TITLE: Investigation of a Translatable Animal Model in Order to Understand the Etiology of Heterotopic Ossification

PRINCIPAL INVESTIGATOR: Dr. Brad M. Isaacson, PhD, MBA, MSF

CONTRACTING ORGANIZATION: Henry M. Jackson Foundation for Advancement of Military Medicine
Bethesda, MD 20817

REPORT DATE: September 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: W81XWH-16-2-0037
Organization: Henry M. Jackson Foundation for Advancement of Military Medicine

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

________________________________________  ____________________________
<table>
<thead>
<tr>
<th>4. TITLE AND SUBTITLE</th>
<th>5. a. CONTRACT NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investigation of a Translatable Animal Model in Order to Understand the Etiology of Heterotopic Ossification</td>
<td>W81XWH-16-2-0037</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>6. AUTHOR(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brad M. Isaacson PhD, MBA, MSF; Dustin L. Williams, PhD; Paul F. Pasquina MD; Roy D. Bloebaum PhD.; Kyle Potter MD; John Shero, MHA</td>
</tr>
<tr>
<td>E-Mail: <a href="mailto:brad.isaacson.ctr@usuhs.edu">brad.isaacson.ctr@usuhs.edu</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Henry M. Jackson Foundation for the Advancement of Military Medicine</td>
</tr>
<tr>
<td>6720-A Rockledge Drive, Suite 100, Bethesda, MD 20817</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.S. Army Medical Research and Materiel Command</td>
</tr>
<tr>
<td>Fort Detrick, Maryland 21702-5012</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>14. ABSTRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Our team has spent the first year of this grant obtaining all of the necessary approvals (primary site and HRPO institutional animal care and use committee (IACUC)), executing a subaward agreement with the University of Utah, procuring supplies, designing and implementing an air impact device (AID), piloting with carcasses, ensuring the noise from our AID could be attenuated and not disturb other animals in the facility, and initiating the beginning phases of animal work. The majority of animals are still being monitored and tissues are being processed for those that have reached the endpoint.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>15. SUBJECT TERMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recruitment, infrastructure, patient identification</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>16. SECURITY CLASSIFICATION OF:</th>
<th>17. LIMITATION OF ABSTRACT</th>
<th>18. NUMBER OF PAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. REPORT U</td>
<td>b. ABSTRACT U</td>
<td>c. THIS PAGE U</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>19a. NAME OF RESPONSIBLE PERSON</th>
</tr>
</thead>
<tbody>
<tr>
<td>USAMRMC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>19b. TELEPHONE NUMBER (Include area code)</th>
<th>19c. NUMBER OF RESPONSIBLE PERSON</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Include area code)</td>
<td>19c. NUMBER OF RESPONSIBLE PERSON</td>
</tr>
</tbody>
</table>
Table of contents

1. Introduction..............................................................................................................4
2. Body.........................................................................................................................4
3. Key Research Accomplishments .............................................................................16
4. Reportable Outcomes and Conclusions ................................................................17
5. References ..............................................................................................................17
6. Appendix ................................................................................................................N/A
1. Introduction
Heterotopic ossification (HO) refers to ectopic bone formation, typically in residual limbs and/or peri-articular regions following trauma and injury.\(^1\) This pathological process manifests outside of the skeleton\(^2\) and is comprised of a hybrid of cortical and cancellous bone.\(^3\) HO was first reported by El Zahrawi (Albucasis) in 1000 C.E. in which he noted that stony hard prominences occasionally developed during fracture healing and demanded urgent removal.\(^4\) While the etiology of HO has not been elucidated in the nearly 1100 years since its initial observance,\(^5,6\) there has been a general agreement in the orthopedic literature that HO is induced from damage to soft tissue and inflammation;\(^5,7\) ectopic bone growth has been most frequently observed after combat-related trauma to service members with blast injuries.\(^8\)

Reviews of orthopedic injuries from Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF) have reported that approximately 70% of war wounds have involved the musculoskeletal system,\(^9\) largely from the use of IEDs and RPGs. Given the intense nature of blast injuries, which require rapid tourniquet use, debridement and surgical intervention, HO has been reported to occur in approximately 63%-65% of wounded service members with limb loss or major extremity injuries.\(^10\)-\(^12\) Reports of recent OIF and OEF combat-related amputees with known HO have indicated that approximately 20-40% of affected patients required surgery to excise their bony masses.\(^12\)-\(^15\) Symptomatic HO may delay rehabilitation regimens since it often requires modifications to prosthetic limb componentry and socket size.\(^13,16\)

Most concerning is that no empirical evidence has indicated a mechanism for quelling or preventing metabolically active HO.\(^1\) Correlative factors such as gender,\(^1,17\) genetics,\(^7,18-20\) bioelectric signals,\(^7\) infection,\(^21\) and age\(^17\) have been associated with ectopic bone growth, but studies have often lacked histologic corroboration and advanced radiologic quantification.\(^22\) Extensive research by our team of military physicians, bone biologists and rehabilitation experts have observed several common factors that may act as catalysts for inducing HO: (1) a blast injury which displaces bone fragments, (2) tourniquet and negative pressure wound vacuums usage at the time of injury and (3) a post-traumatic infection signal. Further, no study to date has included the assessment of these factors individually or in combination using a singular translatable large animal model to determine what clinical catalyst(s) initiates HO.

Ectopic bone formation has been induced in various animal models which include: rats, rabbits, dogs and sheep.\(^23,24\) However, as noted by Kan and Keller, “The incidence of implantation-induced bone formation varies depending upon the material or animal species.”\(^66\) While rats and rabbits are the most commonly used animals for HO research, their MARs are 600% and 40% higher than humans, respectively.\(^25,26\) This is concerning since HO has been well documented to be more metabolically active than non-pathological osseous tissue,\(^1,3,27-30\) and a higher MAR at the start of an experiment (as compared to human bone) could become exacerbated over time, thereby creating more confounding variables and further limiting translational research initiatives. The most practical model, and one that is highly understudied, is the ovine. Sheep have nearly identical MAR levels\(^31\) and bone ingrowth into intramedullary implants\(^32\) as to that of humans and closely replicate the clinical condition. Further, the development of a large animal model (ovine) will address what Forsberg et al. noted in *Burned to the Bone* that “one of the challenges preventing advances in this field has been the lack of robust animal models for HO.”\(^33\)

2. Body
2.1 Carcass Testing for Model Development
The first step in this HO model was to perform carcass testing to fine tune the air impact device (AID) that was selected, develop frames/structures to hold the equipment, and ensure that the percussion blast could be directed toward a hind limb. As such, a support frame was welded together to which carcass legs could be attached (Figure
1). To secure a limb, holes were drilled through the femoral head region and distal tibia, then bolted to the frame. Initial blasts indicated that the drill points created crack propagation and resulted in fracture of the femur. To mitigate the stress points, we secured a brace and placed it in the mid region of the limb to prevent flexion and fracture (Figure 1). Recognizing that this is preliminary data, we envision use of a funnel shaped or other brace that can be secured to a sheep limb in live animal studies to similarly prevent fracture or ligament damage. We will continue to optimize the brace and plan to test blast scenarios in whole sheep carcasses to validate its effectiveness.

As outlined in our IACUCs, there are several sheep groups that will require an incision to be made (Figure 2) prior to the blast being performed so that, for example, periosteum can be disrupted or bone chips can be placed in apposition to the bone. The incision line will be sutured closed prior to the blast. Thus, it was necessary to confirm that when closed, the suture line would remain intact during and after the blast.

As part of this preliminary work, an additional aspect of testing was performed to compare the blast data that was obtained to previously published data wherein blunt force trauma was used in a sheep model with poor outcomes. More specifically, Walton et al. published a study wherein they used a 3.5 kg (~8 lb) weight and dropped it on the midshaft femur region of sheep from a 1 meter height in an attempt to induce HO. An important limitation of their work was that no data was presented that outlined the force created by dropping the 3.5 kg weight. Thus, to determine that force, we dropped a ~3.5 kg weight from a 1 meter height onto a force plate and repeated it n=10 times.
Still shots of the blast scenario are provided (Figure 3) to demonstrate that using the frame and support brace, the limb remained intact and secured. In addition, it was confirmed that when the AID was pressurized to 80 PSI and with a force of approximately 750 N, the suture line remained intact during the blast (Figure 4).

**Figure 3:** Still shots from AID testing on a cadaveric sheep limb. Note the limb is still intact and attached to the metal stand at all times.

**Figure 4:** The suture line that had been secured prior to the blast (Before AID) remained intact after the blast (After AID).

With this model, the objective is not to create a fracture or break bone, but to provide a simulated blast and assess other factors that may contribute to HO formation. As such, it was important to confirm that the blast scenario (with the brace being used) did not fracture bone. Thus, following blast work, femurs were dissected and contact radiographs collected to assess for breaks. As shown in Figure 5, no fractures or damage were observed.
Figure 5: Example radiograph from specimen #3 (Figures 2 – 4) showed no signs of gross fracturing or damage to the femur (blue arrow). The radiolucency seen at the left end of the bone (red arrow) was damage caused by the bolt used to secure the limb to the metal frame. In future tests, the use of a bolt will be eliminated as we continue to optimize the parameters for live animal testing.

Comparative data collected after dropping the ~3.5 kg weight from a height of 1 meter indicated that the blunt force resulted in approximately 180 N of force, whereas the blast resulted in a force of greater than 750 N (Figure 6). Decibel (dB) was also collected for comparison.

Figure 6: Data indicated that an approximately 3.5 kg weight being dropped from a 1 meter height resulted in 180 N of force. In contrast, the blast resulted in greater than 750 N of force.

In summary, these data indicated that the use of an air blast will have greater chance of replicating a battlefield scenario while having greater potential to replicate forces that may be involved in such a blast without compromising bone integrity.

2.2 Noise Attenuation and AID Testing
The IACUC committees also requested that we attenuate the decibel (dB) levels of the AID that we have proposed to use so as not to disturb other animals in the facility. To attenuate these levels on a proof-of-concept basis, we tested polyurethane foam. To test its ability to reduce noise, we placed the materials in varying locations including on the walls and doors of the room and building in which the AID is being tested. All tests were performed with the AID set at a PSI of 80. Initially, to provide a baseline of the dB levels that were made by the AID without attenuation, we performed this testing in a cinderblock-based room with metal ducting, cement floors and steel-framed ceilings (Figure 7).
To measure dB levels without attenuation, a Reed Sound Level Meter (time Weighting 125 milliseconds) was used. Without any form of attenuation and at a distance of 3 feet from the AID, the dB levels had an average of roughly 120 dB, but reached a maximum of 124 dB. Immediately outside of an open door to the room (approximately 7 feet away from the AID), the noise level was 115 dB. Ear protection was worn by personnel to meet OSHA requirements. After obtaining the baseline, to test the attenuation capacity of polyurethane foam, we first lined the walls directly across from the AID with foam pads (Figure 8).

With this foam in place and the door left open, the level of noise immediately outside of the door was 110 dB, an attenuation of 5 dB. The next test was to line the walls and door (Figure 3) of the AID room with polyurethane, then close the door. When this was done, the noise level immediately outside of the room was reduced to 95 dB, an attenuation of 15 dB.

Next, the dB meter was moved 50 feet down the corridor of the building and the same test performed, i.e., foam lined the walls of the room (Figure 8) and the door (Figure 9). The door to the room that had the AID in it was
closed. With the dB meter 50 feet away, the dB level was 88. To provide context to this level of noise, we measured the dB level of a barking dog (behind a closed door) housed at the CMC with the dB meter approximately 50 feet down the hallway. The noise level of barking was between 80 – 85 dB.

The last test we performed to determine the capacity of polyurethane foam attenuation was to remove the polyurethane from the walls, then line the door of the AID room (Figure 9) and the entrance door of the building with polyurethane (Figure 10). The dB levels were measured immediately outside the building’s entrance door. More specifically, the dB meter was placed approximately 4 feet away from the building’s entrance door. With this setup, the dB meter measured the noise levels behind two closed doors that were both lined with polyurethane. This test resulted in a noise level of 64-66 dB. For comparison, a semitruck that was backing up (beeping signals) 30 yards away from the dB meter had a noise level of 72 dB. Similarly, when our group stood in the building and spoke to one another in regular voices with the dB meter 2 feet away, the noise level was 72 dB.

Lastly, for comparison, the noise level behind the two aforementioned closed doors without being lined by polyurethane was 92 dB. As such, when both doors were lined with polyurethane and kept closed, an attenuation of approximately 30 dB could be achieved. And when compared to the baseline dB levels with no doors being closed, an attenuation of approximately 60 dB could be achieved (see above: noise level of the AID without attenuation was 124 dB).

![Figure 10: Entrance door to the building covered with polyurethane foam.](image)

**Table 1:** Representative data of dB readings taken with, for example, number of doors closed, lined or unlined with polyurethane, or left open.

<table>
<thead>
<tr>
<th>Pressure (psi)</th>
<th>Location of dB Meter</th>
<th># of Doors Closed</th>
<th>Foam on Closed Doors (Y/N)</th>
<th>Foam on opposite wall (Y/N)</th>
<th>dB</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>In Room</td>
<td>0</td>
<td>N</td>
<td>N</td>
<td>124</td>
</tr>
<tr>
<td>80</td>
<td>Outside Room</td>
<td>0</td>
<td>N</td>
<td>N</td>
<td>115</td>
</tr>
<tr>
<td>80</td>
<td>Outside Room</td>
<td>1</td>
<td>N</td>
<td>N</td>
<td>98</td>
</tr>
<tr>
<td>80</td>
<td>Outside Building</td>
<td>2</td>
<td>N</td>
<td>N</td>
<td>92</td>
</tr>
<tr>
<td>80</td>
<td>Down corridor 50'</td>
<td>1</td>
<td>Y</td>
<td>N</td>
<td>88</td>
</tr>
<tr>
<td>80</td>
<td>Outside Building</td>
<td>2</td>
<td>Y</td>
<td>N</td>
<td>64</td>
</tr>
</tbody>
</table>

As outlined in Table 1, we were able to determine that with the use of polyurethane foam, dB levels could be attenuated to less than 70 dB by separation of two closed doors that were lined with foam. These levels are similar
to the noise level of human conversation, a beeping truck approximately 30 yards away and lower than the collective barking of dogs.

In addition to the above data, dB levels and attenuation have also been assessed in the CMC facility where the actual procedures are performed. In the operating room (OR), noise levels are approximately 124 dB. Behind three closed doors to the nearest animal room, the dB were 86, but attenuated to less than 60 dB with ear protection. The noise data was assessed and approved by the University of Utah’s Environmental Health and Safety (EH&S) committee as well as the CMC director, Dr. Roger Van Andel. We confirmed that noise levels were below 70 dB in those rooms that contain breeding rodents, which reduces risk that the AID will affect studies that are being performed in surrounding regions. This was an important consideration for neighboring PIs. In short, dB levels were measured carefully by multiple noise meters and multiple individuals to confirm safety to personnel and reduced the risk of disruption to surrounding studies.

2.3 Initial and Current Animal Testing
Following optimization of the AID setup (Figure 11A) the work advanced from in vitro and ex vivo testing to live animals where we could assess factors that may be contribute to HO. All work has been performed under IACUC and ACURO approvals. In the first sheep, a blast only was performed in an effort to make a step-wise approach into the study. A blast only with the AID allowed for assessment as to how sheep would respond to the AID. This also allowed the animal care staff and research team to confirm that the pain management regimen would allow animals to function normally. To date, n=4 sheep have received the blast only treatment and they responded well with the ability to function normally (see Table 2). Each of these 4 sheep reached the endpoint and tissues are being processed to assess for HO formation.

Figure 11: Photographs of the setup to perform AID testing, surgical approach and tourniquet placement. (A) Photo of the AID (blue arrow) used to discharge high-pressure air to a sheep limb. A modular stage (green arrow) is used to support the sheep limb as it is situated below the AID to absorb the shockwave trauma. (B) Photo of how a tourniquet (yellow arrow) is placed on a sheep limb. The purple discoloration in the limb is indicative of loss of blood flow. The incision that can be seen is the region directly over which the AID blasting occurs.
Table 2: Sheep groups (n=5/group are designated for testing), treatments being performed in each group and the number that have been performed to date.

<table>
<thead>
<tr>
<th>Sheep Group</th>
<th>Treatment</th>
<th>Number Worked on to Date (out of n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blast only</td>
<td>n=4</td>
</tr>
<tr>
<td>2</td>
<td>Blast, low number biofilms</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Blast, high number biofilms</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Blast, low or high biofilms, tourniquet, wound VAC</td>
<td>n=4</td>
</tr>
<tr>
<td>5</td>
<td>Blast, tourniquet, wound VAC</td>
<td>n=2</td>
</tr>
<tr>
<td>6</td>
<td>Blast, tourniquet</td>
<td>n=1</td>
</tr>
<tr>
<td>7</td>
<td>Blast, bone chips, periosteal disruption</td>
<td>n=2</td>
</tr>
<tr>
<td>8</td>
<td>Blast, periosteal disruption, bone chips, tourniquet</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Blast, periosteal disruption, bone chips, high or low number biofilm, tourniquet</td>
<td></td>
</tr>
</tbody>
</table>

The overall objective of this study is to assess individual and combinatorial factors that may contribute to HO formation. As such, one of the groups of sheep is designated to receive an AID blast followed by tourniquet placement for a period of 45 minutes. This simulates a situation wherein a wounded warrior may experience a traumatic blast, have a tourniquet placed and be taken to a forward triage unit. To date, n=1 sheep has received this treatment of an AID blast with tourniquet placement (see Table 2 and Figure 11B) and has responded well. This sheep reached its endpoint and tissues are currently being processed to assess for formation of HO.

2.4 Sheep Treated with Biofilms

In the battlefield, wounded warriors who suffer an IED blast and place a tourniquet are also highly likely to have wound sites that are contaminated with bacteria, in particular bacteria that reside in the biofilm phenotype. In natural ecosystems, 99.9% of bacteria preferentially dwell in the biofilm phenotype. Thus, in traumatic injuries, soldiers or civilian patients are likely to be contaminated with bacteria in this phenotype as opposed to planktonic bacteria. In an effort to also simulate a relevant battlefield scenario, several groups of sheep in this study will have biofilm inocula (see Table 2). To date, n=4 sheep have received an AID blast followed by placement of bacterial biofilms that contain approximately $10^7$ colony forming units (CFU; Table 2). This is a clinically relevant number of bacteria that may contaminate a wound site given that soil, sand or dirt can contain up to $10^9$ or $10^{10}$ CFU/g of material. Given that soldiers may be contaminated with dry sand, which will likely have fewer bacteria, a biofilm that contains $10^7$ CFU was selected for inoculation. We have performed work in previous sheep models wherein biofilms are used as initial inocula. Low-grade, chronic infection developed without the need for antibiotic intervention.\textsuperscript{34,35}

To inoculate sheep in this portion of testing, biofilms were grown in the lab on the surface of silica beads and inoculated at the time of surgery. Notably, it was confirmed that well-established biofilms grew well on the surface of the silica beads that were used for inoculation (Figure 12). In the sheep, the silica beads were surgically placed in the same manner, i.e., placed directly onto an exposed region of periosteum. Radiographs have been collected post-op to confirm the location of the beads in the tissue (Figure 13A). In addition, radiographs were collected at 4 weeks, 7 weeks and 12 weeks of the monitoring period. At the 4 week time point, no bone response was detected in the first two sheep. At 7 weeks, it was observed that in one of sheep, the silica beads had settled in...
the muscle tissue and did not maintain contact with the bone (Figure 13B & C). However, in the second animal, the beads were seen radiographically in proximity to the bone (Figure 13D). In the sheep wherein the beads resided in the tissue, little to no bone response was observed, but tissues are currently being processed histologically to determine if a response, i.e., mineralization of tissue surrounding the beads, had occurred. In contrast, in the sheep wherein the beads were in direct proximity to the bone, a significant bone response was observed (Figure 13D). That bone response was even more pronounced by week 12 (Figure 13E). It is recognized that this could be an infection response, but these sheep had no fevers and did not require antibiotics. There is also a pronounced response in the periosteal region away from the beads. MicroCTs have been collected on this limb and indicate that a compelling bone response is occurring (Figure 14) that did not appear to follow a path of classical infection (e.g., moth eaten bone). As we continue to collect data with scanning electron microscopy (SEM) and histology, further indications will determine the type of bone and level of response. There are two sheep still on study in this group.

← Figure 12: SEM image of a silica bead on which biofilm has been grown. Biofilm and accompanying matrix materials grew into three-dimensional structures and were present in large quantities across the surface of the beads.

→ Figure 13: Radiographs of bones in sheep that were inoculated with well-established biofilms. (A) Representative radiograph of a sheep bone at the time of surgery. Arrows point to silica beads that are present in the region. (B) Radiograph taken at 7 weeks. In this sheep, the beads had settled in the muscle region and were estimated to be a several millimeters away from the bone. (C) Radiograph taken at 12 weeks of the same sheep in (B). The beads had not moved and little to no bone response was observed (see arrow). (D) Radiograph taken at 7 weeks. In this sheep, the beads resided in proximity to the sheep bone (see arrow), and appeared to embed in the periosteal region. A significant bone response began to develop by 7 weeks, whereas at 4 weeks no response was observed. (E) Radiograph at 12 weeks of the same sheep in which the beads
were in proximity to the bone (see arrow). By 12 weeks, the bone response was more pronounced. It appeared that cortical thickening had occurred, as well as a response developing adjacent to the bead site. These may be signs of HO formation, and would be consistent with what has been seen in other models wherein bacterial presence enhances HO formation. Data are currently being collected to determine outcomes.

Figure 14: 3D reconstructed Micro-CT images of a biofilm-treated and AID blasted sheep femur. The profile view (Left image) shows the glass beads (yellow arrow) on which biofilm had been grown for inoculation. Biofilms appeared to cause a thickening of the endosteal region (white arrow), along with remodeling bone. (A) Cross-sectional view demonstrated that the proximal region away from the glass beads showed no significant gross bone response. (B) A cross-sectional view of the midshaft region showed bone growth extending around the main bone (red arrow). As discussed below, this was likely due to the presence of biofilm, which may have sparked a tissue repair response. Additional bone growth was observed (blue arrow) extending outside the periosteum where the glass beads were directly in apposition with the bone. If the animal had been monitored longer, we consider that this region may have begun to sprout into HO. (C) Lastly, this cross-sectional view also showed the cortical bone becoming porous (green arrow) as well as thicker in the distal region. This response is similar to what we have seen in human studies where HO has formed (unpublished data).

To date, the biofilm results have initially supported what we discussed in our grant proposal. More specifically, we stated:

“Data have indicated that M1 macrophages (classically activated) are pro-inflammatory and primarily defend against bacterial infections and/or the presence of bacterial components whereas M2 macrophages (alternatively activated) are associated with anti-inflammatory reactions and tissue remodeling. In the case of developing a sheep model of HO formation, it has been suggested that the use of bacteria may be a limitation since bacteria active M1 macrophages, which has the subsequent potential to suppress M2 macrophages. However, as is predominantly the case in animal models of infection, data related to M1 activation have primarily been derived using planktonic bacterial cells which elicit different inflammatory and biological responses compared to bacteria that reside in the biofilm phenotype. More specifically, it is becoming ever more apparent that infection outcomes and inflammatory pathways may differ significantly when biofilms are present, and in particular when they are present initially in a wound site.

Williams et al. and Sinclair et al. have observed that when the same species of bacteria is used as an initial inoculum in the planktonic versus biofilm phenotype in sheep models of infection,
infection outcomes vary drastically. Planktonic-related infection developed more rapidly and aggressively\textsuperscript{38} compared to biofilm-related infections, which developed more slowly and were chronic in nature.\textsuperscript{34,35} Furthermore, multiple groups have shown that in the biofilm phenotype, bacteria may counter the traditional dogma of M1 activation. For example, in a mouse model of biofilm catheter-related infection, Thurlow et al. showed that biofilms of \textit{Staphylococcus aureus} had the ability to circumvent traditional infection pathways, and co-culture with macrophages showed gene expression profiles that resembled activated M2 macrophages.\textsuperscript{39} Thus, as stated by Hanke et al., “Biofilms can skew the immune response to favor anti-inflammatory and profibrotic pathways, which contribute to biofilm persistence.”\textsuperscript{40}

At least in the case we have analyzed so far, the host tissue has appeared to follow a path of remodeling and repair as opposed to moth-eaten, infected bone, which may support a M2 activation pathway. We look forward to collecting the additional sheep data to determine if this will continue to be the case.

2.5 \textbf{Sheep Treated with NPWT}

As part of a battlefield blast scenario, once a wounded warrior has been transported and stabilized to a forward triage unit, within hours or days, depending on the situation, it is likely that they will begin treatment with negative pressure wound therapy (NPWT), i.e., wound vacuum assisted closure (VAC). As such, several groups of sheep in this study have also been treated with NPWT therapy in an effort to model the clinical scenario of a wounded warrior. Prior to moving to live animal work with NPWT, mock surgeries were performed to optimize the approach that would be taken for placing the NPWT tubing, polyurethane foam and track pad (see Figure 15).

![Figure 15: Photos of the mock surgery and placement of the NPWT system prior to moving into live animals. (A) Making an incision in a carcass sheep limb. (B) Placing the polyurethane foam and track pad. (C) NPWT tubing in place. (D) Suture line and exit site sutured and sealed closed. Note: in the live animals, a separate exit site was created to facilitate both the seal and configuration of the tubing. (E) Confirmation that the setup would maintain a seal and negative pressure would be drawn through the line.](image)

Following the mock testing, work on live animals began. In two sheep the same surgical approach outlined above was taken by creating an incision in the midshaft of the femoral region. In this case, the periosteum was not disrupted, and bone chips were not used (these variables will be included in later animal groups). In this group of sheep, the single variable of NPWT was assessed following shockwave trauma. The reason for performing work with NPWT was to first, achieve the objectives and second, to familiarize both our research team and the animal care staff at the animal facility how to handle NPWT units and animals before moving onto more complex groups.
of animals that have multiple variables involved (e.g., periosteal stripping, muscle trauma, bacteria, bone chips and NPWT).

The NPWT system that has been developed has worked well. In short, we have built a platform on a mobile track that attaches to a harness on the animal (see Figure 16). As the animal moves, the entire NPWT system moves with them and we have been able to achieve up to 7 days of NPWT on a sheep at this point. The wound VAC pad that is used was designed to not clog or disrupt suction (Figure 17). The end point for NPWT is 7 days. This has been an important achievement as part of this study. The n=2 two sheep that have received NPWT to date both reached their endpoint and tissues are currently being processed to assess for HO formation.

![Figure 16: Photos of the NPWT system setup. (A) An exit site above the main incision allows the NPWT tubing to leave the affected area and is drawn through (green arrow) to the drainage canister. (B) The NPWT unit and drainage canister (red arrow: unit is a state-of-the-art KCI company unit) sits on a mobile platform and draws 175 mmHg of pressure through the line. The unit has mobility from side to side and back and forth as it is attached to the sheep harness (yellow arrow). Sheep are acclimated to the harness and tubing prior to their surgery day so they get used to it being in place and having one cage of separation from their flock. Positive reinforcement is used to help during the acclimation period.](image-url)
In summary, the research is progressing as planned during this first year and our team is working carefully to schedule surgeries to optimize logistical management of the study, stay on timeline and achieve the objectives. Once the current data are analyzed, work will continue to be performed with additional sheep on the study. We continue to increase our sample sizes and tissue processing is progressing.

2.6 Peer-Reviewed Publications / Conference Abstracts
The first publication related to this work has been drafted and is currently being reviewed by coauthors for submission. It provides an outline of the AID system, its setup and repeatability. The second paper will discuss the pilot work and model development. This is estimated to be drafted in approximately the next 2-3 months as the tissue analysis is completed.

2.7 Literature Review
To ensure that no key information is omitted from future publications, the PI has focused a great deal of time reading a diverse collection of HO literature. An extensive list of articles has been collected and is being reviewed on a daily basis.

3. Key Research Accomplishments
* Achieved full IACUC approval from the University of Utah and HRPO
* Executed a subaward agreement
* Established the surgical model for developing HO
* Ensured the AID blasts could be attenuated
* Developed a NPWT system that can stay in place for multiple days
* Conducted 13 live surgeries
* MicroCT images indicate bone response in at least one group is positive
* Tissues are being processed to determine HO results
* Confirmed that sheep recover from all procedures outlined

4. Reportable Outcomes and Conclusions
No reportable outcomes to date—results are pending

5. References
1. Isaacson BM, Stinstra JG, MacLeod RS, Pasquina PF, Bloebaum RD. Developing a quantitative measurement system for assessing heterotopic ossification and monitoring the bioelectric metrics from electrically induced osseointegration in the residual limb of service members. Annals of biomedical engineering 2010;38:2968-78.
Investigation of a Translatable Animal Model in Order to Understand the Etiology of Heterotopic Ossification
BAA W81XWH-16-2-0037
PI: Brad M. Isaacson, PhD, MBA, MSF  Org: Henry M. Jackson Foundation
Award Amount $958,201

Study Purpose / Deliverables
The objectives of this grant are to analyze three variables that are hypothesized to influence heterotopic ossification (HO) formation. This will be accomplished through a translatable animal model in order to better understand this etiology and improve clinical management for wounded warriors. Developing new methods for assessing HO remains of utmost importance since florid bone growth may result from premature resection and cause additional surgical procedures for injured service members.

Study Aims
• Aim 1: Induce HO in a clinically translatable animal model.
• Aim 2: Analyze resected HO masses from the ovine model with advanced histological techniques (SEM, MAR, microscopy, bone stains).

Timeline and Cost

<table>
<thead>
<tr>
<th>Activities</th>
<th>FY17</th>
<th>FY18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Obtain IACUC approvals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Develop HO model and test factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analyze HO with advanced histology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Determine the MAR of HO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Publish data in peer-reviewed journals</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Estimated Budget ($K)</strong></td>
<td>$852</td>
<td>$106</td>
</tr>
</tbody>
</table>

Updated: 21 September 2017

Accomplishments: * Achieved full IACUC approval from the University of Utah and HRPO, Executed a subaward agreement, Established the surgical model for developing HO, Ensured the air impact device blasts can be attenuated, Conducted 9 live surgeries and HO results are pending, and Achieved 7 days of negative pressure wound therapy in multiple animals.

Goals/Milestones

**FY17 Goals**
☑ Obtain IACUC approvals and execute subaward agreements
☑ Complete study pilots to demonstrate air impact device capability to generate HO
☑ Analyze HO samples using micro CTs, radiographs, SEM, MAR, etc.
☐ Conduct HO model

**FY18 Goals**
☐ Complete HO model and perform detailed histological analysis
☐ Perform parametric / non-parametric statistical evaluations
☐ Disseminate knowledge through the military treatment facilities and publish manuscript(s) detailing the findings

Budget Expenditure to Date: $857,860
Projected Expenditure: $958,201