.
- Histological evaluation was not successful due to issues encountered during embedding. We are attempting to recover the tissues and retry to get this information.

Reportable Outcomes

- None at this stage. Now we have reportable data and are planning to present it at scientific meetings and submit for publication in orthopedic related journals.

Conclusion

- The observations made based on μ CT and mechanical data indicate that modulating the LRP/Wnt signaling pathway with anti-Sost and anti-Dkk-1 monoclonal antibodies enhances new bone formation around allografts in a rat segmental defect model.
- Dkk-1 treatment was found to be effective at earlier time points suggesting accelerated healing and return to function.
- There was an effect of allograft processing protocol where Freeze Dried allografts showed better outcome compared to Fresh Frozen allografts. This has bearings on the handling and processing at tissue bank sites.

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Appendices

- None