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14. ABSTRACT
All animals have reached their pre-determined end point. Micro-CT analysis indicated that the combination of traumatic factors generated ectopic bone growth consistently by 24 weeks. Additional histological analysis is currently being performed to confirm the presence of heterotopic ossification.

15. SUBJECT TERMS
Recruitment, infrastructure, patient identification

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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 improvised explosive devices (IEDs) and rocket propelled grenades (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]

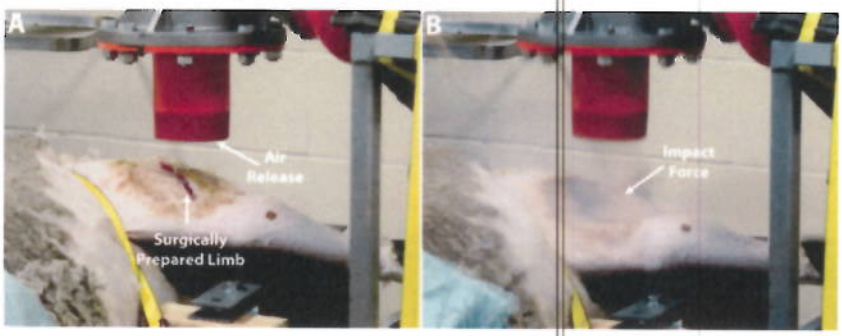
Most concerning is that no empirical evidence has indicated a mechanism for quelling or preventing metabolically active HO.[1] Correlative factors such as gender,[1,17] genetics,[7,18-20] bioelectric signals,[7] infection,[21] and age[17] have been associated with ectopic bone growth, but studies have 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." 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]

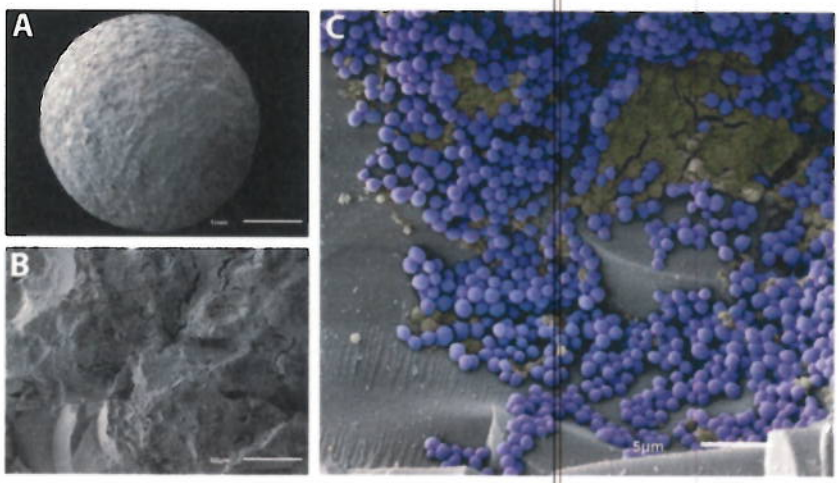
2. Body

2.1 Carcass Testing, Wound VAC Setup, Biofilm Growth and Initiation of Animal Testing

As a brief review, following up on the information from our previous reports, to simulate an IED blast, an air impact device (AID) that is used in the special effects industry was optimized and safety-tested. The AID discharged high-powered bursts of air to the lateral, mid shaft region of ovine femurs to inflict deep tissue trauma (Figure 1). A series of pilot sheep were used first to confirm that the AID procedure and there would not be a bone fracture, thereby allowing sheep to function and maintain mobility. Work then advanced to the larger cohorts of sheep which included various trauma and surgical-related factors including bone disruption, bone chips, tourniquet, using biofilm as an initial inocula (Figure 2), and negative pressure wound therapy (NPWT) (Figure 3). The use of the wound VAC (NPWT) system has been used repeatedly with success in each animal. We have also successfully inoculated the sheep with 10^7 bacteria by way of silicon (Si) glass beads, as well as figured out a safe way to disrupt the periosteum/bone allowing for growth factors to be released from the bone marrow into the adjacent muscle.



←**Figure 1:** (A) Figure demonstrating the impact of the 1100N AID blast (A) Representative image showing a surgically prepared limb prior to an AID blast. The air release opening of the AID was positioned directly above the incision. (B) Still shot showing the effect of the force of the AID blast. The discharge caused the cadaveric sheep limb to concave significantly, suggesting impact to the deep hard tissue.



←**Figure 2:** (A+B) Overhead view of a glass Si bead that was roughened using 60 grit sand paper to allow for better biofilm growth and attachment for inoculation. (C) Higher power view showing the *Staphylococcus aureus* ATCC 6538 biofilm (purple) after 72 hrs of growth on the roughened glass Si bead.

←**Figure 3:** Photography 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 to the drainage canister. (B) The NPWT unit sits upon a mobile platform drawing 175 mmHg of pressure. The unit provides mobility as it is attached to the sheep harness. Mobile backpacks units are also used to provide additional mobility.

2.2 Current Animal Testing

Since the last annual report (10/14/2018) we have performed all remaining surgeries and every ovine reached their end point. This equates to a total of n=36 sheep (Table 1). Note that a n=1 sheep was euthanized early due to a broken leg that was unrelated to the surgery. All sheep have been micro-CT'ed and are being prepared for analysis.

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Table 1: Detailed list of number of sheep and surgical procedure performed.

Sheep List

Groups	Animal #	Time Point	Surgical Trauma
1	n=5	24 Weeks	AID (x5)
2	n=5	24 Weeks	AID (x5) and biofilm
3	n=5	24 Weeks	AID (x5), biofilm and NPWT
4	n=5	24 Weeks	AID (x5), tourniquet and NPWT
5	n=6	24 Weeks	AID (x5), periosteal disruption, transcortical defect and bone fragments
6	n=5	24 Weeks	Periosteal disruption, transcortical defect and bone fragments (no AID)
7	n=5	24 Weeks	AID (x5), periosteal disruption, transcortical defect, bone fragments, tourniquet and biofilm

NOTE: n=1 sheep from Group 5 was euthanized early

2.3 Results

The preliminary micro-CT analysis demonstrated ectopic bone stemming from the posterior side in select groups. More specifically the micro-CT analysis showed that the combination of traumatic factors in Group 7 (all trauma and surgical-related factors excluding NWPT) had the most consistent ectopic bone growth (5 out of 5) within 24 weeks (Figure 4). Bone responses have also been observed in Group 2 (AID and biofilm) (1 out of 5), Group 3 (AID, biofilm, tourniquet, NPWT) (3 out of 5), Group 5 (AID and bone disruption) (2 out of 5) and Group 6 (bone disruption (no AID) (2 out of 5) sheep. Although the bone growth has not been as consistent in these groups compared to the ectopic bone in Group 7. No signs of HO have been detected in Groups 1 or 4 by way of micro-CT analysis. Volume analysis is currently being conducted using the micro-CT scans (Figure 5). Once collected, volume analysis will be compared with the bone morphology by backscatter electron imagining to confirm HO.

PMMA embedded cross-sections (Figure 6) are beginning to be imaged using a Scanning Electron Microscope (SEM) equipped with a Backscatter (BSE) detector. BSE images are able to determine the mineralization of the bone and give distinct grey levels depending on the bones maturity state. The preliminary SEM analysis has confirmed the minimal bone response in Group 1 (Figure 7). The analysis has also revealed sheep from Group 2 revealed an aggressive bone response that didn't follow the classic infection response. Fevers were never present and the sheep did not require antibiotics. The analysis demonstrated sequestrum bone resulting in endosteal thickening and new bone growth extending from the periosteum (Figure 8) on the lateral side of the sheep femur where the Si beads containing bacteria were placed and AID blast occurred. This response is in stark contrast to the sheep's native plexiform bone which could still be seen on the medial side of the femur (Figure 8B). The new bone growth was primarily osteonal remodeling. Further analysis will help determine if this bone response is the beginning stages of HO.

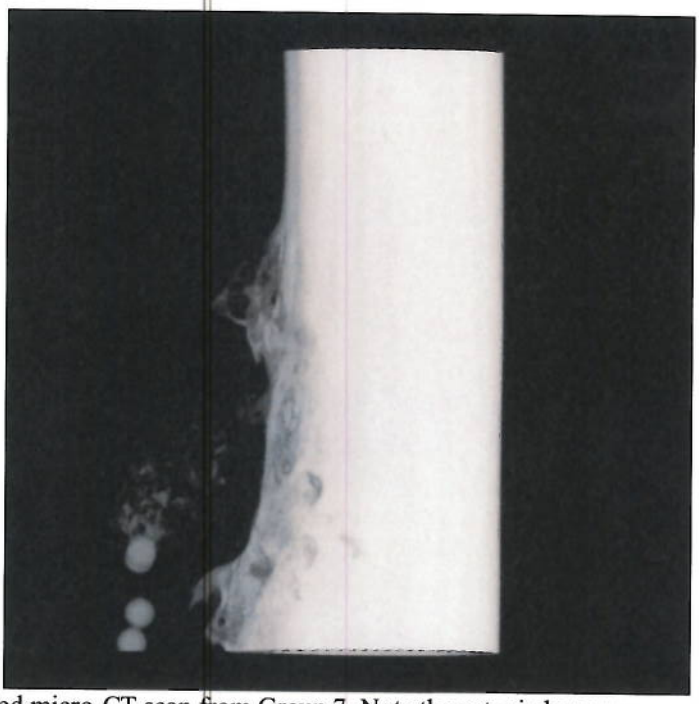


Figure 4: Example 3D rendered micro-CT scan from Group 7. Note the ectopic bone response is very similar to human HO.

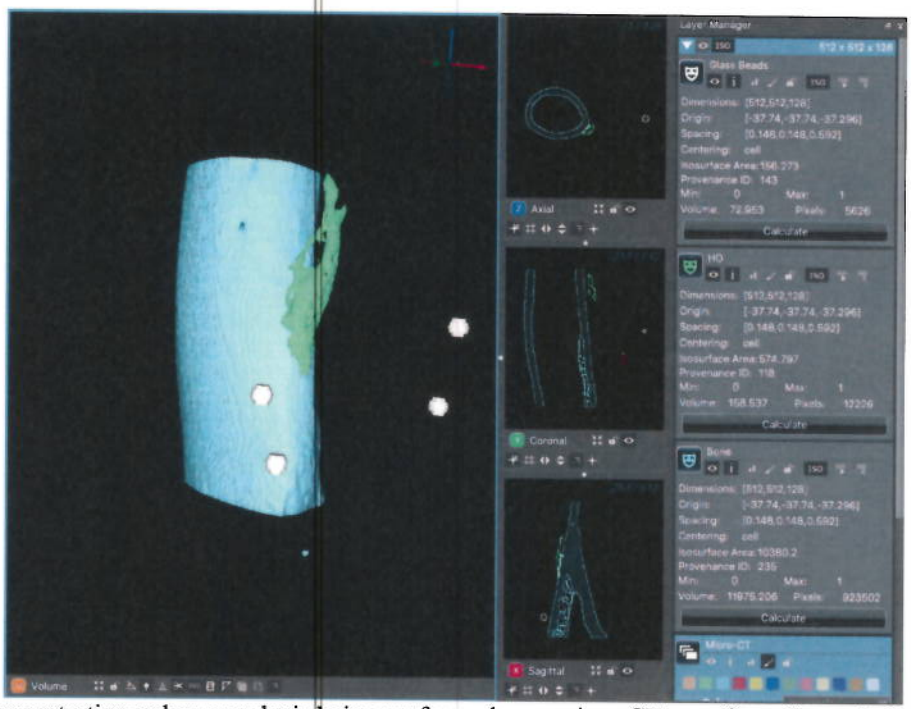
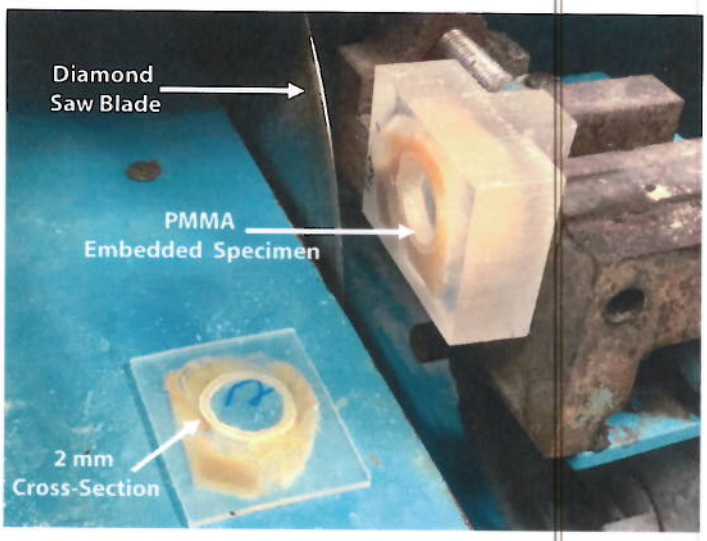
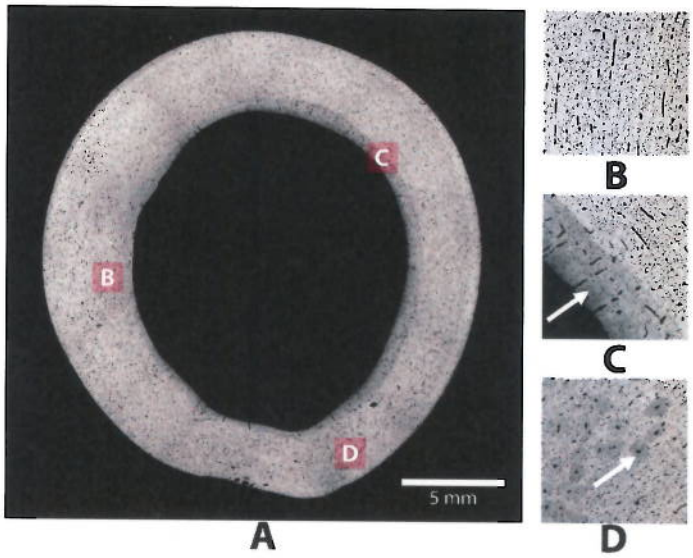


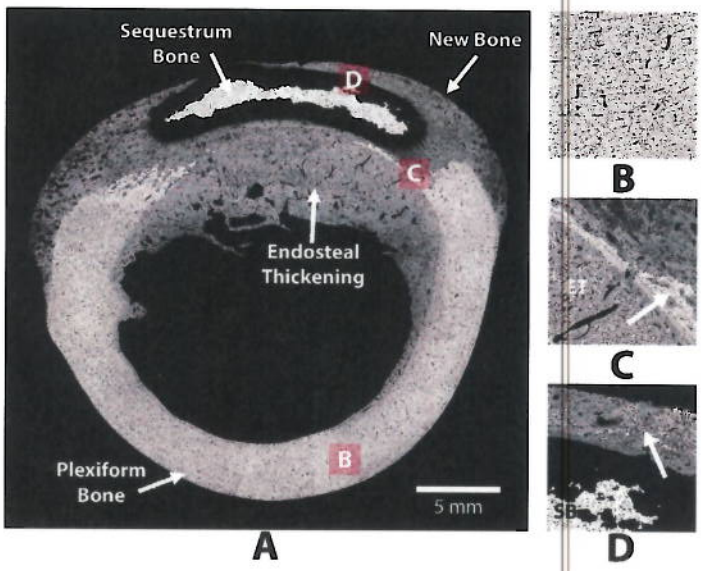
Figure 5: Figure demonstrating volume analysis being performed on a micro-CT scan from Group 7. Note the ectopic bone (green) stemming from the posterior side of the femur (blue). Glass beads (white) used to grow biofilms can be observed in the adjacent muscle.



←**Figure 6:** Image showing a PMMA embedded femur being sectioned into 2mm slices by a custom water cooled saw equipped with a diamond blade.



→**Figure 7:** SEM images of a femur from Group 1. (A) BSE images that have been stitched together using MRICE creating an overhead cross-sectional view. (B) Medial side showing the native plexiform bone structure. (C) Lateral side showing a recently remodeled area in the endosteal region (arrow) where the AID blast occurred. (D) Posterior side showing osteon remodeling (arrows) replacing the plexiform bone.



←**Figure 8:** SEM images of a femur from Group 2. (A) BSE images that have been stitched together using MRICE creating an overhead cross-sectional view. Note the new bone growth extending from the periosteum wrapping around the sequestrum bone. (B) Medial side showing the native plexiform bone structure. (C) Lateral side showing endosteal thickening (ET). Note the limited remaining host bone (arrow) in this region. (D) Lateral side showing new bone growth (arrow) stemming from the periosteum and encasing the sequestrum bone (SB).

2.4 Peer-Reviewed Publications / Conference Abstracts

The first manuscript related to this study was published by JMIR Research Protocols; it outlines the development of the setup and trajectory of particulate following a blast. A second manuscript has been drafted as well. In addition to the manuscripts, abstracts were written and accepted by The Military Health System Research Symposium and the American Society for Microbiology. This work was also selected for a podium presentation by the Orthopedic Research Society. All relevant materials are attached in this final report.

2.5 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 regularly.

3. Key Research Accomplishments to Date

* 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 can be attenuated
* Conducted live surgeries
* Disrupted the bone allowing for growth facture to be released in the adjacent muscle without compromising the sheep's health or ability to move freely
* Achieved 7 days of NPWT
* Successfully inoculated sheep with 10^7 bacteria with a 100% survival rate
* Data is indicating that a combination of factors may result in an ectopic bone response
* All sheep have reached their endpoint

4. Reportable Outcomes and Conclusions

Micro-CT analysis has indicated that the combination of traumatic factors has generated ectopic bone growth consistently by 24 weeks. Additional histological analysis is currently being performed to confirm the presence of heterotopic ossification.

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Original Paper

System Setup to Deliver Air Impact Forces to a Sheep Limb: Preparation for Model Development of Blast-Related Heterotopic Ossification

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Abstract

Background: Heterotopic ossification (HO) is a significant complication for wounded warriors with traumatic limb loss. Although this pathologic condition negatively impacts the general population, ectopic bone has been observed with higher frequency for service members injured in Iraq and Afghanistan due to blast injuries. Several factors, including a traumatic insult, bioburden, tourniquet and wound vacuum usage, and bone fractures or fragments have been associated with increased HO for service members. A large combat-relevant animal model is needed to further understand ectopic bone etiology and develop new pragmatic solutions for reducing HO formation and recurrence.

Objective: This study outlines the optimization of a blast system that may be used to simulate combat-relevant trauma for HO and replicate percussion blast experienced in theater.

Methods: We tested the repeatability and reproducibility of an air impact device (AID) at various pressure settings and compared it with a model of blunt force trauma for HO induction. Furthermore, we assessed the ability of the higher-power air delivery system to injure host tissue, displace metal particulate, and disperse bone chips in cadaveric sheep limbs.

Results: Data demonstrated that the air delivery setup generated battlefield-relevant blast forces. When the AID was charged to 40, 80, and 100 psi, the outputs were 229 (SD 13) N, 778 (SD 50) N, and 1085 (SD 114) N, respectively, compared with the blunt force model which proposed only 168 (SD 11) N. For the 100-psi AID setup, the force equaled a 5.8-kg charge weight of trinitrotoluene at a standoff distance of approximately 2.62 m, which would replicate a dismounted improvised explosive device blast in theater. Dispersion data showed that the delivery system would have the ability to cause host tissue trauma and effectively disperse metal particulate and host bone chips in local musculature compared with the standard blunt force model (13 mm vs 2 mm).

Conclusions: Our data showed that a high-pressure AID was repeatable or reproducible, had the ability to function as a simulated battlefield blast that can model military HO scenarios, and will allow for factors including blast trauma to translate toward a large animal model.

(JMIR Res Protoc 2019;8(2):e12107) doi:10.2196/12107

KEYWORDS

blast; air; sheep; limb; heterotopic ossification

Introduction

Heterotopic ossification (HO) refers to ectopic bone formation, typically in residual limbs or periarticular regions following trauma, surgery, or injury [1]. This pathological process manifests outside the skeleton [2] and comprises a hybrid of cortical and cancellous bone [3]. HO is induced by damage to soft tissue and inflammation [4,5] and has been most frequently observed after combat-related trauma to service members with blast injuries [6].

Reviews of orthopedic injuries from Operation Iraqi Freedom and Operation Enduring Freedom have reported that approximately 70% of war wounds involved the musculoskeletal system [7], largely in part from the use of improvised explosive devices (IEDs) and rocket-propelled grenades. 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 [8-10]. Reports of recent Operation Iraqi Freedom and Operation Enduring Freedom combat-related amputees with known HO have indicated that approximately 20%-40% of affected patients required surgery to excise their bony masses [10-13]. Symptomatic HO may delay rehabilitation regimens, as ectopic bone resection often requires modifications to prosthetic limb componentry and socket size [11,14].

The causative factors of HO development, especially in the case of blast injuries, are not well known. However, it has been hypothesized that contributing factors may include the following: (1) the blast, which generates extensive trauma and potential concomitant brain injury [15]; (2) tourniquet use, which alters local pH and creates a hypoxic environment [16]; (3) the presence of bacteria and biofilms [17]; (4) negative pressure wound therapy that may be used postinjury [9]; and (5) fractured bone, which may be dispersed into the musculature (clinical observations, unpublished data). To identify the various contributing factors for ectopic bone and to provide new evidence-based medicine that may inform clinical guidelines, animal models are currently being developed. However, as noted by Forsberg et al in *Burned to the Bone*, "one of the challenges preventing advances in this field has been the lack of robust animal models for HO" [18].

While rats and rabbits are the most commonly used animals for HO research, their bone growth rates are 600% and 40% higher, respectively, than those of humans [19,20]. This may limit the translatability of this work because HO has been documented to be more metabolically active than nonpathological osseous tissue [1,3,21-24]. Small animal models also cannot accurately reflect combat casualty care because variables such as serial debridement and negative pressure wound therapy must be omitted [25]. The most practical model, and one that is highly understudied, is the ovine model, which has almost identical mineral apposition rates [26] and bone ingrowth into intramedullary implants [27] compared with that of humans. Despite this evidence, only a single study by Walton et al [28] has evaluated HO development in an ovine model; the study results indicated that ectopic bone occurred only 17% of the time. However, Walton et al used blunt force rather than blast trauma, which does not replicate combat conditions, and histological data confirmed that HO formation did not occur. In an effort to address these limitations, preparations are underway to expand HO data collection into a large animal model that includes use of a simulated blast scenario. The first step in this process was to develop a system that could deliver a repeatable and reproducible high-pressure blast. This study outlines the optimization of a simulated blast system that may be translated to a large animal ovine model to assess the development of HO in blast-related scenarios.

Methods

Incident Pressures and Air Impact Device Selection

IEDs are often fabricated from 120-mm artillery rounds and contain approximately 5.8 kg trinitrotoluene (TNT) or its equivalent [29,30]. At a standoff distance of 5.5 m (one of the most commonly used measures for blast assessment), this yields an incident pressure of 110.9 kilopascal (kPa) based on the Kingery-Bulmash blast parameter calculator, which was used for calculating estimated incident pressures in this study [31]. Previous military blast injury models in rodents have utilized pressurized gas systems to mimic IED repercussions [29]. It has been shown that these system types result in incident pressures and other blast parameters, including waveform shape and impulse to detonation, that correlate with IED or other blast outputs [29,32].

In order to more closely simulate an IED or rocket-propelled grenade blast that may occur in theater, and to appropriately translate this to a large animal model, we consulted a special effects pyrotechnics expert who was familiar with the creation of controllable blasts using pressurized gas or air. We identified Martin Tornado Air Cannon with a 4-inch valve (Model BB4-12-28, Martin Engineering, Neponset, IL) as a viable option for simulating a blast. Based on technical sheets, the Tornado system provides rapid depressurization of air within 0.1 seconds [33]. Incident pressures were estimated to range between approximately 174 and 588 kPa (ie, 40-100 psi), consistent with what may be experienced in the range of a battlefield blast setting based on parameters from the Kingery-Bulmash blast parameter calculator [29-31]. The Martin Tornado Air Cannon and its setup, which have been termed the air impact device (AID), were assembled based on manufacturer's recommendations and assessed initially for force output.

Force Plate Testing

Animal limbs and carcasses for this and subsequent analyses were obtained from local butcher shops and from separate Institutional Animal Care and Use Committee-approved studies. To determine force outputs of the AID, NeuLog force plates (Amazon, Seattle, WA, Model Number NUL225) and accompanying sensors were purchased. The AID was secured to a metal cart using industrial strength tie-downs and situated such that the air release opening was directly over a force plate (Figure 1). Tie-down straps were used to secure the device. The NeuLog force plate was adjustable in height and tested at a distance of 2 inches from the AID air release opening to collect force plate data. The force plate was bolted to a custom-made aluminum stand, and tie-downs were used to secure the structure during AID discharge. The force plate was positioned 2 inches from the AID, and data was collected via universal serial bus to a general use Apple MacBook Pro on which NeuLog's publicly available software had been downloaded. The AID was pressurized using a DeWalt fast charge air compressor and tested at pressures of 40, 80, and 100 psi. These settings were assessed experimentally to, in future, determine their ability to cause localized trauma but be within a factor of safety to not cause ovine fractures at this stage of the model. Once pressurized, the AID was discharged. Data were collected with 10 repeat measurements at each psi.

In order to establish baseline force outputs for ovine-induced trauma, we also reproduced the method performed by Walton et al [28], which required a weight of approximately 3.5 kg (head of a sledge hammer) to be dropped from a height of 1 meter. The force of this impact was recorded 10 times for comparison against the AID outcomes. Once force data were collected, testing of the AID system was advanced to *ex vivo* cadaveric sheep limb analysis.

Cadaveric Limb Testing

The AID blast was evaluated on 8 ovine carcass limbs and 4 whole sheep cadavers to characterize the effect of the air blast, to ensure that the force would not generate a localized fracture, and to optimize the surgical model. To ensure that the limb was in the same position between each blast, a support frame was custom welded and a brace secured in the midregion of the limb to prevent flexion and fracture. This was done for disarticulated limbs (Figure 2), as well as whole carcasses (Figure 3). The AID was placed 2 inches from the cadaveric limb (Figure 2) to be consistent with the force plate testing. Bolts were used to attach the limb to the metal frame. Note the metal brace on the back of the limb provided support to the femur and prevented breakage or severe ligament damage from occurring (Figure 2). Radiographs were taken following AID blasts to verify postprocedure bone integrity.

Mock Shrapnel Displacement Testing

IED blast injuries often afflict wounded warriors with shrapnel in the distal limbs. To model this scenario and assess the ability of the AID blast procedure to disperse simulated shrapnel particles into the musculature of cadaveric sheep limbs, a whole carcass was obtained. An incision was made in the midshaft region of a femur. Deep tissue was dissected longitudinally until bone was exposed. A 2.5-g mixture of Cobalt-Chromium (CoCr) beads having a diameter of approximately 0.5 mm was suspended in 5 mL saline solution. The slurry was pipetted over the bone surface. A radiograph was obtained to determine the initial distribution of the CoCr beads (Figure 4).

Figure 1. Setup of the air impact device for force plate analysis.

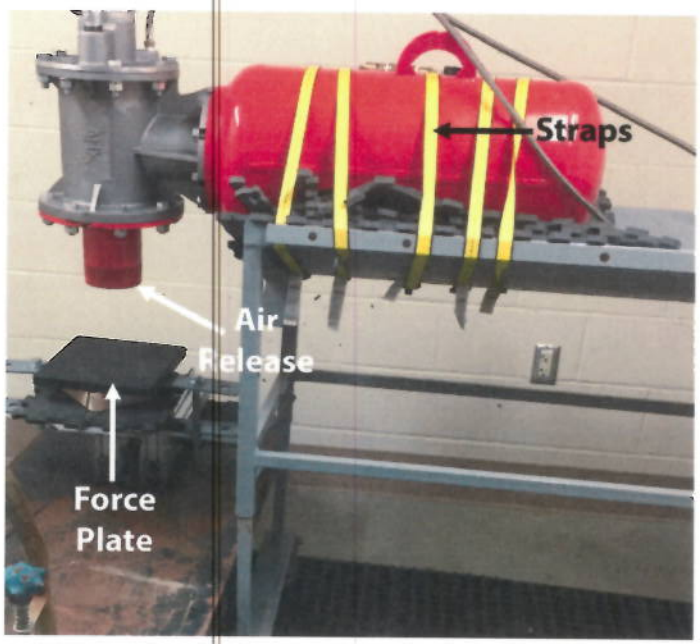


Figure 2. Representative image of a cadaveric sheep limb attached to a metal support frame for air impact device (AID) testing.

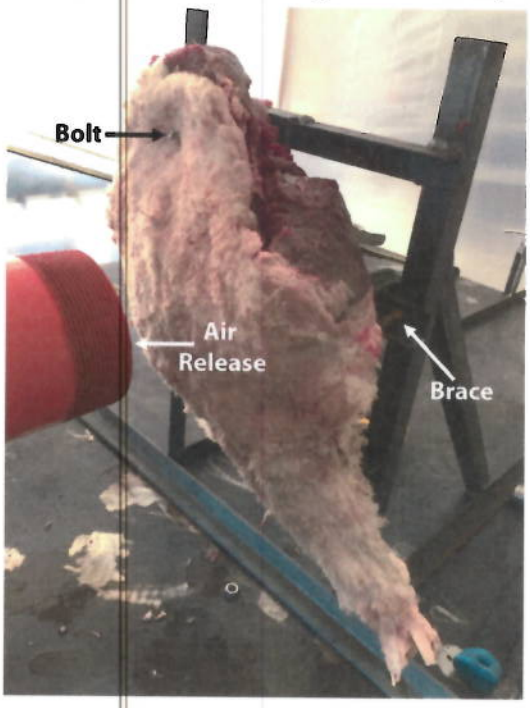
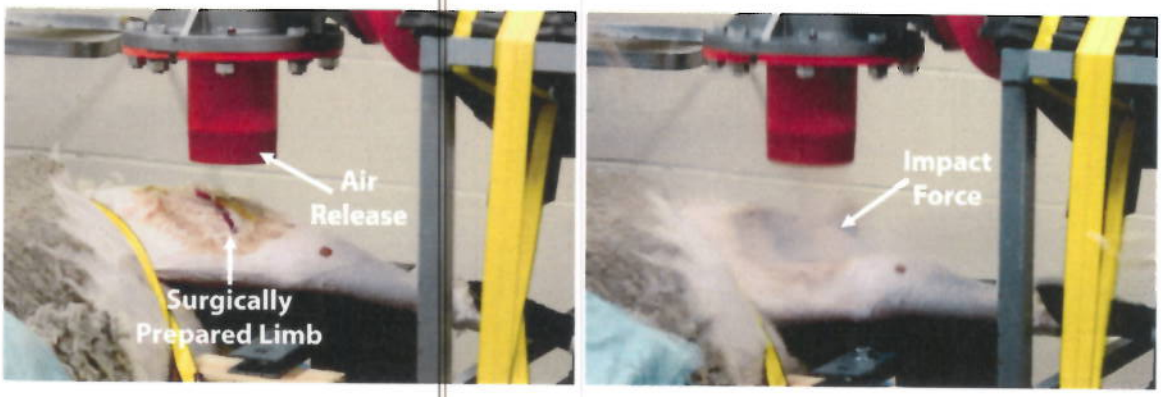


Figure 3. Impact of the air impact device (AID) blast on a cadaveric sheep. Left: Representative image showing a surgically prepared limb prior to an AID blast. The air release opening of the AID was positioned directly above the incision; Right: Still shot showing the effect of the impact force of the AID blast.



Once placement was determined, the midshaft incision was sutured closed and covered with clear adhesive (ie, Tegaderm), and the AID discharge procedure, as outlined above, was performed. To assess for particulate dispersion, the blasting procedure was repeated 5 times. After each blast, a radiograph was obtained to track the displacement of CoCr beads in the deep tissue. This process was repeated in 2 cadaveric limbs.

For comparison, CoCr displacement testing was also performed using the Walton et al [28] method. More specifically, a sheep cadaver was obtained, an incision made in the midshaft region of the femur as described, and a 3.5-kg weight was dropped from 1-m height. Bead placement was again imaged using radiography at time zero and after each drop of the weight to track the movement of the CoCr beads.

In addition to assessing the displacement of CoCr beads, testing was also performed to determine whether the AID could cause host bone chips or fragments to disperse in cadaveric sheep tissue. A mock surgery was performed wherein a bone core of approximately 10 mm was taken from the distal femur. Bone chips were created using a rongeur, mixed with saline to create a slurry, and placed in apposition to the bone in the midshaft of the femur (Figure 5). The sheep was covered with a drape to prevent any contamination that may have been forced though the incision site and to protect equipment in the room (Figure 6). Radiography was obtained after AID blasts (Figure 7).

Figure 4. Radiograph demonstrating that following air impact device exposure or blunt force trauma, femora were intact and not fractured. In addition, images show dispersion of Cobalt-Chromium beads (white).

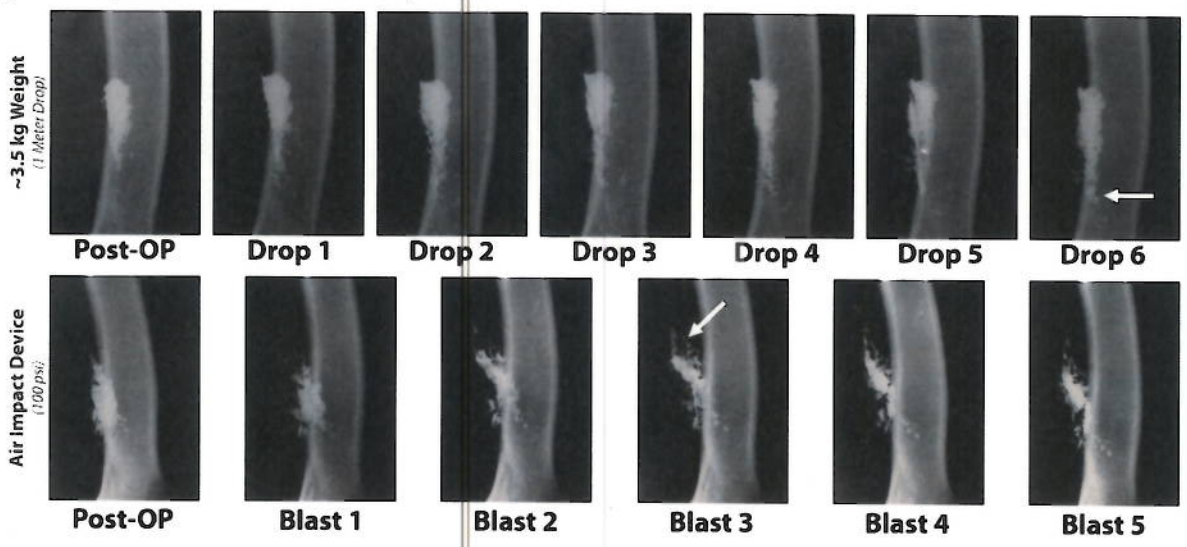


Figure 5. Photography demonstrating a mock surgery on a cadaver sheep for bone chip collection and placement. Left to right: Incision being made toward the distal end of the femur. A 10-mm bone core (arrow) was taken from the distal femur. Bone chips (arrow) were placed on the exposed midshaft of the femur. The incision site was sutured closed. Note that the fascia was also closed by suturing to ensure that the air impact device would not result in surgical site dehiscence.

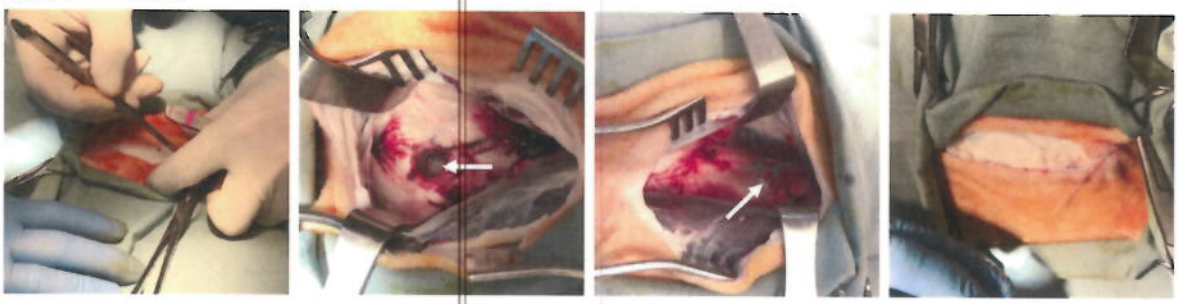


Figure 6. Photography demonstrating the AID blast. Left: A custom limb support created from 80/20 aluminum. This ensured the femur was supported during the lateral air impact device (AID) blast. Right: The final setup of the AID blast over a surgically operated leg.

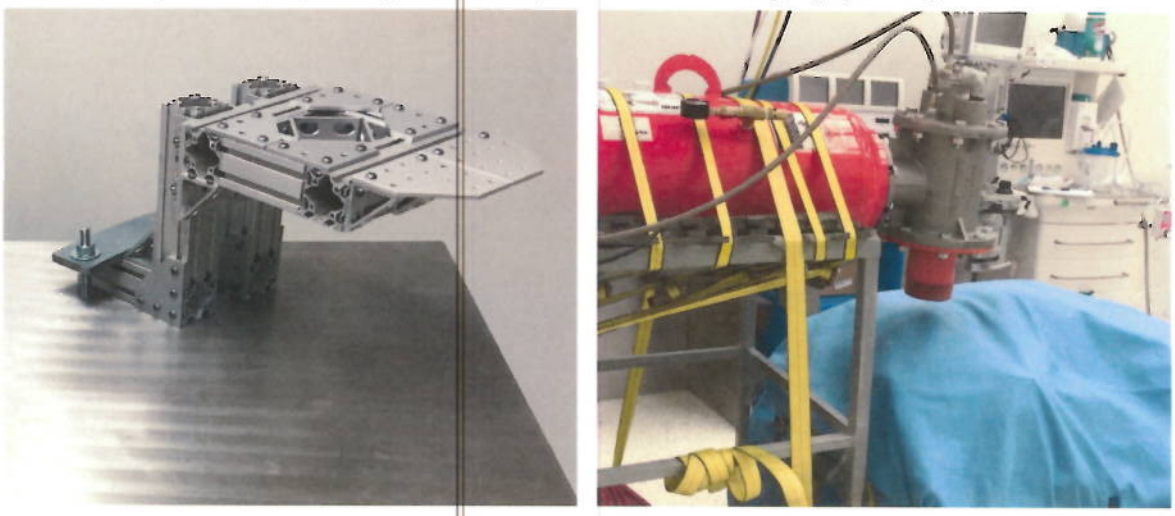
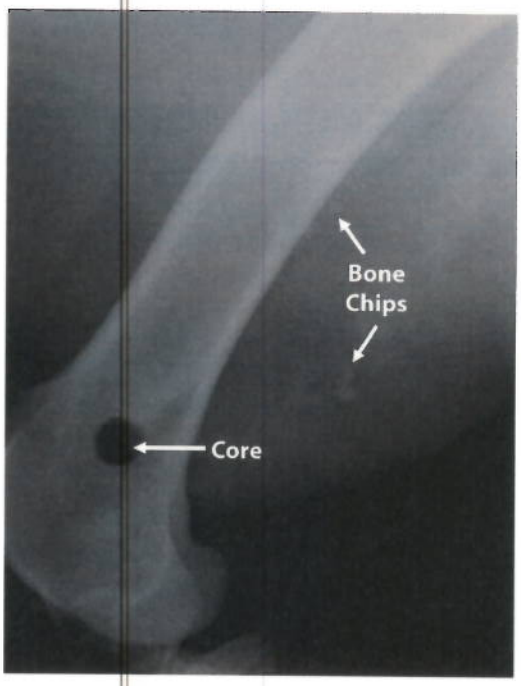


Figure 7. Radiograph obtained after 5 AID blasts revealed that the bone chips placed on the lateral side of the femur had migrated posteriorly as well as into the adjacent muscle tissue.



Results

Force Plate Testing

Results from the force plate portion of testing showed that the air discharge forces of the AID exceeded the force achieved by Walton et al, which required dropping a 3.5-kg weight from a 1-m height (Figure 3). When the AID was pressurized to 40 psi, the air volume was 6.2 cubic feet [33]. At this psi, the incident pressure was 174 kPa, which would equate to a 5.8-kg charge weight of TNT at a standoff distance of approximately 4.5 m [31]. Incident pressure in this case was defined as a free air burst, meaning a burst that had no contact with the ground before striking an object [34]. The force output was 229 (SD 13) N. At 80 psi, the air volume was 10.7 cubic feet [33]. At this psi, the incident pressure was 450 kPa, which would equate approximately to a 5.8-kg charge weight of TNT at a standoff distance of approximately 2.95 m [31]. The force output was 778 (SD 50) N. At 100 psi, the air volume was 12.9 cubic feet [33]. At this psi, the incident pressure was 588 kPa, which would equate approximately to a 5.8-kg charge weight of TNT at a standoff distance of approximately 2.62 m [31]. The force output was 1085 (SD 114) N. Testing did not go higher than 100 psi given that the AID began to have connection leaks at higher pressures.

The force of dropping the 3.5-kg weight was 168 (SD 11) N. Taken together, the data indicated that the AID resulted in a force output that was approximately 7× greater than the dropped weight (Tables 1 and 2) and provided incident pressures that may more closely model an IED.

Cadaveric Limb Testing

Tests from the cadaveric limbs indicated that with a support bar in place (Figure 2), limbs did not fracture. However, it was found that when an incision was present in the leg, the rapid discharge of air opened the incision and created a pocket that compromised the subdermal tissues. To mitigate this outcome, the incision site was covered with durable plastic, such as Tegaderm, which prevented the explosive air from entering the incision site and compromising the musculature. Whole carcass testing was performed in a horizontal plane to more closely simulate a sheep that would be lying on a table for a procedure to be performed.

Table 1. Force plate data output comparisons.

Group	Force output (N)		
	Mean (SD)	95% CI	Minimum, maximum
~3.5-kg weight	168 (11)	159-177	148, 179
AID[a] (40 psi)	229 (13)	217-241	214, 245
AID (80 psi)	778 (50)	745-811	732, 881
AID (1000 psi)	1080 (114)	968-1190	1008, 1252

[a]AID: air impact device.

Table 2. Force plate data statistical comparisons.

Group	*P* value[a]
~3.5-kg weight versus AID[b] (40 psi)	<.001
~3.5-kg weight versus AID (80 psi)	<.001
~3.5-kg weight versus AID (100 psi)	<.001
AID (40 psi) versus AID (80 psi)	<.001
AID (40 psi) versus AID (100 psi)	<.001
AID (80 psi) versus AID (100 psi)	<.001

[a]$P<.05$ is significant.

[b]AID: air impact device.

Mock Shrapnel and Bone Displacement Testing

Results from the mock shrapnel displacement testing showed that the AID discharge procedure dispersed CoCr beads within the musculature of a cadaveric sheep limb (Figure 4). More specifically, groups of beads were tracked and dispersed to a distance of approximately 2.7 mm with each blast that was performed. By the fifth blast, beads resided approximately 13.3 mm distal to their start point. The data also indicated that the sheep limbs were able to withstand multiple sequential blasts. More specifically, radiographs indicated that the limbs did not fracture following multiple AID discharges (Figure 4).

For comparison, the process of dropping a 3.5-kg weight on the limb resulted in minimal movement of the CoCr beads with each sequential hit (Figure 4). Beads primarily tracked parallel to the bone and may have been an artifact from motion during the capturing of the radiographs or as saline drained through the surgical pocket that was created (Figure 4). By the fifth drop of the weight, beads had dispersed by approximately 2 mm or less into the surrounding tissue regions.

Discussion

Principal Findings

The setup of an AID system described herein generated repeatable and reproducible blast of pressurized air that resulted in a force of approximately 1100 N. This may cause significant trauma to local tissue without compromising the underlying skeletal structure of a large animal (which may be critically important for a translatable animal model because lameness and extreme discomfort may necessitate euthanasia). The forces generated in our model were approximately 7× greater than those generated in the blunt force trauma model previously developed to induce ectopic bone [28].

The delivery of a pressurized blast of air was consistent with previous animal studies and incident pressures that may be present in an IED blast [29]. However, the overall goal was not to create massive polytrauma, but rather consistent blasts of air. The AID used in this model also demonstrated that it could effectively disperse metal particulate within the muscle, which would be expected with percussion blasts. Metal beads tracked parallel to the bone following the weight drop, displacing within the soft tissue planes of our intermuscular approach. In contrast, beads that dispersed into the musculature following AID blasts appeared to disperse in a radial pattern created by the pressurized blast of air.

Bone chips or fragments were also found to be affected by the AID. This may be particularly important when the animal model portion of testing begins because in a battlefield-relevant scenario, bone chips or fragments are a common result of blast-related trauma. These data indicate that as this work progresses toward animal modeling, clinically relevant outcomes may be achieved. Current testing has been limited to *ex vivo* analysis. Live animal modeling will be needed to determine whether these data model parameters are safe and effective. *In vivo* data will also reveal whether an approach of highly pressurized air, as opposed to blunt force, will lead to higher rates of HO formation in an ovine model.

Conclusion

HO negatively affects the quality of life for service members and those in the public sector. For example, the pathology can inhibit the ability of those with limb loss to effectively use prosthetic socket systems due to pain as soft tissues compress against bony HO. This in turn delays rehabilitation and, in some cases, requires surgical excision. Methods to better understand the etiology and ectopic bone mitigation will improve clinical outcomes. This study outlines the setup of a high-pressure air blast system to simulate combat-related trauma that may lead to future HO manifestation.

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Conflicts of Interest

None declared.

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Abbreviations

AID: air impact device
HO: heterotopic ossification
IED: improvised explosive device
TNT: trinitrotoluene

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PMID:

Development of a Translatable Large Animal Model in Order to Understand the Etiology of Heterotopic Ossification

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INTRODUCTION: Heterotopic ossification (HO) refers to ectopic bone formation, typically in residual limbs and/or peri-articular regions following trauma and injury. This pathological process manifests outside of the skeleton and is comprised of a hybrid of cortical and cancellous bone. There has been general agreement in the literature that HO is induced from damage to soft tissue and inflammation; ectopic bone growth has been most frequently observed after combat-related trauma to service members with blast injuries. Reviews of orthopedic injuries from Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) have reported that approximately 70% of war wounds have involved the musculoskeletal system, largely from the use of improvised explosive devices (IEDs) and rocket propelled grenades (RPGs). HO has been reported to occur in approximately 63%-65% of wounded service members with limb loss or major extremity injuries. Symptomatic HO may delay rehabilitation regimens since it often requires modifications to prosthetic limb componentry and socket size. Most concerning is that no empirical evidence has indicated a mechanism for quelling or preventing metabolically active HO. Studies have often lacked histologic corroboration and advanced radiologic quantification. Extensive research by our team has observed several common factors that may act as catalysts for inducing HO: (1) blast injury that displaces bone and/or fragments, (2) tourniquet and negative pressure wound therapy (NPWT) usage and (3) a post-traumatic infection signal. 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) initiate and exacerbate HO.

METHODS: Animal work was performed at the University of Utah following local institutional animal care and use committee (IACUC) and external animal care and use review office (ACURO) approvals. To simulate an IED blast, an air impact device (AID) that is used in the special effects industry was optimized and safety-tested. The AID discharged high-powered bursts of air to the lateral, mid shaft region of ovine femurs to inflict deep tissue trauma (Figure 1). A series of pilot sheep were used first to confirm that the AID procedure and there would not be a bone fracture, thereby allowing sheep to function and maintain mobility. Work then advanced to larger cohorts of sheep which included various trauma and surgical-related factors including bone disruption, bone chips, tourniquet, NPWT and using biofilm as an initial inocula. In addition, soft and hard tissue were collected prior to and post-procedure, and blood draws collected at various time points for precision medicine analyses. To date, n=42 surgeries have been performed and n=25 sheep have reached an endpoint up to 24 weeks (Table 1). When the study is finished, Group 1 will have a total of n=10 and the remaining Groups will have n=5 sheep/group. Following AID blast and surgery, all sheep resumed weight bearing activities and were monitored. For those that have been euthanized, the femurs were dissected, micro-CT'ed and processed in polymethyl methacrylate (PMMA) for scanning electron microscopy (SEM) and histological analysis.

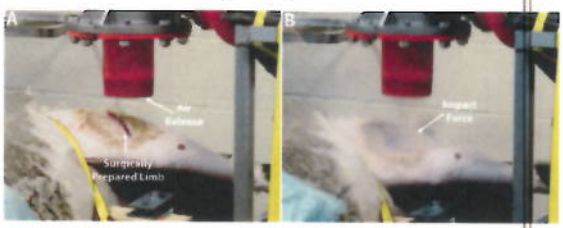
Sheep Group	Trauma	Surgeries Performed	Endpoint Reached
1	AID, Bio, T, P, BC, NPWT	n=10	n=9
2	AID	n=5	n=5
3	AID, Bio	n=5	n=4
4	AID, Bio, NPWT	n=4	n=0
5	AID, T, NPWT	n=3	n=2
6	AID, P, BC	n=5	n=2
7	P, BC	n=5	n=3
8	AID, Bio, T, P,BC	n=5	n=0

Table 1: Sheep groups and surgeries performed to date. **AID**=Blast, **Bio**=10^7 *Staphylococcus aureus* ATCC 6538, **T**=Tourniquet, **P**=Disrupt Periosteum, **BC**=Bone Chips & **NPWT**=Negative Pressure Wound Therapy.

RESULTS SECTION: Preliminary micro-CT analysis has demonstrated that the combination of traumatic factors in Group 1 generated ectopic bone growth consistently within 24 weeks. No signs of HO have been detected in Groups 2 & 5 by way of micro-CT. In Group 3, 1/4 sheep showed signs of ectopic bone. In Group 6, 1/2 sheep demonstrated ectopic b one. Group 7, which did not receive the AID blast showed a periosteal response, but no ectopic bone in the 3/3 that have reached the 24-Week endpoint. Preliminary fluorochrome label analysis has demonstrated distinct double labels in the ectopic bone. The analysis has also revealed minimal remodeling in the adjacent cortex suggesting that the ectopic growth was independent and responsive due to the trauma. The preliminary SEM analysis demonstrated newly formed bone on the lateral and posterior sides of the femur. The observed ectopic growth resembled similar remodeling characteristics of previously analyzed human HO which was a trabecular-like structure with osteon remodeling and hypermineralized regions (Figure 2). The light microscopy analysis confirmed that the ectopic bone was actively remodeling and still advancing into the adjacent tissue/muscle at 24-Weeks. Work is currently ongoing to collect additional data for all groups.

DISCUSSION: Preliminary data has shown consistent ectopic bone growth in a sheep model of a simulated IED blast, in particular when a combination of trauma and battlefield-relevant factors are present. Outcomes have shown similarities to retrieved human HO bone from traumatic blast injuries. Additional animals are being monitored. Lastly, precision medicine components are also currently being collected to assess genetic, protein, cytokine, immune and growth factor responses. Successful demonstration of this translatable animal model will help clinicians understand the etiology of this pathological condition, address a major clinical gap that has been sought after by the DoD, and improve care for combat injured service members.

SIGNIFICANCE/CLINICAL RELEVANCE: A significant portion of wounded warriors who suffer from limb loss also have accompanying morbidity caused by formation of heterotopic ossification. This pathology reduces quality of life, function, increases surgeries and delays rehabilitation. By elucidating the development of HO, further studies can be performed to investigate physical or chemical therapies for prevention and/or treatment.



←**Figure 1:** (A) Figure demonstrating the impact of the AID blast (A) Representative image showing a surgically prepared limb prior to an AID blast. (B) Still shot showing the effect of the force of the AID blast. The discharge caused the cadaveric sheep limb to concave significantly, suggesting impact to the deep hard tissue.

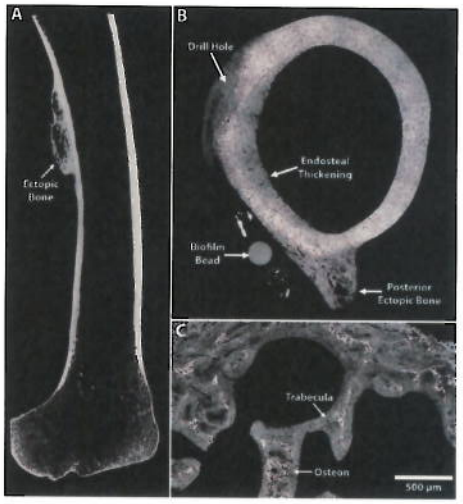


Figure 2: (A) Micro-CT of ectopic bone growth (arrow) extending from the posterior side of a proximal femur from Group 1. (B) Stitched BSE images creating an overhead view showing ectopic bone growth stemming from the drill hole. Ectopic bone was also observed on the posterior side possibly due to the inoculated biofilm by way of Si beads. Note the endosteal thickening due to the biofilm resorbing the periosteum away on the medial side. (C) BSE image showing a trabecular-like structure with osteon remodeling and hypermineralized (white) regions.

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Award #HU0001-11-1-0004; U.S. Army Award, Award #W81XWH-16-2-0037,
The Wounded Warrior Amputee Softball Team Association, and The Geneva Foundation

Developing a Combat-Relevant Translatable Large Animal Model of Heterotopic Ossification

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Background: Heterotopic ossification (HO) refers to ectopic bone formation, typically in residual limbs and/or peri-articular regions following trauma and injury. This pathological process manifests outside of the skeleton and is comprised of a hybrid of cortical and cancellous bone. There has been general agreement in the literature that HO is induced from damage to soft tissue and inflammation; ectopic bone growth has been most frequently observed after combat-related trauma to service members with blast injuries. Reviews of orthopedic injuries from Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) have reported that approximately 70% of war wounds have involved the musculoskeletal system, largely from the use of improvised explosive devices (IEDs) and rocket propelled grenades (RPGs). HO has been reported to occur in approximately 63%-65% of wounded service members with limb loss or major extremity injuries. Symptomatic HO may delay rehabilitation regimens since it often requires modifications to prosthetic limb componentry and socket size. Most concerning is that no empirical evidence has indicated a mechanism for quelling or preventing metabolically active HO. Studies have often lacked histologic corroboration and advanced radiologic quantification. Recent research by our team has identified several common factors that may act as catalysts for inducing HO: 1) blast injury that displaces bone and/or fragments, 2) tourniquet and negative pressure wound therapy (NPWT) usage and 3) a post-traumatic infection signal. Prior to the work performed by our team over the past two years, no study has included the assessment of these factors individually or in combination using a singular translatable large animal model to determine what clinical catalyst(s) initiate and exacerbate HO.

Methods: Animal work was performed at the University of Utah following local institutional animal care and use committee (IACUC) and external animal care and use review office (ACURO) approvals. To simulate an IED blast, an air impact device (AID) that is used in the special effects industry was optimized and safety-tested. The AID discharged high-powered bursts of air to the lateral, mid shaft region of ovine femurs to inflict deep tissue trauma. A series of pilot sheep were used first to optimize the AID procedure and ensure that there would not be a bone fracture, thereby allowing sheep to function and maintain mobility. Work then advanced to larger cohorts of sheep which included various trauma and surgical-related factors including bone disruption, bone chips, tourniquet, NPWT and using biofilm as an initial inocula. In addition, soft and hard tissue were collected prior to and post-procedure, and blood draws collected at various time points for precision medicine analyses. Following AID blast and surgery, all sheep resumed weight bearing activities and were monitored. For those that have been euthanized, the femurs were dissected, micro-computed tomography (CT) scanned and processed in polymethyl methacrylate (PMMA) for scanning electron microscopy (SEM) and histological analysis.

Sheep groups, surgeries performed and endpoints reached:

Pilot Group: (n=3 surgeries, n=3 endpoint reached) Periosteal disruption, autograft bone chips and AID blast (12 week time point)

- **Group 1:** (n=10 surgeries, n=9 endpoint reached) Periosteal disruption, 3 x 2 mm holes drilled in the midshaft femur (to allow growth factors from the medullary canal to surface), autograft bone chips placed at the disruption site, AID blast, biofilm inoculation with Staphylococcus aureus ATCC 6538, wound closure, 45-minute tourniquet, and lastly subdermal placement of negative pressure wound therapy (NPWT). NPWT foam was removed 3-7 days following placement by a secondary surgical procedure.
- **Group 2:** (n=5 surgeries, n=5 endpoint reached) AID blast only
- **Group 3:** (n=5 surgeries, n=4 endpoint reached) AID blast and biofilm inoculation
- **Group 4:** (n=5 surgeries, n=0 endpoint reached) AID blast, biofilm inoculation, tourniquet and NPWT
- **Group 5:** (n=5 surgeries, n=3 endpoint reached) AID blast and NPWT
- **Group 6:** (n=5 surgeries, n=3 endpoint reached) AID blast, periosteal disruption, autograft bone chips, drill holes in midshaft of femur
- **Group 7:** (n=5 surgeries, n=3 endpoint reached) Periosteal disruption, autograft bone chips and drill holes in midshaft of femur. No AID blast.
- **Group 8:** (n=5 surgeries, n=0 endpoint reached) AID blast, periosteal disruption, biofilm inoculation and tourniquet

Results: Preliminary micro-CT analysis demonstrated that the combination of traumatic factors in Group 1 generated ectopic bone growth consistently (100% of cases) within 24 weeks. Ectopic bone was also observed in Group 3 (AID and biofilm) and Group 6 (AID and bone disruption) although has not been consistent (<50%). No signs of HO have been detected in Groups 2, 5 or 7 by way of micro-CT. Micro-CT is currently pending for Groups 4 and 8 and will be completed by May 2019. Preliminary fluorochrome label analysis has demonstrated distinct double labels in the ectopic bone. The analysis has also revealed minimal remodeling in the adjacent cortex suggesting that the ectopic growth was independent and responsive due to the trauma. The preliminary mineral apposition rate has demonstrated the ectopic bone to be 1.7x greater than the host cortical bone. Preliminary SEM analysis on the ectopic bone from Group 1 has demonstrated newly formed bone on the lateral and posterior sides of the femur. Ectopic growth resembled similar remodeling characteristics of previously analyzed human HO which has a hybrid trabecular-like structure with osteon remodeling and hypermineralized regions. Light microscopy analysis confirmed that the ectopic bone was actively remodeling and still advancing into the adjacent tissue/muscle at 24 weeks. Preliminary precision medicine data has indicated a difference exists between Group 1 and Group 7 sheep with specific outcomes currently being analyzed.

Conclusions: Consistent ectopic bone growth was observed in one of the groups tested in a sheep model of a simulated IED blast, in particular when a combination of trauma and battlefield-relevant factors were present. HO that was stimulated herein using our translational model mimicked previous samples retrieved after human HO removal surgery from traumatic blast injuries of service members. Preliminary precision medicine data is being collected to assess genetic, protein, cytokine, immune and growth factor responses, and may reveal more linkages with ectopic bone formation. Successful demonstration of this translatable animal model will help clinicians understand the etiology of this pathological condition, address a major clinical gap that has been sought after by the Department of Defense, and improve care for combat injured service members.

Significance: A significant portion of wounded warriors who suffer from limb loss and combat-trauma also have accompanying HO. This pathology reduces quality of life, decreases function, and delays rehabilitation. By elucidating the development of HO, other studies may be performed to investigate physical or chemical therapies for prevention and/or treatment.

Acknowledgements: Uniformed Services University, Award #HU0001-11-1-0004; U.S. Army Award, Award #W81XWH-16-2-0037, The Wounded Warrior Amputee Softball Team Association, and The Geneva Foundation

Disclosure: The opinions or assertions contained herein are the private view of the author and are not to be construed as official or reflecting the views of the Department of Defense or United States Government.

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Background

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] There has been general agreement in the literature that HO is induced from damage to soft tissue and inflammation;[4,5] ectopic bone growth has been most frequently observed after combat-related trauma to service members with blast injuries.[6] Reviews of orthopedic injuries from Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) have reported that approximately 70% of war wounds have involved the musculoskeletal system,[7] largely from the use of improvised explosive devices (IEDs) and rocket propelled grenades (RPGs). HO has been reported to occur in approximately 63%-65% of wounded service members with limb loss or major extremity injuries.[8-10] Symptomatic HO may delay rehabilitation regimens since it often requires modifications to prosthetic limb componentry and socket size.[11,12] Most concerning is that no empirical evidence has indicated a mechanism for quelling or preventing metabolically active HO.[1] Studies have often lacked histologic corroboration and advanced radiologic quantification.[13] Extensive research by our team has observed several common factors that may act as catalysts for inducing HO: (1) blast injury that displaces bone and/or fragments, (2) tourniquet and negative pressure wound therapy (NPWT) usage and (3) a post-traumatic infection signal. 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) initiate and exacerbate HO.

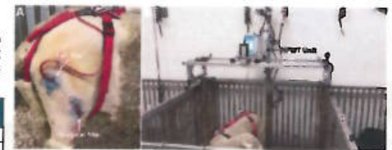
Methods

Animal work was performed at the University of Utah following local institutional animal care and use committee (IACUC) and external animal care and use review office (ACURO) approvals. To simulate an IED blast, an air impact device (AID) that is used in the special effects industry was optimized and safety-tested. The AID discharged high-powered bursts of air to the lateral, mid shaft region of ovine femurs to inflict deep tissue trauma (Figure 1). A series of pilot sheep were used first to confirm that the AID procedure and there would not be a bone fracture, thereby allowing sheep to function and maintain mobility. Work then advanced to larger cohorts of sheep which included various trauma and surgical-related factors including bone disruption, bone chips, tourniquet, NPWT (Figure 2) and using biofilm as an initial inocula (Figure 3). In addition, soft and hard tissue were collected prior to and post-procedure, and blood draws collected at various time points for precision medicine analyses.

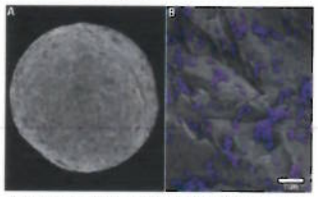
↑Figure 1: (A) Figure demonstrating the impact of the 1100N AID blast (A) Representative image showing a surgically prepared limb prior to an AID blast. The air release opening of the AID was positioned directly above the incision. (B) Still shot showing the effect of the force of the AID blast. The discharge caused the cadaveric sheep limb to concave significantly, suggesting impact to the deep hard tissue.

↓Table 1: Sheep groups and surgeries performed to date. AID=Blast, Bio=10^7 *Staphylococcus aureus* ATCC 6538, T=Tourniquet, P=Disrupt Periosteum, BC=Bone Chips & NPWT=Negative Pressure Wound Therapy.

Sheep Group	Trauma	Surgeries Performed	Endpoint Reached
1	AID, Bio, T, P, BC, NPWT	n=10	n=9
2	AID	n=5	n=5
3	AID, Bio	n=5	n=4
4	AID, Bio, NPWT	n=4	n=0
5	AID, T, NPWT	n=3	n=2
6	AID, P, BC	n=5	n=2
7	P, BC	n=5	n=3
8	AID, Bio, T, P, BC	n=5	n=0

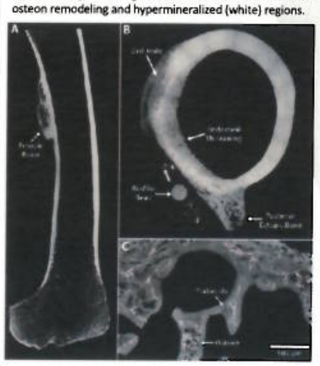


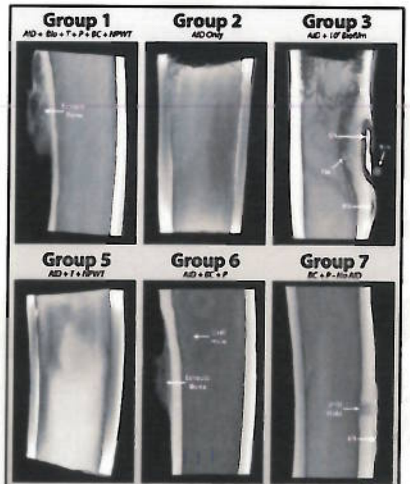
↑Figure 2: Photography of the NPWT system setup. (A) An exit site above the main incision allows the NPWT tubing to leave the affected area and is drawn through to the drainage canister. (B) The NPWT unit sits upon a mobile platform drawing 175 mmHg of pressure. The unit provides mobility as it is attached to the sheep harness. Mobile backpacks units are also used to provide additional mobility.



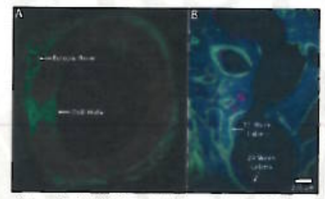
↑Figure 3: (A) Overhead view of a glass Si bead that was roughened using 60 grit sand paper to allow for better biofilm growth and attachment for inoculation. (B) Higher power view showing the *Staphylococcus aureus* ATCC 6538 biofilm (purple) after 72 hrs of growth on the roughened glass Si bead.

↓Figure 6: (A) Micro-CT of ectopic bone growth (arrow) extending from the posterior side of a proximal femur from Group 1. (B) Stitched BSE images creating an overhead view showing ectopic bone growth stemming from the drill hole. Ectopic bone was also observed on the posterior side possibly due to the inoculated biofilm by way of Si beads. Note the endosteal thickening due to the biofilm resorbing the periosteum away on the medial side. (C) BSE image showing a trabecular-like structure with osteon remodeling and hypermineralized (white) regions.



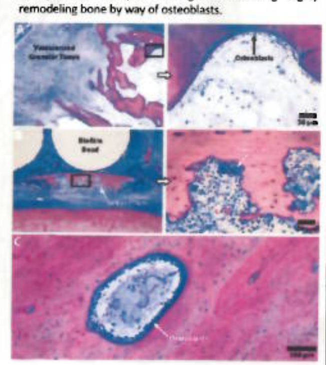


↑Figure 4: 3D reconstructed micro-CT scans. **Group 1** (AID+Bio+T+BC+NPWT) 24-Week femur showing a large mass of ectopic bone growth (arrow) extending from the posterior side of a femur. **Group 2** (AID blast only) 12-Week sheep femur showing no significant signs of any bone changes with the periosteum appearing smooth with no clear evidence of disturbance. **Group 3** (AID+Bio) 24-Week sheep femur shows the glass bead on which biofilm (Bio) had been grown for inoculation. The biofilms appeared to cause a thickening of the endosteal wall (EW) as well as possible sequstem bone (SB) directly below the biofilm beads. Note the periosteal response (PR) in the adjacent bone. **Group 5** (AID+T+NPWT) 12-Week sheep femur coincided with the AID blast only group (Group 2) results with no signs of any gross bone changes by way of micro-CT. **Group 6** (AID+P+BC) 24-Week sheep femur showing a jagged ectopic bong growth on the posterior side of the femur extending into the muscle. **Group 7** (P+BC) 24-Week sheep femur from showing a periosteal response (PR) stemming from the drill hole on the medial side. Note the smooth nature of the PR Compared to the jagged ectopic bone growth from Group 6. The only variable between Group 6 and 7 was Group 7 did not receive the AID blasts.



↑Figure 5: (A) PMMA embedded cross section of a sheep from Group 1 showing the fluorochrome double labels (green). Note the difference in bone remodeling structure of the ectopic bone compared to the drill hole. (B) Higher power view of the ectopic bone showing the calcein green and xylenol orange labels administrated at 12 and 24 wks.

↓Figure 7: Light microscopy micrographs (A) Ectopic bone (pink) growth from a drill hole extending into the adjacent muscle and tissue. Note the vascularized granular tissue (blue) due to the trauma. Higher power view (black box) shows osteoblasts activity on the bone seams. (B) Sequestrum bone adjacent to the biofilm Si beads. Higher power view (black box) shows osteoclast activity on the sequestrum bone. (D) Light microscopy micrographs of the posterior ectopic bone growth showing highly remodeling bone by way of osteoblasts.



Methods (continued)

To date, n=42 surgeries have been performed and n=25 sheep have reached an endpoint up to 24 weeks (Table 1). When the study is finished, Group 1 will have a total of n=10 and the remaining Groups will have n=5 sheep/group. Following AID blast and surgery, all sheep resumed weight bearing activities and were monitored. For those that have been euthanized, the femurs were dissected, micro-CT'ed and processed in polymethyl methacrylate (PMMA) for scanning electron microscopy (SEM) and histological analysis.

Results

Preliminary micro-CT analysis has demonstrated that the combination of traumatic factors in Group 1 generated ectopic bone growth consistently within 24 weeks. No signs of HO have been detected in Groups 2 & 5 by way of micro-CT. In Group 3, 1/4 sheep showed signs of ectopic bone. In Group 6, 1/2 sheep demonstrated ectopic bone. Group 7, which did not receive the AID blast showed a periosteal response, but no ectopic bone in the 3/3 that have reached the 24-Week endpoint (Figure 4). Preliminary fluorochrome label analysis has demonstrated distinct double labels in the ectopic bone. The analysis has also revealed minimal remodeling in the adjacent cortex suggesting that the ectopic growth was independent and responsive due to the trauma (Figure 5). The preliminary SEM analysis demonstrated newly formed bone on the lateral and posterior sides of the femur. The observed ectopic growth resembled similar remodeling characteristics of previously analyzed human HO which was a trabecular-like structure with osteon remodeling and hypermineralized regions (Figure 6). The light microscopy analysis confirmed that the ectopic bone was actively remodeling and still advancing into the adjacent tissue/muscle at 24-Weeks (Figure 7). Work is currently ongoing to collect additional data for all groups.

Discussion

Preliminary data has shown consistent ectopic bone growth in a sheep model of a simulated IED blast, in particular when a combination of trauma and battlefield-relevant factors are present. Outcomes have shown similarities to retrieved human HO bone from traumatic blast injuries. Additional animals are being monitored. Lastly, precision medicine components are also currently being collected to assess genetic, protein, cytokine, immune and growth factor responses. Successful demonstration of this translatable animal model will help clinicians understand the etiology of this pathological condition, address a major clinical gap that has been sought after by the DoD, and improve care for combat injured service members.

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Disclaimer

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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] There has been general agreement in the literature that HO is induced from damage to soft tissue and inflammation;[4,5] ectopic bone growth has been most frequently observed after combat-related trauma to Service members with blast injuries.[6] Reviews of orthopedic injuries from Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) have reported that approximately 70% of war wounds have involved the musculoskeletal system,[7] largely from the use of improvised explosive devices (IEDs) and rocket propelled grenades (RPGs). HO has been reported to occur in approximately 63%-65% of wounded Service members with limb loss or major extremity injuries.[8-10] Symptomatic HO may delay rehabilitation regimens since it often requires modifications to prosthetic limb componentry and socket size.[10,12] Most concerning is that no empirical evidence has indicated a mechanism for quelling or preventing metabolically active HO.[3] Studies have often lacked histologic corroboration and advanced radiologic quantification.[13] Extensive research by our team observed several common factors that may act as catalysts for inducing HO: (1) blast injury that displaces bone and/or fragments, (2) tourniquet and negative pressure wound therapy (NPWT) usage and (3) a post-traumatic infection signal. 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) initiate and exacerbate HO.

METHODS

Animal work was performed at the University of Utah following local institutional animal care and use committee (IACUC) and external animal care and use review office (ACURO) approvals. To simulate an IED blast, an air impact device (AID) that is used in the special effects industry was optimized and safety-tested (Fig. 1). The AID discharged high-powered bursts of air to the lateral, mid shaft region of ovine femurs to inflict deep tissue trauma. A series of pilot sheep were used first to optimize the AID procedure and ensure that there would not be a bone fracture. Work then advanced to larger cohorts of sheep which included various trauma and surgical-related factors including bone disruption, bone chips, tourniquet, NPWT and using biofilm as an initial inocula (Table 1; Figs. 2-3). In addition, soft and hard tissue were collected prior to and post-procedure, and blood draws collected at various time points for precision medicine analyses. Following AID blast and surgery, all sheep resumed weight bearing activities and were monitored. Following 24 Weeks the sheep were euthanized, the femurs were dissected, micro-computed tomography (CT) scanned and processed in polymethyl methacrylate (PMMA) for scanning electron microscopy (SEM) and histological analysis.

Disclosure

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army, the Department of Defense, or the United States government.

MHSRS-19-00435

Main Finding
Heterotopic Ossification Achieved Using a Large Animal Model

Figure 1: Impact of the 1100N AID blast. (A) Representative image showing a surgically prepared cadaveric sheep limb prior to the AID blast. Note the air release opening of the AID was positioned directly above the incision. (B) Still shot showing the effect of the force from the AID blast. The discharge caused the cadaveric sheep limb to remove significantly, suggesting impact AID blast may result in deep tissue trauma needed to hypothetically signal the HO bone response.

[Table 1: Number of sheep per group and surgical trauma performed. AID = Blast, Bio = 10⁷ *Staphylococcus aureus* ATCC 6538, T = Tourniquet, P = Disrupt Periosteum, BC = Bone Chips & NPWT = Negative Pressure Wound Therapy.]

Group	Trauma	Surgeries
Pilot	AID, T, BC	n=2
1	AID, Bio, T, P, BC, NPWT	n=10
2	AID	n-5
3	AID, Bio	n-5
4	AID, Bio, NPWT	n-5
5	AID, T, NPWT	n-5
6	AID, P, BC	n-5
7	P, BC	n-5
8	AID, Bio, T, P,BC	n-5

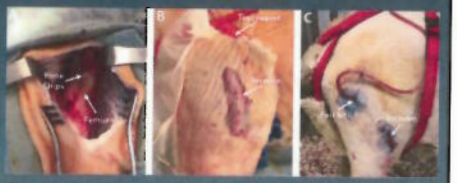

Figure 2: Surgical trauma, AID blast procedure, and NPWT placement. (A) Surgical site with the exposed femur post feathering/roughening. (B) Transparent film allowed for the incision site to remain closed during the AID blast. Note following the AID blast a tourniquet was applied for ~45mins. (C) Exit site above the main incision that allowed the NPWT tubing to be placed subdermal while still being able to attach to the external drainage canister.

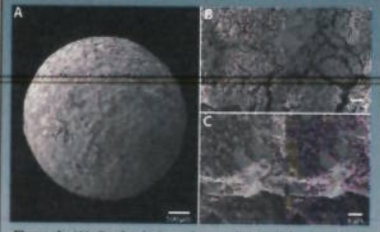

Figure 3: (A) Overhead view of a roughened Si bead used for inoculation. (B) High power view showing the *Staphylococcus aureus* ATCC 6538 biofilm following 72 h of growth. Note the multi-layered biofilm was predominately located within the concaved regions of the Si beads. (C) Higher power view demonstrating the presence of the extra cellar matrix (yellow) on the outer peaks of the Si bead.

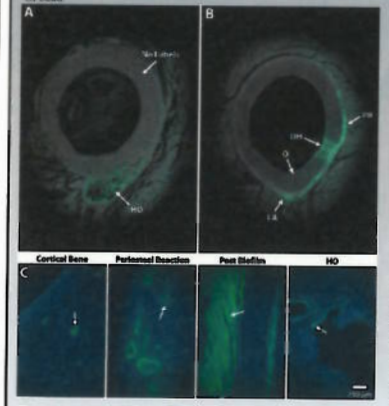

Figure 6: Representative images showing the different bone remodeling structures by way of fluorochrome double labels (green). (A) Overhead view demonstrating HO modeling was independent with minimal effect to the host bone, which demonstrated no labels. (B) Overhead view demonstrating bone modeling at the linea aspera (LA), periosteal reaction (PR) and drill hole (DH) defect regions. Osteonal (O) remolding was also observed mid-cortex. (C) High magnification (100x) view of the different bone remodeling structures. The HO demonstrated similarities of trabecular remodeling with linear double labels at the bone seams.

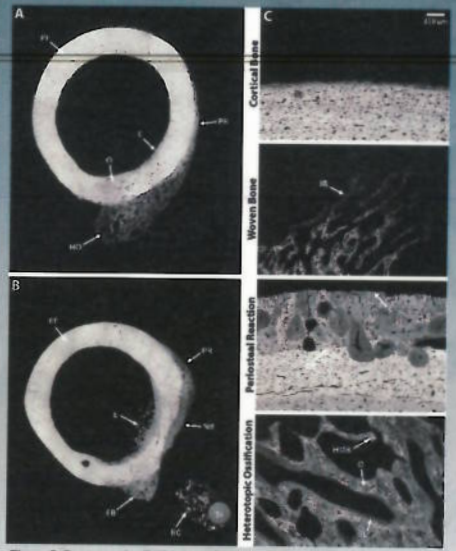

Figure 5: Representative BSE images demonstrating the observed bone responses. (A) Sheep from Group 1 showing HO structured ectopic bone stemming from the posterior side along with osteonal remodeling (O) mid-cortex. Note a smooth periostoal reaction (PR) and endosteal (E) remodeling was observed where the bone was roughened. The anterior and medial regions revealed no signs of a bone response due to the AID blast as the native plexiform (PF) bone structure was still present. (B) Additional sheep from Group 1 demonstrating ectopic bone (EB) growth at the linea aspera interface in addition to new bone growth filling the previously resorbed away cortex due to the inoculated biofilm. This ectopic bone did not have the characteristics as human HO. Note the smooth periosteal response (PR) observed on the lateral side as well as the loose bone chips (BC) around the Si beads (Si). (C) Representative high magnification BSE images of the different observed bone responses. Native sheep cortical bone. Woven bone observed in a 2 weeks specimen demonstrating the immature bone (IB) structure with irregular lacunae and absent of lamellae lines. Periosteal reaction (darker grey) extending from the cortical boundary (CB) on the lateral side of the femur where the bone feathering/roughening occurred. Note the smooth/dense (S) nature of the response in this region. Lastly HO bone growth, which demonstrated a complex hybrid bone structure that resembles human HO. Note the HO bone demonstrated a trabecular-like structure with osteon (O) remodeling, areas of hyper-mineralized bone (HMB), as well as long non-circular lamellae lines (L) at the bone seams.

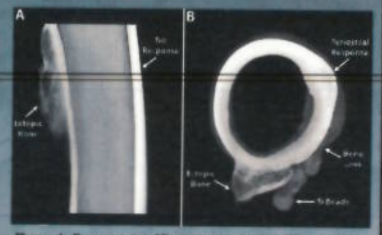

Figure 4: Representative 3D reconstructed micro-CT scans from Group 1 revealing HO on the posterior side of the femur. (A) Sagittal plane showing the posterior HO growth was a similar structure as human HO. (B) Transverse plane again revealing HO present on the posterior side. Note the smooth periosteal response on the lateral side as well as the bone loss adjacent to the Si beads.

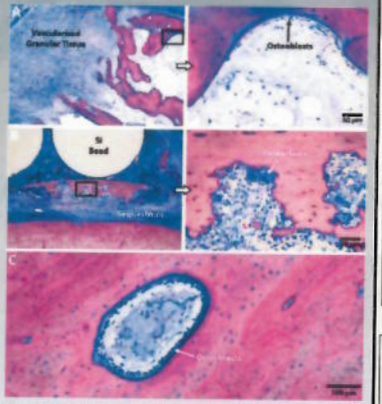

Figure 7: Light microscopy micrographs. (A) Ectopic bone (pink) growth from a drill hole extending into the adjacent muscle and tissue. Note the vascularized granular tissue (blue) due to the trauma. Higher power view (black box) shows osteoblasts activity on the bone seams. (B) Sequestrum bone adjacent to the biofilm Si beads. Higher power view (black box) shows osteoclast activity on the sequestrum bone. (C) Light microscopy micrographs of posterior ectopic bone growth showing highly remodeling bone by way of osteoblasts. Note the abundance of osteocytes present within the lacunae. This suggests the new bone growth was rapid.

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RESULTS

Preliminary micro-CT analysis demonstrated that the combination of traumatic factors in Group 1 generated ectopic bone growth consistently (100% of cases) within 24 weeks (Fig. 4). Ectopic bone was also observed in Group 3 (AID and biofilm) and Group 6 (AID and bone disruption) although has not been consistent (<50%). No sign of ectopic bone has been detected in Groups 2, 5 or 7 by way of micro-CT. Micro-CT is currently pending for Groups 4 and 8. Using advanced histological analysis it was determined that 5 out of 8 (62.5%) of the sheep that reached their predetermined endpoint in Group 1 exhibited similar characterization as human HO bone growth. More specifically the BSE analysis revealed that n=5 sheep contained characteristics of previously analyzed human HO which has a hybrid trabecular-like structure with osteon remodeling and hypermineralized regions (Fig. 5). This correlated with the fluorochrome label analysis which demonstrated distinct linear double labels at the bone seams of the HO bone compared to the circular osteonal remodeling of cortical bone (Fig. 6). The analysis has also revealed minimal remodeling in the adjacent cortex suggesting that the HO growth was independent and responsive due to the trauma. The preliminary mineral apposition rate has demonstrated the HO bone to be 1.7x greater than the host cortical bone. Light microscopy analysis confirmed that the ectopic bone was actively remodeling and still advancing into the adjacent tissue/muscle at 24 weeks (Fig. 7). Histological analysis is currently being performed on the remaining groups in addition to precision medicine data.

CONCLUSIONS

Consistent ectopic bone growth was observed in one of the groups tested in a sheep model of a simulated IED blast, in particular when a combination of trauma and battlefield-relevant factors were present. HO that was stimulated herein using our translational model mimicked previous samples retrieved after human HO removal surgery from traumatic blast injuries of Service members. Preliminary precision medicine data is being collected to assess genetic, protein, cytokine, immune and growth factor responses, and may reveal more linkages with ectopic bone formation. Successful demonstration of this translatable animal model will help clinicians understand the etiology of this pathological condition, address a major clinical gap that has been sought after by the Department of Defense, and improve care for combat injured Service members.

SIGNIFICANCE

A significant portion of Wounded Warriors who suffer from limb loss and combat-trauma also have accompanying HO. This pathology reduces quality of life, decreases function, and delays rehabilitation. By elucidating the development of HO, other studies may be performed to investigate physical or chemical therapies for prevention and/or treatment.

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