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

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CONTRACTING ORGANIZATION: Regents of the University of Michigan

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13. SUPPLEMENTARY NOTES

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 in our preliminary studies. We hypothesize that topical application of apyrase to burn wounds will reduce inflammation, minimize wound progression, 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

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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:90%
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: 70% completed
Subtask 2: Burn analysis of inflammation and progression: 40% completed
Subtask 3: Quantify bacterial load, biofilm and data analysis: 70% completed

What was accomplished under these goals?

Specific Aim 1: Demonstrate that topical apyrase decreases inflammation and wound expansion of partial thickness burns. During the extension of this award, it was necessary to renew the IACUC protocol and seek ACURO approval for the renewal. Animal work was completed for this aim. Results will be restated in the final report.
Subtask 1: Original IACUC Approval: 11/14/2016.
Key accomplishments: The renewal of the IACUC protocol was approved on 09/01/2020 under a new protocol number PRO00009830. Subsequent approval by the ACURO was issued on 09/14/2020. The PI on the renewal was changed from Stewart Wang to Jean Nemzek to streamline the administrative duties associated with animal care.
Subtask 2 and 3: In vivo studies and burn analysis: Completion of the *in vivo* work for Aim I was previously reported in the 2019 annual report.

Specific Aim 2: Validate the anti-microbial properties of topical apyrase in partial-thickness burns. An extension was requested and obtained prior to this reporting year. This year the focus was to process the histological samples from the *Staphylococcus* infected samples as well as conduct the animal experiments with the *Acinetobacter* infection.
Subtask 1: Perform burn model with gram negative and gram positive infection: The cohort of animals with *Staphylococcus* infection was completed previously. Therefore, this year's activities in this aim were devoted to studies of the cohort with *Acinetobacter* infection. In the first quarter, the patient derived strain was obtained. The storage, culture parameters and dilutions for the application of the bacteria to burn wounds were standardized. In the second quarter, the animal experiments were initiated using the methods outlined here.

Thermal injury model: Under isoflurane anesthesia, female Yorkshire mix pigs, approximately 30 kgs, were prepared with a depilatory cream followed by scrubbing with chlorhexidine and 70% ethanol. Thermal contact burns were induced via a 50mm x 50mm copper block, heated to 80°C. Contact was applied for either 20 or 40 seconds, to induce partial thickness burns identified as superficial or deep, respectively. Application force was standardized to 5 pounds of pressure. The corners of the wounds were tattooed for reference points and the wounds were then bandaged with individual pieces of tegaderm followed by padded layers and a protective jacket. The following day, each of the eight wounds were moistened with 0.5 ml saline, then a suspension containing $1x10^6$ log phase *Acinetobacter baumanii* (patient origin) was deposited on the surface of six wounds via a syringe and needle. Each burn was lightly excoriated with the needle during application and then allowed to dry for 15 minutes. The deep and superficial burn wounds were then randomized for topical application of Saline control, Sulfamylon, or Apyrase. The dose of apyrase was doubled to 2.0U compared to previous studies. In subsequent days (1, 3, and 7, 14 and 21), punch biopsies were taken from the center and corner of the square burn injury, 1cm from the tattoo marks followed by immediate re-dosing of topical interventions. The animals were euthanized on Day 21 post-burn.

Significant results or key outcomes: Within the first two quarters, the above protocol was completed on three animals. For the third quarter, there was a mandated interruption of work and temporary shutdown of research activities at the institution. During the fourth quarter, research activities experienced a phased reopening. Studies involving large animal species were allowed to

resume in the later phase, requiring special permissions. The animal experiments were resumed by the Nemzek lab late in the fourth quarter and an experiment is now in progress.

Subtask 2: Burn analysis of inflammation and progression

In this reporting period, the Levi lab finished processing the biopsy samples from the *Staphylococcus* cohort and their final analysis is pending. The biopsy samples from the *Acinetobacter* cohort will be processed when animal studies have been completed. Other assessments of wound healing include determination of percent of healed wound surface.

Methods for area of wound healing: Photographs were taken of each wound prior to biopsies. Using ImageJ software, a blinded operator defined the original area of the wound by drawing straight lines between the tattoo marks at each of the four corners and the area was calculated. The demarcation between the epithelialized and granulation tissues was then traced and the area of open wound calculated. The difference between the two areas was used to determine the percent of healed wound.

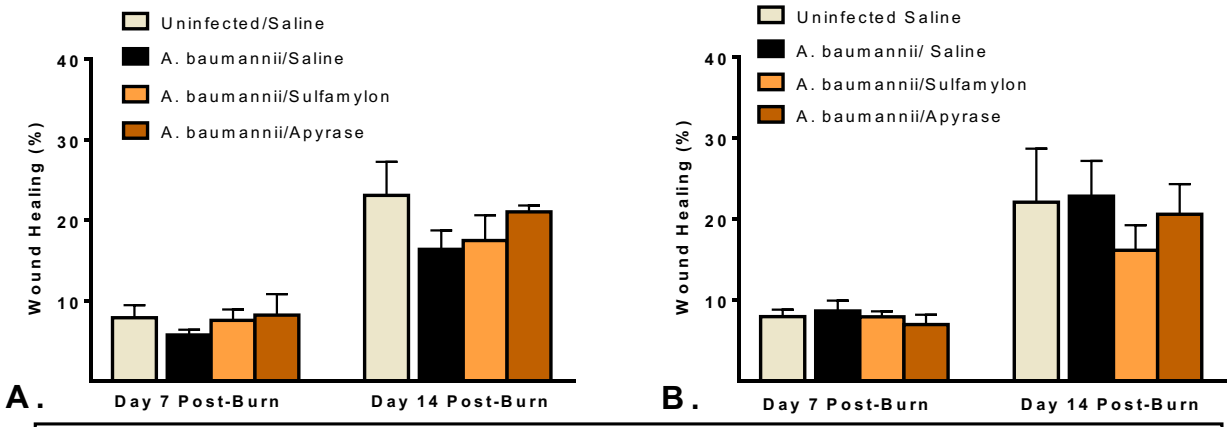

Figure 1. Wound Healing in Acinetobacter baumannii infected burn wounds. A.) Superficial Burns and B) Deep Burns were created by thermal contact. The percent of wound surface covered by epithelium was calcualted at 7 and 14 Days post-burn.

Significant results or key outcomes: Subjective assessment of wounds suggest marked differences in wound characteristics between uninfected and infected wounds at 3 days after infection. By seven days post-burn, epithelialization is evident and quantifiable using the methods described above. By Day 7, in Superficial burn wounds (20second exposure) epithelialization and surface healing of Saline-treated, *A. baumannii* infected wounds appeared slower than in Saline-treated, Uninfected wounds. Treatment with Sulfamylon and Apyrase appeared to increase healing (Figure 1A). The effects at Day14 appeared more pronounced. The uninfected wounds demonstrated a mean of 23% healed area while the infected wounds lagged at 16% healed area. Notably, the Apyrase treated, infected wounds demonstrated 21% healed surface area while the Sulfamylon treated infected wounds were only 17% healed (Figure 1A). The results of treatment in Deep wounds was more difficult to interpret since control infected wounds appeared to heal at the same rate as uninfected wounds. Apyrase had no additional effect while Sulfamylon appeared to retard the epithelialization (Figure 2A). These results are preliminary and represent less than half of the final group size. Statistics will be reported when the full sample size is available.

Subtask 3: Quantify bacterial load, biofilm and data analysis
In this reporting period, quantitative cultures were performed at the time of each biopsy. Data collection is ongoing.

Methods for quantitative cultures: A 3mm biopsy punch was used to obtain a sample for culture. The tissue was 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 37^0C overnight. Dilutions yielding between 30-300 colonies were counted and used to calculate the CFUs/g tissue.

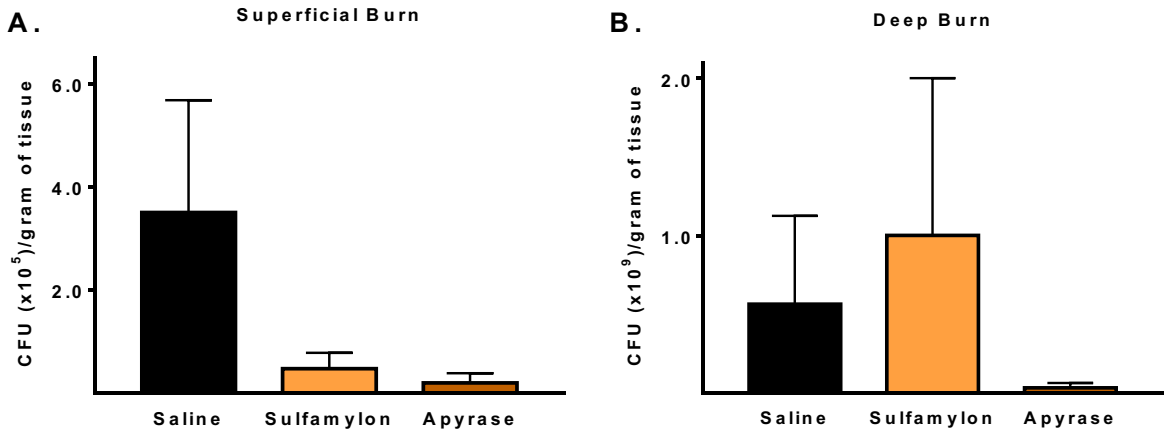

Figure 2. Quantitative bacterial cultures from Acinetobacter baumannii-infected burn wounds.

Significant results or key outcomes: In the *A. baumannii*-infected, Superficial burns the colony counts in both the Sulfamylon and Apyrase treated groups appeared to be less than those in the Saline treated group at 7 days post-burn (Figure 2A). In the *A. baumannii*-infected Deep burns, the Apyrase group produced substantially fewer colony forming units than either the Sulfamylon of the Saline treated groups. However, the elevation of values in the Sulfamylon group may be due to a single outlier. These data will be evaluated when all animal experiments have been completed to determine if extreme values are valid. Statistics will be completed when the entire set of experimental data from the animal work is available and appropriate sample size achieved.

Goals not met: We have not yet completed the cohort of animals infected with the Gram negative bacteria. However, this work is on track to be completed within two months.

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. 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 may be more pronounced in the presence of Gram negative infection. 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. In the past year, the global health crisis required a temporary disruption in the work, We have extended our study duration to insure that the remaining work can be concluded. The trajectory could be disrupted if additional restrictions related to social distancing are implemented at this institution or if personnel are absent due to illness. In that event, we will resume studies as soon as possible. No further delays are expected at this time.

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

Jean Nemzek, DVM, MS
Benjamin Levi, MD
Chuanwu Xi, MD

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