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14. ABSTRACT: In this proposal we hypothesized that using a minced skeletal muscle autorgraft to fill a volumetric muscle defect in Yucatan Minipigs subjected to a segmental bone defect (SBD) combined with volumetric muscle loss (VML) would 1) improve fracture healing of the SBD; and 2) improve muscle function. We have operated on 5 pigs with isolated SBD; 7 pigs with SBD combined with VML; and 5 pigs with SBD and VML treated with minced skeletal muscle autograft (SMA). Our findings showed that SMA improved bone healing achieving union in 3 of 4 pigs and currently the 5th pig is on a healing trajectory. In contrast 5 of 6 VML pigs have developed nonunions and the 7th pig is still under evaluation. SMA has not improved muscle function at the time of sacrifice at 3 months but muscle strength was still improving in SMA pigs compared to VML pigs.

In summary, SMA improved bone healing and may improve muscle function. Detailed biochemical and histological analysis of the tissue will be conducted during a no-cost extension period from 11.1.2019 to 10.31.2020.

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1. INTRODUCTION: We investigated minced skeletal muscle autografting (SMA) in a miniature swine model to determine how well SMA improves bone healing and restores in vivo muscle function. The purpose of the investigation is to establish efficacy of muscle grafting in a pre-clinical model to translate into human use. We used a miniature swine model that created a 25 mm segmental bone defect (SBD) that led to delayed bone healing (negative controls). The experiment tested how the addition of a 7.0 gram volumetric muscle loss (VML) defect adjacent to the SBD impaired bone healing (positive controls). Subsequently, we determined how treating the VML with minced SMA restored bone healing in the segmental bone defect (experimental group). Finally, we determined how minced SMA affected in vivo muscle function in the experimental group compared to positive control animals with a SBD and adjacent VML which was untreated, and with negative control animals with isolated SBDs.

2. KEYWORDS

Segmental bone defect; Volumetric muscle loss; Skeletal muscle autografting; Nonunion; Mangled Limb; Muscle function

3. ACCOMPLISHMENTS:

Overall Project Summary and Major Goals: The project involved using a porcine SBD model with Yucatan Minipigs (YMPs) to determine how VML would affect bone healing. Subsequently, we quantified how treating the VML defect with minced SMA improved bone healing. In addition, we quantified how an isolated SBD, a SBD with an adjacent VML defect, and an SBD with a VML defect treated with SMA affected in vivo muscle function.

The final numbers of pigs that were successfully carried to sacrifice at three months included 5 YMPs with an isolated SBD [denoted SBD], 7 YMPs with a SBD and VML defect that was untreated [denoted VML] and 6 YMPs with a SBD and VML defect that was treated with minced SMA [denoted SMA]. Two pigs in the SBD group and one pig in the SMA group were lost due to early catastrophic fracture of their surgical limbs in the first two weeks after surgery.

We quantified bone healing by serial radiographs at monthly intervals and calculated modified Radiographic Union Score for Tibia Fractures (mRUST) as a measure of bone healing. In addition, we performed CT scans on the bone defects to validate plain xray results. Muscle function was quantified by monthly measurements of maximal dorsiflexion torque of the anterior compartment of the leg using a custom-built testing apparatus. Monthly testing was carried until sacrifice. Muscle samples were collected at sacrifice for histologic and biochemical analyses. The objectives of the project included:

1. Quantify the effects of VML on bone healing (Major Task 1: **Completed**)
2. Quantify the effects of a SBD on muscle healing and function (Major Task 2: **Completed**)
3. Quantify the effects of VML on muscle healing and function (Major Task 2: **Completed**)
4. Quantify the effects of minced SMA on bone healing (Major Task 4: **Completed**)
5. Quantify the effects of skeletal muscle autografting on muscle healing and function (Major Task 4: **Completed**)

Note, Major Task 3 which was centered around collecting serial wound aspirates was discontinued when the animal model was modified early in the project.

Key Research Accomplishments: In summary we have accomplished the following:

1. We quantified how VML impaired bone healing in a porcine SBD.
2. We quantified how minced SMA improved bone healing in a porcine SBD with adjacent VML.
3. We quantified how an isolated SBD impaired muscle function in a porcine SBD model.
4. We determined that the overwhelming cause of muscle weakness in the porcine SBD/VML model was the presence of the SBD. This was an unanticipated finding. The presence of VML adjacent to the SBD, whether treated or untreated did not affect muscle function.
5. We quantified tissue-level biochemical changes secondary to minced SMA in the VML defect.

Overall Accomplishments Details

1. *VML impaired bone healing.* (Figure 1). We used the modified radiographic union score for tibia fractures (mRUST) to quantify bone healing. Three observers made two randomized and independent observations of cortical healing scores on each of the four cortices (anterior; posterior; medial; lateral). Cortical scores are integers from 1 to 4 as follows: 1 = no callus observed; 2 = callus observed but no bridging callus; 3 = bridging callus; 4 = healed and remodeled bone. The overall mRUST is the sum of the four individual cortical scores and ranges between 4 and 16. Individual cortical scores of $\geq$ 3 are predictive of healing. Overall scores of $\geq$ 11 have moderate correspondence to bone healing and $\geq$ 13 are highly predictive of bone healing.

   Total mRUST scores and scores for each cortex were calculated at monthly intervals. Overall mRUST scores at three months were 10.7 (+/- 1.4) in SBD specimens compared to 9.4 (+/- 2.2) in VML specimens although the decrease was not statistically significant ($p = 0.187$) (Figure 1 upper left panel). Individual specimen trajectories (Figure 1 upper right panel) demonstrated that 4 of 5 SBD specimens (black lines) were $\geq$ 10.7 indicating a likely healing trajectory. In contrast, only 2 of 7 VML specimens (green lines) had individual healing trajectories.

   The majority of impaired healing secondary to VML occurred in the anterior cortex which was immediately adjacent to the location of the muscle defect (Figure 1; bottom left panel). The divergence in healing occurred between two and three months after injury with the three month anterior cortical score measuring 2.7 (+/- 0.7) in SBD specimens compared to 1.9 (+/- 0.7; $p = 0.003$). The VML defect had minimal effect on healing on the posterior, medial or lateral cortices (Figure 1 bottom row of panels).

   CT scanning confirmed mRUST results (Figure 2). Three of five SBD had bridging bone across at least one cortex and a fourth had nearly bridged with a small (< 0.1 mm) gap on reconstructed images. The top two images depict an SBD specimen with a clearly bridged lateral and anterior cortex (specimen 9117; upper left; mRUST = 12.3)) and the specimen with a very small residual gap on both CT images (specimen 9375; upper right; mRUST = 10.7). In contrast, only two of seven VML animals had bridging bone on CT scanning. One of the two healed VML specimens

is depicted on the bottom left panel (specimen 9409; mRUST = 12.3). The bottom right panel of CT images depicts the specimen with the highest mRUST score (specimen 9399; mRUST = 9.7) of the five unhealed VML specimens.

2. *Minced SMA improved bone healing in specimens with volumetric muscle loss.* (Figure 3) Minced SMA improved overall bone healing at both two months and three months after injury. At two months, SMA improved overall mRUST scores from 8.0 (+/- 1.5) to 9.2 (+/- 1.8; $p = 0.054$). By three months SMA improve overall mRUST scores from 9.4 (+/- 2.2) to 11.1 (+/- 2.6; $p = 0.050$). Individual trajectories in the upper right panel demonstrated three of six SMA specimens clearly healed at $\geq$ 12 and two more specimens $\geq$ 10 with potential healing trajectories. Again, the majority of healing benefit was observed in the anterior cortex immediately adjacent to the muscle defect and site of autografting (Figure 2 bottom left panel) measuring .

   CT scanning supported xray results with four of six SMA specimens with at least one cortex bridged (Figure 4). A fifth specimen was nearly bridged (gap < 100 to 200 microns). One specimen was not bridged. In contrast specimens with untreated VML defects had bridging cortex on only two of seven specimens and the other five specimens had no cortical bridging, all with large gaps. The top row of Figure 4 shows SMA specimens including four with bridging bone (spec numbers 63033, 62118, 61136 and 67063). Note on specimen 67063 adjacent images documented bridging of the circled piece of callus to the proximal segment (red dashed circle left image) and the distal segment (red dashed circle right image). Specimen 70015 had a minimal gap at three months. In contrast, only two VML specs had bridging bone and a third specimen, 9399, had a small gap.

3. *Isolated Segmental Bone Defects affect in vivo muscle strength (Figure 5).* While we anticipated that creating the VML defect would reduce muscle strength, we also demonstrated that over a three-month interval, an isolated 25.0 mm SBD with no muscle injury also had significant effects on muscle strength. In vivo dorsiflexion maximal torque was reduced to less than 25% of initial strength at one month after injury in SBD animals. By two months and three months, maximal dorsiflexion torque had improved to 46% and 55% of original intact strength. However strength deficits in SBD animals were just as severe as VML and SMA animals demonstrating that the presence of a SBD had an overwhelming effect on muscle strength.

4. *Minced skeletal muscle autografting did not improve muscle strength in specimens with SBDs and VML (Figure 5).* Muscle strength deficits in VML vs. SMA groups were not different at any time point. Animals treated with minced SMA also did not have strength improvements compared to the VML specimens. At three months, maximum muscle torque in VML and SMA animals were 53% and 49% of intact muscle strength respectively. Again, these strength deficits were similar to animals with isolated bone defects and no muscle injury (SBD animals; 55%)

5. *Minced Skeletal Muscle Autografting increased several muscle-based proteins in the healing tissue samples, but all three groups had significant fibrosis in the muscle adjacent to the SBD (Figure 6).* In a biochemical series of tests, we determined that minced SMA had greater concentrations of Myoblast Determination Protein-1 (MyoD), Tubulin β2, and nicotinic acetylcholine receptors (nACR) compared to SBD and VML specimens. In addition, trends toward greater concentration of myogenin were also measured.

   There were no differences in a panel of a panel of inflammatory cytokines or chemokines between any of the groups at three months.

6. *There was extensive fibrosis in all three groups on histologic analysis at 3 months.* All three groups demonstrated substantial fibrotic changes in muscle histologic features in retrieval specimens (Figure 7) demonstrating that a SBD without a direct muscle injury had profound effects on muscle content in adjacent muscle.

Training and Professional Development

In the course of the project, we had one undergraduate student, nine medical students and two postdoctoral students work on the project. The project offered a wide variety of opportunities for professional development for all of the participants. Everyone involved received training in animal surgery which encompassed all facets from presurgical preparation to postsurgical care. All of the medical students and the two postdoctoral students scrubbed into surgery and assisted the cases. Our two postdoctoral students did the majority of the surgical closing and wound care.

In addition, all of the team members assisted in muscle function testing. This was a fairly complex process involving computer-assisted data acquisition and precise instrumentation to facilitate accurate and reproducible data. Our two postdoctoral students took the lead on this part of the experiment.

Dissemination of Results

Standard venues for dissemination of findings including peer reviewed manuscripts and meeting abstracts were used to disseminate results.

4. IMPACT

Impact on Primary Discipline

The primary impact of this work is that it has demonstrated in a large animal model of a severe limb injury that minced skeletal muscle autografting improves bone healing. This is impactful for both Orthopaedic researchers and clinicians. Unfortunately, in this model, the muscle autografting did not improve strength. Our next step will be to investigate methods to improve functional muscle strength using SMA in VML defects with SBDs. We are developing adjunctive therapies to

mitigate the extensive fibrosis that occurs in the healing region. Interestingly, we did quantify how much an isolated bone defect affects adjacent muscle function both in injured and uninjured muscle. Fibrosis was clearly the pathologic component that affected muscle function the most and therapies to mitigate this will be investigated. Specimens with isolated bone injuries and no direct muscle injury had substantial fibrosis in the muscle also. This clearly depicts that bone-muscle interaction in the setting of a severe injury has reciprocal and significant effects on both tissues.

Our work is also impactful as it has established, to our best knowledge, the first large animal model that simultaneously has both a SBD and VML injury. This will be impactful for future DoD-based research as these are hallmark features of mangled limbs from blast injuries. We gained a lot of practical knowledge as we move forward with the model for future investigations, but the knowledge to date will help us and other interested investigators survey treatments for these devastating injuries.

Impact on Other Disciplines

The world of regenerative medicine is notably broad and our approach offers 1) a new large animal model to survey regenerative methods to grow bone and muscle; and 2) a novel approach to generate new skeletal muscle.

Impact on Technology Transfer

Nothing to report

Impact Beyond Science

Nothing to report.

5. CHANGES/PROBLEMS

Experimental Problems and mitigation strategies.

The primary problem we had was in model development. Early in the course of the experiment we were having consistent infections in the SBD manifesting through the surgical incision. We eliminated the custom spacer from the SBD and the infections were mitigated. In our foundational work, we did not have this problem and still do not fully understand the source of the problem. Fortunately, our SBD specimens healed at a rate consistent with our foundational work (McKinley TO et. al. Mil Med. 2020 Nov 26:usaa516. doi: 10.1093/milmed/usaa516. Online ahead of print.). All of the specimens that had the SBD spacer were eliminated from analyses and we started the experiment over without the spacer.

We also had several catastrophic early hardware failures. These clearly appeared to result from the pigs torqueing their legs while standing. All of the pigs that had early failure were > 80 Kg at the time of surgery. Two measures were instituted to mitigate this. We waited until small (< 70 Kg) pigs were available and we changed the floor configuration of the housing cages. Again, these pigs were eliminated from analyses.

Changes that had a significant impact on expenditures.

As noted above, we had to purchase additional animals due to problems with infection leading to modifying the model (loss of our first eight animals) and catastrophic failures of internal fixation (3 animals). The Principal Investigator covered the cost of additional animals with other funds

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Nothing to report.

6. PRODUCTS

Publications, conference papers, and presentations

Manuscripts

A. The primary results manuscript is under preparation. We are awaiting the final histology results to be complete. This will be one expanded manuscript that will include both bone and muscle data and will be targeted at several high-impact journals (Science and Translational Medicine will be the initial submission site).

B. McKinley TO, Natoli RM, Fischer JP, Rytlewski JD, Scofield DC, Usmani R, Kuzma A, Griffin KS, Jewell E, Childress P, Shively KD, Chu TG, Anglen JO, Kacena MA. Internal Fixation Construct and Defect Size Affect Healing of a Translational Porcine Diaphyseal Tibial Segmental Bone Defect. Mil Med. 2020 Nov 26:usaa516. doi: 10.1093/milmed/usaa516. Online ahead of print

C. McKinley TO, Childress P, Jewell E, Griffin KS, Wingerer A, Tucker A, Gremah A, Savaglio M, Warden S, Fuchs R, Natoli RM, Shively KD, Chu TG, Anglen JO, Kacena MA. Bone Morphogenetic Protein-2 Rapidly Heals Two Distinct Critical Sized Segmental Diaphyseal Bone Defects in a Porcine Model. In Revision Mil Med 2021.

Presentations

A. Todd O. McKinley MD; Benjamin T. Corona PhD; Melissa Kacena MD; Caio Staut MD; Stephen Mendenhall MD; Nicholas Diggins BS; Alexander Brinker BS; Roman Natoli MD PhD Skeletal Muscle Autografting Improves Bone Healing But Did Not Improve Muscle Function in a Porcine Model of Volumetric Muscle Loss with a Segmental Bone Defect. MHSRS 2021 Orlando

B. Natoli RM, Kacena MA, McKinley TO. Development and Characterization of a Porcine Model of Combined Segmental Bone Defect and Volumetric Muscle Loss to Examine

Longitudinal Muscle Strength Changes Resulting from Injury. Indiana Center for Musculoskeletal Health Bone and Muscle Interaction Symposium. Indianapolis Aug 2019

C. McKinley TO, Anglen J, Shively K, Brinker A, Childress P, Natoli R, Kacena M. Developing a Porcine Critical Sized Bone Defect Model for Mangled Limb Translational Research. Military Health System Research Symposium. Orlando. 2019

D. Natoli R, Childress P, Anglen J, Chu TG, Wininger A, Shively K, Kacena M, McKinley TO. BMP2 Accelerates Healing and Prevents Infections in Two Porcine Critical Sized Defect Models. Orthopaedic Trauma Association. Denver 2019

E. McKinley TO, Natoli RM, Kacena MA, Brinker A, Staudt C. Mendenhall S. Skeletal Muscle Autografting Improves Bone Healing In Pigs with Segmental Bone Defects and Volumetric Muscle Loss. OTA Annual Meeting Nashville, TN 2020

Technologies, Techniques, Patents, Inventions and Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals Working on the Project

A. Undergraduate Students (1)
Name(s): Alexander Brinker
Project Role: Assisted in all surgical aspects, muscle testing and postmortem tissue harvest
Time on project: 24 months

B. Medical Students (9)
Name(s): Amir Tucker; Adam Gremah; Zack Campbell; Zachary Gunderson; Anthony Perugini; Seungyup Sun; Michael Savaglio, Nicholas Diggins, Luke McVeigh.
Project Role: Surgical Assistance and postmortem tissue harvest
Time on project: 6 to 24 months

C. Postdoctoral Students (2)

Names: Stephen Mendenhall (PGY5 Neurosurgery); Caio Staut (PGY3 Orthopaedic Surgery)
Project Role: Surgical Assistants and lead role in functional muscle testing
Time on project: Dr. Mendenhall 12 months; Dr. Staut 24 months

Partnering Organizations

We have established direct collaboration with Drs. Christopher Dearth and Stephen Goldman at the Uniformed Services University with this project. Based on the findings of this study, Drs. Dearth and McKinley submitted a proposal which has been successfully funded by the Peer Reviewed Medical Research Program to investigate adjunctive therapies to reduce fibrosis and improve muscle function in the VML defect. Also in this collaboration, muscle biochemistry and histology analyses for the current project were conducted in Dr. Dearth's laboratory.

8. Special Reporting Requirements

Collaborative Awards: nothing to report

Quad Chart: attached

9. Appendices

Figures 1 through 7 and figure captions attached.