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14. ABSTRACT

In this project, we aim to develop a sprayable, antimicrobial, and anti-inflammatory hydrogel-based wound dressing for rapid treatment of second and third degree burns in austere combat zones until an appropriate care unit can surgically treat the patient. This hydrogel includes hydrolyzed collagen (gelatin) as the bioactive base component, hyaluronic acid as the anti-inflammatory component, and peroxide microparticles as the antimicrobial component. Our sprayable hydrogel dressing will be useful in the pre-treatment of deep dermal and full thickness wounds. It will have quick and easy administration features, allow for on-site management of the burn wound rapidly, maintain a physical barrier, and be non-toxic to the tissue. The sprayable dressing can remain intact up to two weeks if necessary, it will protect the wound from mechanical effects, and prevent infection. The goals will be accomplished by pursuing the following aims:

Specific Aim 1: To synthesize a sprayable antimicrobial hydrogel-based wound dressing and characterize the material properties and antimicrobial efficiency.
Specific Aim 2: To evaluate the cytocompatibility of the sprayable hydrogel dressings.
Specific Aim 3: To evaluate the effectiveness of the sprayable wound dressing *in vivo*.

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1. INTRODUCTION

In the Y3 annual technical report, we focused on the analysis and evaluation of the *in vivo* experiments in Pig #2 and Pig#3. We have synthesized oxygen-generating hydrogel dressings, carried out and evaluated histology stainings for the biopsied samples from the animal experiment. We also imaged and analyzed the data. Moreover, we have synthesized the base hydrogels, gelatin methacrylate (GelMA) and methacrylated hyaluronic acid (HAMA).

We prepared the sprayable hydrogel dressing using pristine GelMA, and 15 mg/mL CaO_2 in GelMA for the Pig#2. We also added 1 mg/mL of catalase to the sterilized GelMA solution and incorporated the CaO2 homogeneously within the 3D of the antimicrobial wound dressing. At the UMass Chan Medical School, our clinical collaborator's Team has created the 2nd and 3rd degree burn wounds on the Pig#2 and applied the dressing by spraying on the burns. The burns were created using brass blocks on the skin. The Pig #2 was treated in double blind fashion with no dressing (control), commercial burn dressing (WaterJel) and our sprayable anti-microbial hydrogel dressing. Each experimental condition was repeated in duplicate. The summary of the experimental conditions that were tested in Pig#2 is given in the table below.

We carried out H&E staining and Masson's trichrome staining, Chloroacetate Esterase staining, H&E staining for the bacteria colonies, Ki-67 staining for proliferation, imaged and analyzed the data, gene expression analyses via quantitative reverse transcription polymerase experiments for the biopsied samples.

Table 1. Experimental conditions for Pig#2.

2nd degree burn	Blank	Blank	WaterJel	WaterJel	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)
3rd degree burn	Blank	Blank	WaterJel	WaterJel	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)

Based on the results we obtained from the Pig#2, we proceeded with the Pig#3 experiments. Below is a summary of the conditions tested in Pig#3. The sprayable hydrogel dressing contained 15 mg/mL CaO_2, 10% GelMA,1% HAMA, 1 mg/mL of catalase for the Pig#3. After creating the 2nd and 3rd degree burn wounds on the Pig#3, the wounds were infected using 1:1 ratio of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, and the dressings were applied via spraying on the burns. Each experimental condition was repeated in triplicate.

Table 2. Experimental conditions for Pig#3 with bacterial infection conditions.

2nd degree burn	Blank	Blank	Blank	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)
3rd degree burn	Blank	Blank	Blank	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)

The experimental results are summarized in the "Detailed progress and results" section below.

2. KEYWORDS

Second-degree burn, third-degree burn, sprayable hydrogel dressing, wound pretreatment, battlefield burn injuries, antibacterial, anti-inflammatory.

3. ACCOMPLISHMENTS

What were the major goals of the project? (Goals to be accomplished and status.)

<u>Specific Aim 1:</u> *To synthesize a sprayable antimicrobial hydrogel-based wound dressing and characterize the material properties and antimicrobial efficiency (months 1-30)*

- STATUS: started Y1Q1, will be completed Y3Q2

Goal 1: Complete synthesizing each component of the dressing formulation. (months 1-3)

- STATUS: Completed. We will continue material synthesis on an "as needed" basis during the project.
- Synthesized GelMA and obtained CaO_2.
- Compared and determined the single spray volume of different types of spray systems.

- Characterized the properties of sprayable GelMA solution with different concentrations, and determined the suitable concentrations based on spray conditions on commercially available pork skin tissue (ex vivo model).
- Fabricated crosslinked GelMA dressing with different polymer concentrations and compared with WaterJel® (commercially available control product) to determine suitable concentrations.
- Synthesized the anti-inflammatory component (methacrylated hyaluronic acid (HAMA)) of the sprayable wound dressing.

Goal 2: Obtain IACUC/ACURO approvals for the pig burn model. (months 1-9)

- STATUS: Completed
- STATUS: The IACUC protocol has expired on 08/23/2023. Our collaborator at the UMass Medical School has applied to renew the IACUC protocol. We will then renew the ACURO approval.

Goal 3: Combine the components of the dressing and perform material characterization (e.g. swelling, degradation, porosity, mechanical tests).

- STATUS: currently in progress (started in month 4) (months 3-9). We will continue material characterization on an "as needed" basis during the project.
- Identified a UV crosslinker for the sprayable hydrogel dressing and tested different polymer concentrations of GelMA for crosslinking times (10% w/v) and added other components, calcium peroxide (CaO_2) with different concentrations (0-12 mg/mL in 10% w/v GelMA).
- Performed experiments for swelling and degradation profiles.

Goal 4: Demonstrate sustained oxygen delivery up to two weeks. (months 6-12)

- STATUS: currently in progress (started in month 6)
- Performed experiments for oxygen release kinetics using 0-12 mg/mL CaO_2 in 10% w/v GelMA.

Goal 5: Conduct tests for antimicrobial activity of the dressing. (months 6-12)

- STATUS: currently in progress (started in month 6)

Goal 6: Optimize the dressing formulation based on *in vivo* results. (months 9-42)

- STATUS: yet to start

<u>Specific Aim 2:</u> To evaluate the cytocompatibility of the sprayable hydrogel dressings (months 6-45)

- STATUS: started Y1Q3, will be completed Y4Q3

Goal 1: Evaluate growth, cytotoxicity, and functionality assays against the dressing. (months 6-24)

- STATUS: currently in progress (started in month 6)
- Performed experiments by culturing Human Dermal Fibroblast cells in oxygen-generating 10% w/v GelMA-based hydrogels (with 0-12 mg/mL CaO_2) and assessed whether there is cytotoxicity from the sprayable gels using LDH and Alamar Blue assays.

Goal 2: Conduct degradation tests and wound healing genes expressions for the dressing in vitro using primary human dermal fibroblasts up to two weeks. (months 12-45)

- STATUS: yet to start

<u>Specific Aim 3:</u> To evaluate the effectiveness of the sprayable wound dressing in vivo (months 9-48)

- STATUS: started Y1Q4, will be completed Y4Q4

Goal 1: Demonstrate the biocompatibility of the dressing in pigs. (months 9-18)

- STATUS: currently in progress
- Performed the pilot pig (Pig#1) experiment and generated 2nd and 3rd degree burn wounds to test the sprayable composite dressing *in vivo*

- Performed the Pig#2 experiment and tested the sprayable composite dressing for 2nd and 3rd degree burn wounds *in vivo*
- Performed the Pig#3 experiment and tested the sprayable composite dressing for 2nd and 3rd degree burn wounds *in vivo*

Goal 2: Demonstrate the efficiency of the dressing and optimize it for pre-treatment of second- and third-degree burns. (months 9-48)

- STATUS: currently in progress

What was accomplished under these goals? (Detailed progress and results.)

Specific Aim 1: To synthesize a sprayable antimicrobial hydrogel-based wound dressing and characterize the material properties and antimicrobial efficiency (months 1-42)

In the Y3 annual technical report, we synthesized the base hydrogel gelatin methacrylate (GelMA) and the anti-inflammatory component methacrylated hyaluronic acid (HAMA)).

Key Findings or Accomplishments:

- Bioactive hydrogels were synthesized using gelatin (hydrolyzed collagen) and methacrylated gelatin-(GelMA) was obtained.
- Composite hydrogels were created using the bioactive GelMA, anti-inflammatory methacrylated hyaluronic acid HAMA, and the antimicrobial component calcium peroxide.

Specific Aim 2: To evaluate the cytocompatibility of the sprayable hydrogel dressings (months 6-45)

In the Y3 annual technical report, Dr. Camci-Unal's team has improved the *in vitro* system and worked on optimizing the hypoxic cell cultures.

To improve our *in vitro* cell culture system, we have encapsulated primary human dermal fibroblasts (HDFs) in 5% (w/v) GelMA in 96-well plates. We then placed our hydrogel dressing formulations on top of the 3D encapsulated HDFs, which provided a biomimetic system that is similar to the setting that we use *in vivo* where we have the cells inside the wound and the dressing is placed on top of the wound. We then measure the metabolic activity of the HDF cells encapsulated in 5% GelMA (w/v). The calcium peroxide component (CaO_2) was included in the 10%GelMA or 10%GelMA+1%HAMA hydrogels in the wound dressing formulation (Figure a).

The addition of HAMA shows an added benefit by the increase in the metabolic activity of the HDFs. We will continue optimizing the 96-well system for *in vitro* characterization of our dressing formulations.

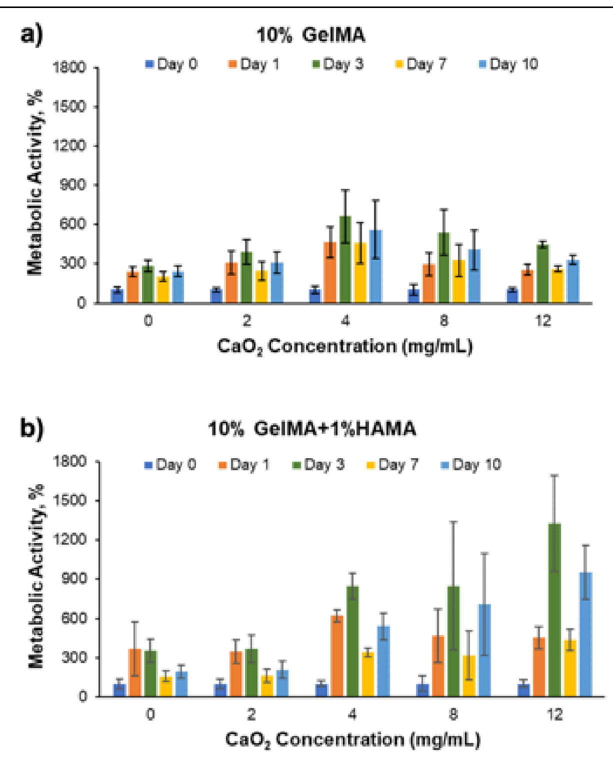


Figure a. Metabolic activity of the HDF cells encapsulated in 5% GelMA (w/v) hydrogels. An Alamar Blue assay was performed to measure the metabolic activity of the primary HDFs. The effect of adding the HAMA component is provided in part b.

Key Findings or Accomplishments:

- The composite hydrogels were characterized for their biological properties by Alamar Blue metabolic activity assays.
- Increasing metabolic activity was observed over time.

Specific Aim 3: To evaluate the effectiveness of the sprayable wound dressing in vivo (months 9-48)

In this reporting period, we have analyzed and evaluated the remaining *in vivo* experiments for Pig #2 and analyzed data from Pig#3.

Table 1. Experimental conditions for Pig#2.

2nd degree burn	Blank	Blank	WaterJel	WaterJel	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)
3rd degree burn	Blank	Blank	WaterJel	WaterJel	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)

Details of the animal surgery:

We prepared the sprayable hydrogel dressing (15 mg/mL CaO_2 in 10% GelMA-1%HAMA) for the Pig#2. The photoinitiator (PI) (Irgacure 2959) was dissolved in PBS at 70°C with a concentration of 0.5% (w/v) to obtain the PI solution. 10% (w/v) GelMA, 1% HAMA (w/v) and 2% (v/v) antifoaming agent were dissolved in PI solution and the solution was sterilized by the 0.22 μm syringe filter. Catalase was added to the GelMA solution at 1 mg/mL concentration and allowed to dissolve. CaO_2 was mixed with the polymer solution at 15 mg/mL concentration to obtain the antimicrobial wound dressing.

We inflicted the 2nd and 3rd degree burn wounds on a swine model and applied the dressing by spraying on the burns. The Yorkshire swine weighing 55 kg was used in the Pig#2 experiment. The pig was bred in a pathogen-free facility and housed at the University of Massachusetts Medical School, in accordance with the Guide for the Care and Use of Laboratory Animals. All experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School, and in accordance with the National Institute of Health, Public Health Service, Federal, DoD, Army, USAMRMC, and international regulatory guidelines for animal care.

The animal was sedated with a combination of 2 mg/kg Telazol and 2 mg/kg xylazine by intramuscular injection. The endotracheally intubated pig was kept under anesthesia with isoflurane 1-2% in an operating room in a prone position for the duration of the experiment. Oxygen saturation and heart rate were measured with pulse oximeter ear sensors, and respiratory rate, mucus membrane color, and rectal temperature were also monitored throughout the procedure. The flank and back hair were clipped, and a sterile field produced using skin prep with soap, chlorhexidine, and poviodone-iodine. Circular areas for burning were outlined with a marking pen. A custom made brass block was preheated on a hot plate to 100°C and measured by a thermometer. The brass block contacted with skin for 17.5s for the second degree burn and 32.5s for the third degree burn.

The Animal was treated in double blind fashion with no dressing (control), commercial burn dressing (WaterJel), and our anti-inflammatory and antimicrobial hydrogel dressing. The blank control burn areas were covered with polyurethane occlusive dressing (Tegaderm). The WaterJel was sprayed on the wounds and covered by Tegaderm immediately after spraying. The hydrogel-based liquid wound dressings were sprayed on the wounds with help of custom-made stencils to prevent spilling. The polymer solutions were crosslinked with using the UV light. A second layer of hydrogel dressing was created on top of the first layer using the same method. The wound was covered by Tegaderm immediately after crosslinking for the second layer. The animal was wrapped with a tubular net to prevent manipulation by the animal subject. Burn wounds were carefully monitored by clinical observation for any sign of infection daily. Post-procedural pain was treated with buprenorphine IV and fentanyl patch transdermally.

The biopsy punches were taken for each wound on day 1, 7, and 14. The animal was maintained under anesthesia as described above. Tegaderm was removed from the wound and a quarter of the wound area was defined for biopsy. Six biopsy punches were performed for each wound with the wound dressing remaining on day 1 and 7. On day 14, the dead skin and the dressing on top of it was removed and the biopsy punches was taken from the tissue underneath. Half of the biopsy samples from each wound each time point were placed in cassettes and fixed in formalin at room temperature for histology analyses. The removed skin and dressing on day 14 was also preceded for histology. The other half of the samples were placed in the RNase-free 1.5 mL tubes and frozen with dry ice for further PCR analyses. The new Tegaderms were applied for the wounds on days 1 and 7 after the biopsy punches. The post-operation care on days 1 and 7 was the same as mentioned above.

Details of the Histology experiments:

The biopsy samples were embedded in paraffin according to the manufacturer’s protocols. The pig tissue sections were obtained using a microtome with a thickness of 10 μm. The tissue sections were then baked at 65 °C for 60 min and incubated in xylene twice for 10 min for dewaxing. The samples were then rehydrated in absolute ethanol, 95% ethanol, and 75% ethanol gradient consecutively for 2 min incubation time for each. Following, the samples were treated in ethylenediaminetetraacetic acid (EDTA) solution pH 9.0 and boiled for 10 mins. The samples slides were removed and rinsed with distilled water after cooling to room temperature. Then, 3% (v/v) solution of hydrogen peroxide was used to treat the samples 15 min to block the endogenous peroxidase. Afterwards, the H&E staining was performed according to the manufacturer's instructions. The hematoxylin-stained samples were treated with 1% hydrochloric acid and re-blued in absolute ethanol and rinsed with tap water. The reversed order of ethanol gradients was used to dehydrate the stained samples before mounting.

We have performed H&E staining and Masson's trichrome staining on the biopsies obtained from blank control, WaterJel, and antimicrobial gels (AMG) on days 1 and 7 (Figures 1-4). We have demonstrated the tissue structures on these samples.

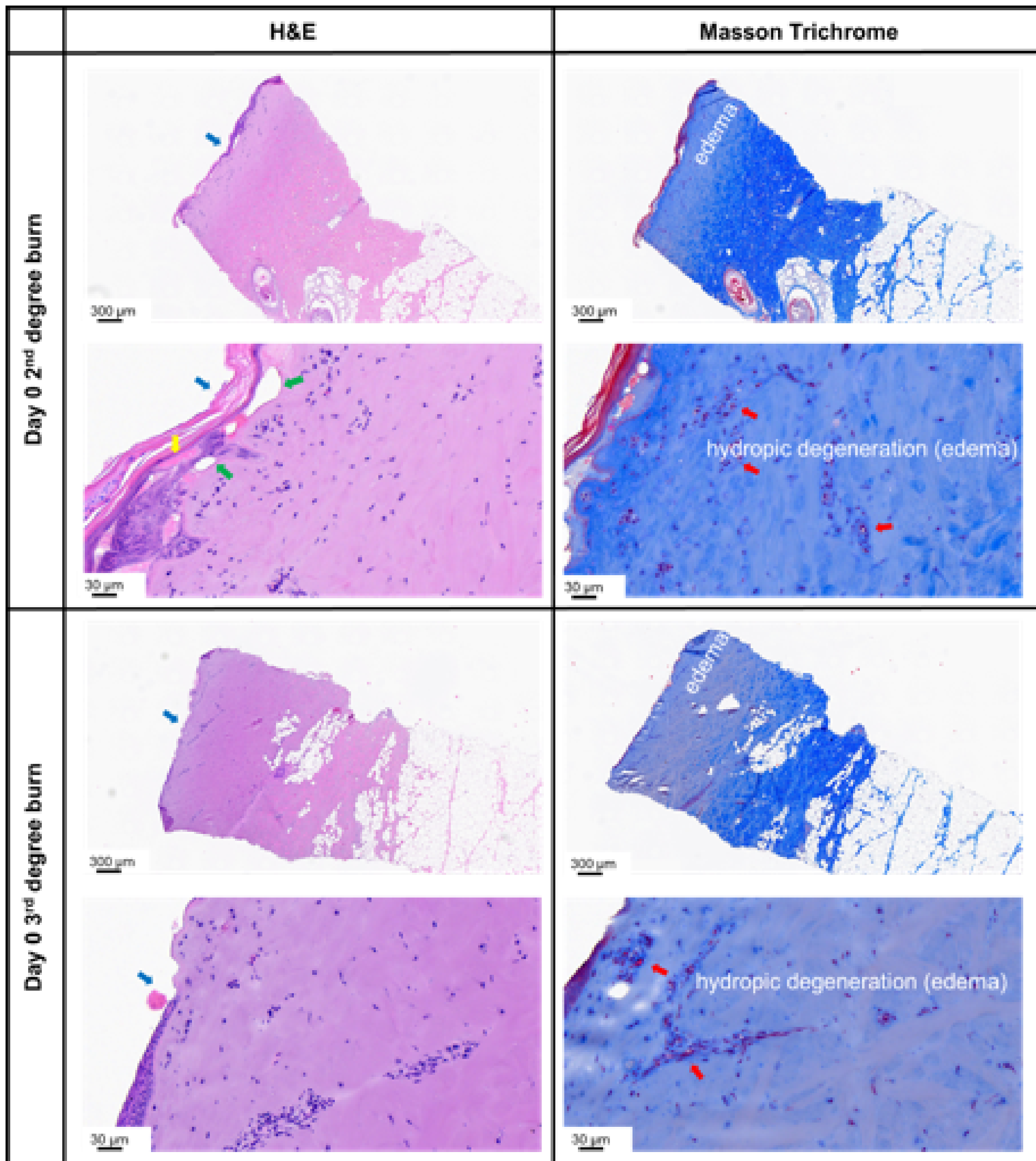


Figure 1. H&E and Masson trichrome staining of 2nd and 3rd degree burn samples on day 0 of blank control for Pig#2. The damage and loss of epidermis and basal cell and basement membrane deformation were observed in both 2nd and 3rd degree burns (blue arrows). Exudate (yellow arrows) and blisters (green arrows) are the signs of burn wounds. The vascular smooth muscle cells are stained red (red arrows) in Masson trichrome staining. The light stain in Masson trichrome staining represents the hydropic degeneration of dermis layer.

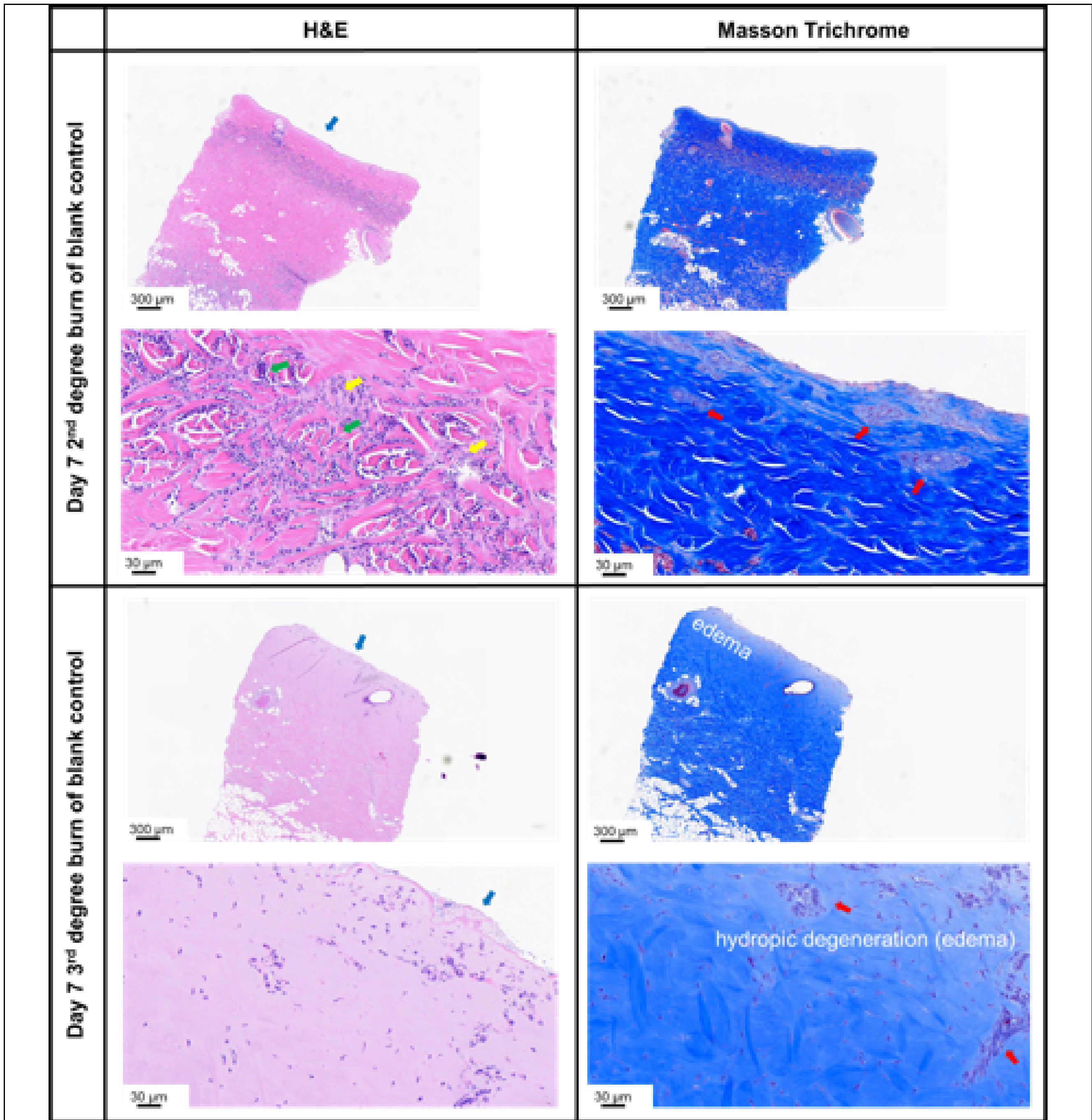


Figure 2. H&E and Masson trichrome staining of 2nd and 3rd degree burn samples on day 7 of blank control for Pig#2. The burn damage and the loss of epidermis are indicated in the 2nd and 3rd degree burns (blue arrows). The lymphocytes, plasmacytes, neutrophils, and fibroblasts (green arrows) are observed in the 2nd degree burn samples. They formed a band close to dermis. Necrosis and pyocytes can be seen in the band (yellow arrows). The vascular smooth muscle cells are stained to red color (red arrows) in Masson trichrome staining. The Masson trichrome staining shows the hydropic degeneration of the dermis layer.

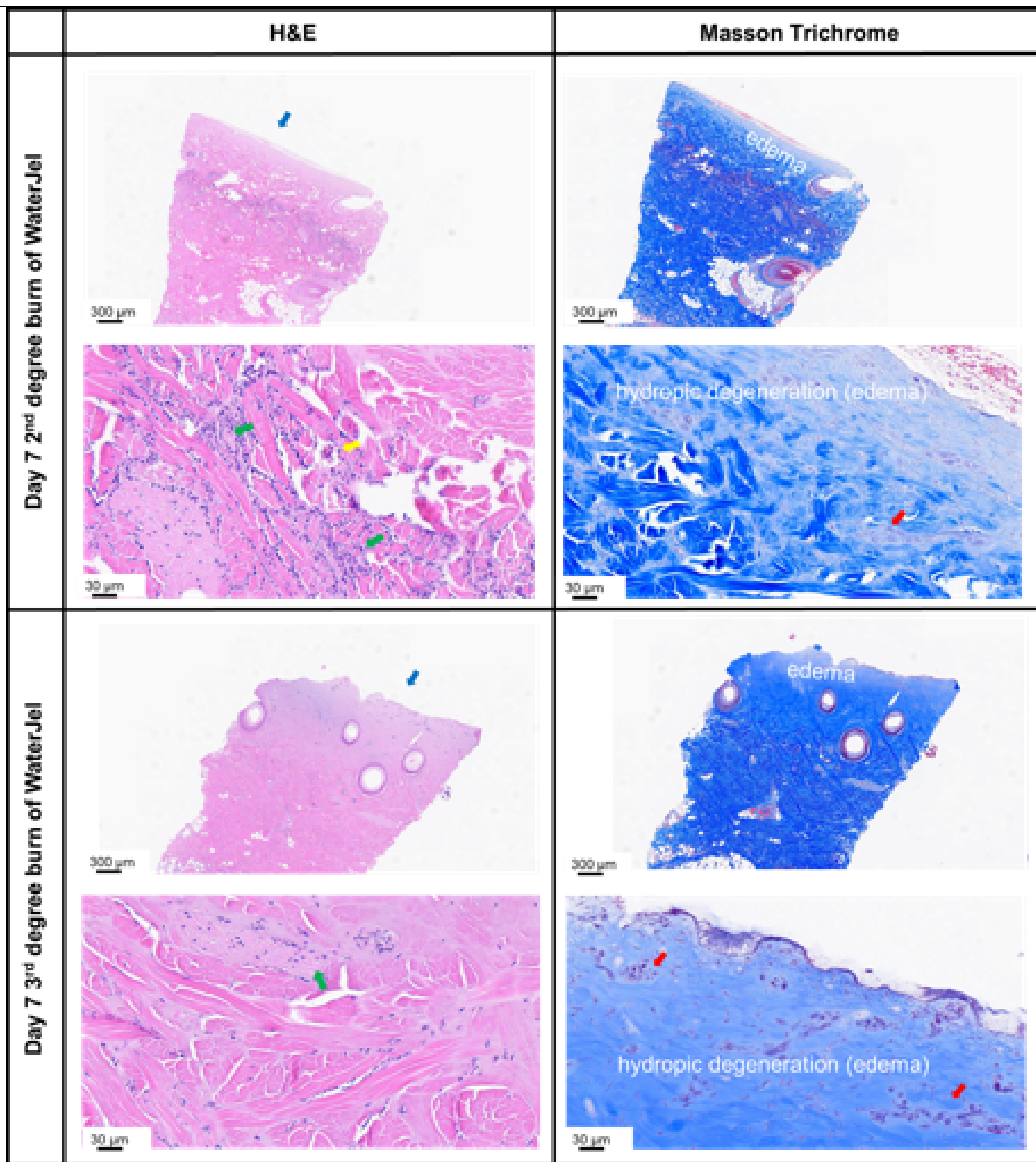


Figure 3. H&E and Masson trichrome staining for the 2nd and 3rd degree burn samples on day 7 for the control WaterJel samples for Pig#2. WaterJel is commonly used for burn treatment in the battlefield. The damage and loss of epidermis were observed in the 2nd and 3rd degree burns as indicated by the blue arrows. The ymphocytes, plasmacytes, neutrophils, and fibroblasts (green arrows) are seen in the 2nd and 3rd degree burn samples where they formed a band close to dermis in the 2nd degree burn samples. Necrosis can be seen in the band as indicated by the yellow arrows. The vascular smooth muscle cells are stained red (red arrows) in the Masson trichrome staining which demonstrates the degeneration of the dermis layer.

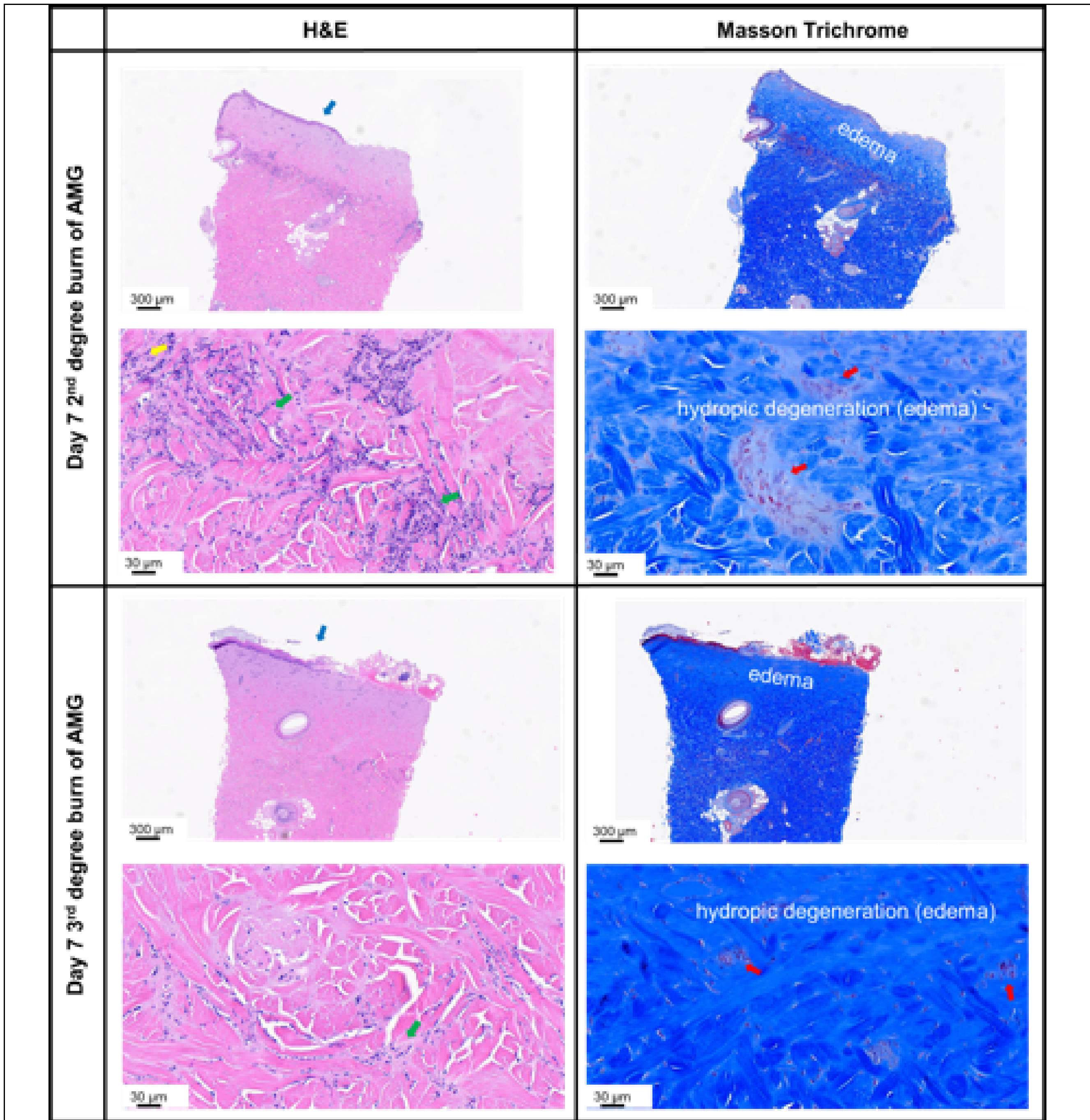


Figure 4. The H&E and Masson trichrome staining of 2nd and 3rd degree burn samples on day 7 for the AMG samples for Pig#2. The damage and the loss of epidermis were observed in both 2nd and 3rd degree burns (blue arrows). The lymphocytes, plasmacytes, neutrophils, and fibroblasts (green arrows) were present in both 2nd and 3rd degree burn samples where they formed a band close to the dermis layer in the 2nd degree burn samples. Necrosis is present in the band (yellow arrows). The vascular smooth muscle cells are shown in red color by red arrows in the Masson trichrome staining. The Masson trichrome staining shows the hydropic degeneration of the dermis layer.

In the histology samples, we have seen the loss of the epidermis layer from the blank control and WaterJel groups in all time points. The AMG group was able to maintain the most of the epidermis layer which was comparable to the day 0 structure. We have confirmed from the day 0 samples that the epidermis layer was completely damaged in the 2nd and 3rd burn samples as indicated by the presence of deformed basal cells. The AMG samples were able to preserve the damaged epidermis on day 7.

We have performed the H&E staining and Masson's trichrome staining on the biopsies obtained from blank control, WaterJel, and antimicrobial gels (AMG) on day 14 (Figures 5-7). We have demonstrated the tissue structures on these samples.

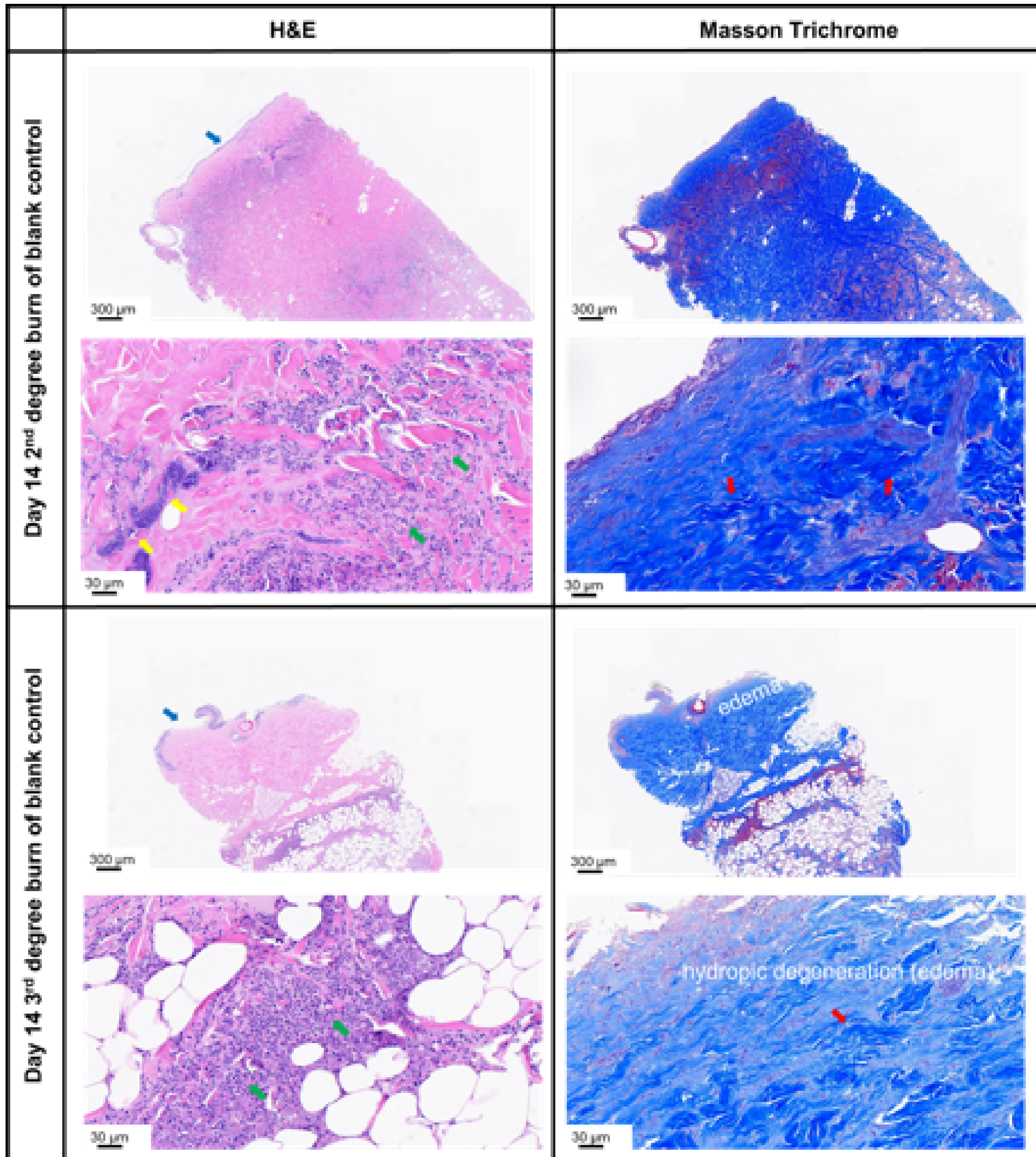


Figure 5. The H&E and Masson trichrome staining for the 2nd and 3rd degree burn samples on day 14 of blank control for Pig#2. The damage and loss of epidermis were observed in both 2nd and 3rd degree burns (blue arrows). Lymphocytes, plasmacytes, neutrophils, and fibroblasts (green arrows) are the observed in both 2nd and 3rd degree samples and they formed a band close to dermis in 2nd degree and deep in subcutaneous region in 3rd degree. Necrosis and bacteria colonies can be seen in the band (yellow arrows). The deformed collagen fibers (red arrows) are observed in Masson trichrome staining. The light stain in Masson trichrome staining represents the hydropic degeneration of dermis layer.

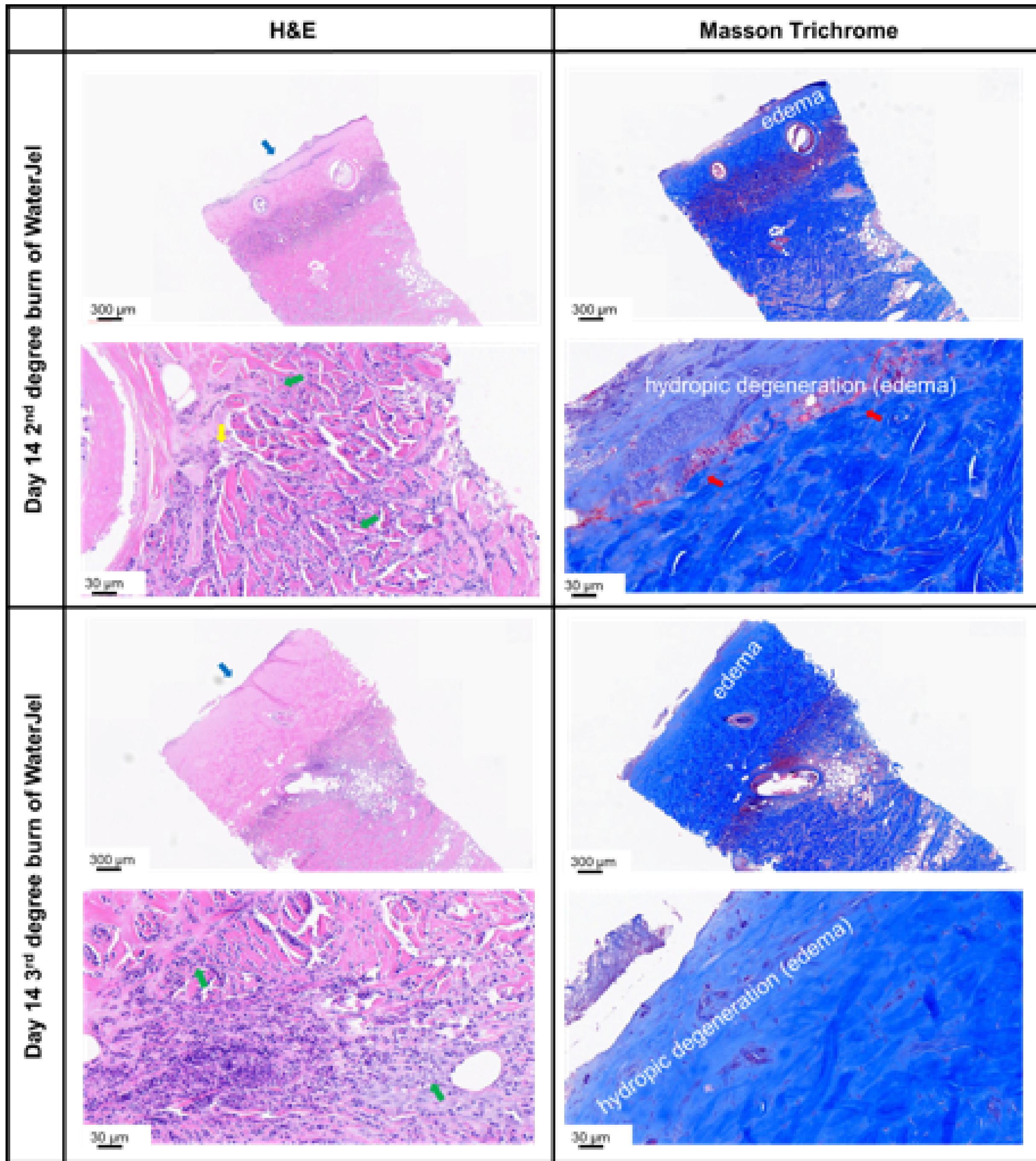


Figure 6. The H&E and Masson trichrome staining for 2nd and 3rd degree burn samples on day 14 for the commercially available control WaterJel samples for Pig#2. The results are similar to those obtained from the non-treated blank control. The damage and loss of epidermis were observed in both 2nd and 3rd degree burns as shown by the blue arrows. Lymphocytes, plasmacytes, neutrophils, and fibroblasts are indicated by the green arrows. They are observed in both 2nd and 3rd degree samples forming a band close to dermis in 2nd degree samples and deep in subcutaneous region in 3rd degree samples. Necrosis can be detected in the band as shown by the yellow arrows. The inflammatory cells are stained red as shown by the red arrows in Masson trichrome staining. The light stain in Masson trichrome staining represents the degeneration of dermis.

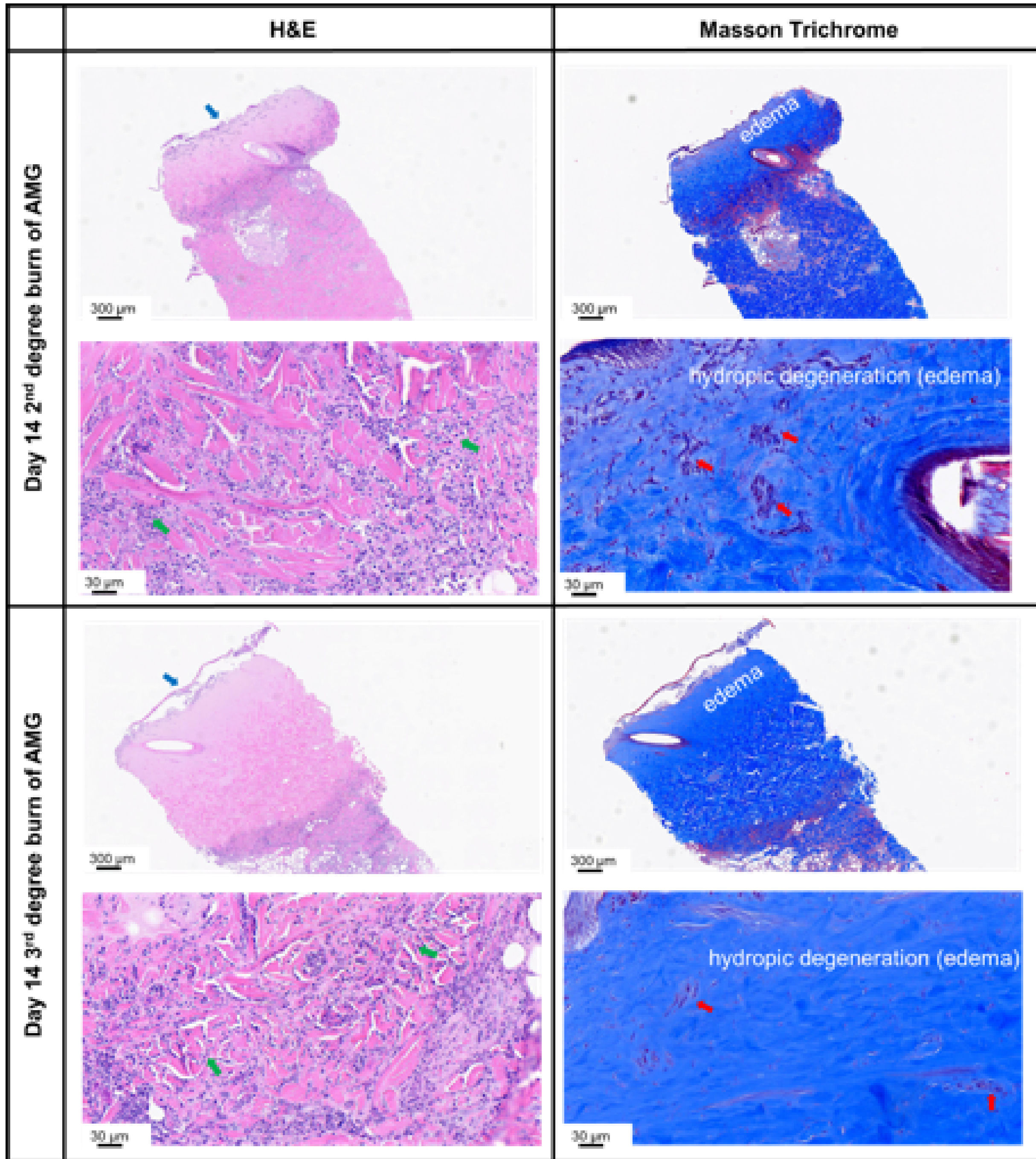


Figure 7. The H&E and Masson trichrome staining for the 2nd and 3rd degree burn samples on day 14 for the AMG samples for Pig#2. There was damage and loss of epidermis in the 2nd and 3rd degree burns (see blue

arrows). Lymphocytes, plasmacytes, neutrophils, and fibroblasts (green arrows) are the observed in both 2nd and 3rd degree samples. These cells formed a band close to dermis in 2nd degree samples and in deep subcutaneous layer in 3rd degree samples. The vascular smooth muscle cells can be seen in red color indicated by red arrows in Masson trichrome staining. Similar to the previous figures, the light stain in Masson trichrome staining indicated the hydropic degeneration of the dermis layer.

In both 2nd and 3rd degree burn groups, the dermis layer showed significant edema caused by the burn damage. The edema caused by 3rd degree burn was significantly deeper than that of the 2nd degree burn. The edema became smaller over time due to the loss of epidermis and the vascularization in the dermis. The reduction of edema occurred the most in the blank control group, and the least for the AMG group. Due to the loss of epidermis in the blank control and WaterJel groups after day 0, the wound dried as the Tegaderm allowed gas exchange, and the edema was reduced quickly due to the drying. The AMG was able to prevent the wound from drying, however, the edema was not reduced by the circulatory system as efficient as drying. On the other hand, the edema is not a sign of permanent cell damage, and the reduction of edema is not a sign of tissue repair. The AMG can provide the wound the most protection that prolongs the time limit from the initial burning to surgery.

In all 2nd degree burn conditions, there was a cell band emerged from day 7 in the dermis layer. There are mainly lymphocytes, plasmacytes, neutrophils, and fibroblasts in the band, and necrosis is observed. The most severe condition is in blank control group that the pyocytes and bacteria colonies are observed (Figure 5). The AMG group had the least inflammatory reaction on day 7 among all groups. The result shows the anti-inflammatory property of the AMG that contains 1% hyaluronic acid. The cell bands are not observed on day 7 in all 3rd degree burn conditions and emerged on day 14 for all conditions, and they were in a significant deeper level of dermis or deeper in the subcutaneous layer compared with 2nd degree burn groups. The deeper edema and greater dermic damage might be the reason of the delayed inflammatory response and the inflammatory level may be an indicator of the depth of damage.

In all conditions, the rate of neutrophils is lower, and rate of lymphocytes is higher in the cell bands from 3rd degree burn groups compared with them from 2nd degree groups (Figure 8). The 3rd degree burn shows closer to chronic inflammation while 2nd degree burn shows acute. Our AMG formulation was able to alleviate the acute inflammation in 2nd degree burn and showed similar result for 3rd degree burn compared with blank control and WaterJel.

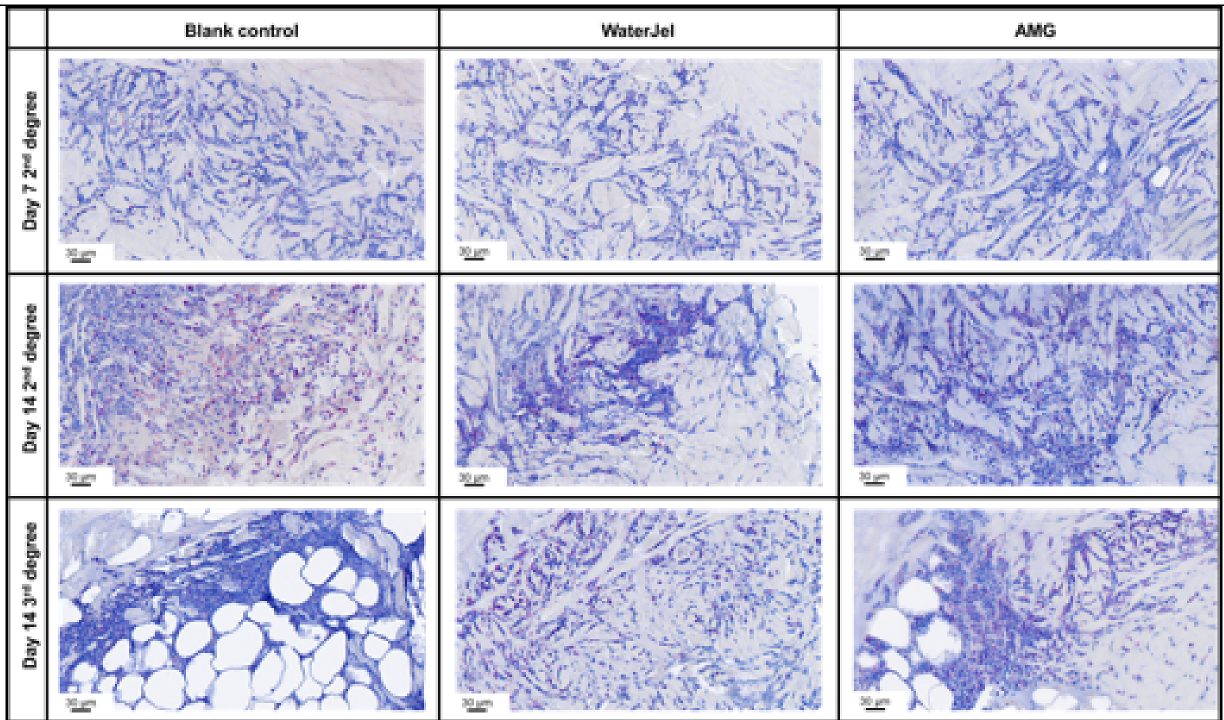


Figure 8. The Chloroacetate Esterase staining for the cell bands for Pig#2. The neutrophils are stained in red color. There was an increased in the amount of neutrophils from day 7 to day 14 in all groups from 2nd degree burn condition. On day 14, there were less neutrophils from 3rd degree burn groups compared with the 2nd degree burn groups.

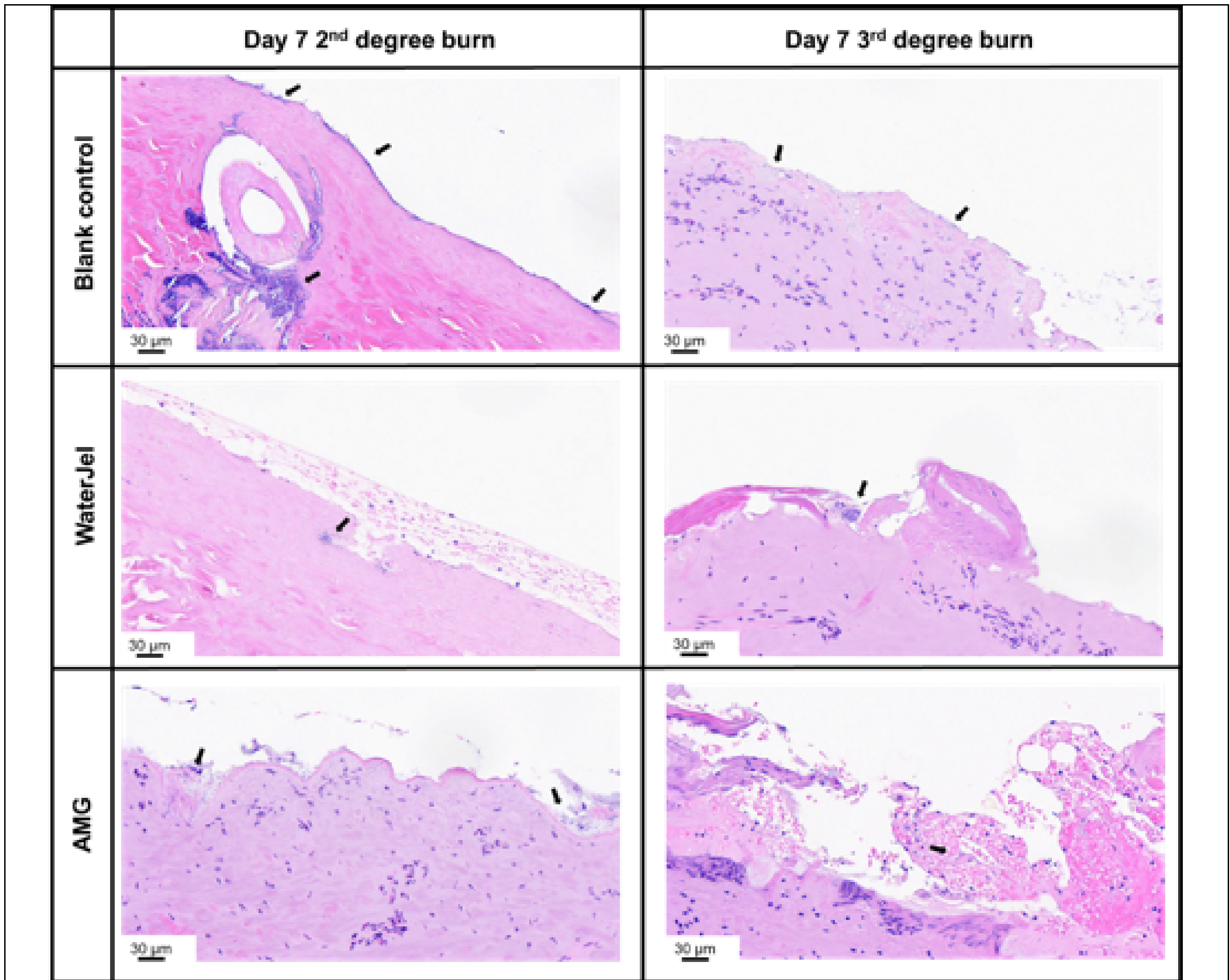


Figure 9. The H&E staining for the bacteria colonies (black arrows) on day 7 for Pig#2. Bacterial colonies are observed in all conditions. The non-treated blank control showed the most colonization among all groups in both 2nd and 3rd degree burn wounds.

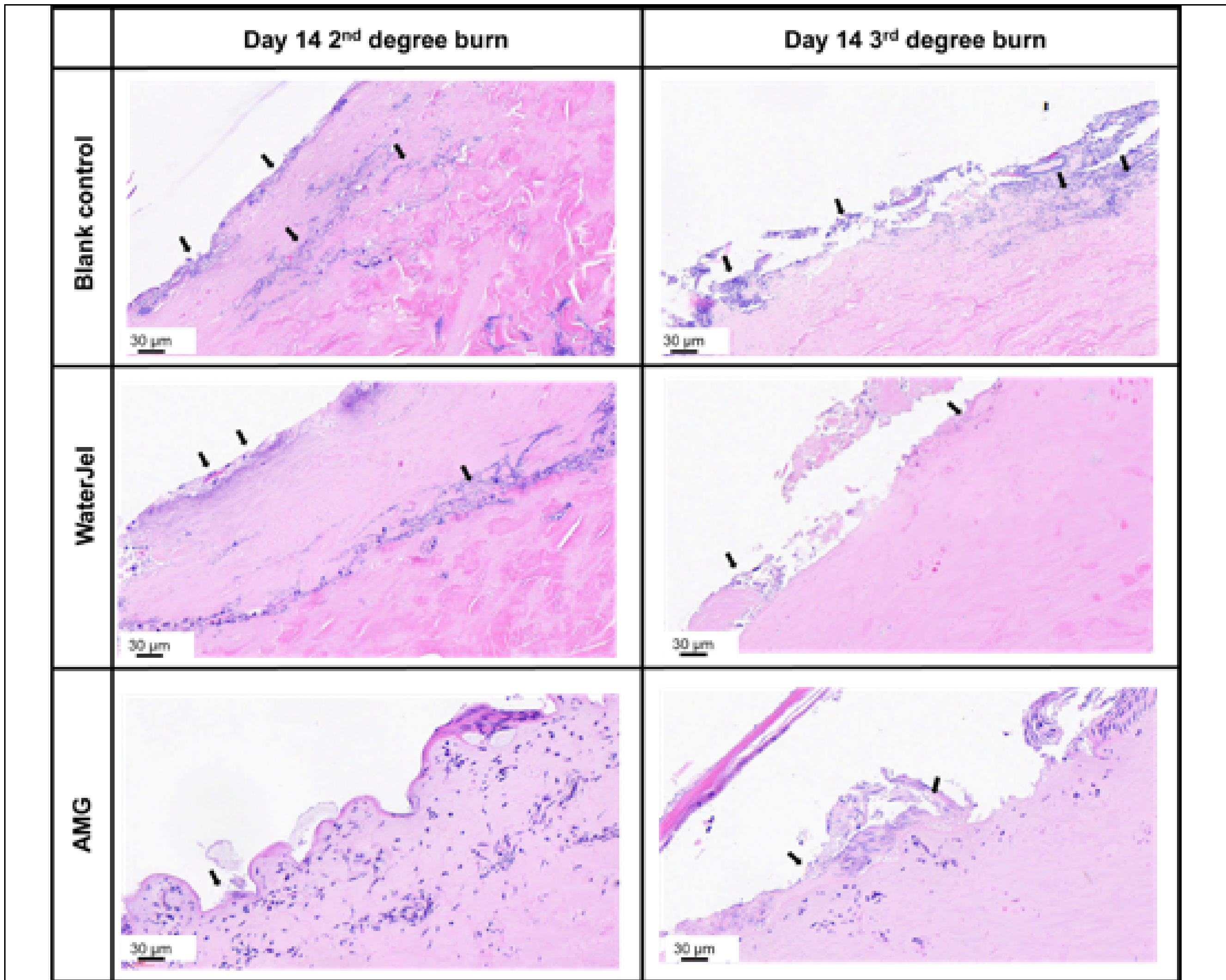


Figure 10. The H&E staining for the bacteria colonies (blank arrows) on day 14 for Pig#2. Some bacterial colonies are observed in all of the experimental groups. The non-treated blank control and WaterJel samples showed significantly more colonization than the AMG group in both 2nd and 3rd degree burn wounds.

We have observed bacteria colonies from day 7 to day 14 in all conditions in H&E staining (Figures 9-10). The blank control samples were found to be infected on day 7 while only small colonies were found for WaterJel and AMG groups. Both WaterJel and AMG showed antimicrobial activity compared with the blank control, however. In both 2nd and 3rd degree burn conditions, the bacteria invaded into deeper dermis layer in blank control and WaterJel samples and the area of the colonies became larger on day 14. The AMG showed superior antimicrobial property at this time point that the colonies were restricted on the surface of samples from both 2nd and 3rd degree burn wounds and the area remain similar to day 7.

The overall results from the Pig#2 experiments demonstrated the anti-inflammatory and antimicrobial properties of the AMG. The results also indicated that the AMG dressing can also prevent the wound from drying. The WaterJel control serves similarly to AMG when comparing with the non-treated blank control, however, the AMG was able to provide continuous protection for the wound up to day 14 while WaterJel can only be effective up to day 7. We will investigate the gene expression for inflammation and regeneration to better understand the roll of the AMG for the burn wound. The effects of AMG for subcutaneous tissue and infected wound are still unknown, which will be studied in the upcoming surgeries.

We have performed Ki-67 staining to evaluate the proliferating cells in the burn wounds. The biopsies were taken from 2nd and 3rd degree burn treated with Blank control (BC), WaterJel (WJ), and antimicrobial hydrogel (AMG) groups on day 0, 7, and 14.

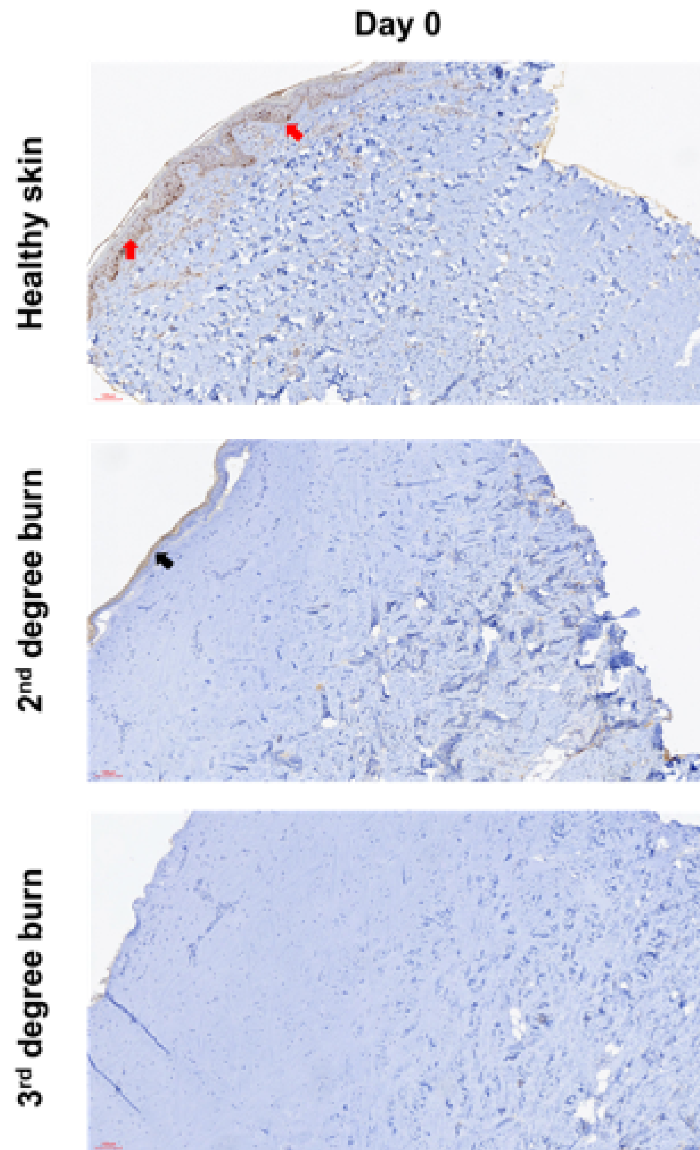


Figure 11. Ki-67 staining on **day 0** for the biopsies taken from the healthy skin, 2nd degree burn, and 3rd degree burn for Pig#2. The proliferating cells are stained in brown color (shown by red arrow). Small amount of non-specific staining was observed in the area without the cells (shown by black arrow).

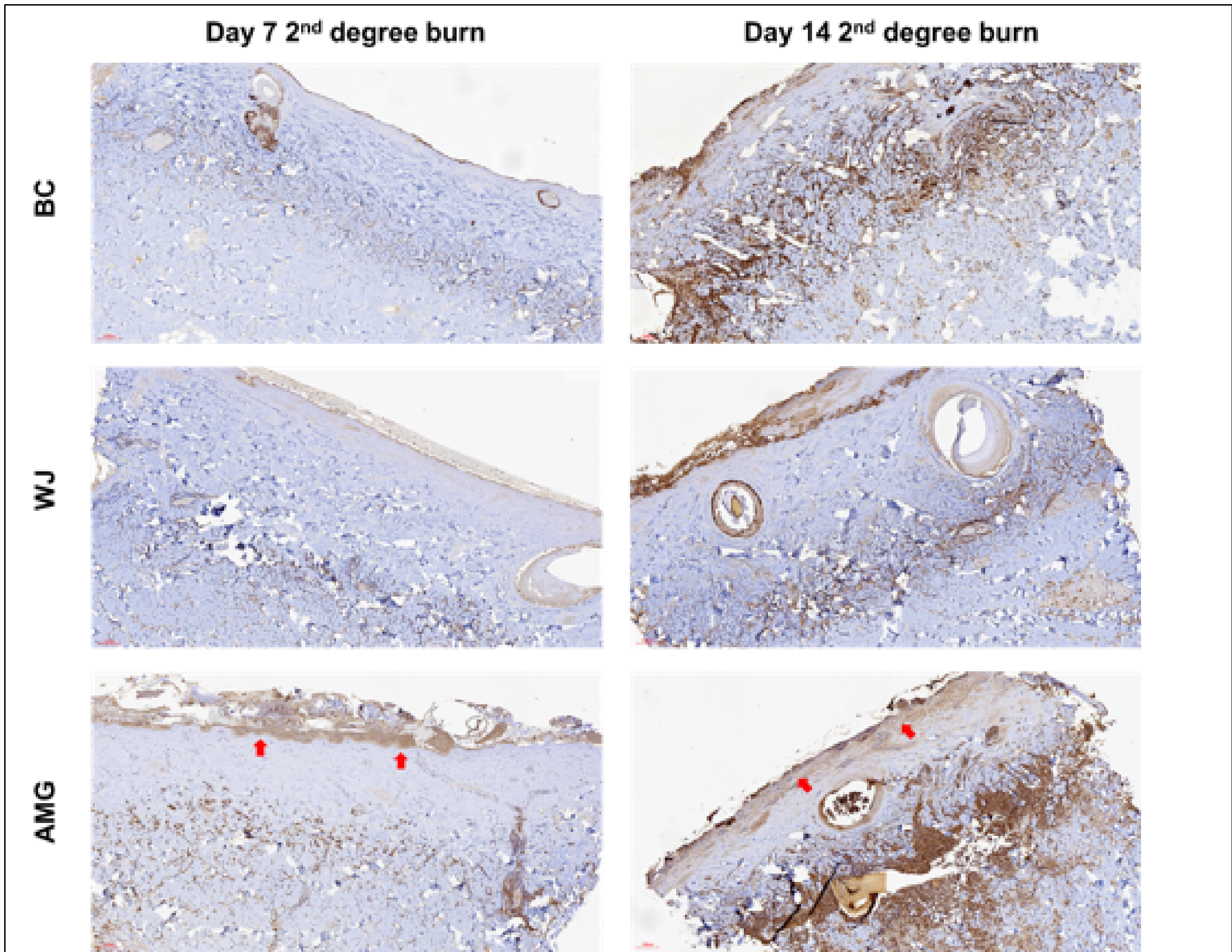


Figure 12. The Ki-67 staining for 2nd degree burns on **days 7 and 14** for the biopsies taken from BC, WJ, and AMG samples for Pig #2. The inflammatory cells are stained showing in the dermis. Possible reepithelization occurred in the AMG group (shown by red arrow) indicating the advantage of our antimicrobial sprayable hydrogel dressing on tissue healing.

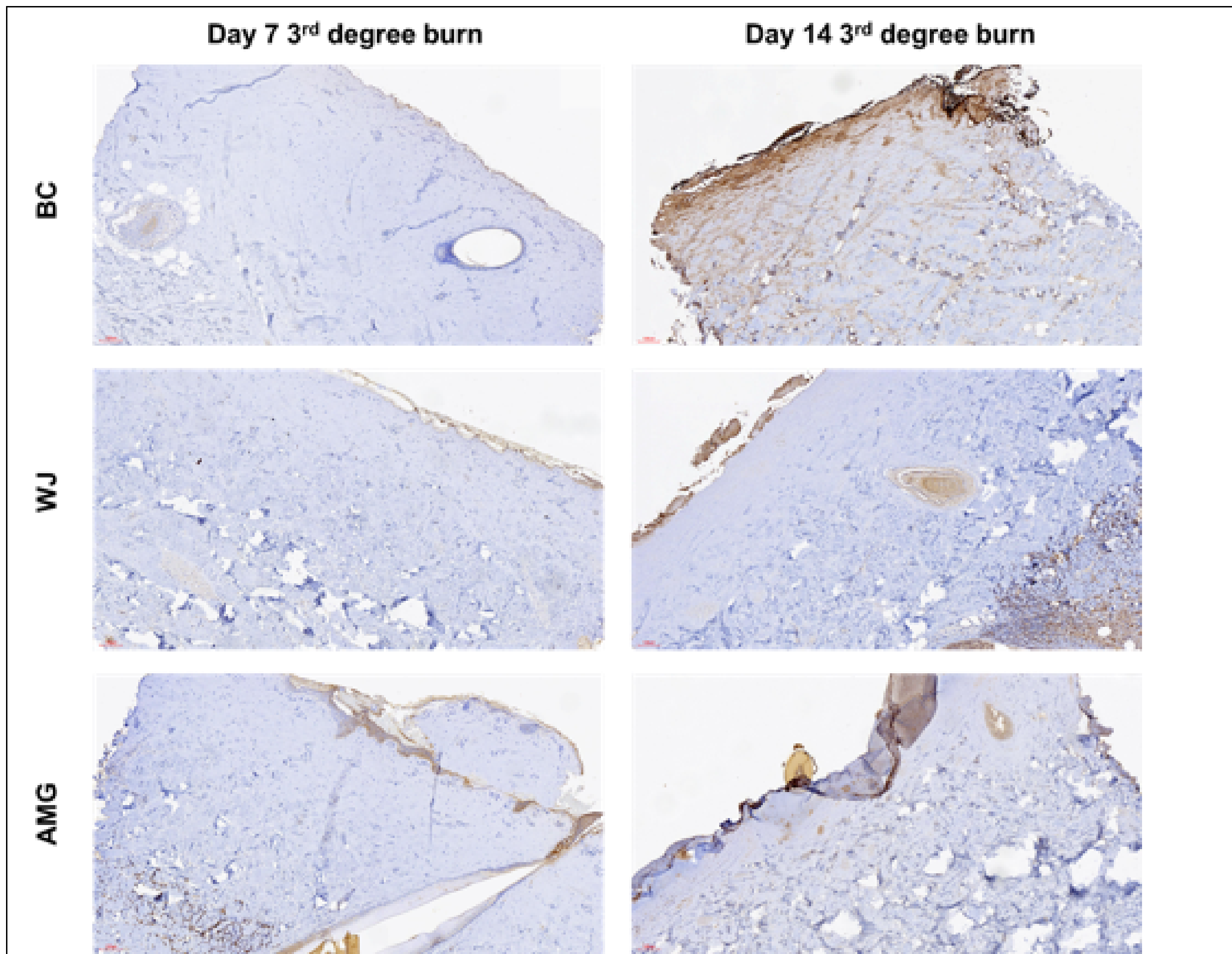


Figure 13. Results from the Ki-67 immunohistochemistry staining for 3rd degree burns on **days 7 and 14** for the biopsies obtained from the BC, WJ, and AMG samples for Pig #2. Blank control is BC, WaterJel is WJ, and antimicrobial hydrogel is AMG. There are smaller number of proliferating cells in the 3rd degree burn wounds, as expected.

In the healthy skin sample (Figure 11), the most proliferating cells are the squamous cells in the basal layer. The proliferation and arrangement of squamous cells are important indications for reepithelization after burn. In the 2nd and 3rd degree burn samples on day 0, proliferating cells were completely damaged. It is confirmed that all proliferating cells in later stages were recruited from surrounding tissues.

In the 2nd degree burn wounds (Figure 12), the lymphocytes, neutrophils, and plasma cells are clearly seen and well stained in the images from day 7 and 14. This is expected since there will be cells remaining in the dermis. However, the evidence of reepithelization is not found in BC and WJ samples. Comparing with BC and WJ, AMG samples showed the inflammation cells closer to the epidermis and showed the basal layer with similar cell arrangement and staining to the healthy tissue. Our sprayable dressing AMG was able to better protect and regenerate the 2nd degree burn wounds.

In the 3rd degree burn wounds (Figure 13), the stained inflammatory cells were far away from the epidermis and no sign of regeneration was observed. This is also expected due to the deep damage of cells that 3rd degree burn requires surgery treatment. The Ki-67 staining also confirmed the PCR results for 3rd degree burn (Figure 14).

To analyze the wound healing and evaluate the inflammation of the burn wounds, we have performed RT-qPCR on VEGF, IGF-1, TNF, IL-6, and CCL-2 genes (Figure 5). The biopsies were taken from 2nd and 3rd degree burn treated with Blank control (BC), WaterJel (WJ), and antimicrobial hydrogel (AMG) groups on day 0, 7, and 14. The biopsy samples were stored in 1 mL RNAlater solution to preserve RNA. The samples were then immersed in lysing/binding buffer from the RNAqueous kit (Invitrogen, Carlsbad, CA, USA) and were homogenized in the buffer for 1 min. The RNA was subsequently isolated according to manufacturer's protocol. RT-qPCR analysis was performed using the Verso One-Step RT-qPCR kit according to the manufacturer's protocol. The PCR instrument used was the CFX Connect Real-Time system (Bio-Rad, Hercules, CA, USA).

The primers used for RT-qPCR were as follows: VEGF (forward, 5'–GCTTCCTACAGCACAACAAATG–3'; reverse, 5'–AAATGCTTTCTCCGCTCTGA–3'), CCL2 (forward, 5'–CATAAGCCACCTGGACAAGAA–3'; reverse, 5'–AGGGCAAGTTAGAAGGAAATGA–3'), IGF1 (forward, 5'–GAGGGAGTTCAGGAAACAAGAA–3'; reverse, 5'–TAACTCGTGCAGAGCAAAGG–3'), IL-6 (forward, 5'–CGGTCTTGTGGAGTTTCAGATA–3'; reverse, 5'–CTGGATCAGTGCTTTGGTACT–3'), TNF (forward, 5'–CCTACTGCACTTCGAGGTTATC–3'; reverse, 5'–ACGGGCTTATCTGAGGTTTG–3'), and GAPDH (forward, 5'–GTGACACTCACTCTTCCACTT–3'; reverse, 5'–CCTGTTGCTGTAGCCAAATTC–3'). The ΔΔCT values were generated from the housekeeping gene GAPDH and day 0 values from 2nd or 3rd burn wounds.

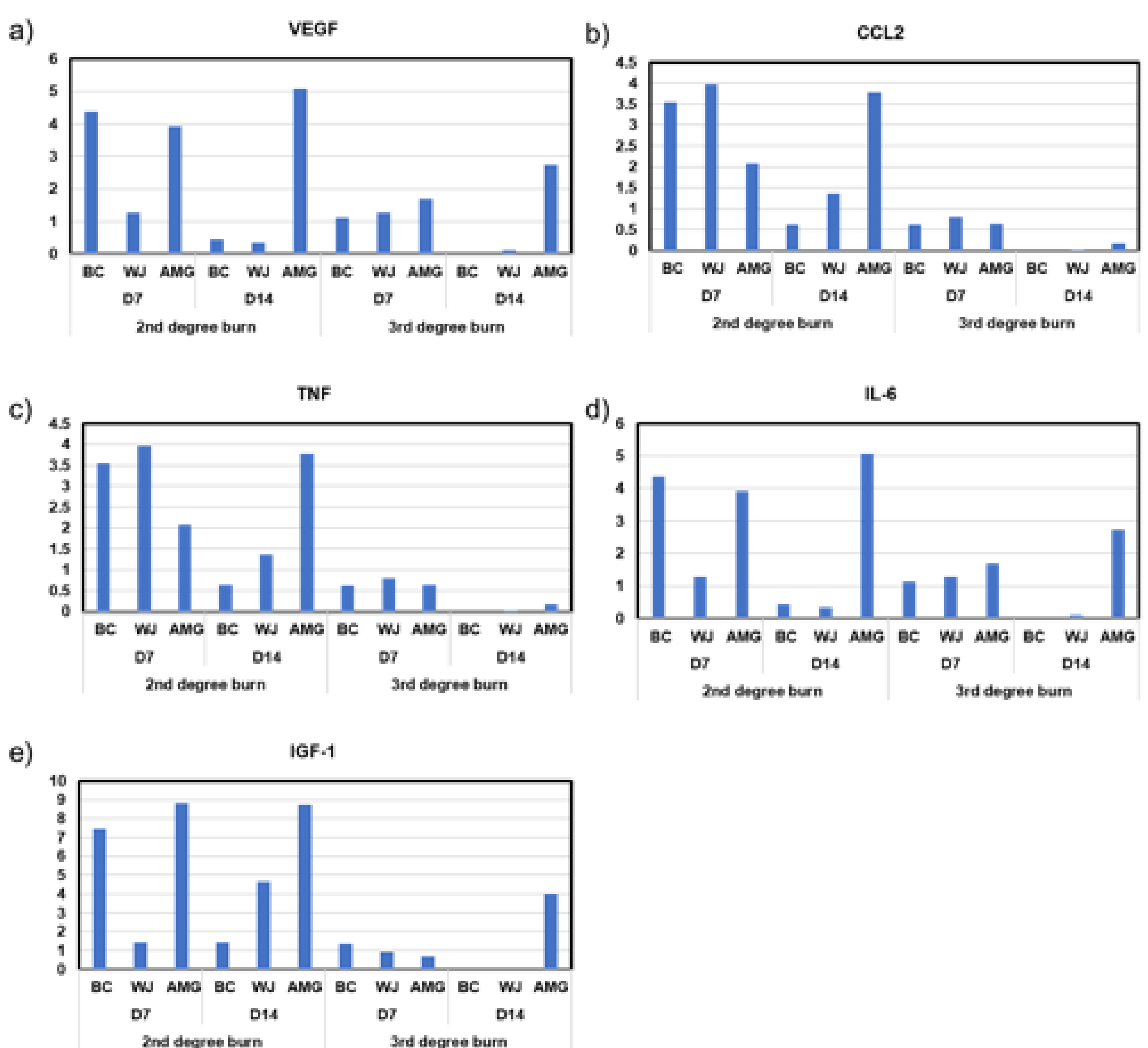


Figure 14. Gene expression profile of the wound healing markers in burn wound biopsies for Pig #2. $2^{-\Delta\Delta CT}$ represents the quantitative fold change in the plot. Gene expressions of a) VEGF, b) CCL2, c) TNF, d) IL-6 and e) IGF-1 were quantified for the 2nd degree burns on days 7 and 14, and 3rd degree burns on days 7 and 14.

On Day 7, VEGF was upregulated in the 2nd degree burn wounds, which represents the healing process from remaining endothelial cells and granulation tissue (Figure 14). CCL2, IL-6, and IGF-1 genes showed a similar expression trend compared to VEGF, representing and active wound healing for 2nd burns. CCL2, IL-6, and IGF-1 were also involved in the inflammation and stimulate the migration and proliferation of fibroblasts, keratinocytes, and endothelial cells.

Among all groups, the sprayable AMG showed the most upregulated IGF-1 expression on day 7. Our gel is able to preserve the remaining live tissue showing in histology, and therefore can create an active environment that facilitates rapid healing. On day 14, the downregulated VEGF expression in BC and WJ groups showed the damage of the tissue, and which was supported by the presence of pyocytes in histology. The higher expression of CCL2 and IGF-1 in the WJ group compared with suppression of expression in BC group indicating severe inflammation in the WJ group and inhibition of healing and damage in the BC group. On day 14, all marker genes remained at high expression levels demonstrating that the regeneration process continued from day 7 to day 14 by the help of our sprayable AMG dressing.

The gene expressions for 3rd burn wounds from BC and WJ groups were mostly down regulated on days 7 and 14. This is expected due to the complete damage of the epidermic and dermic layers. Our wound dressing was designed to protect the 3rd degree wound from deterioration before tissue graft surgery. Intriguingly, the IL-6 and IGF-1 expression from the AMG group were upregulated on day 14 that may be due to the inflammatory cells migrating from the subcutaneous tissue, according to the histology findings. The AMG may contribute to a delayed regeneration without causing a severe inflammation and abscess.

Based on our promising data, we continued characterizing the *in vivo* response to our hydrogels with Pig #3. With this experiment, we are studying the effects of our AMG dressings for subcutaneous tissue and infected wound.

Table 2. Experimental conditions for Pig#3 with bacterial infection conditions.

2nd degree burn	Blank	Blank	Blank	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)
3rd degree burn	Blank	Blank	Blank	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)	15 mg/mL CaO_2 (AMG)

In Pig#3, the formula of the hydrogel is 10% GelMA, 1% hyaluronic acid, 1 mg/ml catalase, 15 mg/ml CaO_2 in PBS. A 100 microL of bacteria solution was applied on each wound 5 min after inducing the burn in the wounds. The polymer solutions were crosslinked with UV for 150s. AMGs were reapplied on day 7. We have performed H&E and Masson Trichrome stainings using the biopsies obtained from blank control (BC) and AMG on day 0, 7, and 14. We will continue our analysis with the Chloroacetate Esterase and KI-67 stains in the next reporting period.

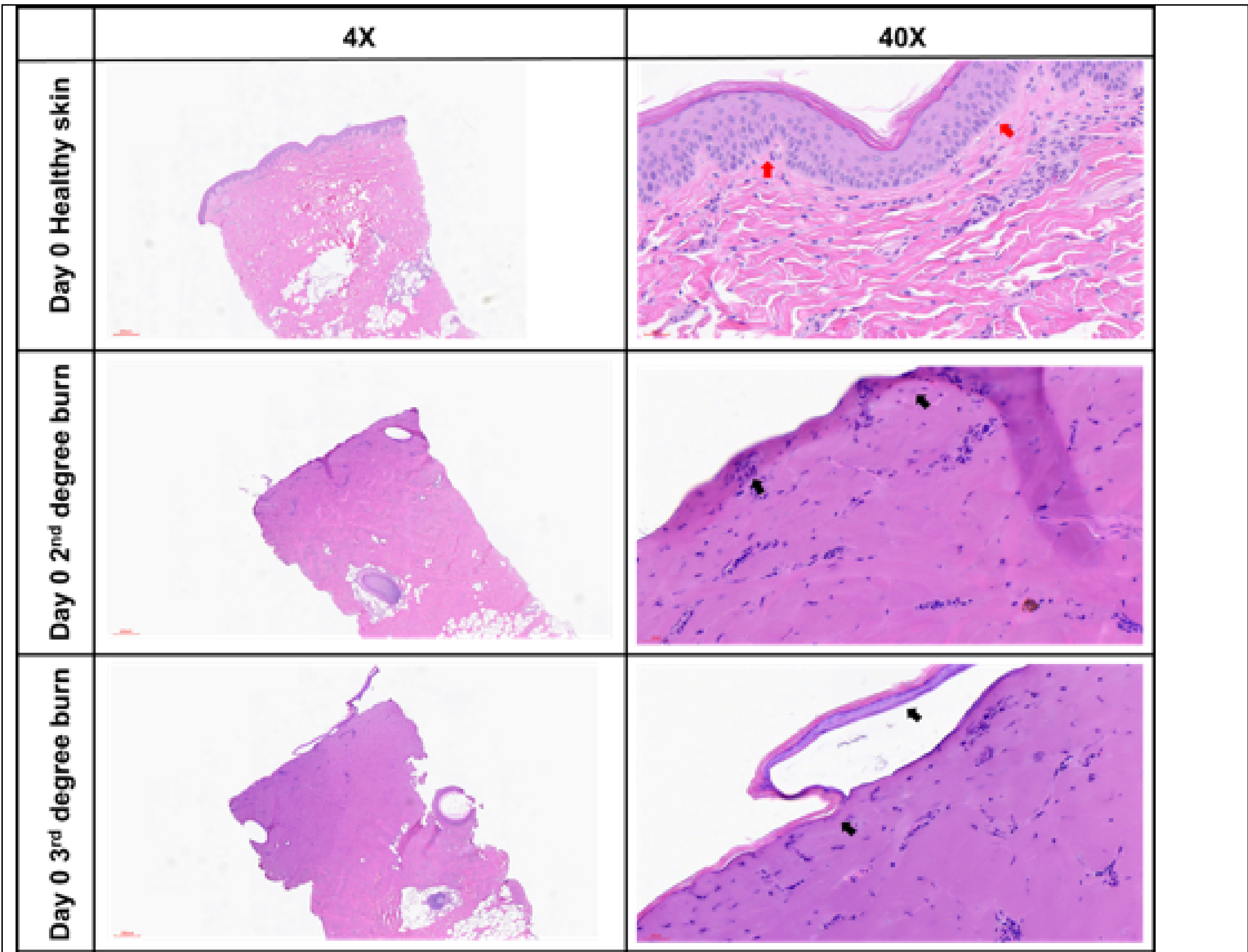


Figure 15. H&E staining of healthy skin, 2nd, and 3rd degree burn samples on day 0 for Pig#3. The squamous cells in basal layer have been observed (red arrows) in healthy skin sample. The signs of burn wound (exudation, deformation, and blister) can be seen in both 2nd and 3rd degree burn wounds (black arrows).

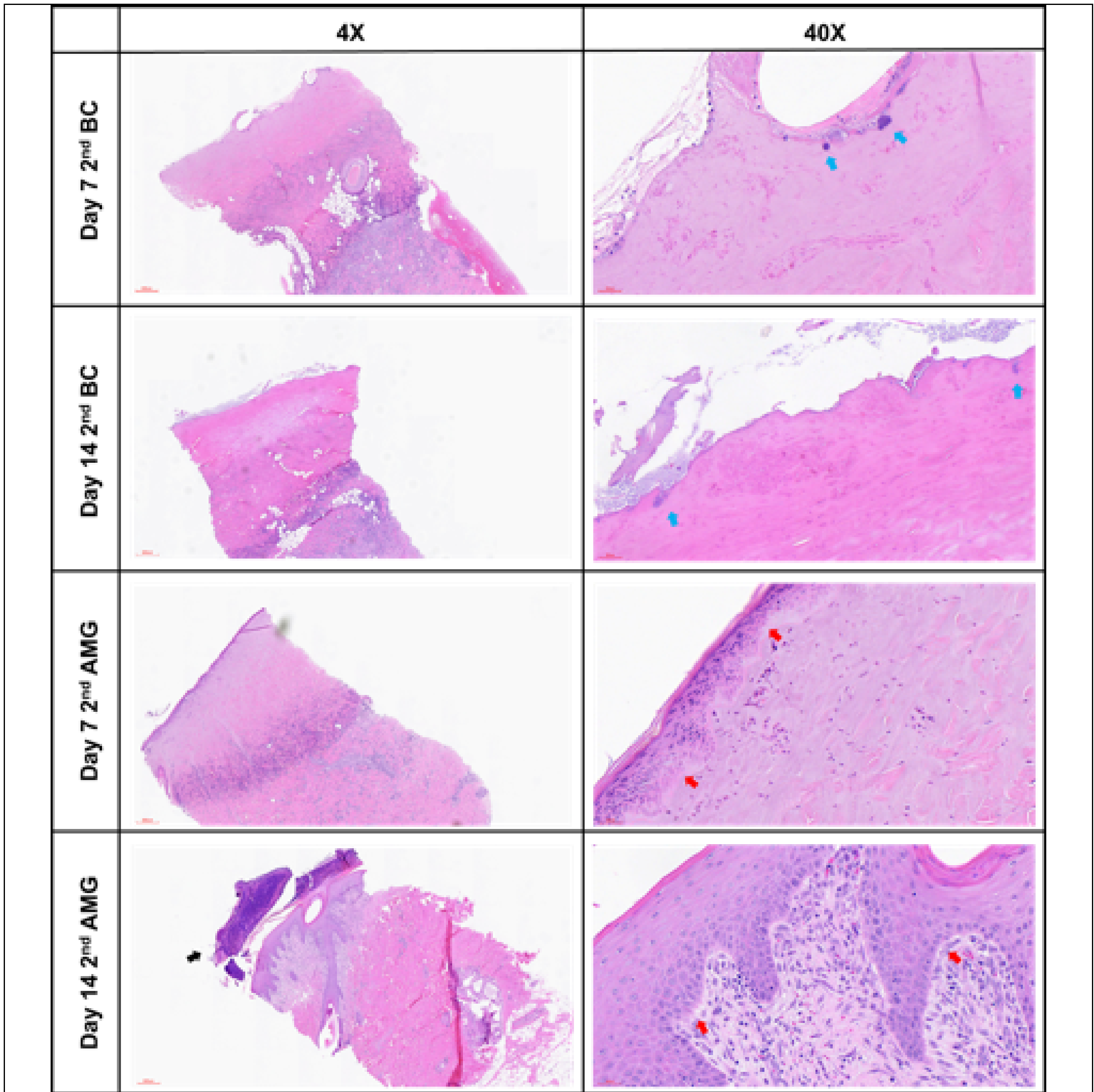


Figure 16. H&E staining of 2nd degree burn samples on days 7 and 14 for Pig#3. The bacterial colonies has been found in BC samples on both day 7 and 14 (blue arrows). The squamous cells in basal layer have been observed (red arrows) in AMG samples on both day 7 and 14. The damaged scab (black arrow) was shown in AMG sample on day 14 and reepithelization occurred underneath.

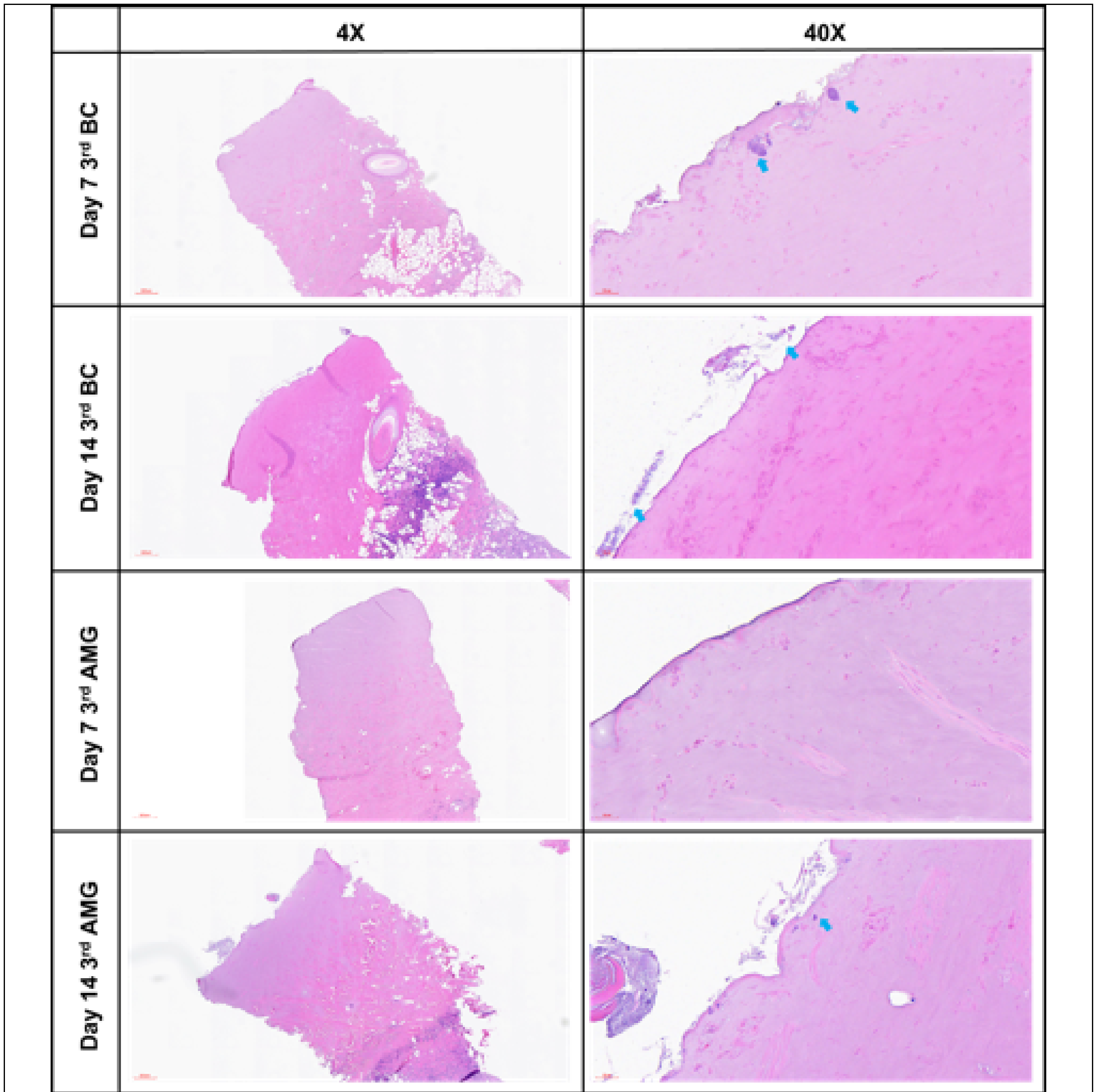


Figure 17. H&E staining of 3rd degree burn samples on days 7 and 14 for Pig#3. No sign of reepithelization and repair can be seen in 3rd degree burns. The bacterial colonies have been found in BC samples on both day 7 and 14, and in AMG samples only on day 14 (blue arrows).

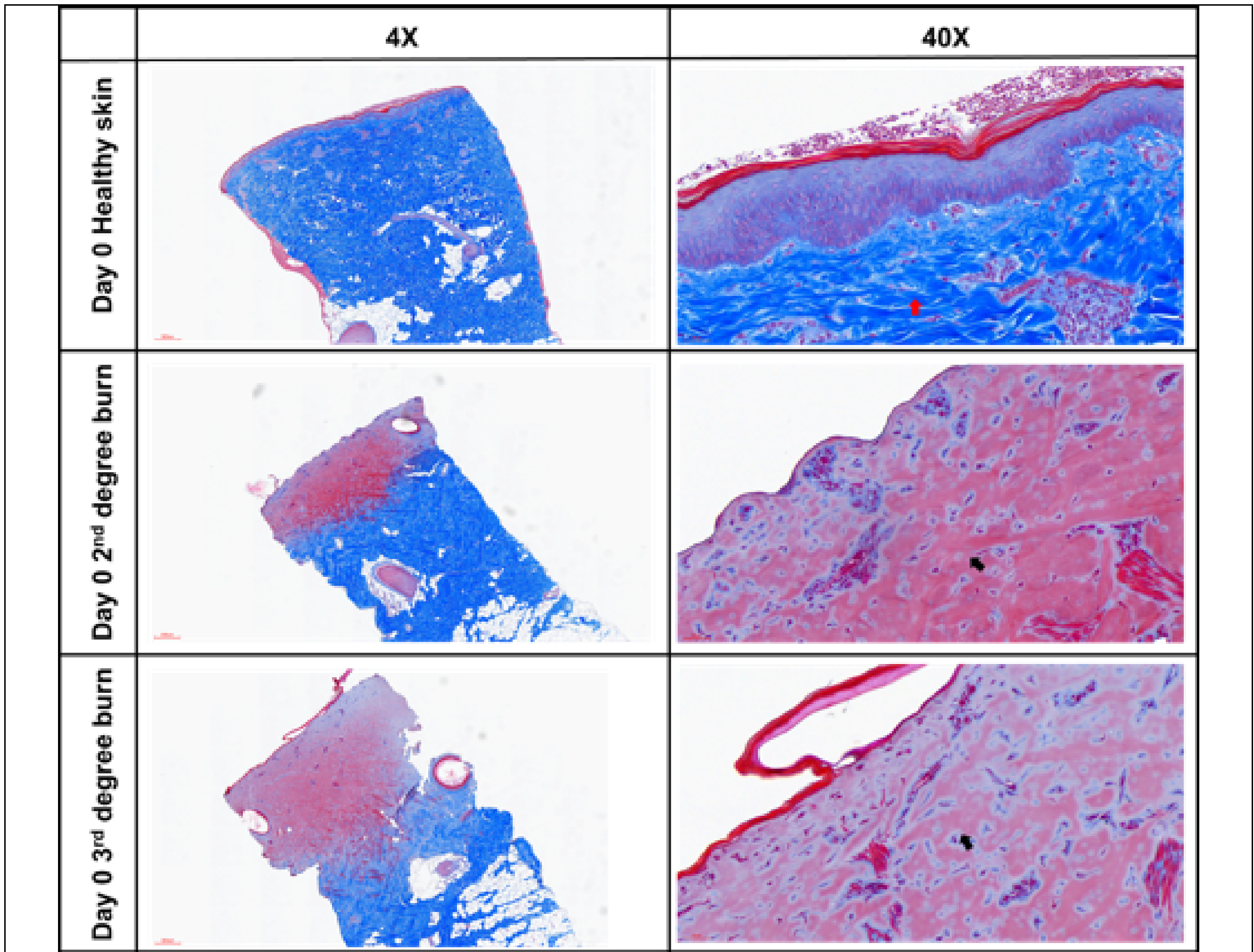


Figure 18. Masson trichrome staining for the healthy skin, 2nd, and 3rd degree burn samples on day 0 for Pig#3. The healthy (red arrow) and damaged (black arrows) collagen fibers were observed in healthy skin and burn wounds, respectively. The depth of burn damage was greater for 3rd degree burn wounds compared with 2nd degree burn wounds.

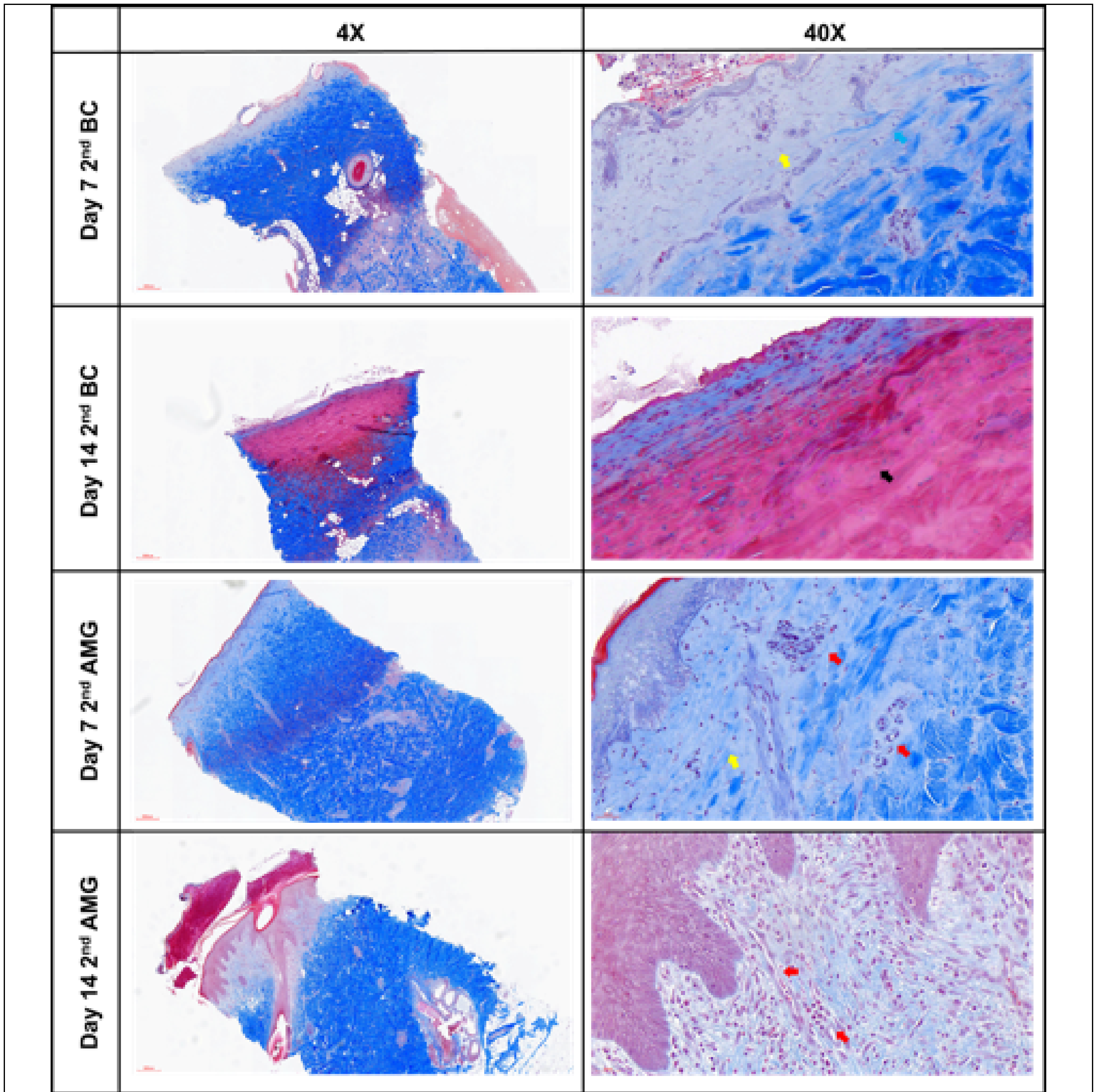


Figure 19. Masson trichrome staining of 2nd degree burn samples on days 7 and 14 for Pig#3. The hydropic degeneration (edema) showed in BC and AMG samples on day 7 (yellow arrows). The edema was absorbed on day 14 and the damaged collagen fibers showed again in BC samples (black arrow). Vascular smooth muscle cells were present in the AMG samples (red stained) on days 7 and 14 (red arrows) demonstrating vascularization.

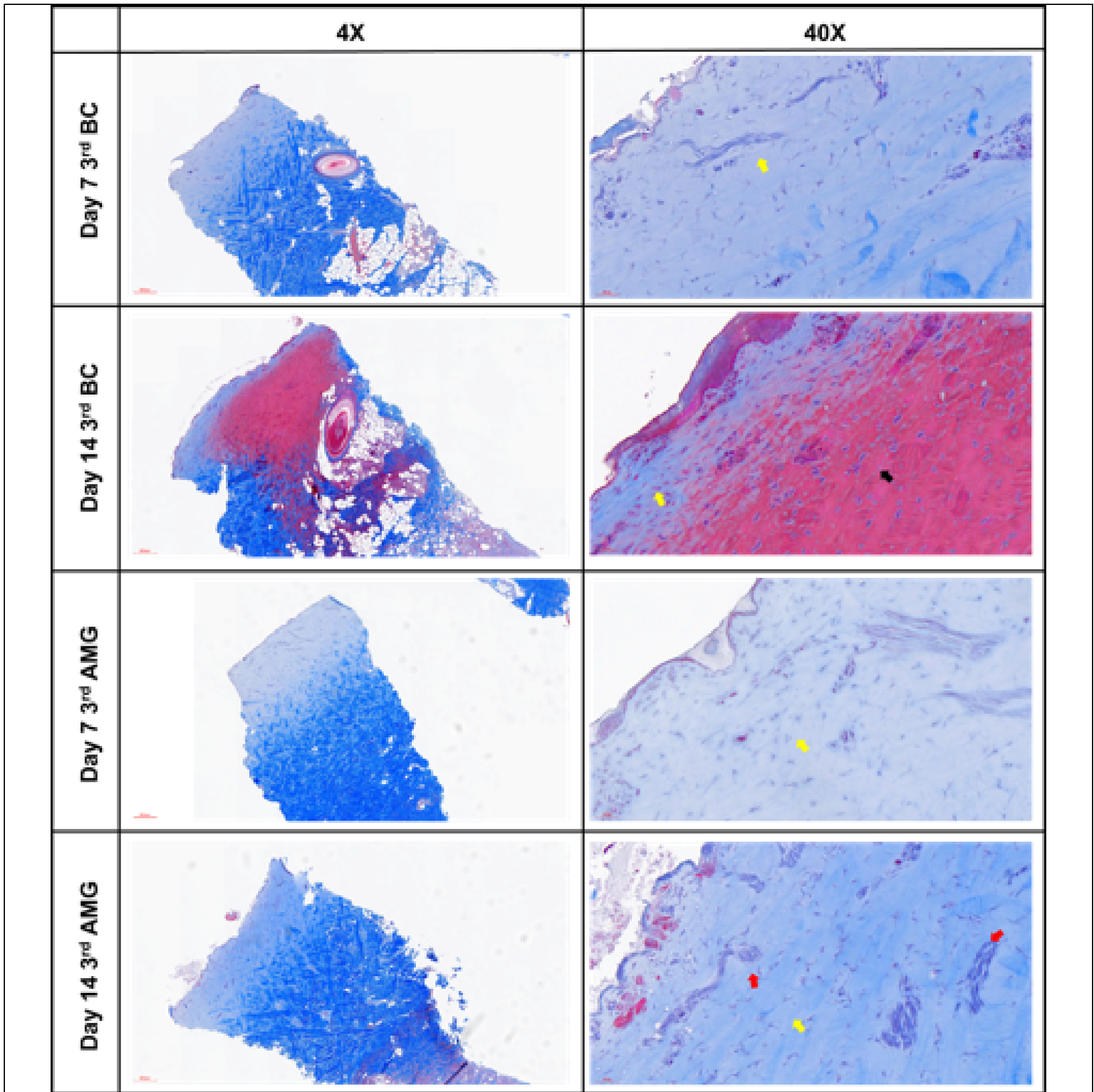


Figure 20. Masson trichrome staining of 3rd degree burn samples on days 7 and 14 for Pig#3. The hydropic degeneration (edema) showed in all samples and all time points (yellow arrows). The edema was absorbed on day 14 and the damaged collagen fibers showed again in BC samples (black arrow). The red stained vascular smooth muscle cells were observed in AMG samples on day 14 (red arrows), which shows vascularization.

Key Findings or Accomplishments:

- The composite sprayable hydrogels were used in the Pig#2 and Pig#3 experiments.
- The biocompatibility and efficiency of the anti-inflammatory composite hydrogel was tested in Pig#2 and Pig#3.
- Biopsies were collected on days 0, 7, and 14 for histology analysis, cell proliferation, and PCR for Pig#2 and Pig#3.
- No infection occurred in any of the experimental conditions for Pig#2.
- Infected wounds were studied in Pig#3. We will continue analyzing our data in the next reporting period.

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

This project has provided opportunities for post-doc and graduate students to carry out technical literature search and hand on research experience on developing wound dressings. The researchers and students have learned about *in vitro* and *in vivo* strategies for the design and fabrication of wound dressing for treatment of 2nd and 3rd degree burns. The project also provided the PI of the project (Dr. Camci-Unal) student mentoring and student training opportunities. She had a chance to mentor the graduate students, interact with them one-on-one, and provide them scientific training, which helped with professional development.

How were the results disseminated to communities of interest?

Nothing to report in this period.

Plans for the next reporting period to accomplish the goals

In the next reporting period, we will continue analyzing our data from the Pig#3. We will carry out (H&E), evaluate cell proliferation to study wound progression in the Pig#3. We are also going to continue characterizing our composite wound dressings using the biopsied samples, analyze presence of bacteria due to introduction of infection in the wounds, perform gene expression experiments via PCR. We will also use a high resolution scanning electron microscope (SEM) to evaluate our wound dressings and the presence of bacteria.

We will also synthesize GelMA and HAMA components of the sprayable wound dressing. We will improve our *in vitro* cell cultures by making changes to the system where the 3D encapsulated human dermal fibroblasts get exposure to the wound dressing similar to the in vivo setting. We will optimize the cell cultures in a hypoxia chamber to see the effects of antimicrobial component in the composite dressings. To achieve this goal, we will perform *in vitro* experiments by using primary human dermal fibroblasts (HDFs) in our composite wound dressing formulations. We will also assess the *in vitro* degradation behavior of the composite hydrogels.

4. IMPACT

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

We expect that our project will accelerate progress in medical research field for military members after traumatic combat-related burn injuries. In addition to burn wounds, we anticipate that our composite sprayable dressing can also be useful for healing of other wounds that are commonly seen in the war zones.

What was the impact on other disciplines?

In addition to pre-treatment of burn wounds, our sprayable dressing is expected to be useful in the future in plastic surgery, dermatology, aesthetics, and chronical wounds in patients from military as well as general public.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Our research provides a solution for the existing problem of lack of efficient methods to treat second and third degree deep tissue burns by non-burn specialists in the war zones. Both medical and non-medical first responders can easily spray our hydrogel dressing on the burned tissue to provide full protection immediately. The application of the sprayable dressing is conveniently simple and does not require highly trained personnel to administer. Our long-term vision includes providing successful treatment for deep burn wounds for the Service members, Veterans, and those within the general public. We anticipate that our findings will contribute to the society by improving the public knowledge about burn wound healing by the end of the project.

5. CHANGES/PROBLEMS

IMPORTANT REMINDER – Award recipient organization is required to obtain prior written approval from the awarding agency Contracting/Grants Officer whenever there are significant changes in the project or its direction such as significant change in scope or the Statement of Work (e.g. removal, change, or addition of aims/tasks or animal model change), change in PI or key personnel, reduction of 25% FTE, or significant change in budget.

Changes in approach and reasons for change

Nothing to Report.

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

The IACUC protocol has expired on 08/23/2023. Our collaborator at the UMass Medical School has applied to renew the IACUC protocol. The ACURO approval will be renewed once the IACUC is renewed. We will not perform new animal experiments until the IACUC protocol and ACURO approvals are renewed.
We do not anticipate problems with animal protocol renewal.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects

No human use research is involved.

Significant changes in use or care of vertebrate animals

TOTAL PROTOCOL(S): 1

PROTOCOL (X of Y total):
IACUC Protocol Number: PROTO202000052
ACURO Protocol Number: MB190127.e001
Protocol PI: Raymond Dunn, MD
Protocol Site: University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655
Protocol Title: Pig Sprayable Wound Dressing Study
Number of Animals Approved for Use: 10, Yorkshire pigs
IACUC INITIAL APPROVAL DATE: 08/24/2020 (expires 08/23/2023)
The IACUC protocol has expired on 08/23/2023. Our collaborator at the UMass Medical School has applied to renew the IACUC protocol. We will not perform new animal experiments until the IACUC protocol and ACURO approvals are renewed.
ACURO INITIAL APPROVAL DATE: 10/15/2020
The ACURO approval will be renewed once the IACUC is renewed. We will not perform new animal experiments until the IACUC protocol and ACURO approvals are renewed.
RENEWAL APPROVAL DATES:
- Due 08/24/2021

AMENDMENTS:
- None.

ADVERSE EVENTS OR UNANTICIPATED PROBLEMS:
- None.

Significant changes in use of biohazards and/or select agents

No biohazard or select agent research is involved.

6. PRODUCTS

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: Gulden Camci-Unal
Project Role: Principal Investigator of the project (PI)
Researcher Identifier: 0000-0003-4258-844X
Nearest person month worked: 1 summer month per year is the official effort of the PI. She has been actively working on the project during each academic month.
Contribution to Project: Dr. Camci-Unal is responsible for the overall coordination and supervision of all aspects of the study including planning, design and implementation of the project as well as being responsible for training and supervision of the graduate students, and ensuring the quality of data collection and management over time. Dr. Camci-Unal has closely worked with the graduate student Ms. Ilayda Firlar and Dr. Xinchen Wu in the experiments and data evaluation.

Name: Mine Altunbek
Project Role: Post-doctoral researcher
Researcher Identifier: N/A
Nearest person month worked: 4.5
Contribution to Project: The primary responsibilities of the post-doc Dr. Mine Altunbek was to help synthesizing and characterizing polymers, perform *in vitro* experiments, and help with evaluating *in vivo* data. She read literature examples, analyzed them, had regular discussions with Dr. Camci-Unal, and prepared data for the progress report.

Name: Xinchen Wu
Project Role: Graduate Student
Researcher Identifier: N/A
Nearest person month worked: 0
Contribution to Project: The primary responsibilities of the graduate student Dr. Xinchen Wu was to help with *in vivo* data analysis and evaluation. He analyzed related literature, had regular discussions with Dr. Camci-Unal, and prepared data for the progress reports.

Name: Mert Gezek
Project Role: Graduate Student
Researcher Identifier: N/A
Nearest person month worked: 0
Contribution to Project: The primary responsibilities of the graduate student Mr. Mert Gezek was to help with *in vivo* data analysis and evaluation. He analyzed experiments, had regular discussions with Dr. Camci-Unal, and prepared data for the progress report.

COLLABORATING ORGANIZATION: University of Massachusetts Medical School

Name of the PI of the collaborating organization: Raymond Dunn
Project Role: Sub-contract PI
Researcher Identifier:
Nearest person month worked: 0.1
Contribution to Project: Dr. Raymond Dunn is responsible for the coordination and supervision of the pig study in Aim 3 of this project including planning, design and implementation of the animal experiments in collaboration with Dr. Camci-Unal, training and supervision of the residents and medical students, and ensuring the quality of data collection and management.

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

There has been a change in the active other support of the PI of the project, Dr. Gulden Camci-Unal. She received a new grant. The change in Dr. Camci-Unal's active other support does not impact the effort on this project. Currently, there is no overlap with person efforts or scientific content in the active support.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

QUAD CHART

9. APPENDICES

A Sprayable Antimicrobial Wound Dressing for Burn Treatment in the Battlefield

Proposal # MB190127, Award # W81XWH-20-1-0521

PI: Gulden Camci-Unal, PhD **Org:** University of Massachusetts, Lowell **Award Amount:** $500,000

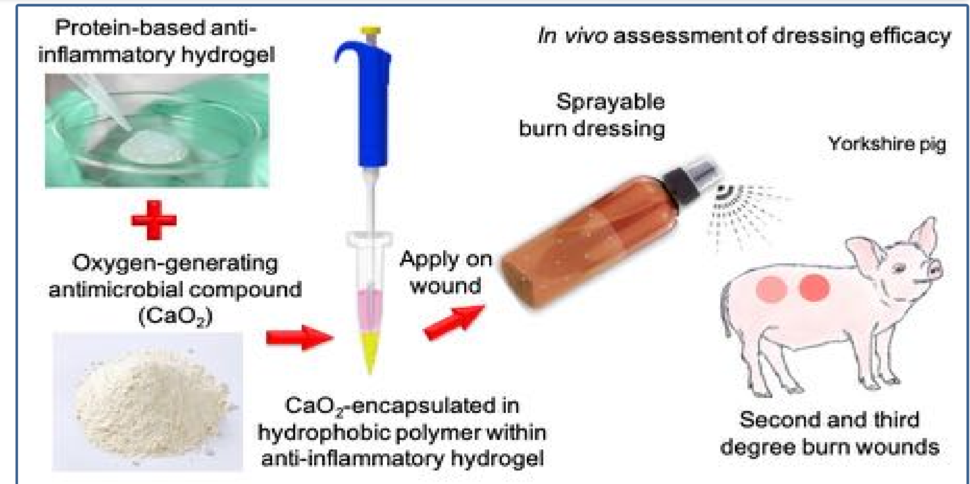
Study/Product Aim(s)

Develop a sprayable antimicrobial hydrogel-based wound dressing for burn.

- Aim 1: Synthesize a sprayable antimicrobial hydrogel-based wound dressing and characterize the material properties and antimicrobial efficiency (months 1-42)
- Aim 2: Evaluate the cytocompatibility of the sprayable hydrogel dressings (months 6-45)
- Aim 3: Evaluate the effectiveness of the sprayable wound dressing in vivo (months 9-48)

Approach

We are developing a sprayable, antimicrobial, and anti-inflammatory hydrogel wound dressing for rapid pre-treatment in austere combat zones for second and third degree burns until an appropriate care unit can surgically treat the patient. Our product will easily be applied to the wound by non-medical or medical first responders. The outcomes of the proposed research will provide burn solutions closer to the point of injury for wounded Service members and Veterans.



Accomplishment: We synthesized different components of the composite hydrogel dressings, performed material characterization, *in vitro* experiments as well as *in vivo* experiments for pig#1, pig#2, and pig#3.

Timeline and Cost

Activities CY	20	21	22	23	24 – NCE
Aim 1-Synthesize hydrogel dressing formulation					
Aim 2-Assess biocompatibility					
Aim 3-Evaluate dressing efficacy in a pig model					
Estimated Budget ($500K)	**$10K**	**$170K**	**$80K**	**$120K**	**$120K**

Updated: September 2023

Goals/Milestones

CY20-21 and CY23-24 Goal – Synthesize and characterize sprayable antimicrobial hydrogel-based wound dressing

☐**X** Complete synthesizing each component of the dressing formulation. Synthesize as needed during the project.

☐**X** Obtain IACUC/ACURO approvals for the pig burn model

☐ Complete first iteration of the dressing formulation and characterize it

☐ **X** Demonstrate sustained oxygen delivery for up to 2 weeks-in progress

☐ **X** Conduct antimicrobial activity tests-in progress

CY21-22 and CY23-24 Goal – Evaluate cell responses to sprayable dressing

☐ **X** Conduct growth, cytotoxicity, and functionality assays-in progress

☐ Conduct degradation tests using primary human dermal fibroblasts

CY22-23 and CY23-24 Goal – Determine efficacy of sprayable dressing

☐ **X** Conduct pig burn model-in progress

☐ Demonstrate biocompatibility of dressing, efficiency, and optimize dressing in pig burn model with second and third degree burns

Comments/Challenges/Issues/Concerns

- We received a 1-year No Cost Extension (NCE).
- We will renew the IACUC and ACURO for one more year. We will not carry out a new animal experiment until the IACUC and ACURO renewals are approved.

Budget Expenditure to Date

Projected Expenditure: $370K

Actual Expenditure: $341K