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

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14. ABSTRACT Since the last annual report, we have performed surgeries on an additional n=21 animals, which equates to a total of n=34 sheep having been worked on with n=22 euthanized at the predesignated end point. Micro-CT analysis has indicated that the presence of biofilm contributes to a bone response as long as there are placed in close proximity to the bone. Micro-CT analysis has also demonstrated that disruption of the periosteum and drill holes through the cortex allowing for growth fracture to be released in the adjacent muscle in addition to the AID blast may result in ectopic bone. Work on additional animals are being performed and will continue to be collected to assess timelines as to when HO begins to form in the various groups of animals. Tissue processing is underway for histological analysis of those animals that have reached their endpoint and will continue to be performed on the back end of these studies to further confirm formation of HO in the various groups.					
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Table of contents

1. Introduction.....	4
2. Body.....	5
3. Key Research Accomplishments	8
4. Reportable Outcomes and Conclusions.....	9
5. References.....	9
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 skeleton² 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}

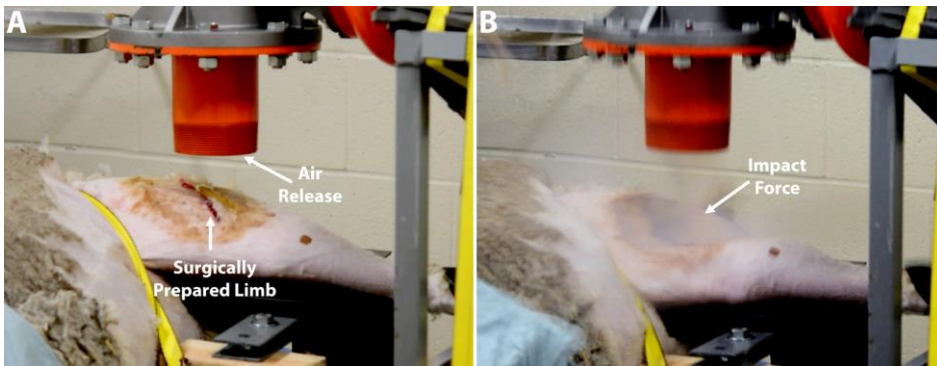
Most concerning is that no empirical evidence has indicated a mechanism for quelling or preventing metabolically active HO.¹ Correlative factors such as gender,^{1,17} genetics,^{7,18-20} bioelectric signals,⁷ infection,²¹ and age¹⁷ have been associated with ectopic bone growth, but studies have often 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 levels³¹ and bone ingrowth into intramedullary implants³² 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

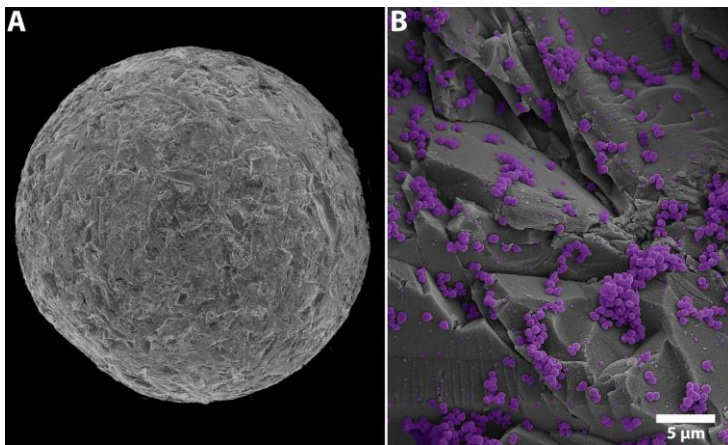
2. Body

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 (see report dated 9/20/2017), 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) 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 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.

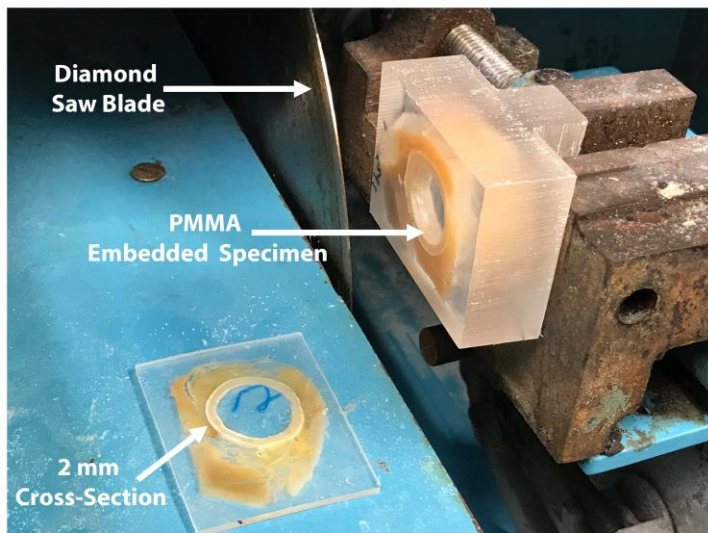
2.2 Current Animal Testing

Since the last annual report we have performed surgeries on an additional n=21 animals (see Table 1). This equates to a total of n=34 sheep having been worked on with n=22 euthanized at the predesignated end point, n=1 was euthanized early due to a broken leg that was unrelated to the surgery and n=11 are still currently running. The AID blasted limbs from the n=22 sheep are being processed for embedment in polymethyl methacrylate (PMMA). The 1st set of limbs from Group 1 are currently being sectioned and prepared for analysis (Figure 4).

Table 1: Sheep groups (n=5/group are designated for testing), treatments being performed in each group and the number that have been performed to date.

Sheep Group	Treatment	Surgeries	Euthanized	Currently Running
1	AID Blast Only	4	4	0
2	AID Blast, 10 ³ Biofilm			
3	AID Blast, 10 ⁷ Biofilm	5	5	-
4	AID Blast, 10 ⁷ Biofilm, Wound VAC	4	0	4
5	AID Blast, Tourniquet, Wound VAC	5	2	3
6	AID Blast, Tourniquet	1	1	0
7	AID Blast, Bone Chips, Periosteal Disruption	6	3	3
8	Bone Chips, Periosteal Disruption	4	3	1
9	AID Blast, 10 ⁷ Biofilm, Tourniquet, Bone Chips, Periosteal Disruption	5	5	0
Total		34	23	11

2.3 Preliminary Results



←**Figure 4:** Image showing a PMMA embedded femur being sectioned into 2mm slices by a custom water cooled saw equipped with a diamond blade.

The preliminary data has indicated that the sheep in Group 1 that received an AID blast only, had no gross signs of any bone response by 12 weeks (Figure 5A). This outcomes was the same for the sheep in Groups 5 & 6 (Figure 5C). The sheep from Group 3 have showed signs of the beginning of possible ectopic bone growth (Figure 5B) stemming from the periosteum. It is recognized that the response in Group 3 could be an infection response, but these sheep had no fevers and did not require antibiotics. There was also a pronounced response in the periosteal region away from the beads and indicate that a compelling bone

response is occurring 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. The sheep from Group 7 showed promising signs of jagged ectopic bone growth (Figure 5D) stemming from the posterior side and advancing into the muscle. In contrast the sheep from Group 8 who surgeries were identical to Group 7 except for no AID blast showed only a smooth periosteal response (Figure 5E) adjacent to the drill holes. This is a very interesting finding and may prove the

importance of the AID blast to inflict deep muscle trauma to help promote HO growth. The n=22 euthanized sheep limbs are currently being processed for histological analysis to see if bone remodeling or early signs of HO formation is present in the mid-shaft of the femur. The first PMMA cross-section from Group 1 has been imaged using a Scanning Electron Microscope equipped with a Backscatter (BSE) detector which gives a much higher resolution image than the micro-CT scans (Figure 6). The BSE images are also able to determine the mineralization of the bone and give distinct grey levels depending on whether the bone is mature host bone (light grey) or new recently remodeled bone (darker grey). The preliminary SEM analysis revealed endosteal remodeling (Figure 6C) on the lateral side of the sheep's femur where the AID blast occurred. The posterior side demonstrated osteon remodeling (Figure 6D), this is in stark contrast to the sheep's native plexiform bone that could still be seen on the medial side of the femur (Figure 6B). Further analysis will help determine if this bone response observed is a direct result of the AID blast.

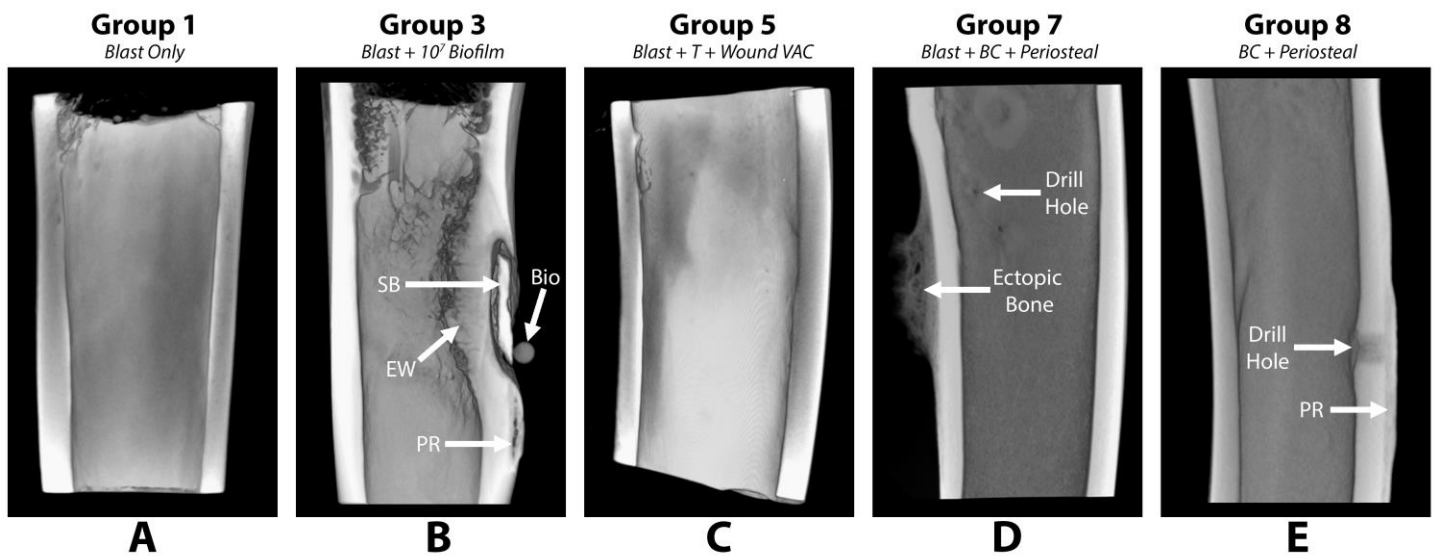
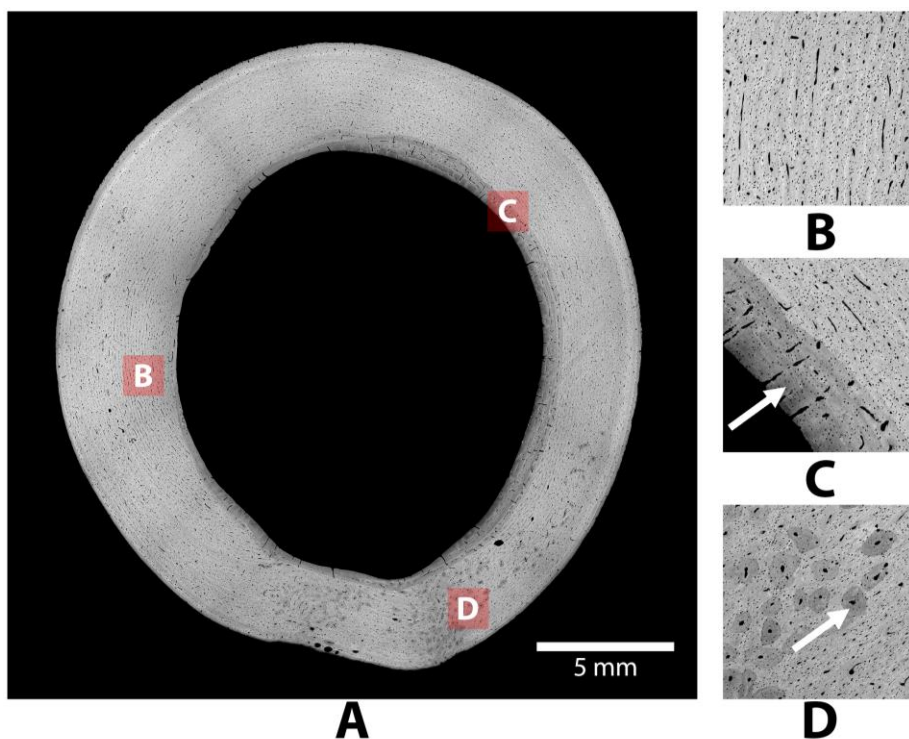


Figure 5: 3D reconstructed Micro-CT images (A) 12-Week sheep femur from Group 1 (AID blast only). The coronal plane showing no significant signs of any bone changes with the periosteum appearing smooth with no signs of disturbance. (B) 24-Week sheep femur from Group 3 (AID blast + biofilm). The coronal plane 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 sequense bone (SB) directly below the biofilm beads. Note the periosteal response (PR) in the adjacent bone. It is unclear if this is the beginning of HO or simply a periosteal response. Further histological analysis is needed by way of SEM and light microscopy. (C) 12-Week sheep femur from Group 5 (AID blast + ~45min Tourniquet + Wound VAC). The Group 5 femurs coincided with the blast only group (Group 1) results with no signs of any gross bone changes by way of micro-CT. (D) 24-Week sheep femur from Group 7 (AID blast + bone disruption). The parasagittal plane shows a jagged ectopic bone growth on the posterior side of the femur extending into the muscle. Note the drill holes on the lateral side of the bone were still visible at 24-Weeks. (E) 24-Week sheep femur from Group 8 (bone disruption only). The coronal plane 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 7 (D).



←**Figure 6:** 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.

In summary, the research is progressing as planned and our team is working carefully to schedule surgeries to optimize logistical management of the study, stay on timeline and achieve the objectives. Data is currently being analyzed and work will continue to be performed with additional sheep on the study.

2.4 Peer-Reviewed Publications / Conference Abstracts

The first manuscript related to this has been submitted to JMIR Research Protocols. This outlines the development of the setup and trajectory of particulate following a blast. A second manuscript is being drafted to provide details and outcomes of the first phase of model development. In addition to the manuscripts, an abstract was written and accepted by The Military Health System Research Symposium outlining all work completed to date. A 2nd abstract was also accepted to the American Society for Microbiology. A 3rd abstract has been submitted to the Orthopaedic Research Society and is currently being reviewed.

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 n=34 live surgeries and HO results are pending
- * Data is indicating that a combination of factors, as opposed to individual factors, may result in bone response
- * Achieved 7 days of NPWT in multiple animals
- * Successfully inoculated sheep with 10^7 bacteria with a 100% survival rate.
- * Disrupted the bone allowing for growth factor to be released in the adjacent muscle without compromising the sheep's health or ability to move freely.
- * Demonstrated jagged ectopic bone growth.

4. Reportable Outcomes and Conclusions

Micro-CT analysis has indicated that the presence of biofilm contribute to a bone response as long as there are placed in close proximity to the bone. Micro-CT analysis has also demonstrated that disruption of the periosteum and drill holes through the cortex allowing for growth fracture to be released in the adjacent muscle in addition to the AID blast may result in ectopic bone. Work on additional animals are being performed and will continue to be collected to assess timelines as to when HO begins to form in the various groups of animals. Tissue processing is underway for histological analysis of those animals that have reached their endpoint and will continue to be performed on the back end of these studies to further confirm formation of HO in the various groups.

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

BAA W81XWH-16-2-0037

PI: COL (ret) John Shero, MHA, Brad M. Isaacson, PhD, MBA, MSF
Amount \$958,201

Org: Henry M. Jackson Foundation

Award

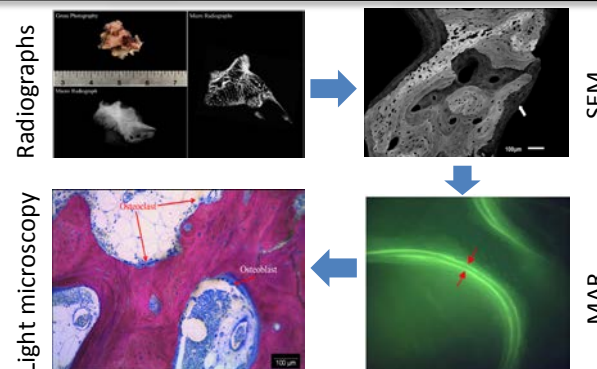


Study Purpose / Deliverables

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).



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 25 live surgeries and HO results are pending, and Achieved 7 days of negative pressure wound therapy in multiple animals.

Timeline and Cost

Activities	FY	17	18
Obtain IACUC approvals			
Develop HO model and test factors			
Analyze HO with advanced histology			
Determine the MAR of HO			
Publish data in peer-reviewed journals			
Estimated Budget (\$K)		\$852	\$106

Updated: 12 October 2018

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: \$943,085

Projected Expenditure: \$958,201