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TITLE: Apyrase: A Portable Treatment to Prevent Burn Progression and Infection

PRINCIPAL INVESTIGATOR: Stewart C. Wang, MD, PhD

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, MI 48109-5677

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Apyrase: A Portable Treatment to Prevent Burn Progression and Infection

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6. AUTHOR(S)
Stewart Wang, MD, PhD
Jean Nemzek, DVM, MS
Benjamin Levi, MD
Chuanwu Xi, PhD
EMAIL: stewartw@med.umich.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Regents of the University of Michigan
Kathyrn DeWitt
503 Thompson St
Ann Arbor, MI48109-1340

8. PERFORMING ORGANIZATION REPORT NUMBER

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14. ABSTRACT
Definitive treatment of burns often requires surgical excision and grafting. However, the facilities and personnel needed for this may not be acutely available in the combat casualty care arena. This creates the need for interim care strategies that would promote healing and prevent infection until more definitive treatment can be provided. Topical apyrase, an adenosine triphosphate (ATP) hydrolyzing enzyme, has local anti-inflammatory and anti-microbial characteristics that proved beneficial and eliminate infection without local toxicity. In the first aim of the study, we developed a porcine model of partial thickness burn injury to compare the effectiveness of two dosages of apyrase with a standard method of treatment and the in vivo work was finished in this annual reporting period. Serial biopsies, wound measurements and photographs were taken over time to assess inflammation and healing responses. Final results are pending. Work on Specific Aim II involving infected burn wounds began in this reporting period as well. Optimal bacterial growth conditions were defined and inoculum size determined for the infection experiments. Wounds were infected and treatments applied one day after burn and then daily for 4 days. Most notably, blinded, assessments of wound characteristics suggest that infected wounds treated with apyrase more closely resemble uninfected wounds at Day 3 post burn than do infected burns treated with either saline of sulfamylon. The results of culture and biofilm studies will provide more quantitative assessments and will be available at the conclusion of the in vivo studies.

15. SUBJECT TERMS
Inflammation, Thermal, Healing, Antimicrobial, Enzyme, Infection, Biofilm

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
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TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

In the management of thermal injury, the major goals of initial, non-surgical treatment are reduction of local inflammation, prevention of wound progression, and inhibition of bacterial infection. However, at this time, there are no effective treatments to reduce wound progression and the emergence of resistant bacteria has threated the efficacy of antibiotic therapy. In this study, we propose that apyrase, an ATP hydrolytic enzyme, would fulfill the unmet need for an effective topical treatment for burn injury. Excessive extracellular ATP (eATP) released from injured tissues acts as a danger-associated molecular pattern, triggering inflammatory responses, and eATP also promotes biofilm formation in several strains of bacteria. Apyrase hydrolyzes ATP to ADP and phosphate which has effectively controlled the inflammatory response in our previous work in mouse models of thermal injury and associated complications. This current study will further examine the use of apyrase in a relevant porcine model. The study is designed to quantify healing, inflammation, and bacterial infection of burn wounds to compare the efficacy of apyrase with that of controls and standard of care topical therapy. The application of apyrase will be tested in partial thickness burns and repeated in burns with concurrent bacterial contamination. Outcome measures to be assessed include gross wound characteristics, histology, inflammation, and bacterial colony counts. These measures will be used to assess the known anti-microbial, anti-inflammatory, and pro-healing effects of apyrase and act as a step towards translation of this treatment into burn wound therapy. Ultimately the goal is to improve recovery time, reduce costs, and improve outcomes for many burn patients.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Inflammation, Thermal, Healing, Antimicrobial, Enzyme, Infection, Biofilm

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1: Demonstrate that topical apyrase decreases inflammation and wound expansion of partial thickness burns.
Subtask 1: UCUCA Approval: 11/14/2016
Subtask 2: Burn analysis for inflammation and progression: pending
Subtask 3: Conduct design of experiments analysis using pig model: 100% completed
Aim 2: Validate the anti-microbial properties of topical apyrase in partial-thickness burns.
Subtask 1: Perform burn model with gram negative and gram positive infection: 50% completed
Subtask 2: Burn analysis of inflammation and progression: 40% completed
Subtask 3: Quantify bacterial load, biofilm and data analysis: 50% completed

What was accomplished under these goals?

Specific Aim 1: Demonstrate that topical apyrase decreases inflammation and wound expansion of partial thickness burns. During year two, we completed all the *in vivo* work for this specific aim. Measurements of wound contraction and epithelialization were recorded and analyzed. In year three, histology samples were processed and preliminary results obtained. Final analysis will be completed during the approved extension of grant period.

Subtask 1: UCUCA Approval: 11/14/2016

Subtask 3: Conduct design of experiments analysis using pig model

Description of methods used: Pigs (25-30kg, female Yorkshire-cross, pigs were acclimated to facilities for at least 5 days. The pigs were sedated with telazol/xylazine and maintained with isoflurane via face mask. Analgesia was provided with one pre-emptive buprenorphine injection and placement of a buprenorphine patch prior to procedure. Eight burn wounds were created with a heated copper block (5 x 5cm, 80°C) for either 20 (Superficial) or 40 (Deep) seconds under 5 pounds of pressure. The corners of the burns were tattooed. Treatments were randomly assigned to wounds on either side of the animal and applied with a spray bottle (saline, sulfamylon, 0.5 Units apyrase and 1.0 Units Apyrase). The wounds were covered with telfa pads and Tegaderm followed by padding, protective bandaging and a jacket. Bandages were changed and treatments re-applied on post-burn days 1, 2, 3, 4, 7, and 14. On days 1,3,7, 14 and 21 post-burn, two (3mm) biopsies were obtained (one located centrally and one in a corner of the wound) and wounds were swabbed for ATP analysis. The animals were euthanized on Day21 and the entire wound resected for histology.

Key Accomplishments: The animal work for this aim was completed

Subtask 2: Burn analysis for inflammation and progression

During this period, we focused on measurements of wound depth at various time points.

Description of methods used: Biopsy samples from the corner (1cm from tattoo mark) and center of each wound were fixed in formalin. Standard sectioning and H&E staining were performed and sections were analyzed for burn depth based on collagen alterations as previously described (PMID: 19200248). The images were analyzed with FIJI/ImageJ (NIH) to calculate average burn depth, a parameter robust to variations introduced by rete ridges. Results were reported as average depth of wounds for each treatment on superficial and deep burns (Figure 1A and B).

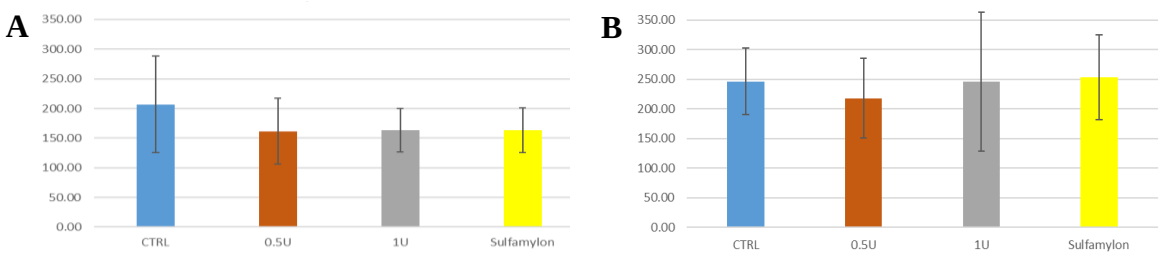


Figure 1. Preliminary assessment of burn depth. Depth of injury was measured with Image J software on (A) Superficial and (B) Deep burn wounds treated with either saline (CTRL), 0.5U Apyrase, 1.0U Apyrase or Sulfamylon. Results expressed as mean ±SD. n = 5/group

Key Findings or Accomplishments: Final histology results are pending, however the results from Day 7 after burn wound suggest that burn depth (Figure A and B) was dependent upon duration of exposure to thermal insult. The results suggest that burn depth in superficial wounds is reduced by treatment with either dose of apyrase and by sulfamylon as compared to control

wounds. However, the difference is not statistically significant with the current sample size of completed histology. For deeper wounds, there is no significant difference between control and treatment groups. These findings suggest only slight effects on depth of burn injury suggesting the ability of Apyrase to slow wound progression may be limited. It is possible a higher dose or greater frequency of early administration may be needed to affect wound progression.

Aim 2: Validate the anti-microbial properties of topical apyrase in partial-thickness burns.

Subtask 1: Perform burn model with gram negative and gram positive infection

In year two, in vitro studies were done to define the log phase growth and optimal conditions for producing the *Staphylococcus* wound infection. In year three, the *in vivo* studies of wounds infected with *Staphylococcus* were conducted.

Description of methods used: The methods for these studies were identical to those above with some exceptions. After initial burn wound, the wounds were dressed without treatment. The following day, either an inoculum of $1x10^6$ log phase bacteria or equal volume of saline was placed on the surface of the wound. This was allowed to dry for 15 minutes, then the randomized treatments (saline, sulfamylon or 1.0 Units Apyrase) were applied. On days designated for biopsy harvest, a total of three, 3mm biopsies were obtained, one from the corner of each wound was placed in formalin for histology, two from the center of each wound were placed in sterile vials for quantitative culture and biofilm analyses.

Key Accomplishment: The *in vivo* studies were completed in all animals (n=8). Planning and scheduling for the infection studies with the Gram negative infection have been initiated.

Subtask 2: Burn analysis of inflammation and progression

In year three, the data from *in vivo* studies including the wound measurements, visual assessments and determination of epithelial growth were examined.

Description of methods used: Wounds were covered by individual pieces of tegaderm and wound characteristics were assessed by blinded observers for characteristics such as erythema, discoloration, and exudate. Photographs were taken of each wound. Applying ImageJ to each photo, a blinded observer defined the original area of the wound by using the tattoo marks on each corner. Then the unhealed area of the wound was outlined and the area calculated.

Key Accomplishments: Subjective assessment of wounds suggest that tegaderm and routine wound care isolated the wounds from each other over the first week of bandage changes. Differences in wound characteristics between uninfected and infected wounds and infected wounds given different treatments were particularly evident 3 days after infection. Some differences were still noted on Day 7 but wounds appeared similar on Days 14 and 21. This suggests the effects of apyrase on infection may be greatest with early application and continued daily application may be beneficial. Epithelialization and surface healing increased over time in all groups (Figure 2). Although most notable in 21 day uninfected wounds, there were no significant differences in the percent of wound area healed among any of the groups. Apyrase appeared to be most effective in the Deep burn wounds (Figure 2B). The wide standard deviations suggest data should be reviewed for outliers and the measurements may be hampered by the presence of eschar and exudate, left undisturbed if possible during the course of the experiment. Subjectively, the 21 day measurements may be the most accurate due to less exudate and the loosening of eschar. Going forward additional measurements will be performed on wounds debrided post mortem. Coupled with other data, the results suggest that more frequent treatment or larger doses may be in order.

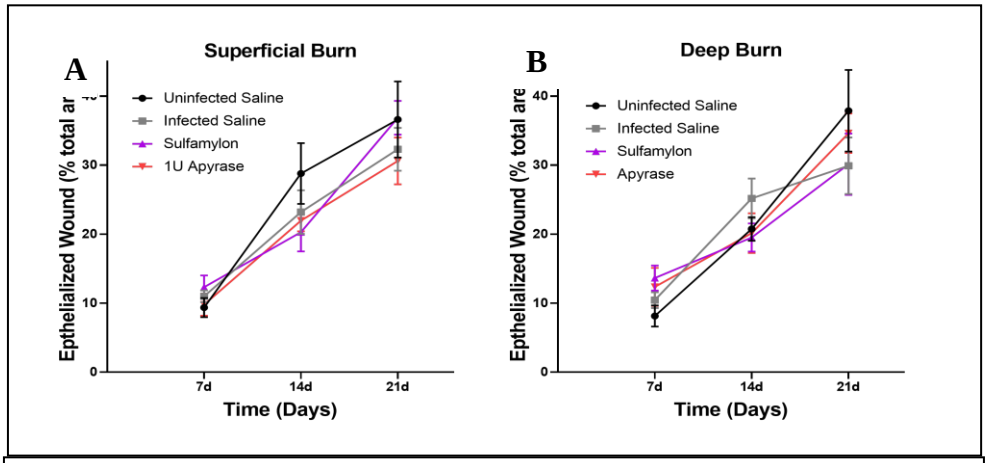


Figure 2. Analysis of wound healing and epithelial coverage. Photographs were used to determine the area of healed wound by analysis with Image J software. The area of the original wound was defined by tattoo mark and the healed area was demarcated at the visual border between granulation bed and epithelium. The healed area of (A) Superficial and (B) Deep burn was expressed as a percent of the original wound size. Results expressed as mean ±SD. n = 7/group.

Subtask 3: Quantify bacterial load, biofilm and data analysis

To date, the quantification of bacterial load has been done for the Gram positive infection studies. Complete histology results are pending for that group.

Description of methods used: Vital staining of biopsy samples was performed to determine the live bacteria present and presence of biofilm. Additional tissue samples taken at each time point were weighed, placed in 5ml of PBS and homogenized for 90 seconds. Serial dilutions of the homogenate were plated on blood agar and allowed to dry for 15 minutes. The plates were then inverted and incubated at 37ºC overnight. Dilutions yielding between 30-300 colonies were counted and used to calculate the CFUs/g tissue.

Key Findings: Tissue samples from some infected wounds were stained to detect live bacteria. The presence of live (green) cocci was noted however the bacteria colonize at several levels within the tissue making attempts at quantification difficult (Figure 3A). Further, staining for biofilm revealed significant background staining of tissues and even hair (Figure 3B). A fluorescent antibody against *Staph* organisms was also used to better define the bacteria, however quantification of the biofilm has proven difficult in comparison to quantitative culture.

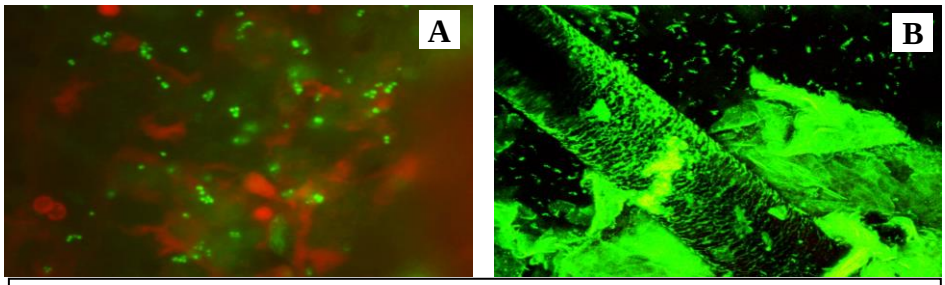


Figure 3. Bacterial load and biofilm analysis. Representative photomicrographs of (A) live/dead stain (green and red, respectively) of biopsy from infected burn wound and (B) Background staining confounding biofilm quantification.

Quantitative cultures revealed no statistically significant differences between the treatment groups at any time of the points (Figure 4). However, for the Deep burn wounds, there is a trend with Apyrase producing counts similar to those of uninfected wounds while the saline and sulfamylon treated wounds are higher. This effect is lost on Day 7. As with the histology data, these data suggest that a five-day regimen of treatment followed by two days without treatment, may negate the positive effects of Apyrase.

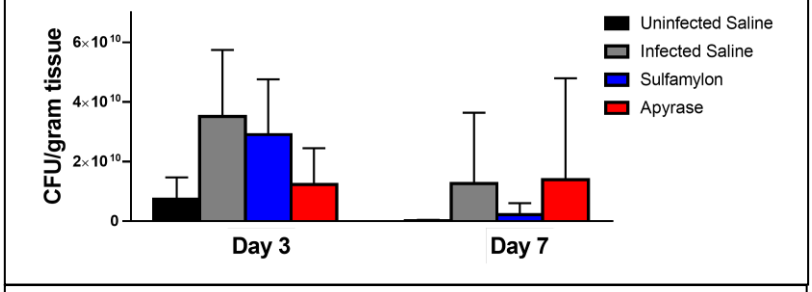


Figure 4. Quantitative cultures of burn wounds. Biopsy samples of Deep burns taken on Days 3 and 7 were weighed, serially diluted and cultured on blood agar for 18 hours. Colonies were counted and expressed as mean ± SEM. n= 6/group

Goals not met: We have not yet completed the cohort of animals infected with the Gram negative bacteria. A no cost time extension has been initiated in order to finish this work. The detection of biofilm on tissue samples has been difficult. The irregular surface and thickness of tissue samples as compared to *in vitro* samples makes microscopy difficult. In addition, in the infection model, Tegaderm was used to cover the wound. With removal of the Tegaderm, the upper layer of the wound is disturbed to varying degrees. This confounds the ability to quantify the biofilm.

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

Nothing to report

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

A no cost time extension was requested and granted. With the extension, we will finish the final cohort with *A. baumanni* and finish the observational, histological and bacteria quantification. The results to date suggest that an alternate strategy is needed for the dosing of apyrase and we will submit an animal use amendment to increase the dose and frequency of application during the Gram negative infection studies. A final assessment of all results will be performed and reported.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Preliminary results suggest trends for apyrase to reduce bacterial load and improve healing in infected wounds. This could impact the early treatment of wounds under austere conditions when definitive treatment is delayed.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not

previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Over the past three years, there were some administrative and facilities delays of the animal work. We have extended our study duration to insure that the remaining work can be concluded. No further delays are expected.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**

None

Books or other non-periodical, one-time publications.

None

Other publications, conference papers, and presentations.

None

- **Website(s) or other Internet site(s)**

None

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

None

- **Inventions, patent applications, and/or licenses**

None

- **Other Products**

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Stewart Wang, MD, PhD	No Change
Jean Nemzek, DVM, MS	No Change
Benjamin Levi, MD	No Change
Chuanwu Xi, MD	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

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

Single PI report

9. APPENDICES

Appendix A: quad chart

Apyrase: A Portable Treatment to Prevent Burn Progression and Infection

MB150237

W81XWH-16-1-0574

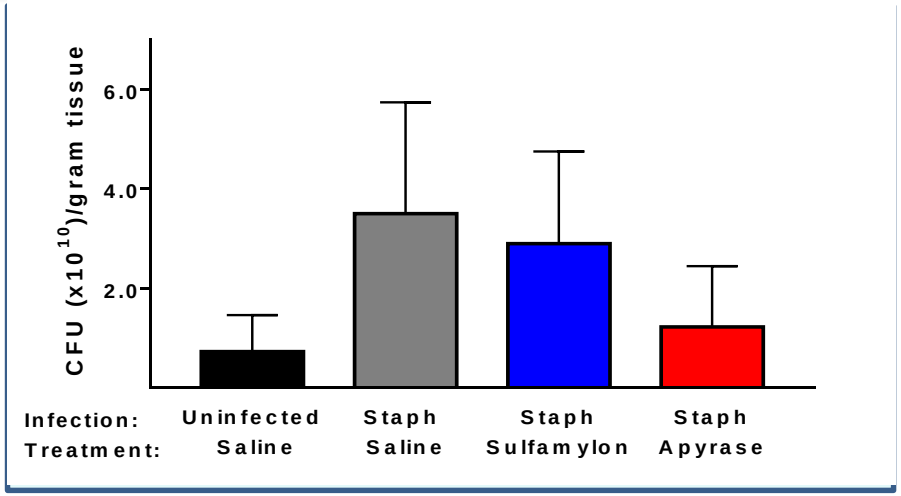
PI: Wang, Stewart C. **Org:** University of Michigan **Award Amount:** $750, 000

Study/Product Aim(s)

- Specific Aim I: Demonstrate that apyrase decreases inflammation and wound expansion of partial thickness burns without toxicity.
- Specific Aim II: Validate the anti-microbial properties of apyrase in partial-thickness burns.

Approach

Apyrase will be tested in a porcine model of multiple partial thickness burns produced by standardized thermal contact. First, an optimal dose will be determined by comparing inflammation and wound progression after treatment with apyrase. The optimal dose will be evaluated for evidence of local and systemic toxicity. Finally, the antimicrobial effects of apyrase will be tested in burns infected with bacteria.



Apyrase reduced bacterial load early (Day3) in the course of Staph infection of deep burn wounds.

Timeline and Cost

Activities CY	16	17	18	19
Demonstrate effectiveness				
Validate anti-microbial properties				
Infection studies				
Data analysis & Preparation for possible clinical trials				
Estimated Budget ($K)	**$250**	**$250**	**$250**	

Updated: 08/31/2019

Goals/Milestones

CY17 Goal – Demonstrate topical apyrase decreases inflammation and wound expansion of partial thickness burns

100% Animal Use Approval (completed 11/16)

100% Design of experiment analysis using pig model

90% Burn analysis for inflammation progression

CY18 & Goal – Validate the anti-microbial properties of topical apyrase in partial-thickness burns

50% Perform burn model with gram negative/positive infection

50% Burn analysis of inflammation and progression

CY19 Goal – Infection studies and future directions

50% Quantify bacterial load, biofilm and data analysis

Comments/Challenges/Issues/Concerns

No Cost Time Extension Requested July 26, 2019

Budget Expenditure to Date

Projected Expenditure: $750,000

Actual Expenditure: $481,530