Validation of a Novel Rodent Model to Test Anti-scarring Therapeutics

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Abstract: Scar contracture is a debilitating disease that affects many people worldwide. There are currently no effective preventative medical treatments. A pivotal step to attaining the goal of developing a treatment is the testing of anti-scarring agents in preclinical hierarchical animal models of human scarring. Methods. A 2-cm x 2-cm, full-thickness, excisional wound was created in the rats’ mid-scapular area. Three experiments were performed. The first experiment determined the optimal dressing in wound contraction. The second experiment developed upon the results of the first experiment, and determined how anatomic site of osmotic pump implantation affected wound healing. The third experiment determined how the size of osmotic pump affected wound healing. Wound healing parameters including rate of wound contraction, systemic and local toxicity, proliferation, collagen architecture, and collagen production were assessed. Results. The results of the present study showed that covering the wound with Tegaderm™ (3M Health Care, St. Paul, MN) alone had the most linear wound contraction rate with the smallest standard error of the mean. Implantation of all osmotic pump sizes, when implanted intraperitoneally, was tolerated and did not interfere with wound healing. In contrast, subcutaneous implanted pumps caused significant discomfort in the rats. Conclusion. Implantation of an osmotic pump intraperitoneal in the rat excisional wound model, where the wound is covered with Tegaderm, provides for a reproducible, accurate, preclinical animal model to study anti-scar contracture treatments.

There are more than 2.4 million American burn victims and more than 40 million scar patients worldwide annually. The costs of scarring exceed $12 billion annually. The incidence of large joint scar contractures in major burns is approximately 40%. Currently, an effective, preventative anti-scar contracture treatment does not exist. There is an unmet need to develop an anti-scarring therapy. A pivotal step to attaining this goal is the testing of anti-scarring agents in preclinical hierarchical animal models of scarring. In this investigation, a novel rodent model of wound healing that can be used for future wound healing investigations was validated.

It is important to use animal models that closely mimic the human condi-
The rodent is the most commonly used animal for initial preclinical animal testing. The reason for this is because scarring in the rodent shares many similarities to humans, the animals are easy to handle and house, are inexpensive, are readily available, and can be genetically modified. The rodent enables investigators to determine mechanism of action, demonstrate proof of concept, elucidate pharmacodynamic profiles, and clarify pharmacokinetic drug interactions. There are limitations to the rodent model and there are also other animal models that share many general characteristics with humans in skin anatomy and scarring formation, and thus are commonly used, like the red Duroc pig and the skin grafted pig model, but their size and difficulty in handling, the cost of housing, and the lack of genetic flexibility compared to the rat model makes rodent models more favorable for scar contracture studies.

There are several different approaches to causing cutaneous scarring in rodents, which recapitulate the clinical scenario in humans, but the excisional wound model is considered the best and most widely used method to study scar contracture. This is because the cells that cause wound contraction (fibroblasts and myofibroblasts) are the same cells that cause scar contracture. In the rodent wound contraction model, a segment of dorsal parascapular dermis is excised, the wound is dressed, an anti-scarring agent is administered to the rodent, and the wound is allowed to contract closed over 2 to 3 weeks. An accurate animal model with minimal variability that serves in preclinical testing should also elucidate which topical wound dressing is the best to use for rodent studies. The reason for this is because wound dressings variably affect wound closure. Wound dressings protect the wound from infectious agents and maintain a moist healing environment. Dressings that have been widely used in animal models are Tegaderm (3M Health Care, St. Paul, MN), a semicocclusive transparent dressing, Algisite (Smith & Nephew, St. Petersburg, FL), and Allevyn (Smith & Nephew), both which are absorptive dressings. These three dressing types are the most commonly used in rodent studies because they are also widely used in treating human patients.

In animal studies, drugs can be administered per oral, topical, via subcutaneous (sc) or intraperitoneal (ip) injection, or intravenously. Another method of dosing that has recently become widely used is the Alzet® Osmotic Pump (Durect Corp, Cupertino, CA). Osmotic pumps are advantageous because they allow for continuous, sustained delivery of known duration and flow rates without the need for repetitive intervention. Osmotic pumps can be implanted into rats in either the sc space (flank, back, or abdomen) or ip. Osmotic pumps have been used in multidisciplinary research. Osmotic Pumps have been used in cardiovascular research, cancer research, and neuroscience, among the other fields of contemporary research. It is unknown whether the intervention associated with pump implantation affects wound contraction and general animal welfare, as this would be assessed by weight changes, abnormal rodent behavior like irritability, abnormal respiratory rate, biting and scratching of the implant site, and macroscopic appearance of the wound. Stress is a known retardant of wound healing. Previous studies have demonstrated that stress activates the hypothalamic-pituitary-adrenal axis and corticosterone release. The anti-inflammatory and immunosuppressive effects of corticosterone impair inflammation and wound healing kinetics.

The purpose of the study is to determine how dressing, osmotic pump size, and implantation location affects wound healing. Full-thickness excisional wounds were created on the rat dorsum and wound dressings were applied (Tegaderm, Algisite, and Allevyn). Alzet Osmotic Pumps (small, medium, and large) were implanted sc in the flank or ip and wound healing parameters including rate of wound contraction (wound size), animal welfare (grooming, irritability, and quality of the wound), proliferation (Ki67 IHC), collagen architecture, and collagen production (Masson’s trichrome staining) were assessed. Local and systemic toxicity were analyzed. The outcomes from this study have validated a novel preclinical animal model to study and develop anti-scarring therapeutics.

**Methods**

**Osmotic pumps.** Alzet Osmotic Pumps are miniature, infusion pumps for the continuous dosing or labora-
Pumps that deliver the solution for 4 weeks were used. The three sizes studied were the small sized 1004 model (1.5 cm length, 0.5 mL volume, 0.11 µL/hr flow rate, 4 weeks delivery duration), the medium sized 2004 model (3 cm length, 1 mL volume, 0.25 µL/hr flow rate, 4 weeks delivery duration), and the large sized 2ML4 model (5.1 cm length, 6.5 mL volume, 2.5 µL/hr flow rate, 4 weeks delivery duration).

Rodent model of wound healing. This experimental protocol was approved by the Institutional Animal Care and Use Committee of Duke University School of Medicine. The 10- to 12-week-old (200 g–225 g) Wistar Han female rats were anesthetized with inhaled 2% isoflurane and their dorsal surface shaved followed by a depilatory agent. The 10- to 12-week-old Wistar Han female rat was chosen because rapid animal growth can counteract wound contraction and this strain of rat has slow growth kinetics. An excisional wound (2 cm x 2 cm) was created on the rat dorsum between the scapular angles (Figure 1A). The full-thickness skin segment was resected up to but not through the panniculus carnosus. The panniculus carnosus was not excised because in preliminary studies resection of the panniculus carnosus led to large variability in healing rates. The wound was covered with Tegaderm (Figure 1B) secured with medical adhesive spray for 4 days, then left uncovered. Each osmotic pump was filled with saline and implanted either subcutaneously into the flank (Figure 1C) or intraperitoneally through a distinct incision separate from the wound (Figure 1D). The flank incision was closed with interrupted 3-0 nylon sutures. In the ip group, the peritoneum was closed with 4-0 absorbable sutures and the abdominal cavity with 3-0 nylon sutures. This procedure was followed for all the rats involved in the study. There were a total of three experiments with n = 15 rats per group for the first two and n = 20 for the last experiment.

**Experimental Design**

Experiment 1 was designed to determine the optimal wound dressing that led to the least variability in wound contraction. Three groups of rats (n = 5 per group {a, b, and c}) were studied and wounds were covered with (a)
Tegaderm alone, (b) Algisite and Tegaderm, and (c) Allevyn and Tegaderm. The three dressings were chosen for comparison because they are commonly used in the clinical practice to treat open wounds.

Experiment 2 developed on the data obtained from experiment 1 and then determined how anatomic site of pump implantation affected healing. Three groups of rats (n = 5 per group; for each of groups a, b, and c) were studied: (a) control animals, no pumps implanted, (b) sc pump implantation, and (c) ip pump implantation. The pumps were implanted in a distinctly different site of the body through separate incisions than where the wounds were created.

Experiment 3 built upon the data from experiment 2 to further determine how pump size affected healing. Four groups (n = 5 per group [a, b, c, and d]) of rats were studied: a) control group, no pumps implanted; b) small pumps implanted ip; c) medium pumps implanted ip; d) large pumps implanted ip.

Wound Analysis and Assessment

Starting on day 0 and then every other day, wound size was measured by gravitational planimetry. The wound margins were traced onto acetate paper, cut out, and weighed. Wound area was calculated as a percent of original and remaining wound size: \[
\text{Remaining} = \left(\frac{\text{remaining}}{\text{original}}\right) \times 100
\]

A wound was considered completely closed when the wound area was close to or equal to zero. Animals were weighed and assessed for signs of discomfort, including grooming, irritability, and healthy appearance of the wound, as described in Table 1. Pictures of the wounds were taken to assess gross appearance and normal healing. Dressings were reapplied after measurements and observations were completed.

Histology. On the last day of the experiment, the animals were euthanized using pentobarbital (250 mg/kg intraperitoneal injection). The wounds were excised, including a 5-mm margin of normal skin around the edges of the wound, and fixed in 10% formalin. The samples underwent histological processing for hematoxylin and eosin (H&E) and Goldner’s modification of Masson’s trichrome stain.

Immunohistochemistry. Formalin fixed, paraffin-embedded tissue blocks were sectioned at 5 µm–10 µm. Appropriate positive and negative controls were performed for each antigen assayed. Slides were counterstained with hematoxylin. The sections were incubated with: rabbit anti-Ki-67 (1:200 dilution, monoclonal, RM-9106-S, Thermo Scientific, Fremont, CA), overnight at 4˚C. Tissue staining was visualized using the avidin biotinylated enzyme complex system (Vectastain Elite ABC, Vector, Burlingame, CA) and 3,3’-diaminobenzidine substrate chromogen solution (Dako, Carpinteria, CA). The number of Ki-67 positive nuclei were counted per 40x power field.

Statistical Analysis

Results are expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using ANOVA. A value of \( P < 0.05 \) was considered statistically significant.

Results

Experiment 1: Effects of Wound Dressing on Closure. To evaluate how wound dressing would affect wound healing, three different wound dressings were used: (a) Tegaderm alone, (b) Algisite and Tegaderm, and (c) Allevyn and Tegaderm. Wound size was measured by gravitational planimetry and results are shown in Figure 2A. There was statistically significant difference among the three groups \( (P < 0.05) \) on days 2, 3, 4, 6, 7, 9, 11, and 16. The group of rats that had their wounds covered

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Table 1. Summary of observer assessment for the duration of the experiments.
with Tegaderm alone had a more linear contraction rate, after the first two days of a rapid contraction. Moreover, the same group also displayed smaller SEM, rendering the results from this group the more reproducible with a smaller inter-animal variability. Additionally, wounds covered with Tegaderm alone were closed 3 days earlier (18 days vs. 21 days in the other two groups). All the rats in experiment 1 gained weight throughout the experiment duration. There were no wound infections or other signs of distress as assessed by observing rat behavior.

**Experiment 2: Effects of Pump Location on Wound Closure.** To evaluate how pump location would affect wound healing, rats were divided into three groups. Group (a) controls with no pumps implanted, Group (b) osmotic pumps filled with saline implanted sc in the rat’s flanks, Group (c) osmotic pumps filled with saline implanted ip. Wounds were closed by day 16. The wound contraction rates were similar between the controls and the two groups with the Alzet Osmotic Pumps implanted. \((P > 0.05; \text{Figure 3A})\). Furthermore, all the rats in the experiment gained weight throughout the duration of study. (Figure 3B).

In the group with the pump implanted sc, one rat died of infection at the implantation site and the others developed wound dehiscence or skin necrosis either due to pressure at the implantation site or the rats’ attempts to remove the pump by biting the skin overlying the pump. On the contrary, the rats tolerated the ip implantation.

**Experiment 3: Effects of Osmotic Pump Size on Wound Closure.** All wounds were covered with Tegaderm only, as it was determined as the most reliable dressing type by experiment 1. Group (a) served as controls and no pumps were implanted. The remaining groups had the osmotic pumps implanted ip, as it was determined as the most reliable site of implantation from experiment 2. Group (b) had the small osmotic pumps (1004 model)

![Figure 2](image)

**Figure 2.** Comparison chart showing the wound contraction rate between groups: (a) Tegaderm alone (black bars), (b) Algisite and Tegaderm (white bars), and (c) Allevyn and Tegaderm (gray bars). Contraction rate in Tegaderm alone group was more linear. Tegaderm alone group had a lower standard error of the mean.

![Figure 3](image)

**Figure 3.** A) Comparison chart showing the wound contraction rate between groups: (a) Controls, no pumps implanted (black bars), (b) sc pumps (gray bars), and (c) ip pumps (white bars). All groups had similar wound contraction rates with no statistically significant difference \((P > 0.05)\). B) All rats gained weight throughout the experiment except one from ip group (b) that died of infection in the site of implantation. sc: subcutaneous, ip: intraperitoneal.
Figure 4. A) Comparison chart showing the wound contraction rate in the three groups: (a) Controls, no pumps implanted (black bars), (b) large pumps implanted ip (gray bars), (c) medium pumps implanted ip (white bars) and small pumps implanted ip (textured bars). All groups had similar wound contraction rate with no statistical significant difference, ($P > 0.05$). B) All rats gained weight throughout the experiment.

Figure 5. Histological and Immunohistochemical evaluation of control, large, medium, and small pump groups. Hematoxylin and eosin showed all groups had high cellularity, including fibroblasts lymphocytes, macrophages (e = epidermis, d = dermis, 20x magnification, scale bar = 200 µm). Masson’s trichrome stain for collagen revealed a random alignment of collagen fibers in scar tissue. This pattern was similar in all four groups (10x magnification, scale bars = 500 µm). Ki-67 staining showed no difference in proliferation between the four groups (black arrows pointing at positive nuclei, 40x magnification, scale bar = 100 µm).
implanted ip, Group (c) had the medium size osmotic pumps (2004 model) implanted ip and Group (d) had the large osmotic pumps (2ML4 model) implanted ip.

Wounds were closed by day 14. The wound contraction rate was similar between the control and experimental groups ($P > 0.05$; Figure 4A). Additionally, all the rats participating in the experiment gained weight throughout the experiment (Figure 4B).

### Histological Evaluation of the Effects of Osmotic Pumps on Wound Healing

H&E staining was used to evaluate rodent dermal healing. The wound showed fibroblastic proliferation with lymphohistocytic infiltrates, the fibroblasts were arranged parallel to the epidermis surface, and neovascularization was displayed perpendicular to the epidermis. Wound sections were also subjected to Masson’s trichrome staining and examined for presence of scar tissue. Wounds healed with an obvious scar (Figure 5) indicated by loss of hair follicles and densely packed, disorganized collagen. The intensity of the trichrome stain demonstrates increased collagen content around fibroblasts in the scar area. No difference was observed in Masson’s trichrome staining between the control, osmotic pump bearing groups, and pumps of different sizes (Figure 5).

Wound tissue was stained for Ki-67 to assess cell proliferation. Nuclear immunoreactivity to Ki-67 antibody had a dark brown granular appearance (Figure 5). The positive nuclei were counted per 40x power field and a Ki-67 proliferating index of $76 \pm 20$ was found for the control group, $60 \pm 11$ for the large pumps group, $60 \pm 13$ for the medium pumps group and $76 \pm 2$ for the small pumps group. The difference in proliferation between the four groups was not significant ($P = 0.072$; Figure 6).

### Discussion

The optimal preclinical animal model for studying scar contracture would be reproducible, accurate, with minimal variability in scarring parameters between replicates. In this study, a logical series of experiments were performed to validate an optimal preclinical rodent model for studying the mechanisms of scar contracture and for the development of antiscarring agents where compounds can be delivered through osmotic pumps. The effects of wound dressing type, osmotic pump size, and implantation location were analyzed under the following wound healing parameters: wound contraction rates, systemic and local toxicity, proliferation, and collagen architecture and production.

The results of the present study show that absorptive dressings Allevyn and Algisite interfere with wound contraction rate, but Tegaderm did not. This is an important differentiating factor between dressings especially because each of the dressings have been used in previous animal wound studies without careful consideration of its effects on healing.18–21 Of the dressings studied, Tegaderm combines the benefits of wound coverage with a reproducible, linear wound contraction rate and minimal variability between animals, resulting in a reliable model of wound healing study.

Implantation of the largest size osmotic pumps ip is a reliable route of drug delivery that does not interfere with the wound healing process. All pump sizes (small, medium, large) were tolerated by the rats, without interfering with the wound healing process, as shown by the similar pattern of wound healing and weight range between all the pump groups and the controls. This is in contrast to sc implanted pumps where implantation caused significant discomfort in the rats. The rats were constantly biting the skin overlying the pump, rendering the flank an unfavorable site for implanting the pumps. This is important because it is known that stress modulates tissue repair and wound healing.32–46 Implanting an osmotic mini-pump ip at the time of wounding is a process that can be easily followed. The pump ensures systemic delivery of the compound under investigation with minimal rodent stress,
as would otherwise be caused by other routes of repetitive dosing. The pump also facilitates direct delivery of the compound, at a constant rate, into the blood stream. This avoids complicating factors such as the first pass effect that is present in oral delivery or maintenance of the compound into the animal as can occur by topical applications in open wounds.

The female Wistar-Han rat was used for two reasons. The rat is more docile than other strains, and at 10- to 12-weeks of age when most wound healing studies are performed, the rodent weight and size is relatively static. This is important because an animal’s growth could counteract the wound contraction rate. The dorsum of the rat for wounding was used so that the rodent would not be able to reach and manipulate the healing wound. The full-thickness wounding resembles the loss of tissue often found in clinical practice and the 2 cm x 2 cm size of the wound gives the advantage of a relatively big wound that can be analyzed by biochemical, histologic, and immunohistochemical methods. Gravitational planimetry is a highly accurate, simple approach to quantify contraction.

There are limitations to this model in terms of scarring. Normally cutaneous injury initiates a well coordinated sequence of complex processes that lead to wound repair. This dynamic process of wound healing has three phases: inflammation (2–5 days), proliferation (2 days–3 weeks), and remodeling (3 weeks–2 years). The length of study in the authors’ model (2–3 weeks) facilitates the study of the inflammatory and proliferative phases of repair, but not remodeling. Nonetheless, one aspect of the remodeling phase, contracture formation, is modeled by wound contraction, which occurs in the proliferative phase in this model. Both contractures and wound contraction are believed to occur by similar mechanisms of action, namely fibroblast and myofibroblast mediated remodeling of the extracellular matrix. Rodents have loose skin and contraction plays a significant role in wound closure. They also possess a subcutaneous tissue called panniculus carnosus muscle, which also contributes to contraction and collagen formation. There is no animal model to directly study contracture formation. Although it would be beneficial to develop a contracture model, it would be impractical because contractures develop across joints, their quantification is subtle, measured in limitation of joint range of motion, and they take months to form. Rudolph et al. studied skin graft contraction in 1979 where excisional wounds were either left open to heal, were covered with split-thickness skin grafts, or received full-thickness skin grafts. He demonstrated that a split-thickness skin graft contracts up to 40% of its initial size but that the normal variation between animals was up to 20%. Given this large inter-animal variability, based on a power-analysis it would take the study of 25 rats/group to determine a 10% reduction in contractures. In the present study, contraction rates had a variation between animals, approximately 10% and based on a power-analysis that would use this variation, it would take the study of seven rats/group to detect a 10% reduction in contractures. This significantly smaller population size highlights the importance of the present study, which gives reproducible results in a cost-effective manner. Once a potential anti-scar contracture agent in rodents is identified the next step in pre-clinical drug development would be the use of the red Duroc pig. Both the red Duroc pig and human skin create scars that are thicker than uninjured skin, contain nodules of abundant and disorganized collagen fibers, as well as demonstrate elevated mast cell and myofibroblast counts. Another contemporary animal model for scar contracture study is the skin graft contraction in the pig. Henrichsen et al. created full-thickness wounds in the pigs back and left them heal either by covering them with a split-thickness skin graft or by second intention, covered by a dressing. Median healing time in grafted wounds was 12 days and 30 days in the secondarily healing wounds. This data shows that a grafted wound in pigs does not behave as a chronic delayed wound that will eventually result in hypertrophic scar.

**Conclusion**

This study validates a novel model for studying scar contracture. It is a fundamentally important advance in the field of wound healing and fibrosis because it enables future uniform studies. Potential anti-scarring compounds that are found to be effective in the rodent model can then be further tested in higher order animals whose skin structure more closely resembles humans, such as the pig.
References


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