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TITLE: Sequential versus Combined Medical Therapies as a Novel Heterotopic Ossification Prevention Strategy

PRINCIPAL INVESTIGATOR: LCDR Carolyn Gosztyla, MD

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6. AUTHOR(S)
LCDR Carolyn Gosztyla, MD
Dr. Stephen Kaba

E-Mail: carolyn.e.gosztyla.mil@mail.mil/ stephen.a.kaba.ctr@mail.mil

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Naval Medical Research Center
503 Robert Grant Ave. Silver Spring MD 20910

Uniformed Services University of the Health Sciences
4301 Jones Bridge Rd, Bethesda MD 20814

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14. ABSTRACT
The long-term treatment of military warfighters who sustain severe battlefield blast-related extremity injuries and/or multiple limb amputations is one of the major challenges for military healthcare providers. A common complication facing modern combat casualties is the extra-skeletal development of bone within damaged/healing tissue resulting in soft tissue heterotopic ossification (HO). HO is more prevalent in military trauma, occurring in approximately 65-67% of amputations and nearly 62% of limb sparing procedures. Importantly, clinicians describe HO as the single most important barrier to meaningful functional mobility, independence, and return to military service. In the proposed research, we will use our physiologic model of blast and extremity trauma-induced heterotopic ossification (HO) to test and investigate two drugs (Palovarotene and Rapamycin) which are FDA approved and are currently used clinically for various indications, so that our goal of reaching clinical trials in human patients within five years can become realistic. Our physiologic model of blast-related HO extremity injury incorporates many of the same critical injury patterns detected in combat service members causalities with acute extremity injuries/amputation, including blast overpressure exposure, a comminuted femur fracture, and crush injury to the surrounding musculoskeletal tissue.

15. SUBJECT TERMS
Combat related amputations, heterotopic ossification, fracture healing

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TABLE OF CONTENTS

1. INTRODUCTION:

With the combined effects of improved body armor, tourniquets, aggressive resuscitation techniques, and improvements in the training of medical personnel, an increased number of service members survive to reach tertiary combat care units. The physiological insults produced by blast overpressure trauma results in severe extremity injury trauma accounting for approximately 70% of all combat-related war injuries. Compared to previous conflicts, there is a relative decrease in lower extremity and torso wounds, with a concomitant increase in the head, neck and upper extremity wounds. Heterotopic ossification (HO), the development of ectopic bone within non-skeletal tissues, affects two thirds of patients with high-energy blast-related injuries and more specifically HO develops in 30% of injured service members with complex upper extremity wounds wherein the residual limb is amputated. In the civilian trauma setting, HO is most commonly encountered following musculoskeletal trauma, severe burns, spinal cord injury or following acetabular or elbow surgery. In contrast, combat-related blast injuries are heterogeneous and complex, in regard to severity, distribution, mechanism of injury and level of contamination. These devastating multisystem wounds involving lacerations, crush injury, burns, fractures, systemic overpressure exposure on tissues, traumatic brain injury, extensive soft tissue, neural, vascular and osseous destruction, amputations, ischemia reperfusion injury and infection provide significant clinical management and tissue reconstruction challenges.

The clinical complications of HO formation include lifestyle-limiting pain, skin ulceration and poor tolerance of prosthetic wear, where conservative interventions such as multi-modal pain regimens, physiotherapy and prosthetic modification fail to alleviate symptoms and prevent surgical excision in approximately 41% of patients. Surgical excision of mature HO, although largely definitive, can be wrought with complications and when successful, can result in further delays of rehabilitation

The clinical impact of upper extremity HO differs from that of lower extremity HO based on the differing functions of the upper and lower extremities. The lower extremity functions in weight-bearing and locomotion, and bone-spikes of HO can greatly interfere with comfortable socket wear. The upper extremity exists to move the hand in space, and an injured upper extremity with 2-opposable fingers and decreased range of motion at the wrist and elbow is still often more functional than a prosthetic. Preservation of length and distal function guide the treatment of upper extremity injuries, while creation of a functional limb with robust soft tissue envelope for weight bearing is the goal of treating lower extremity injuries. As such, HO in the upper extremity effects the function differently, not just through limiting prosthetic tolerance (which is usually greater), but also by inhibiting range of motion (ROM) for positioning the terminal device in space for fine motor activities and activities of daily living. Upper extremity HO can also limit function and signal capture from muscle groups necessary for myoelectric device control.

2. KEYWORDS:

Combat related amputations, heterotopic ossification, fracture healing

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Regulatory Tasks (months 0-3)
Subtask 1: Submission of IACUC Applications.
Subtask 2: Submission of IACUC protocol for ACURO and DoD approval of animal studies.
Milestone 1 - ACURO approval. (completed 12OCT2016)
Research Tasks
Specific Aim 1 - Demonstrate the *in vitro* effect of Palovarotene and Rapamycin on the growth and osteogenic and angiogenic differentiation of muscle-derived rat MSCs (months 4-8). 100% completed.

A. Isolate and characterize rMSCs from skeletal muscle of naïve Sprague Dawley rats.
   - Immune-phenotype and functionally characterize the isolated rMSCs. (completed 21DEC2016)
   - Establish a working inventory of stock rMSCs to conduct Specific Aim-1. (completed 21DEC2016)

B. Determine the dose response for Rapamycin and Palovarotene in vitro.
   - Determine optimal dose and combination of Rapamycin, Palovarotene to significantly attenuate osteogenic and endotheliogenic differentiation. (completed 30NOV2017)
   - Assay gene expression of osteogenic, chondrogenic and angiogenic markers for the optimal dose of the drug
   - Quantitate the effects of drugs alone and/or in combination on cellular mineralization, endothelial cell vessel formation and cartilage at optimal and cytotoxic doses. (Completed).

Milestone 2 – Completion of *in vitro* studies and data analysis by end of the 9th month. Determination of the best-of-breed angiogenic/osteogenic inhibitor and/or combination.
Specific Aim 2 - To investigate the effects of multiple drug combination strategies in preventing HO formation in blast-related extremity injury model of HO (9-15 months). 50% completed.

A. Determine the efficacy of different combinations of Rapamycin and RAR- γ agonist in attenuating HO formation
   - Determine the optimal doses of Rapamycin and RAR- γ agonist that radiographically demonstrate the most significant attenuation of HO at 12 weeks. (completed 30OCT2017)
   - Histological and molecular analysis of wound healing/repair and tissue chondrogenesis,

Milestone 3 – Determination of best-of-breed treatment strategy to prevent HO formation. Submission of manuscript entailing the combined *in vitro* and *in vivo* optimal doses and time dependent efficacy of Rapamycin and Palovarotene in attenuating osteogenic, chondrogenic and endotheliogenic differentiation.

Specific Aim 3 - Assess the effect of Rapamycin and Palovarotene independently and/or concomitantly on fracture healing (months 15-22). 80% completed.

A. Test the effects of RAR- γ agonist and Rapamycin alone and in combination on fracture healing.
    - Monitor fracture healing radiographically using micro-CT (2, 3, 6, and 8 weeks post injury). (100% complete).
    - Access the progression and quantitate fracture callus volume following prophylactic treatment of the rats, using microCT and biomechanical testing.
    - Histological and molecular assessment of early fracture healing/repair.(100% complete).

Milestone 4 – Completion of *in vivo* studies assessing the effects of therapeutic strategies on fracture healing. Submission of a peer-reviewed manuscript covering these findings (months 22-24). (100% complete)

What was accomplished under these goals?

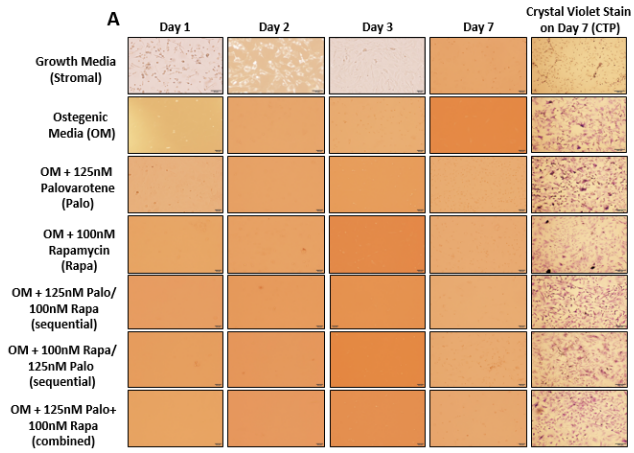
In vitro drug combinations experiments using mesenchymal stem cells (MSCs). We used old frozen bone marrow derived Sprague Dawley rats MSCs (rMSCs) obtained from SA1.

1. Assessed the efficacy of different drug combinations of Palovarotene and Rapamycin on the growth and osteogenic differentiation of rMSCs:
   a. Effect of sequential treatment of Palovarotene, followed by rapamycin (**P/R**) on connective tissue progenitor (CTP) frequency and osteogenic differentiation *in vitro*

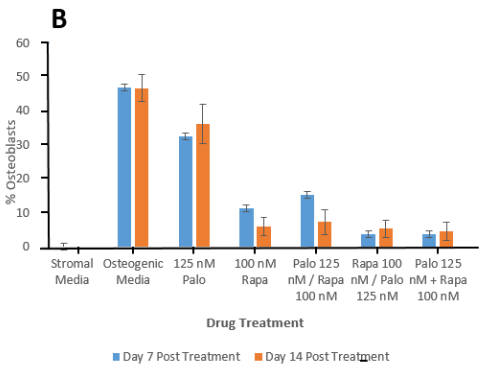
   b. Effect of sequential treatment of rapamycin, followed by Palovarotene (**R/P**) on connective tissue progenitor (CTP) frequency and osteogenic differentiation *in vitro*

   c. Effect of combined treatment of Palovarotene and rapamycin (**PR**) (simultaneous) on connective tissue progenitor (CTP) frequency and osteogenic differentiation *in vitro*

***In vitro* effect of sequential and combined treatment of Palovarotene and Rapamycin on the growth and osteogenic differentiation of bone marrow-derived rat mesenchymal stem cells (rMSCs)**



A: Growth and osteogenic differentiation of rMSCs in growth (stromal) and drug treated osteogenic media



B: Percent of osteoblast cell population in growth (stromal) and drug treated osteogenic media

Figure 1: *In vitro* effect of sequential and combined treatment of Palovarotene and Rapamycin on the growth and osteogenic differentiation of bone marrow-derived rat mesenchymal stem cells (rMSCs)

Previously isolated bone-derived rat mesenchymal stem cells (rMSCs) from the naïve donor rats (2nd passage) were seeded in triplicate at a density of $1x10^3$ cells/well in 6-well plates in normal growth media (stromal) supplemented with 10% FBS, 100 U/ml penicillin, and for 24 h at 37°C in fully humidified 5% CO_2 in air atmosphere. For the differentiation study group, normal growth media was changed to osteogenic media and supplemented with different combinations of Palovarotene (125 nM) and/or Rapamycin (100 nM) with media changes every 3 days. After 7 or 14 days, adherent cell colonies were rinsed twice with PBS, fixed with 100% methanol for 5 min at room temperature, air-dried, stained with Crystal violet solution for 5 min, and then rinsed with distilled water to remove residual dye. Connective tissue progenitor osteoblast (CTP-O) colonies were counted using light microscopy by a reader (TAD) that was blinded to the treatment groups.

A: Shows the growth and osteogenic differentiation of rMSCs in stromal and osteogenic media with/without Palovarotene and/or Rapamycin treatments. **B:** Alteration of CTP frequency and osteogenic differentiation after sequential and/or combined Palovarotene and Rapamycin treatment.

Palovarotene alone decreased osteogenic connective tissue progenitor (CTP-O) the least, while non-treated osteogenic media did not show any observable decrease *in-vitro*. Treating rMSCs first with Rapamycin followed Palovarotene 30 min later (Rapa/Palo) as well as simultaneous treatment of both drugs showed the least percentage in osteoblast population *in-vitro*.

What opportunities for training and professional development has the project provided?

Andrew Yurko, MSc, – Research Associate, designed and executed the cell culture study. Improving ability to plan studies, assist in cell culturing, and perform analysis of harvested specimens.

Sohaib Alvi – Research Assistant, helped in the design and mentored cell culture during the course of the study. Improving ability to plan studies, assist in cell culturing, and perform analysis of harvested specimens.

How were the results disseminated to communities of interest?

Nothing to report at this time, analysis on-going.

What do you plan to do during the next reporting period to accomplish the goals?

1. Refine assessment of efficacy of different drug combinations for Rapamycin and Palovarotene in attenuating ectopic bone formation.
    a. Repeating *in vivo* combination drug experiments of Rapamycin and Palovarotene with original Rapamycin distributor.
        i. Palovarotene (PODs 0-7) + Rapamycin (PODs 7-14) (50% completed).
        ii. Rapamycin (PODs 0-7) + Palovarotene (PODs 7-14) (50% completed).
        iii. Histological and molecular characterization of changes in wound healing/repair and tissue chondrogenesis, angiogenesis, and osteogenesis.

2. In vitro assays
    a. Testing combination treatments on naïve and injured cells.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Early results suggest that rapamycin may inhibit early fracture healing which should be considered if further efforts are made to utilize rapamycin as a prophylactic agent for heterotopic ossification: particularly in the setting of the multiply injured combat patient

What was the impact on other disciplines?

The potential increased risk of delayed fracture healing should be considered for transplant patients using rapamycin in their immunosuppressant regimen.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

- The old (expired) animal use protocol has been replaced with a newly approved animal use protocol.
- We have secured a replacement computer for the mCT scanner; currently awaiting engineers from manufacturer (Bruker) for software updates and installation of computer. The mCT scanner is an essential equipment for this project and once it is fixed, we can start the animal models.

Actual or anticipated problems or delays and actions or plans to resolve them

- We have secured a replacement computer for the mCT scanner and currently working with Bruker (the manufacturer) engineers to undertake software updates and install the computer.
- We will start the animal models and in vivo studies as soon as the mCT is fixed.

Changes that had a significant impact on expenditures

Nothing to report.

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	LCDR Carolyn Gosztyla, MD
Project Role:	PI
Nearest Person month worked:	7
Contribution to the Project:	New PI
Name:	Stephen Kaba
Project Role:	Senior Scientist
Nearest Person month worked:	8
Contribution to the Project:	No change
Name:	Patrick Benoit, MD
Project Role:	General Surgeon
Nearest Person month worked:	7
Contribution to the Project:	Scientific management and analysis
Name:	Andrew Yurko
Project Role:	Surgical and Laboratory Research Assistant
Nearest Person month worked:	8
Contribution to the Project:	Assist with animal manipulation, monitoring and sample collection & analysis
Name:	Crystal Leonhardt
Project Role:	Surgical and Laboratory Research Coordinator
Nearest Person month worked:	8
Contribution to the Project:	No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.