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14. ABSTRACT
The **purpose** of this project is to identify predictive markers of heterotopic ossification in an established animal model that would forecast development of heterotopic ossification (HO) in humans soon after injury. Blast procedures have been completed on all 30 animals (Groups I & II) in the year 1 SOW and 45 animals (Groups III – V) in the year 2 SOW. All animals were biopsied and have been sacrificed according to protocol schedule. Groups I and II animals were also followed with scheduled routine radiographs to monitor progression of HO. Specimen samples are under analysis for gene and protein level expressions with the Nesti partnering molecular biology lab. In year 3, early-appearing gene and protein biomarkers will be identified by analyzing correlations with radiographic HO and will be compared to gene expression signatures in existing human tissue samples known to have gone on to develop HO.

15. SUBJECT TERMS
Heterotopic ossification, blast injury, amputation, bone formation, animal model, rat model, gene expression, protein expression, biomarkers

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Introduction:

Heterotopic ossification (HO), characterized by the pathologic formation of mature bone in the soft tissues, is a frequent complication following high energy orthopaedic trauma. HO is prevalent in patients with severe extremity war-time wounds; specifically, its incidence is reported as high as 57% in patients that sustain a poly-trauma blast injury [1]. Complications related to HO in residual limbs following blast amputation include pain, overlying skin and muscle breakdown, poor fitting and functioning of prosthetic limbs, reoperation for amputation revision, and impaired limb function that delays or limits rehabilitation [2-6]. Current treatments to prevent HO are limited to mitigation rather than prevention. Furthermore, removal of heterotopic bone after it has formed can be difficult; this frequently requires resection of substantial amounts of soft tissue and risks injury to adjacent neurovascular structures that are often intimately associated with the ectopic bone. Hence, it is preferable to address the issue of HO before it begins. Prevention of HO in residual limbs is needed to offer amputation survivors the best possible quality of life and return to function. We have developed a validated blast amputation animal model and confirmed that it replicates the human condition with respect to formation of HO. The current studies are directed at identifying early-appearing biomarkers in the animal model that predict the occurrence of HO in our experimental animals and may well similarly predict the development of HO in the human condition. Patients exhibiting biomarkers predictive of exuberant HO formation can then be identified before the disease process begins and treated prophylactically.

Keywords:
Heterotopic ossification, blast injury, amputation, bone formation, animal model, rat model, gene expression, protein expression, biomarkers

Overall Project Summary:

Current objectives: **All 75 hind-limb blast amputation procedures under Specific Aims 1 & 2 in year 1 & 2 SOW (Groups I – V) have been completed, and all 150 specimens from both amputated and contralateral control limbs have been collected.** Group I and II animals (15 each) were followed with serial radiographs to monitor progression of HO and sacrificed at 24 weeks post-blast, per protocol. Group I animals underwent bilateral muscle biopsy procedure at two weeks, while Group II animals underwent biopsy procedure at four weeks. Group III – V animals (15 each) were biopsied at 24 hours, 72 hours, and 72 hours, respectively, and sacrificed at the same time of biopsy procedures, as per protocol. Group III and IV animals underwent standard wound care with bulb syringe irrigation prior to wound closure following blast amputation while Group V animals underwent pulsed lavage irrigation prior to wound closure. All the biopsy specimens have been sent to the Nesti partner lab for analysis. They were processed to collect total RNAs and protein lysates for identifying both gene- and protein-level biomarkers and will be compared to gene expression signatures in existing human tissue samples known to be characteristic for the formation of heterotopic ossification.

Results: HO progression has been assessed and graded between immediate post-blast and post-mortem radiographs on Group I & II animals. **Radiographic HO data acquired from Group I & II animals are included in supplemental appendix, #1.**

Biomarker analysis, from animal biopsy specimens to identify molecular predictors of HO, was performed by the Nesti partner lab. qRT-PCR and Western blot analysis was carried out to examine the expression of fibrosis markers, such as TGF-b1, Col1al, Acta2, Smad3, and fibronectin. **Biomarker expression data are included in supplemental appendices, #2 & 3.**

Progress and Accomplishments: The project is on schedule as proposed and implemented at our institution. All hind-limb blast amputation procedures on 75 animals have been completed, as well as related scheduled biopsies as specified under Specific Aims 1 & 2. The harvested specimens have been sent to the Nesti partner lab and are currently undergoing RNA profiling using an osteogenesis PCR array to examine the correlation of osteogenic marker expression with radiographic HO findings. Human tissue sample collection from wounded service personnel as specified under Specific Aim 3 will start when the partnering PI obtains IRB approval. Currently, the USUHS online IRB submission system, IRBnet, is transitioning to a new system and is expected to be online by March 2016. This results in a delay in the IRB approval process. However, the continuing review for the IRB has been submitted by partnering PI Nesti and is currently under review.

Key Research Accomplishments: Animal experiments completed on schedule and processing ongoing with data becoming available on a rolling basis as tissue samples are processed and analyzed. Correlation analysis will be performed as data collection from samples is more complete.

Conclusion: Research work is on schedule as proposed and planned. Research conclusions and clinical importance will be determined, as data and analysis are complete in year 3. Nothing further to report.

Publications, Abstracts, and Presentations: Nothing to report.

Inventions, Patents and Licenses: Nothing to report.

Reportable Outcomes: Nothing to report.

Other Achievements: The experience and training provided by this award during the prior year directly contributed to the successful hiring of the past research resident to a position in the Orthopaedic residency at the Medical University of South Carolina.

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Appendices:

1. HO radiographic data – Group I & I animals.

Rat #	Biopsy Time	Post-op Radiographic Measurements (mm)		Postmortem Radiographic Measurements (mm)		%L	%W	HO Grade			HO Severity Score
		Length	Width	Length	Width			L	W	Overall	
1	2 weeks	8.85	10.9	11.3	12.1	27.7	11.0	moderate	mild	moderate	2
2	2 weeks	13.7	8.98	15	12.3	9.5	37.0	mild	moderate	moderate	2
3	2 weeks	11.6	16.8	13.1	17.8	12.9	6.0	mild	mild	mild	1
4	2 weeks	12.4	10.2	14.7	10.5	18.5	2.9	mild	mild	mild	1
5	2 weeks	15	6.66	12.7	7.38	-15.3	10.8	mild	mild	mild	1
6	2 weeks	15	11.9	12.6	7.94	-16.0	-33.3	mild	mild	mild	1
7	2 weeks	12.7	9.36	11.9	10.3	-6.3	10.0	mild	mild	mild	1
8	2 weeks	15.8	7.35	19.3	9.04	22.2	23.0	mild	mild	mild	1
9	2 weeks	8.87	10.8	10.9	12.1	22.9	12.0	mild	mild	mild	1
10	2 weeks	9.83	17.5	15.4	13	56.7	-25.7	severe	mild	severe	3
11	2 weeks	8.01	10.6	5.82	8.31	-27.3	-21.6	mild	mild	mild	1
12	2 weeks	9.89	7.64	8.31	9.88	-16.0	29.3	mild	moderate	moderate	2
13	2 weeks	9.36	10.7	14.8	9.82	58.1	-8.2	severe	mild	severe	3
14	2 weeks	14.9	9.04	15.9	8.63	6.7	-4.5	mild	mild	mild	1
15	2 weeks	10.1	10.7	12.7	10.8	25.7	0.9	moderate	mild	moderate	2
16	4 weeks	8.93	8.13	11.4	8.35	27.7	2.7	moderate	mild	moderate	2
17	4 weeks	15.8	11.1	17	11	7.6	-0.9	mild	mild	mild	1
18	4 weeks	11.5	12.6	8.41	12.7	-26.9	0.8	mild	mild	mild	1
19	4 weeks	7.39	8.29	5.81	11.8	-21.4	42.3	mild	moderate	moderate	2
20	4 weeks	13.5	10.3	15.2	11.6	12.6	12.6	mild	mild	mild	1
21	4 weeks	15.1	8.05	15.3	15.3	1.3	90.1	mild	severe	severe	3
22	4 weeks	16.7	8.29	19.7	16.2	18.0	95.4	mild	severe	severe	3
23	4 weeks	13.5	12.8	14.2	11.1	5.2	-13.3	mild	mild	mild	1
24	4 weeks	9.25	14.8	10.7	18.6	15.7	25.7	mild	moderate	moderate	2
25	4 weeks	19.7	10.6	16.8	16.2	-14.7	52.8	mild	severe	severe	3
26	4 weeks	15	7.81	17	9.68	13.3	23.9	mild	mild	mild	1
27	4 weeks	10.1	20	13.3	19.6	31.7	-2.0	moderate	mild	moderate	2
28	4 weeks	9.41	9.09	9.11	9.25	-3.2	1.8	mild	mild	mild	1
29	4 weeks	20	8.26			0.0	0.0	mild	mild	mild	1
30	4 weeks	15.8	7.19	13.9	15.7	-12.0	118.4	mild	severe	severe	3

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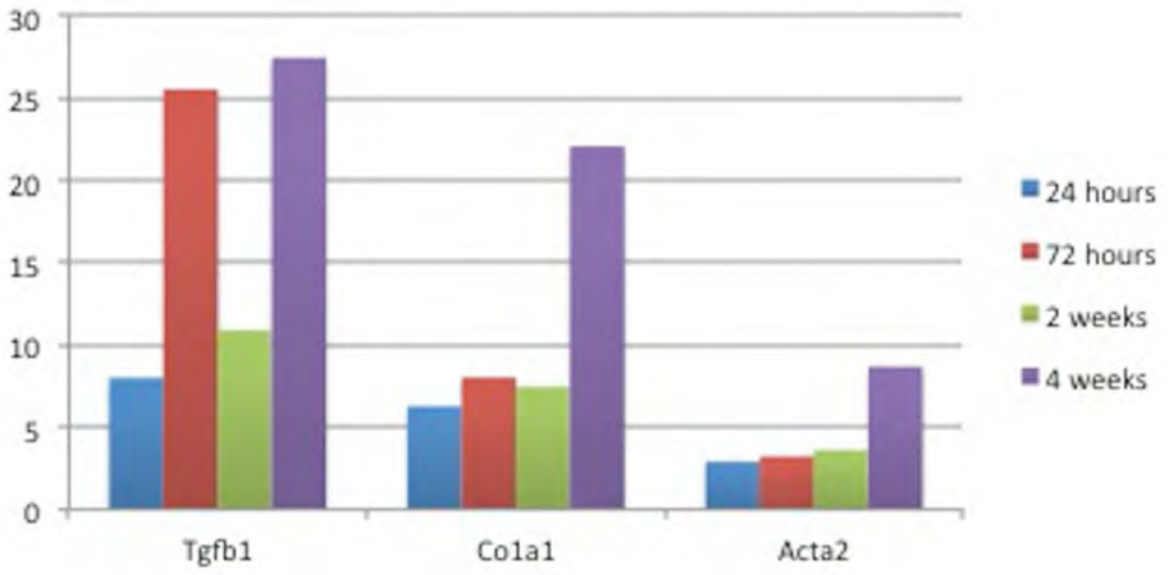
2. Biomarker expression of animal biopsy specimens by qRT-PCR (provided by Nesti partner lab).

Biopsy Time points	Fold Changes		
	Tgfb1	Co1a1	Acta2
24 hr BBS	8.005	6.286	2.917
72 hr BBS	25.533	8.034	3.236
2 week BBS	10.9	7.462	3.622
4 week BBS	27.446	22.058	8.704



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3. Biomarker expression of animal biopsy specimens by western blot (provided by Nesti partner lab).