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TITLE: Use of Tranexamic Acid to Reduce Tissue Edema and Prevent Burn Wound Conversion

PRINCIPAL INVESTIGATOR: Damien Carter, MD

CONTRACTING ORGANIZATION: MaineHealth, Portland, ME

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Damien Carter, MD

E-Mail: Damien.Carter@mainehealth.org

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MaineHealth
22 Bramhall Street
Portland, ME 04102

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14. ABSTRACT

The purpose of the experiments performed during the past year was to elucidate the effect of tranexamic acid (TXA) on burn wound conversion in a rat burn wound conversion model. The comb burn injury model is well validated in the literature for this purpose – particularly at 7 days post injury which represents the gold standard for this experiment. Our studies clearly demonstrated that burn wound conversion was reduced by treatment of rats with intraperitoneal (IP) TXA at this time point. Furthermore, TXA demonstrated efficacy at 24, 48 and 72 hours after comb burn injury. Additionally, we were able to demonstrate that less mitochondrial DNA – an established marker of tissue damage – was reduced at 24 and 48 hours. This finding indicates that TXA has significant cell protecting activity.Experiment #2 demonstrated that these protective effects were maintained with a more severe concomitant burn injury and that tissue regeneration was significantly upregulated. Burn-induced SIRS was also down regulated and tissue edema as well as pulmonary inflammation reduced. These findings clearly demonstrate that Tranexamic acid reduces burn wound conversion and has cell protective properties in the rat burn wound conversion model when compared to no treatment. In the context of severe burn injury, TXA reduces SIRS, burn wound conversion, tissue edema, capillary leak and pulmonary infiltration. Our findings suggest TXA may be an ideal therapy for burn injured warfighters in the forward deployed setting.

15. SUBJECT TERMS
Burn wound conversion, Tranexamic acid, SIRS, comb burn, tissue edema

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
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1. **INTRODUCTION:**

Burn wound conversion is the process where superficial partial thickness burns convert into deep partial and full thickness burn injuries. This conversion process increases morbidity and often requires surgical intervention to achieve timely wound healing. Thus, therapeutic interventions that may prevent secondary progression and cell death in burn-injured tissue is desirable. Recent work by our group and others has established that tranexamic acid (TXA) has significant anti-inflammatory properties that may ameliorate the root causes of burn wound conversion. **Hypothesis**: TXA treatment of burn injuries will reduce burn wound progression, capillary leak, and cutaneous tissue and organ edema.

2. **KEYWORDS:**

Burn wound conversion, Tranexamic acid, SIRS, comb burn, tissue edema

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Specific Aim 1: Determine the effect of TXA on burn wound conversion (months 1-21)
- STATUS: started Y1Q1, completed Y1Q4.

Specific Aim 2: Assess the capacity of TXA to attenuate burn-induced tissue edema (months 13-30)
- STATUS: Started Y2Q1, Completed Y3Q1.
- All tissue analysis completed for this specific Aim in Y3Q2
- Results were positive for both specific Aims 1 & 2.
- Publication submitted for review with Burns journal Y3Q4 based on specific aims 1&2.

Specific Aim 3: Determine the effects of TXA on burn induced SIRS and assess its ability to improve mitochondrial function after burn (months 23-36)
- STATUS: started Y3Q3.
- Burn-induced SIRS milestone completed Y3Q4
- Mitochondrial function milestone incomplete. Application for no cost extension approved through 12/2023 to complete this portion of the study.

What was accomplished under these goals?

Specific Aim 1: Determine the effect of TXA on burn wound (months 1-21)
All experiments were completed and reported as preliminary data in the Y1Q3 report. During Q4, We completed processing of harvested tissues and completed data analysis. This more complete data set confirmed our preliminary findings. Our data demonstrate a benefit of intraperitoneal administered TXA in ameliorating burn wound conversion in the comb burn model at 48hrs, 72 hrs and 7 days (figure 1) post-injury when compared to no treatment.

Key Findings or Accomplishments:
Findings: Briefly, based on H&E and photographic evaluation of the ischemic zones, there appears to be a benefit to TXA administration. Ischemic zones had less evidence of necrosis visually and microscopically. During Q4, we also found that IHC evaluation confirmed these results. We will combine this data with the findings of experiment #2 and begin writing a manuscript for publication in a major journal in Winter 2023.

Specific Aim 2: Assess the capacity of TXA to attenuate burn-induced tissue edema (months 13-30)
All experiments completed for experiment #2 which addresses specific aim 2 and partially specific aim #3. Analysis completed for all tissues in these experiments. Our results are summarized graphically in the appendix to this report. Briefly, TXA effectively reduced burn wound conversion even in this more severe injury model. IHC evaluation of the zone of stasis exhibited significantly more evidence of tissue repair as measured by proliferation and regeneration markers (Ki-67 & CTHRC-1). Finally, TXA clearly reduces both lung tissue edema and capillary leak.
Specific Aim 3: Determine the effects of TXA on burn induced SIRS and assess its ability to improve mitochondrial function after burn (months 13-36)
These experiments were performed on the most severe injury model of this project (60%TBSA). There was no assessment of burn conversion in this model. The results of experiment 2 confirmed reduction in capillary leak, burn-induced SIRS and lung tissue inflammation. These results are also summarized graphically in the appendix to this report.
The second major milestone remains incomplete regarding mitochondrial function assessment. We applied and have been granted a no cost extension to complete this task through December of 2023. Briefly, in preparation to the Seahorse studies of the effects of TXA on mitochondrial respiration in burned animals, we assessed the respiratory capacity of isolated mitochondria, using mitochondria freshly prepared from mouse liver. Unfortunately, the initial attempts using a standard Percoll gradient-based method of mitochondria isolation gave us mitochondria of low quality with damaged respiratory activity. Only during the recent month, when we switched to an expensive commercial kit for mitochondrial isolation, we got the preparations of decent quality. The mitochondria isolated using this kit responded to TXA added in vitro, at 100 □g/ml by a significant increase of spare respiratory capacity (Figure 1, Seahorse X96 instrument at the Physiology Core of MHIR was used to study mouse liver mitochondria). Now, we plan to use this method of mitochondria isolation to study the effects of TXA administration in vivo after burn injury upon mitochondrial respiration.

Describe opportunities for training and professional development

Nothing to report

How were the results disseminated to communities of interest?

We submitted a manuscript summarizing the findings from Specific aims 1 and 2 described above. This was submitted to the Burns journal. It is currently under review. The information from these experiments has been presented at the Shock Society Annual meeting, the North American Burn Society, Northeast Region Burn Conference and at several internal research presentation forums. With the completion of specific aim #3 during the no cost extension, we anticipate submission of a manuscript for publication and further scientific presentations on the ramifications of TXA to mitochondrial respiration and bioenergetics.

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

We are in the no cost extension time period. This will be focused on completion of burn experiments (60% TBSA) with isolation and analysis of mitochondrial function which appears to be enhanced by TXA after severe burn injury.

Briefly, the initial attempts using a standard Percoll gradient-based method of mitochondria isolation gave us mitochondria of low quality with damaged respiratory activity. Only during the recent month, when we switched to an expensive commercial kit for mitochondrial isolation, we got the preparations of decent quality. The mitochondria isolated using this kit responded to TXA added in vitro, at 100 □g/ml by a significant increase of spare respiratory capacity (Figure 1, Seahorse X96 instrument at the Physiology Core of MHIR was used to study mouse liver mitochondria). Now, we plan to use this method of mitochondria isolation to study the effects of TXA administration in vivo after burn injury upon mitochondrial respiration.

We used the mass-spectrometry analysis (performed at the Proteomics and Lipidomics Core of MHIR) to identify the effects of TXA administration on the protein composition of blood plasma, 6 h after 35% TBSA burn of rats. We found a 5.9 fold increase of cathepsin B plasma content by burn ($p=0.0004$) comparatively to control. TXA treatment resulted in a 5.4 fold decrease of cathepsin B content comparatively to burn ($p=0.003$). The assessment of cathepsin B content in plasma using a commercial ELISA kit confirmed mass-spectrometry results (Figure 2). Cathepsin B is a lysosomal protease, which is released form damaged cells. The role of cathepsin B in a number of pathologies has been reported. We will expand on this work and have summary information available at the completion of the no cost extension time period.

4. IMPACT:

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

Our findings provide convincing evidence of the anti-inflammatory properties of tranexamic acid. Importantly, these properties have significant clinical effect beyond the well-known anti-fibrinolytic properties of this drug and include burned-tissue repair and reduction of burn wound conversion, reductions in the SIRS response and reduced capillary leak. Further studies will elucidate the mechanism of action in addition to the impact on mitochondrial biogenesis and dysfunction.

What was the impact on other disciplines?

Nothing to Report

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:

No cost extension was approved to allow completion of specific aim 3. As described above, we needed to sort out some technical issues with mitochondrial isolation in order to proceed with this group of experiments. That has now been sorted and we will begin experiments in August 2023. Final results anticipated in November 2023.

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

See above. Expect to complete remainder of project during the no cost extension period which ends December 2023.

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

TOTAL PROTOCOL(S): 1
PROTOCOL (X of Y total):
IACUC Protocol Number: 2301
ACURO Protocol Number: MB190064.e001
Protocol PI: Damien Carter, MD
Protocol Site: MaineHealth
Protocol Title: Use of Tranexamic Acid to reduce tissue edema and prevent burn wound conversion
Number of Animals Approved for Use: 252
IACUC INITIAL APPROVAL DATE: 03/13/2020 (expired 03/13/2023)
ACURO INITIAL APPROVAL DATE: 05/13/2020
RENEWAL APPROVAL DATES:
- *03/14/2023*
AMENDMENTS:
- Amendment 1 approved 05/13/2020
ADVERSE EVENTS OR UNANTICIPATED PROBLEMS:
- None.

Significant changes in use of biohazards and/or select agents

Not applicable

Significant changes in use or care of vertebrate animals

Not applicable

6. PRODUCTS:

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

Journal publications.

Nothing to report

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

Nothing to report

Other publications, conference papers and presentations.

Nothing to report

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

Nothing to Report

- **Technologies or techniques**
  Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Nothing to Report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Damien Carter
Project Role: PD/PI
Researcher Identifier:
Nearest person month worked: .49 (Based on salary cap of
Contribution to Project: Dr. Carter supervises the entire project, designs the studies and performs animal experiments.

Name: Igor Prudovsky
Project Role: Co-PD/PI
Researcher Identifier:
Nearest person month worked: 2.4
Contribution to Project: Dr. Prudovsky is responsible for design and supervision of biochemical and histological studies. He performed animal experiments together with Dr. Carter.

Name: Doreen Kacer
Project Role: Research Associate
Researcher Identifier:
Nearest person month worked: 1.67
Contribution to Project: Responsible for performing biochemical and histological studies.

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

COLLABORATIVE AWARDS:

QUAD CHARTS:

Use of Tranexamic Acid to Reduce Tissue Edema and Prevent Burn Wound Conversion

Proposal # MB190064, Award # W81XWH-20-1-0474

PI: Damien Wilson Carter, MD **Org:** MaineHealth **Award Amount:** $500,000

Study/Product Aim(s)

- Burn wound conversion is the process where superficial partial thickness burns convert into deep partial and full thickness burn injuries. This conversion process increases morbidity and often requires surgical intervention to achieve timely wound healing.
- Thus, therapeutic interventions that may prevent secondary progression and cell death in burn-injured tissue is desirable.
- Recent work by our group and others has established that tranexamic acid (TXA) has significant anti-inflammatory properties that may ameliorate the root causes of burn wound conversion.

Hypothesis: TXA treatment of burn injuries will reduce burn wound progression and cutaneous tissue and organ edema.

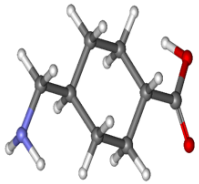
Aims:.1. Determine the effect of TXA on burn wound conversion; 2. Assess the capacity of TXA to attenuate burn-induced tissue edema; 3. Determine the effects of TXA on burn-induced SIRS and mitochondrial dysfunction.

Approach

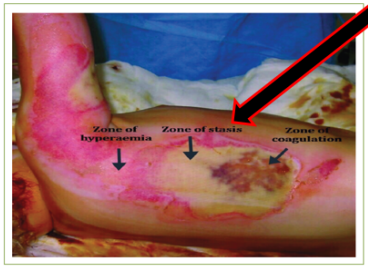
Wild type Sprague-Dawley rats will be subjected to the following types of burn: 1. comb burn injury; 2. comb burn injury + an adjacent 30% TBSA burn; 3. 60% TBSA scald burn injury. **For each type of burn, four experimental groups will be studied: (i) sham; (ii) burn, no treatment; (iii) burn, TXA given once post-injury, and (iv) burn, TXA given daily until sacrifice**. Tissues will be analyzed by histology and immunohistochemistry to determine if TXA effectively reduces burn wound conversion. Additional analysis will evaluate TXA effect on tissue edema formation, mitochondrial health and burn-induced systemic inflammation.

Experimental Groups	No treatment (#)	Single dose TXA(#)	Daily TXA(#)
Sham	(6)	(6)	(6)
Comb burn injury	(6)	(6)	(6)
30%TBSA Burn + Comb burn injury	(6)	(6)	(6)
60%TBSA Burn	(6)	(6)	(6)

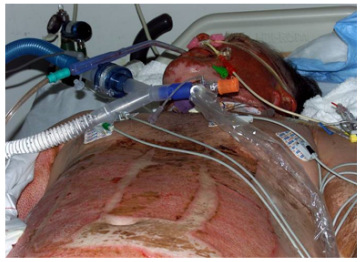
Sacrifice at 12h, 24h, 72h, 7days



Tranexamic Acid



Preserves the zone of stasis



Reduces edema and burn-induced SIRS

Timeline and Cost

Activities CY	20	21	22	23
Approval of animal procedures Preparation for experiments				
Study of TXA effects on comb burn injury				
Study of TXA effects on comb burn + adjacent 30% TBSA injury				
Study of TXA effects on 60% TBSA burn injury				NCE
Estimated Budget ($500K)	**$166,538**	**$166,667**	**$166,795**	

Updated: 7/13/2023

Goals/Milestones

CY20 Goal – Preparation for experiments, and initial studies

CY21 Goals –Elucidation of TXA effects on wound conversion with animals and small burns - **completed**

CY22 Goal – Elucidating TXA effects in animals with intermediate and severe burns in context of burn wound conversion - **completed**

CY23 Goal –Finalizing the study and preparing the report and publications

☐Finish the studies of TXA effects in animals with severe burns

☐Prepare the final report

☐Prepare the application for preclinical studies using a pig model.

☐**Comments/Challenges/Issues/Concerns**

- Experiment #1 & #2 Completed. TXA efficacy demonstrated in both models. Manuscript submission for these findings: Feb 2023.
- Experiment #3 – Underway. 50% complete. No Cost Extension (NCE) granted.

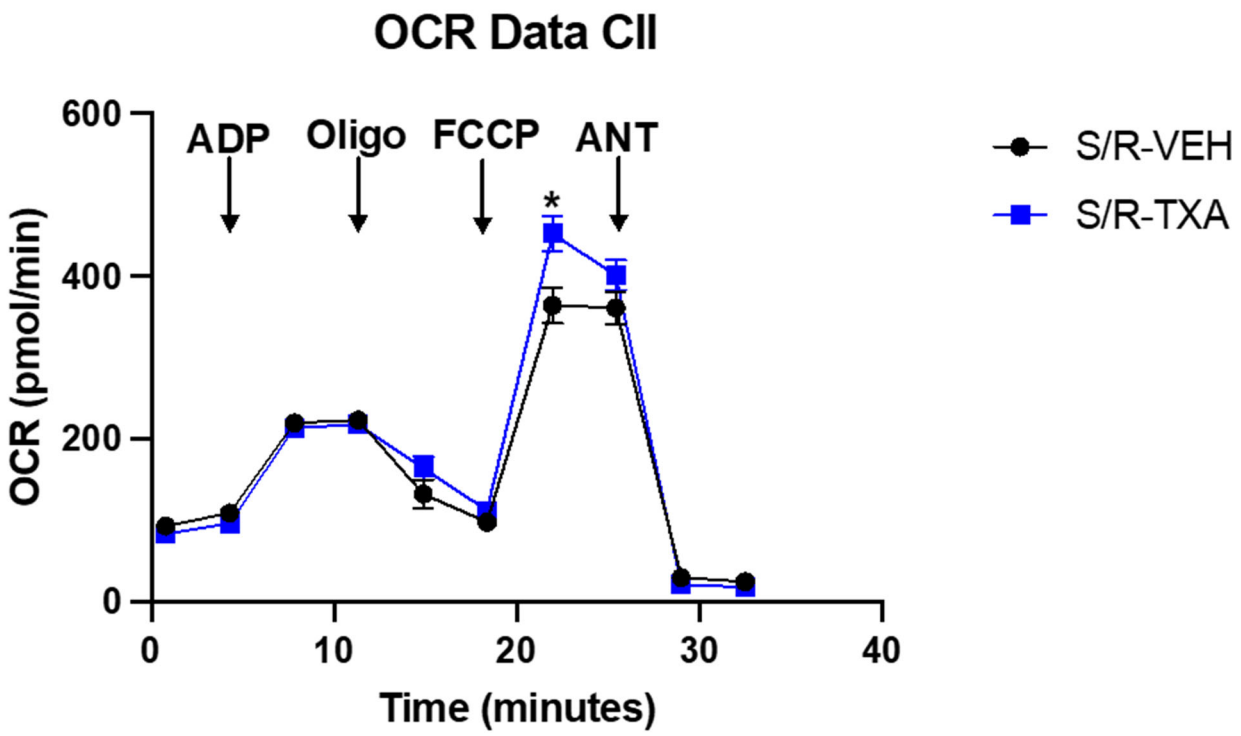
Budget Expenditure to Date

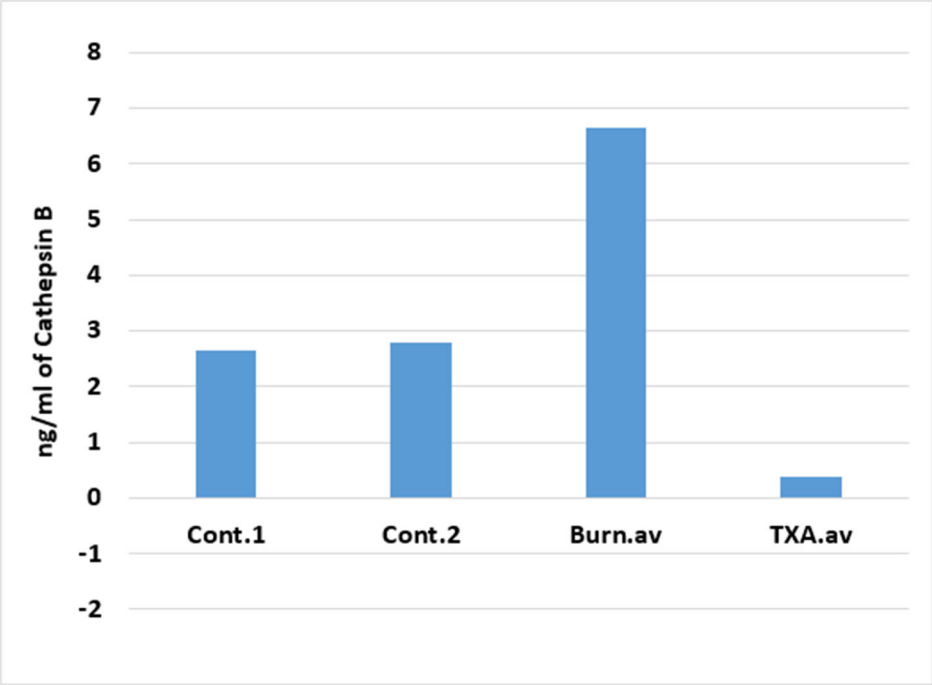
Projected Expenditure: $500,000

Actual Expenditure: $492,665.74

9. APPENDICES:

Recent mitochondrial isolation data (Referring to Question #4)

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5
4
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1
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-2
Cont.1
Cont.2
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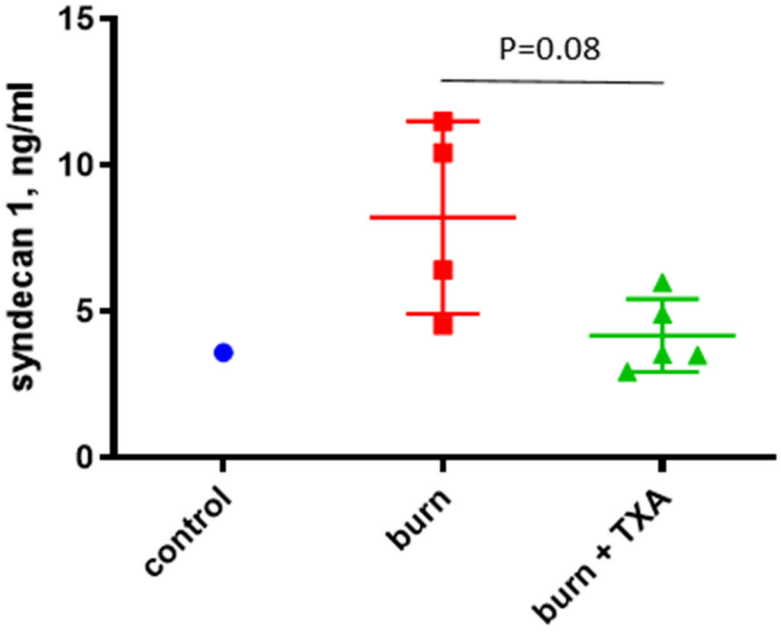
TXA.av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15
10
5
0
P=0.08
control
burn
burn + TXA

Tranexamic Acid Reduces Inflammation, Edema and Burn Wound Conversion in a Rodent Model

Prudovsky I, Kacer D, Lindner V, Rappold J, Carter DW

Authors:
Igor Prudovsky, PhD[a]
Doreen Kacer, MS[a]
Volkard Lindner, MD, PhD[a]
Joseph Rappold, MD, FACS[a,b]
Damien Wilson Carter, MD, FACS[a,b]

Affiliations:

a. Maine Health Institute for Research, 81 Research Drive, Scarborough, Maine, 04074, USA
b. Maine Medical Center, 22 Bramhall Street, Portland, Maine, 04105, USA

Correspondence Author:
Damien Wilson Carter, MD, FACS
Assistant Professor of Surgery, Tufts University SOM
Burn Medical Director, MMC Burn Service
Department of Surgery
Maine Medical Center
Portland, Maine
Email: dcarter@mmc.org

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We used the services of Molecular Phenotyping and Histopathology Cores (both supported by NIH COBRE grant P20GM121301) and Animal Facility at our institution.

Author Contributions: IP and DWC were the primary contributors to the conception, design, data analysis and interpretation of results. DWC, DK, VL, and IP performed data collection and analysis. IP and DWC drafted the manuscript. All authors contributed to revising the manuscript and critical review.

Tranexamic Acid Reduces Inflammation, Edema and Burn Wound Conversion in a Rodent Model

Burn wound conversion is the observed process where superficial partial thickness burns convert into deep partial or full thickness burn injuries. This conversion process often requires surgical excision to achieve timely wound healing. Unfortunately, the pathophysiology of this phenomenon is multifactorial and poorly understood. Thus, a therapeutic intervention that may prevent secondary progression and cell death in burn-injured tissue is desirable. Recent work by our group and others has established that tranexamic acid (TXA) has significant anti-inflammatory properties in addition to its well-known anti-fibrinolytic effects. This study investigates TXA as a novel therapeutic treatment to mitigate burn wound conversion and reduce systemic inflammation. Sprague-Dawley rats were subjected to a hot comb burn contact injury. A subset of animals underwent a similar comb burn with an adjacent 30%TBSA contact injury. The interspaces represent the ischemic zones simulating the zone of stasis. The treatment group received injections of TXA (100mg/kg) immediately after injury and once daily until euthanasia. Animals were harvested for analyses at 6 hours and 7 days after injury. Full-thickness biopsies from the ischemic zones and lung tissue were assessed with established histological techniques. Plasma was collected for measurement of damage associated molecular patterns (DAMPs), cytokines and other inflammatory markers. Animals treated with TXA demonstrated reduced burn wound conversion and decreased burn-induced systemic inflammatory response syndrome (SIRS) response. Lung inflammation and capillary leak was also significantly reduced by administration of TXA after injury. Future research will elucidate the underlying anti-inflammatory properties of TXA responsible for these findings.

Keywords: Tranexamic acid, burn wound conversion, zone of stasis, comb burn, SIRS, edema

BACKGROUND

Burn wound conversion is the observed process where superficial partial thickness burns convert into deep partial and even full thickness burn injuries. This process is well characterized by the Jackson zones of injury, which posits three zones of injury in a burn wound.[1] The central, irreversibly damaged area is the zone of coagulation or necrosis. The surrounding damaged, but threatened region is called the zone of stasis. The outermost zone is the most recoverable, the zone of hyperemia. The zone of stasis is known to have damaged, but viable skin cells and thus remains a target of potential therapeutic intervention. Unfortunately, the pathophysiology of burn injury creates a deleterious environment for these injured cells. This often leads to an increase in the area of non-viable skin. Clinically, this process occurs over hours to days after a burn injury as the clinician waits for the burn wound to "declare". This conversion process often requires eventual surgical excision to achieve timely wound healing, thus complicating management of burn wounds that might otherwise have healed without surgical intervention. The pathophysiology of this phenomenon is multifactorial and poorly understood.[2] Thus, therapeutic interventions that may prevent secondary progression and cell death in burn-injured tissue are desirable. Recently, the etiology of burn wound conversion has been attributed to a complex combination of prolonged inflammation, ischemia, excessive tissue edema and reactive oxygen species (ROS) damage to injured but viable cells in the zone of stasis.[2] More recently, the roles of autophagy and apoptosis have been demonstrated as central to the process of burn wound conversion.[3,4]

Our group has been studying the anti-inflammatory effects on burn injury of tranexamic acid (TXA), the most popular anti-fibrinolytic agent, used in hemorrhagic trauma and cardiac surgery patients. Our studies were prompted by clinical data indicating that beneficial effects of TXA transcend hemorrhage control and include the suppression of burn-induced SIRS and the development of tissue edema.[5,6] Specifically, we found that in animals with burn injury TXA suppresses the release of mitochondrial DAMPs into the bloodstream and reduces pulmonary infiltration of inflammatory cells.[7] We have also shown that TXA stimulates mitochondrial respiration and mitochondrial biogenesis in the culture of endothelial cells and protects the integrity of the endothelial monolayer.[8] The use of TXA to attenuate burn wound conversion has not previously been studied. However, there is sufficient laboratory evidence to suggest that this agent may be efficacious in mitigating the deleterious effects of burn pathophysiology as described above and improve tissue repair in the zone of stasis. In the present study, we used two rat comb burn models to assess the effects of TXA administration on burn wound healing and the inflammatory response to burn injury.

METHODS

Experimental animals

All experiments were performed in accordance with the guidelines set forth by the National Research Council in the "Guide for the care and use of laboratory animals". Approval for the experimental protocol was obtained from the Institutional Animal Care and Use Committee at our institution.

Age- and weight-matched male Sprague-Dawley rats (Charles-River Laboratories; Wilmington, MA) weighing 250 g to 300 g were allowed to acclimate for 1 week before being used in any experiment. Animals had unrestricted access to standard chow and water throughout the course of the study. Using STATA 8.0 and assuming a power of 0.9 and alpha of 0.05, we determined that 5 animals per experimental group would be sufficient for these experiments. Control groups (no burn) included 2 animals.

Rat burn procedures

The comb burn injury was performed according to Singer et al. using a 150-g brass comb preheated to 100°C, to create four rectangular burns, separated by three unburned interspaces on each side of the back.[9] The interspaces represent the ischemic zones surrounding the central necrotic core. The interspaces are not directly injured; however, they typically undergo progressive ischemia over the initial 24 to 48 hours to the point where most are necrotic. These areas represent the zone of stasis. Sham (control) animals underwent an identical procedure, with application of a room temperature comb.

A 30% TBSA full thickness burn was created by using a heated metal column to create a 'stamp' like burn that is approximately 30%TBSA on the dorsum of each animal according to Zheng et al.[10] As a modification of the comb burn model, a single row of comb burns was made adjacent to the dominant burn wound.

Before burn procedures, animals were anesthetized by isoflurane inhalation and the dorsal hairs clipped. After burn injuries, animals were resuscitated by intraperitoneal (i.p.) injection with lactated Ringer's solution according to the following formula: 4ml x (%TBSA burn) x Weight (kg). They also received a subcutaneous buprenorphine injection (0.03 mg per kg weight). Control animals were subjected to identical anesthesia, resuscitation and buprenorphine injection. TXA (100mg/kg body weight) or vehicle was administered i.p. immediately after the burn injury and repeated daily together with buprenorphine SC injection for the 7 day sacrifice experiments. The comb burn model is well validated in multiple studies in rodents and swine with a 7 day sacrifice and evaluation.[2]

Evaluation of burn wound conversion

Animals undergoing the burn injury were observed and photographed for evidence of interspace necrosis prior to sacrifice at 7 days post injury. Full thickness ischemic zones were harvested, formalin fixed and paraffin sections were prepared for histological evaluation using standard H&E staining. The photomicrographs of H&E-stained sections were taken in a blind manner with a 4x objective. Image J software was used to determine the % of dermis sections occupied by highly eosinophilic necrotic areas.

Immunohistochemical study of burned skin

Full thickness samples of burned skin were harvested and formalin fixed for analysis of cellular regeneration and proliferation activity. Paraffin sections of skin were prepared by the Histopathology Core at our institution and used for immunoperoxidase detection of regeneration marker CTHRC1 (rabbit monoclonal antibody, clone Vli55).[11]

Plasma preparation and determination of nuclear DNA content in plasma

Rats were sacrificed by isoflurane inhalation to effect 6 hours after burn. Blood was obtained through heart puncture and transferred to Eppendorf tubes containing 50 µL of 0.5 M EDTA Platelet-free plasma was prepared by two consecutive cycles of centrifugation, each at 2,000g for 10 minutes.

Plasma DNA was isolated from plasma using a Qiamp DNA Blood Mini Kit (Qiagen ?51106). Manufacturer's instructions were followed using 150 µL of PBS added to 50 µL of plasma sample. Fifty microliters of nuclear DNA was eluded from the columns. Plasma levels of nuclear DNA were determined using quantitative PCR. Five microliters of the isolated DNA was analyzed using Biorad iQ Sybr Green Supermix and primers to rat GAPDH gene (Forward: 5'-ACTCCCATTCTTCCACCTTTG-3'; Reverse: 5'-CCCTGTTGCTGTAGCCATATT-3') and cycled 40 times on a Biorad CFX real-time machine.

Preparation of RNA from liver and qRT-PCR

Livers were harvested from animals sacrificed 6 h after burn and cut into approximately 0.5cm long pieces, snap frozen in liquid nitrogen and stored at -70ºC until processed. The tissue piece was placed in a pre-cooled Eppendorf tube on dry ice until ready to process, then placed on wet ice briefly, and, before thawing, 50 µl of Trizol reagent was added to the tube and immediately homogenized for 20 seconds using a motorized pestle mixer (Cole Parmer; Vernon Hills, IL). An additional 350 µl of Trizol was then added and homogenized for additional 20 seconds or until no large tissue particles were visible.

The tubes were spun in a 4^0C microfuge at 10,000 rpm for 6 minutes, supernatant containing RNA collected and transferred to new tube. Total RNA was extracted from each mouse organ using a Direct-zol RNA miniprep Plus kit (Zymo Research; Tustin, CA) according to the manufacturer's protocol, with the exception of an extra 200 □l wash before eluting the RNA in 60 µl of DNase/RNase-free water. 5 µl of the purified RNA was run on a 1% bleach/1.5% TXA agarose gel to evaluate RNA integrity. Total RNA was quantified using a Nanodrop 2000c instrument and 1.5 □g samples were reverse transcribed using the AzuraQuant cDNA Synthesis kit (Azura Genomics; Raynham, MA) according to manufacturer's instructions. Afterward, the cDNA pool was diluted 9-fold for internal standard and 5-fold for genes of interest with DNase/RNase-free water and stored at −80°C.

Quantitative real-time (RT) PCR was carried out in 96-well plates using the AzuraQuant Green Fast qPCR LoRox Master Mix on a Biorad CFX 96 system. RT-qPCR was performed with a final volume of 20 µl containing 10 µl of 2 × AzuraQuant Green Master Mix, 0.5 µl each of 10 µM forward and reverse primer solutions, 6 µl diluted cDNA, and 3 µl of ultrapure water. The optimized thermal cycling conditions were as follows: 95 °C for 10 min, 95 °C for 15 s, and 55 °C for 60 s, for 40 cycles; 65 °C for 5 s, and 95 °C for 30 s, for 1 cycle. For each run, an ultrapure water negative control was included as well as a no-Reverse Transcriptase sample for genomic DNA contamination determination. All reactions were performed as technical triplicates, and an analysis of melting curves was performed in each reaction. The relative expression levels of the genes were normalized using TATA-binding protein (TBP) expression as a housekeeping gene control.

The following primers were used:

IL1β

Forward: 5'-AAGACAAGCCTGTGTTGCTGAAGG-3'

Reverse: 5'-TCCCAGAAGAAAATGAGGTCGGTC-3'

TNFα

Forward: 5'-GTCGTAGCAAACCACCAAGC-3'

Reverse: 5'-TGTGGGTGAGGAGCACATAG-3'β-actin (loading control)

Forward: 5'-AAGTCCCTCACCCTCCCAAAAG-3'

Reverse: 5'-AAGCAATGCTGTCACCTTCCC-3'

Determination of neutrophil infiltration of lungs

Rat lungs were collected from sacrificed animals 6 hours after burn and fixed overnight in 10% neutral formalin, followed by storing for 24 hours in 70% ethanol, followed by tissue processing, embedding in paraffin and sectioning at 5µm. Immunoperoxidase staining of neutrophil marker myeloperoxidase (MPO) (Thermo Fisher Scientific, Waltham, MA) and counterstaining with

hematoxylin. The numbers of MPO positive cells per x40 objective field were counted in blind manner.

Assessment of capillary leak in lung tissue
The effect of TXA upon endothelial permeability was assessed using Evans blue dye. At the end of the experiment (6 h after burn) the animals were injected intravenously with 0.2 ml of 1g/L Evans blue in PBS. After thirty minutes, cardiac puncture was performed on isoflurane-anesthetized animals and the pulmonary artery was flushed with 5mL of heparinized PBS. Rats were then perfused through the exposed aorta for 10 minutes with heparinized PBS. The lungs were collected and then extracted for 48 h in formamide. The content of extracted formamide was measured fluorimetrically (excitation 475 nm, emission 660-720 nm).

RESULTS

TXA Reduces burn-induced ischemic zone necrosis
Daily TXA administration after burn injury reduced necrotic conversion of the ischemic zones at 7 days after 10% burn injury (Figure 1A). The H&E staining of skin sections demonstrated a significant decrease of necrotic areas, which demonstrated a strong homogeneous eosin staining (Figure 1B).
CTHRC1 is a secreted protein expressed by activated fibroblasts, especially in the tissues undergoing repair and in its absence tissue repair is impaired.[12,13,14] We found that daily TXA treatment increased the abundance of CTHRC1 positive fibroblasts in the dermis of burn wounds 7 days after burn (Figure 1 C). Similar to the 10% comb burn model, TXA also reduced the necrotic conversion after 30% burn (Figure 2A,B).

TXA suppresses the systemic release of nuclear DNA and reduces proinflammatory cytokines expression after burn injury
The increase of DAMP in the bloodstream is a typical early systemic response to severe injury including burns.[15] In a recent publication, cell-free nuclear DNA levels were found to correlate with the early inflammatory response after severe trauma.[16] We used circulating nuclear DNA as a surrogate for generalized systemic DAMP release. Using qPCR, we found that 6 hours after 30% burn TXA treatment causes a significant decrease of nuclear DNA plasma levels (Figure 3A).

Similarly, burn injury has been shown to increase the expression of TNF□ in mouse liver.[17] We found a similar effect in rats 6 hours after burn, and it was significantly suppressed by TXA treatment (Figure 3B). In addition, TXA suppressed the burn-induced increase of IL1□ gene expression in rat livers (Figure 3C).

TXA reduces neutrophil infiltration to lungs

Similar to other traumas, severe burn results in massive infiltration of neutrophils to lungs.[18] Using immunohistochemistry with antibodies against the neutrophil marker myeloperoxidase, we found that TXA significantly suppresses neutrophil invasion in lung tissue 6 hours after burn (Figure 4).

TXA suppresses the burn induced vessel leakage

Severe burn drastically increases vessel leakage resulting in edema and immune cell infiltration in lung tissues.[19] Six hours after burn, the perfused rat lungs exhibited red-brownish color as a result of erythrocytes leakage from the vessels (Figure 5A). The lung coloration was reduced to almost control level in the animals treated with TXA. The study of burn-induced vessel leakage using Evans blue also demonstrated that it was suppressed by TXA (Figure 5B).

DISCUSSION

Burn wound conversion remains a complex and poorly understood phenomenon in burn care. Generally, the etiology of burn wound conversion has been attributed to a complex combination of prolonged inflammation, ischemia, excessive tissue edema and reactive oxygen species (ROS) damage to injured but viable cells in the zone of stasis.[2] More recently, the roles of autophagy and apoptosis have been demonstrated as central to the process of burn wound conversion.[2,3,4] Given the centrality of mitochondrial dysfunction to the pathophysiology of cell death in the zone of stasis[2], we hypothesized that the anti-inflammatory and mitochondrial supportive properties of TXA might make this agent an ideal treatment for burn wound conversion.[4,5,6,7,8]

In addition to the long established efficacy of TXA in hemorrhagic shock and, more recently, cardiac surgical patients, multiple studies demonstrate that the effects of TXA transcend its anti-fibrinolytic activity.[20] For instance, TXA treatment of cardiac surgery patients attenuates inflammation, in particular, inflammatory gene expression in leukocytes.[21,22] In addition, TXA application decreases the hemorrhage-associated shedding of glycocalyx from gut epithelium of mice and hydrogen peroxide-induced shedding of glycocalyx from endothelial cells in microfluidics culture.[5,6]

Recent literature has shown that early systemic inflammatory signaling after burn injury is induced by a large group of circulating molecules collectively referred to as DAMPs and is responsible for the initial propagation of SIRS.[23] Our group has recently shown that TXA decreases the release of certain DAMPs such as mitochondrial DNA (mtDNA) from neutrophils and endothelial cells and enhances mitochondrial respiration in the endothelial cells in vitro.[8] In addition, TXA exhibited systemic anti-inflammatory effects, including suppression of mitochondrial DNA (mtDNA) release and macrophage infiltration in the lungs in a murine burn model.[7]

In this study, we have clearly demonstrated that TXA has the potential to ameliorate the process of burn wound conversion. We were able to demonstrate this efficacy in a traditional comb burn model and when combined with a severe burn injury. Our findings in these two discrete models are important considerations for relevance to varied %TBSA burn injuries in the clinical setting. We also demonstrated that TXA appears to improve tissue repair and regeneration within the ischemic zones as evidenced by CTHRC1 expression. Our findings also demonstrated significant reduction of a potent DAMPs, circulating nuclear DNA and the decrease of burn-stimulated expression of genes coding for two potent proinflammatory cytokines, TNFα and IL1β, 6 hours after 30% TBSA burn. These results demonstrate cell protective and anti-inflammatory effects of TXA in a severe burn injury model. One of the major pathological effects of the severe burn is a drastic increase of permeability of endothelial monolayer lining vessels, which results in the enhanced extravasation of inflammatory cells, ROS generation and tissue edema. We found that 6 h after 30% TBSA burn injury, TXA significantly suppresses the burn induced invasion of neutrophils to lungs and normalizes the permeability of lung vessels. Furthermore, these data suggests that the anti-inflammatory effects of TXA ultimately result in improved healing and tissue repair after burn

injury. These data are largely consistent with our previous TXA research using in vitro and mouse models discussed above.

In these studies, TXA has demonstrated a clear capacity to improve wound healing and tissue repair while also exhibiting a powerful ability to reduce DAMPs release, expression of inflammatory cytokines and lung tissue capillary leak. From a practical standpoint, systemic TXA is a well-established therapeutic with an acceptable side effect profile. This agent could easily integrate with standard burn care practices and be utilized in prolonged out of hospital care settings as well (i.e. rural, forward deployed military theaters, etc.). The systemic administration also makes it more practical when compared with other topical treatments evaluated in the preclinical setting for burn wound conversion treatment.[9,24] Additionally, the systemic effects of TXA suggest that this agent could be useful as a fluid resuscitation adjunct.

One important limitation to consider is that TXA has pro-thrombotic effects that complicate its potential use as an agent for this indication. While acute administration (soon after injury) may be reasonable, continued administration over several days might be expected to increase venous thromboembolic events in the clinical setting.[25] As a result, we have undertaken an in-depth investigation of the mechanisms underlying the pro-mitochondrial energetic and anti-inflammatory properties of this agent.[26] A detailed discussion of our preliminary findings in this area is beyond the scope of this manuscript, but offers the potential to use alternative agents to achieve the same effects without pro-thrombotic activity.

CONCLUSION

Animals treated with TXA demonstrated reduced burn wound conversion and decreased burn-induced SIRS response. Lung inflammation and vessel leakage was also significantly reduced by administration of TXA after injury. TXA demonstrated a capacity to improve tissue repair in the zone of stasis while also reducing systemic inflammation. TXA is a well-established therapeutic with an acceptable side effect profile that could easily integrate into current burn care management practices. Future research will elucidate the underlying anti-inflammatory and mitochondrial protective properties of TXA responsible for these findings.

References Cited

1. Singh V, Devgan L, Bhat S, Milner SM. *The pathogenesis of burn wound conversion*. Annals of Plastic Surgery 2007; 59:109-15. PMID: 17589272
2. Salibian AA, Rosario ATD, Severo LAM, Nguyen L, Banyard DA, Toranto JD, Evans GRD, Widgerow AD. *Current concepts on burn wound conversion-A review of recent advances in understanding the secondary progressions of burns*. Burns. 2016 Aug; 42 (5):1025-1035. PMID: 26787127
3. Levine B, and Kroemer G. *Autophagy in the pathogenesis of disease*. Cell 2008; 132:27-42. PMID: 18191218
4. Gravante G, Filingeri V, Delogu D, Santeusanio G, Palmieri MB, Esposito G, et al: *Apoptotic cell death in deep partial thickness burns by coexpression analysis of TUNEL and Fas*. Surgery. 2006; 139:854-855. PMID: 16782446
5. Peng Z, Ban K, LeBlanc A, Kozar RA. *Intraluminal tranexamic acid inhibits intestinal sheddases and mitigates gut and lung injury and inflammation in a rodent model of hemorrhagic shock*. J. Trauma Acute Care Surgery. 2016 Aug; 81(2):358-65. PMID: 27027557

6. Diebel LN, Martin JV, Liberati DM. *Early tranexamic acid administration ameliorates the endotheliopathy of trauma and shock in an in vitro model*. J Trauma Acute Care Surg. 2017 Jun; 82(6):1080-1086. PMID: 28328682
7. Carter DW, Prudovsky I, Kacer D, T. Soul, C. Kumpel, K. Pyburn, M. Palmeri, R. Kramer, J. Rappold, MD . *Tranexamic Acid Suppresses the Release of Mitochondrial DAMPs and Reduces Lung Inflammation in a Murine Burn Model*. Journal Trauma & Acute Care Surgery. 2018 Dec. 26 (EPUB); PMID: 30589751
8. Prudovsky I, Carter DW, Kacer D, Palmeri M, Soul T, Kumpel C, Pyburn K, Barrett K, DeMambro V, Alexandrov I, Brandina I, Kramer R, Rappold J. *Tranexamic acid suppresses the release of mitochondrial DNA, protects the endothelial monolayer and enhances oxidative phosphorylation*. J. Cell Physiology. 2019 Apr 2 (EPUB). PMID: 30941770
9. Singer AJ, Taira BR, Lin F, Lim T, Anderson R, McClain SA, et al. *Curcumin reduces injury progression in a rat comb burn model*. Journal of Burn Care & Research: Official Publication of the American Burn Association 2011; 32:135-42. PMID: 21088615
10. Zheng B, Shen C, Sun J, Guo W, Jin Y, Niu Y. *Developing a Simple Burn Model in Rats of Different Ages*. J. Burn Care Research. 2019 Apr 29. pii: irz072. PMID: 31111883
11. Stohn JP, Perreault NG, Wang Q, Liaw L, Lindner V. Cthrc1, a novel circulating hormone regulating metabolism. PLoS One. 2012; 7(10):e47142. Epub 2012 Oct 8. PMID: 23056600
12. Ruiz-Villalba A, Romero JP, Hernández SC, Vilas-Zornoza A, Fortelny N, Castro-Labrador L, San Martin-Uriz P, Lorenzo-Vivas E, García-Olloqui P, Palacio M, Gavira JJ, Bastarrika G, Janssens S, Wu M, Iglesias E, Abizanda G, de Morentin XM, Lasaga M, Planell N, Bock C, Alignani D, Medal G, Prudovsky I, Jin YR, Ryzhov S, Yin H, Pelacho B, Gomez-Cabrero D, Lindner V, Lara-Astiaso D, Prósper F. *Single-Cell RNA Sequencing Analysis Reveals a Crucial Role for CTHRC1 (Collagen Triple Helix Repeat Containing 1) Cardiac Fibroblasts After Myocardial Infarction*. Circulation. 2020 Nov 10; 142(19):1831-1847. Epub 2020 Sep 25. PMID: 32972203
13. Zhang Y, Enhejirigala, Li Z, Song W, Yao B, Li J, Wang Y, Duan X, Yuan X, Huang S, Fu X. *The role of CTHRC1 in hair follicle regenerative capacity restored by plantar dermis homogenate*. Biochem Biophys Res Commun. 2021 Sep 24; 571:14-19. Epub 2021 Jul 20. PMID: 34298337
14. Duan X, Yuan X, Yao B, Song W, Li Z, Enhejirigala, Kong Y, Wang Y, Fu X, Huang S. *The role of CTHRC1 in promotion of cutaneous wound healing*. Signal Transduct Target Ther. 2022 Jun 15; 7(1):183. PMID: 35701414
15. Pantalone D, Bergamini C, Martellucci J, Alemanno G, Bruscino A, Maltinti G, Sheiterle M, Viligiardi R, Panconesi R, Guagni T, Prosperi P. *The Role of DAMPS in Burns and Hemorrhagic Shock Immune Response: Pathophysiology and Clinical Issues*. Review. Int J Mol Sci. 2021 Jun 29; 22(13):7020. PMID: 34209943
16. Stortz JA, Hawkins RB, Holden DC, Raymond SL, Wang Z, Brakenridge SC, Cuschieri J, Moore FA, Maier RV, Moldawer LL, Efron PA. *Cell-free nuclear, but not mitochondrial, DNA concentrations correlate with the early host inflammatory response after severe trauma*. Sci Rep. 2019 Sep 20; 9(1):13648. PMID: 31541163
17. Idrovo JP, Boe DM, Kaahui S, Yang WL, Kovacs EJ. *Hepatic inflammation after burn injury is associated with necroptotic cell death signaling*. J Trauma Acute Care Surg. 2020 Oct; 89(4):768-774. PMID: 33017135

18. Chai JK, Cai JH, Deng HP, Zou XF, Liu W, Hu QG, Shen CA, Yin HN, Zhang XB, Chi YF, Ma L, Feng R. *Role of neutrophil elastase in lung injury induced by burn-blast combined injury in rats.* Burns. 2013 Jun; 39(4):745-53. Epub 2012 Sep 19. PMID: 22999209.
19. Vigiola Cruz M, Carney BC, Luker JN, Monger KW, Vazquez JS, Moffatt LT, Johnson LS, Shupp JW. *Plasma Ameliorates Endothelial Dysfunction in Burn Injury.* J Surg Res. 2019 Jan; 233:459-466. Epub 2018 Sep 22. PMID: 30502286.
20. Morrison JJ, Dubose JJ, Rasmussen TE, Midwinter MJ. *Military Application of Tranexamic Acid in Trauma Emergency Resuscitation (MATTERs) Study*. Archives of Surgery. 2012 Feb; 147(2):113-9. PMID: 22006852
21. Robertshaw HJ. *An anti-inflammatory role for tranexamic acid in cardiac surgery?* Critical Care. 2008; 12(1):105. PMID: 18254939
22. Later AF, Sitniakowsky LS, van Hilten JA, van de Watering L, Brand A, Smit NP, Klautz RJ. *Antifibrinolytics attenuate inflammatory gene expression after cardiac surgery.* J. Thoracic Cardiovascular Surgery. 2013 Jun; 145(6):1611-6. PMID: 23332183
23. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, Brohi K, Itagaki K, Hauser CJ. *Circulating mitochondrial DAMPs cause inflammatory responses to injury*. Nature. 2010 Mar 4; 464(7285):104-7. PMID: 20203610
24. Eski M, Ozer F, Firat C, Alhan D, Arslan N, Senturk T, et al. *Cerium nitrate treatment prevents progressive tissue necrosis in the zone of stasis following burn*. Burns: Journal of the International Society of Burn Injuries 2012; 38:283-9. PMID: 22015201
25. Russo RM, Lozano R, Ruf AC, Ho JW, Strayve D, Zakaluzny SA, Keeney-Bonthrone TP. *A Systematic Review of Tranexamic Acid-Associated Venous Thromboembolic Events in Combat Casualties and Considerations for Prolonged Field Care*. Mil Med. 2022 Oct 31:usac317. PMID: 36315470.
26. Prudovsky I, Kacer D, Zucco VV, Palmeri M, Falank C, Kramer R, Carter D, Rappold J. *Tranexamic acid: Beyond antifibrinolysis*. Transfusion. 2022 Aug; 62 Suppl 1:S301-S312. Epub 2022 Jul 14. PMID: 35834488.

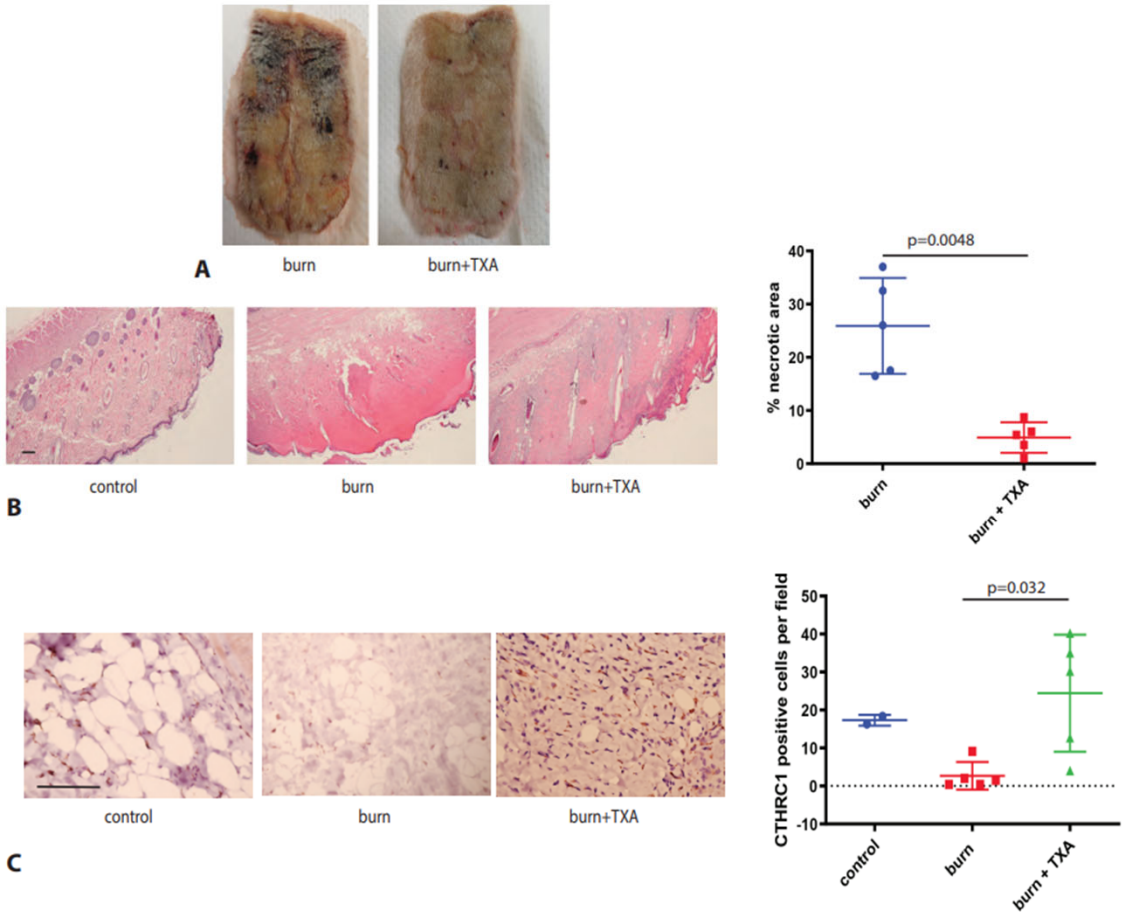

A
burn
burn+TXA
B
control
burn
burn+TXA
C
control
burn
burn+TXA
p=0.0048
% necrotic area
40
30
20
10
0
burn
burn + TXA
p=0.032
CTHRC1 positive cells per field
50
40
30
20
10
0
-10
control
burn
burn + TXA

Figure 1

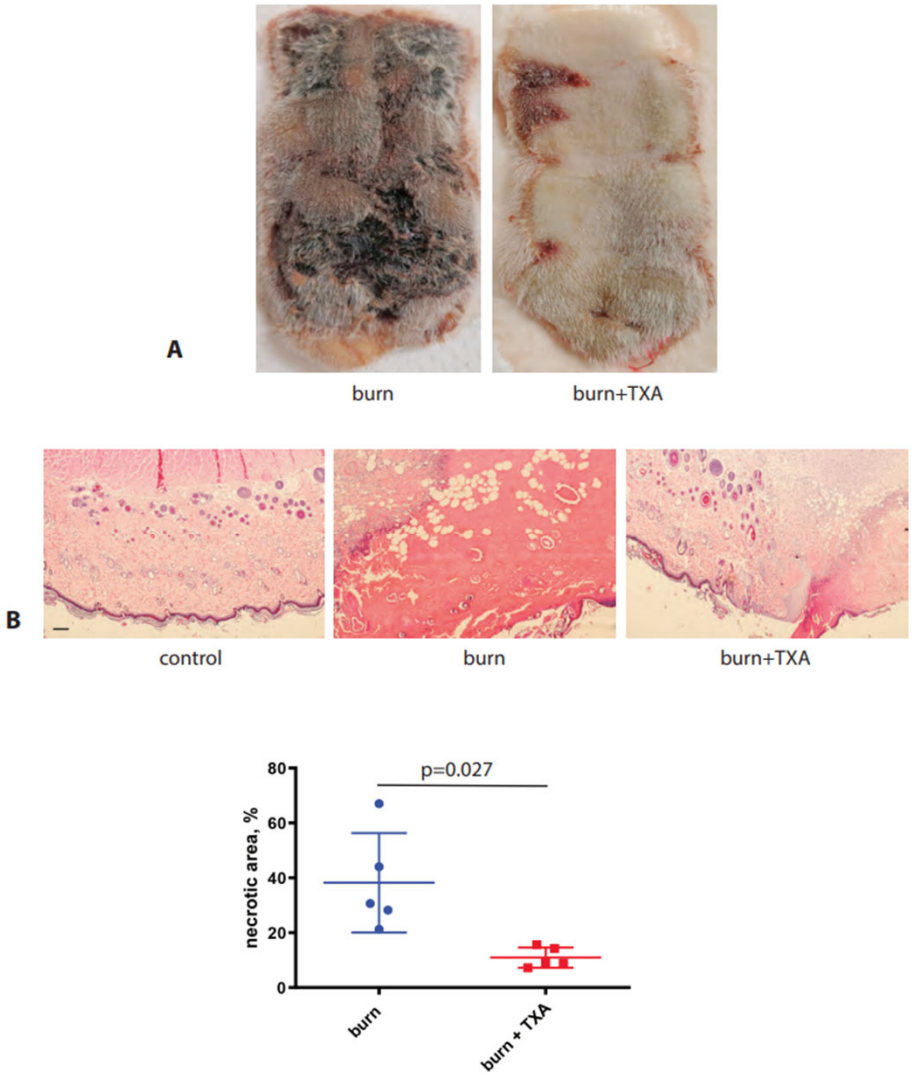

A
burn
burn+TXA
B
control
burn
burn+TXA
p=0.027
necrotic area, %
80
60
40
20
0
burn
burn + TXA

Figure 2

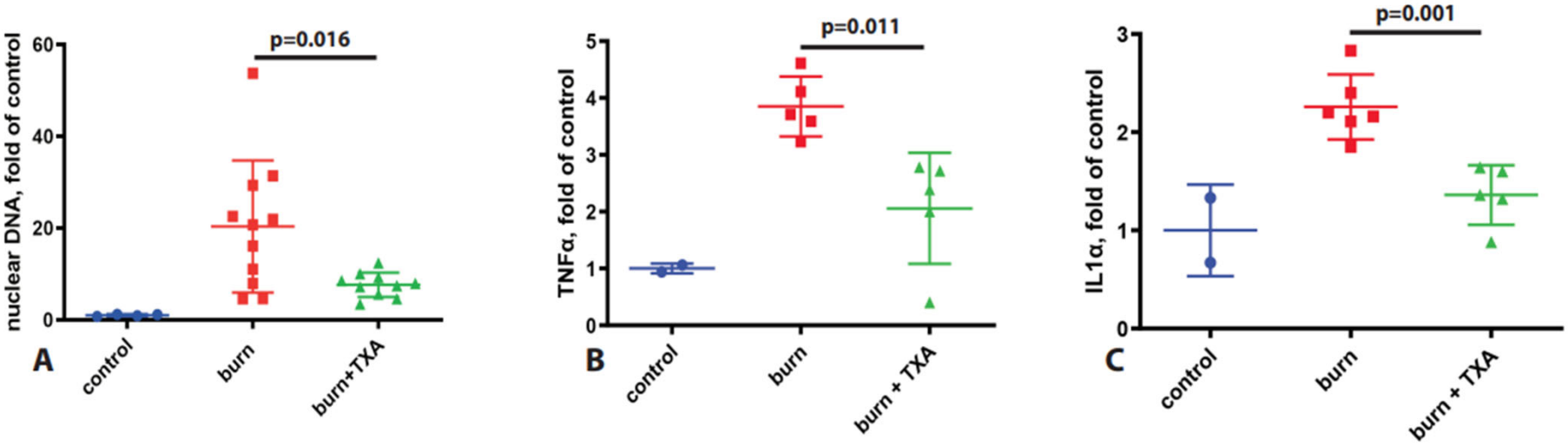

nuclear DNA, fold of control
60
40
20
0
p=0.016
A
control
burn
burn+TXA
TNFα, fold of control
5
4
3
2
1
0
p=0.011
B
control
burn
burn + TXA
IL1α, fold of control
3
2
1
0
p=0.001
C
control
burn
burn + TXA

Figure 3

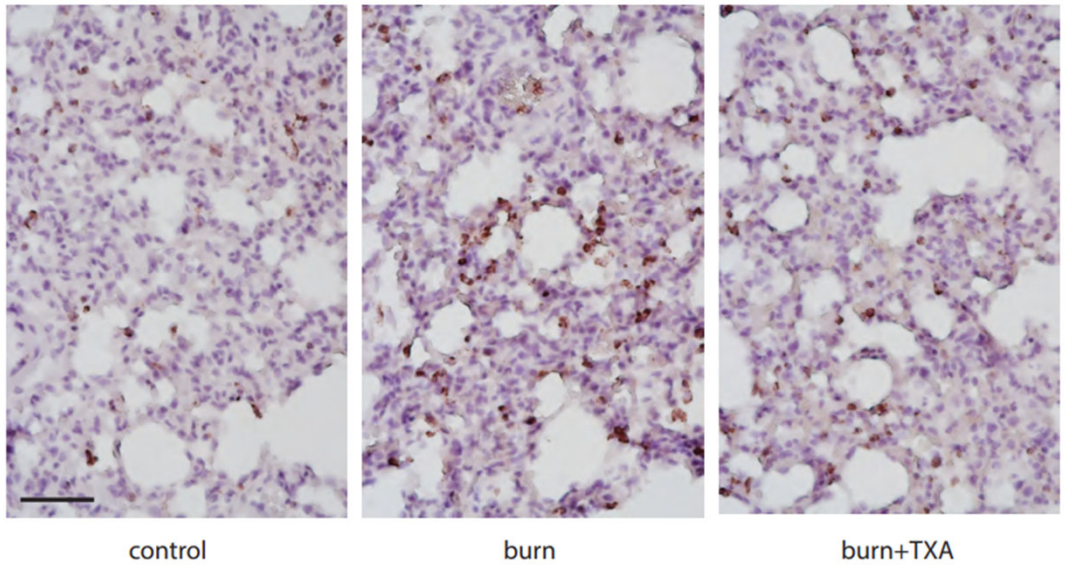
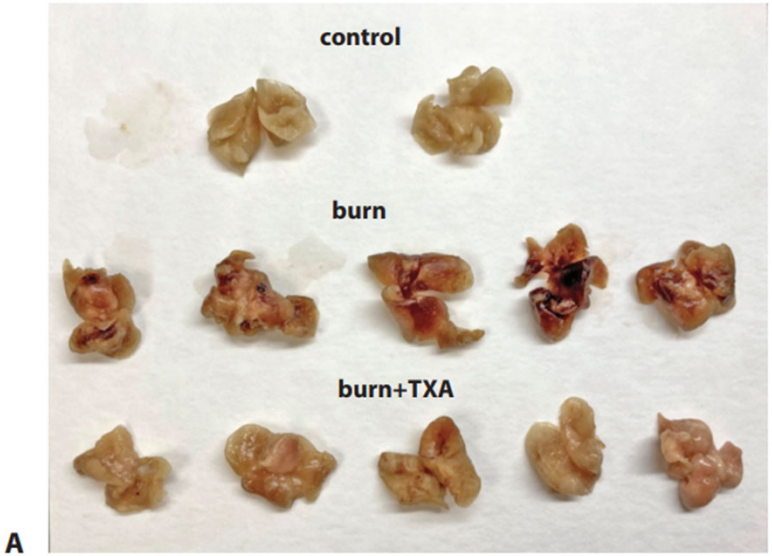
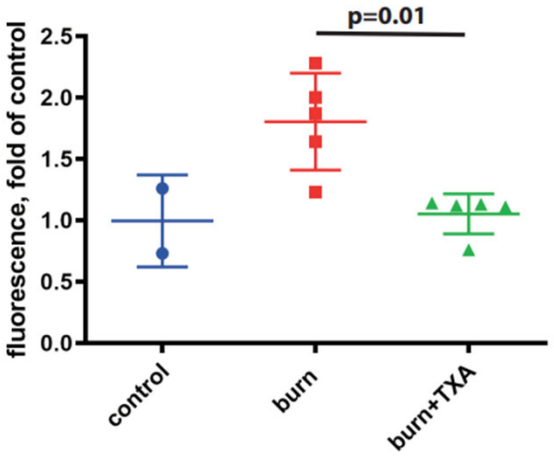

control
burn
burn+TXA
neutrophils per field
100
80
60
40
20
0
p=0.003
control
burn
burn+TXA

Figure 4


control
burn
burn+TXA

A


p=0.01
fluorescence, fold of control
2.5
2.0
1.5
1.0
0.5
0.0
control
burn
burn+TXA

B

Figure 5