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Sling Training with Positive Reinforcement to Facilitate Porcine Wound Studies

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Domestic swine have become important large animal models for dermatologic and wound studies owing to the similarity of their skin architecture to that of human skin. To improve on current porcine wound protocols and accomplish postoperational daily wound care or treatment in a welfare-centered, low-stress setting, we developed a unique sling-training program using a commercially available Panepinto-like sling in combination with positive reinforcement of desired behaviors. Training using these methods is initiated during the acclimation period of 7–10 days before the initial surgical manipulation and continued throughout project-specific treatments for the duration of the study. Using this protocol, daily treatments can be administered without additional anesthesia while the animals rest in the sling with the administration of simultaneous nutritional enrichment. This low-stress handling program successfully facilitates the postoperational treatments and wound care without the use of potentially confounding anesthesia or sedation. It has a wide range of potential applications in translational medicine and in data acquisition from a resting state where baseline readouts of unstressed animals can be achieved.

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INTRODUCTION

A preclinical large animal model is often required for clinical translation of potential wound therapeutics to examine efficacy and address safety concerns. Although rodent wound models are commonly used, the porcine wound model is noted by the Food and Drug Administration for developing treatments for humans with impaired healing (FDA Wound Healing Clinical Focus Group, 2001). Domestic (Yorkshire-cross) and miniature (Yucatan and Gottingen breeds) swine are established porcine models for wound studies (Stricker-Krongrad et al., 2016) owing to the similarity of the skin architecture, such as the thickness of the epidermis and dermis and insulation by fat but not by fur (Meyer et al., 1978), and cellular wound healing processes that are similar to those in humans (Lindblad, 2008). In loose skinned animals such as in rodents, wounds heal primarily by contraction of the panniculus carnosus structure (Lindblad, 2008; Sullivan et al., 2001), whereas porcine skin heals primarily by re-epithelialization, regenerated granulation tissue, and contraction, depending on the depth of the wounds, similar to that in human skin (De Coninck et al., 1996; Dyson et al., 1988; Lindblad, 2008; Sullivan et al., 2001). Domestic swine are relatively affordable laboratory animals, and the skin area is large enough to allow the control and multiple treatment groups to be performed simultaneously on the same pig. This preserves statistical significance while reducing the need for increased numbers of research animals, an important tenet for the care and use of animals in research (Russell and Burch, 1992).

Physical methods of restraint, for example, nose snaring (Damm et al., 2000), have been used on pigs, but the restraint device and the examination process create a stressful experience for both the animals and the handlers in addition to giving rise to animal and personnel safety and ergonomic concerns with an awake animal (American Veterinary Medical Association, 2020; Huang et al., 2017; Swindle et al., 1994). Chemical methods of restraint such as anesthesia or sedation are also other options that can mitigate procedural stresses as the pigs are either not awake or are tranquilized, respectively (Smith and Swindle, 2008). However, there are inherent challenges and safety concerns associated with the use of pharmaceuticals such as potentially difficult intratracheal intubation and malignant hyperthermia in certain breeds. In addition, vital signs monitoring while under sedation or anesthesia is indicated. An alternative recommended by veterinarians for noninvasive or minimally invasive procedures is the sling with positive reinforcement. The reinforcement works well because pigs are sentient, social, and intelligent animals (Fox et al., 2015; Grandin, 1986).

The Panepinto sling was introduced as a humane, minimal-stress restraint method to handle Yucatan miniature swine in the 1960s (Panepinto et al., 1983). A well-trained pig can be hand or leash guided or can voluntarily walk onto the sling area and then be lifted for physical examination. Although sling training has been reported for subcutaneous or

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intranasal drug administration (Durkes and Sivasankar, 2017; Zeltner, 2013), its use has not been reported in skin wound studies. The goal of this work was to optimize the sling technique for short-term skin wound studies. In this paper, we report a modified protocol to train young, domestic pigs during the acclimation period (7−10 days) before wounding surgery, so to preclude the subsequent use of anesthesia and reduce restraint-associated stressors during daily wound treatments.

METHODS AND DESCRIPTION

Porcine training

The porcine training and wounding protocol are in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011) guidelines and were approved by the UC Davis Institutional Animal Care and Use Committee. Five young female pigs (*Sus scrofa domesticus*, weighing 27−40 kg, aged 3−4 months) from a Yorkshire/Landrace-cross background closed colony were acquired from the Swine Teaching and Research Center at the University of California, Davis (Davis, CA). The pigs were singly housed (temperature controlled at 21 °C or 70 °F, 12-hour light and dark cycles) with a minimum of 1.4 m^2 per 15 ft^2 space per pig and environment enrichment (Kong toys [Kong Company, Golden, CO], 90 cm of 3 per 8 gauge chain link, bowling balls, and pig balls). Pig feed (Nutrena brand, Country feed grower and finisher pig feed, 16% crude protein pellet [Cargill, Minnetonka, MN]) was supplied at 650−700 g per animal twice a day (regular diet) or once daily during the required treatment and wound care days to control the total food intake when morning treats (1.5−2 kg per animal per training session, discussed in the following paragraph) were given during the training period. Water was available ad libitum.

The pigs were acclimated for 7−10 days, during which daily, food-driven training lasting approximately 30−60 minutes each day was used to first habituate the pigs to novel food items and treats and to the laboratory staff and then to expose them to the portable Panepinto-like sling (swine or sheep restraint sling complete frame, catalog #SF H1PU, Lomir, Malone, NY) (Durkes and Sivasankar, 2017; Grandin, 1986; Sørensen, 2010). While being hand fed with fresh fruit slices (apples, cantaloupe, and peaches) and syringe fed with palatable, high-value foodstuffs such as yogurt, apple sauce, pumpkin soup, or cat food gruel (Dannon yogurt, Mott's apple sauce, freshly cooked pumpkin blended 50:50 with water and heavy cream, and Friskies mixed grill mince, made into a gruel 50:50 with water) using 60 ml syringes (60 ml catheter-tip syringe without needles, catalog #309620, BD, San Jose, CA), the pigs were initially led into the neutral, open hallway area of the vivarium between housing stalls where the flattened, cotton sling cover was relaxed squarely on the ground with a floor mat underneath.

To additionally decrease the stress from the imposing stature of standing humans (Grandin, 1986), the novel laboratory members were seated at eye level to the pig in key areas proximal to the lifting handles, with a key laboratory member providing continuous syringe feeding of the various aforementioned foodstuffs. After the pig was led to stand stationary over the precut peripheral limb openings, a coordinated and simultaneous holding and wrapping of the pig with the sling cover was done. Each pig was trained gradually to be wrapped with the sling cover with all the limbs in the holes and then to be lifted off the ground from seconds to minutes until the pig could be comfortably placed on the sling frame for 15−30 minutes daily, all done while continuing to syringe feed the pig (Figure 1a and b). For both initial acclimation into the sling and subsequent uses for postoperational treatments, persistent peripheral limb motion was observed commonly but was reduced with continued use of the sling. At the end of the training session, the pig was lifted off from the sling frame, placed on the floor mat again, released gently from the sling cover, and guided back to the housing stall with the food treats. The sling set was rinsed to clean off any food residue after daily uses.

Blood collection, wounding surgery, and wound care

The pigs were fasted for 12 hours before the surgery and were induced under tiletamine and zolazepam injection (Telazol 4−8 mg/kg, intramuscular injection, Zoetis Inc, Parsippany, NJ) or ketamine (10−30 mg/kg) with xylazine (2 mg/kg, intramuscular injection). Each pig was intubated endotracheally and maintained under isoflurane (0.5−4% in 100% oxygen, inhalation). The heart rate, respiratory rate, and body temperature were monitored, and Ringer's lactate solution was given (5−10 ml/kg/h, intravenous injection [Vetivex Veterinary Lactated Ringer's Injection; Dechra Pharmaceuticals PLC, Northwich, UK]) during the surgery. To compare the stress-induced catecholamine concentrations in plasma before and after the sling training, pig blood samples were obtained immediately after anesthesia induction on the surgery day (day 0 samples, after 7−10 sessions of preoperative training, 30 minutes per session per day) and on the tissue collection day (day 15 samples, after 21−24 sessions of preoperative and postoperative sling lifting, 30 minutes per session per day). Whole blood (10 ml) was collected through the ear vein in EDTA tubes and kept on ice at the surgery day before the wounding procedure and at the 15-day endpoint before euthanization. The plasma samples were isolated by centrifuging the blood at 1,500*g* for 10 minutes and stored at −80 °C for the HPLC analysis.

In total, 12−18, 16-mm circular, full-thickness wounds (2 cm^2 each, 3−5 cm apart) were generated on the back skin of each pig. The wounds in the control group were treated with wound scaffolds (Integra Wound Matrix Thin Skin, catalog #54051T, Integra LifeSciences, Plainsboro, NJ) with daily application of saline (300 µl). The wounds in the treated groups were applied with timolol and hypoxia-preconditioned human mesenchymal stem cells seeded in the wound scaffolds and daily timolol solution (0.047 mg in 300 µl saline), following a similar protocol that has been used to successfully improve healing in murine wounds (Yang et al., 2020). Detailed analysis of the experimental wound healing outcomes is presented elsewhere (Yang, unpublished data, 2021). All the wounds were dressed with wound veil (Conformant 2 Wound Veil Sheet, Smith & Nephew, Watford, United Kingdom) and bolster foam (Optifoam Basic Non-Adhesive Dressing, Medline, Northfield, IL) and sealed with Tegaderm Transparent Film Dressings (3M, Maplewood, MN). To protect the wounded area, the entire back of the pig was covered with foam pads (Reston Self-Adhering Foam Dressing Pad, 3M) and a tear-resistant coat (modified from a spandex sheep slinky with an opening on the dorsal side held closed with Velcro and extra foaming around the axillary and inguinal areas to minimize pressure sores). A fentanyl patch (5 µg/kg/h for 72 hours) was applied to each pig for postoperative pain management.

Figure 1. Pig sling-training and wound treatment. (**a**) Positive reinforcement is used to lead the pig to the correct position on the sling. Once in position, the sling is lifted around the pig's legs. (**b**) Pig is resting comfortably on the sling while in the frame with continuous treats. (**c**) A modified, tear-resistant coat and cushion foam pads were applied to protect the wounded area. (**d**) Daily postoperational wound care. The dressings were replaced, and the wound treatments were injected onto the wound surface while the pig rested in the sling. The identification tags and ear notches of the pigs have been obscured.

In this study, daily postoperational wound care, including bandage removal, administration of topical medications to the wound bed, and rebandaging, was performed for 15 days, after which, the pigs were humanely euthanized (100 mg/kg intravenous pentobarbital injection). No additional postoperational anesthesia was administered.

Catecholamine measurement

Catecholamines in pig plasma were measured using ion-pairing ultra HPLC with amperometric detection following purification by phenylboronic acid silica solid-phase extraction. The mobile phase consisted of 4:96 acetonitrile:phosphate citrate buffer, pH = 6.0. The total concentrations of phosphate and citrate buffer were 100 mM each, and the mobile phase also contained a total of 0.1 mM EDTA and 600 mg/l sodium octanesulfonic acid; the pH of the aqueous portion was adjusted before the addition of acetonitrile. Catecholamines were separated on an Acquity UPLC BEH C18 column (1 mm inner diameter × 100 mm length, 1.7 mm particle diameter, Waters, Dublin, Ireland) at a flow rate of 50 ml/min at 37 °C. A 10 ml full-loop injection was used. The applied potential for the glassy carbon working electrode was 0.46 V versus the silver−silver chloride reference electrode, with a noise filter of 0.5 Hz and a range of 1 nA/V.

For phenylboronic acid silica solid-phase extraction clean-up of 500 ml plasma was first spiked with 20 ml internal standard solution (37.5 nM epinine in water) and 10 ml antioxidant solution (1.25% ascorbic acid + 2 mM EDTA in water) and was mixed well. The sample was then mixed with 500 ml of 0.100 N ammonium sulfate buffer, pH = 8.5, and vortexed again. This loading solution was applied to a conditioned MonoSpin PBA cartridge (800 ml capacity, GL Sciences, Tokyo, Japan), and the cartridges were rinsed with 500 ml ultrapure water and eluted with 50 ml of 1% (v/v) acetic acid in water. A short dry spin was used between the sample loading, washing, and elution steps and after the final elution step to remove residual liquid from the sorbent. All the centrifugation steps were performed at 1,000*g*.

The linear range of the instrument detection for norepinephrine, epinephrine, and epinine (the internal standard) was 1,000−15,000 pM. Because plasma samples were concentrated 10-fold before analysis, this corresponds to a range of 100−1,500 pM catecholamines in the plasma. The concentration of the lowest calibrator (1,000 pM) was taken as the instrument's lowest limit of quantitation. The instrument limit of detection was 388 pM for norepinephrine and 409 pM for epinephrine, corresponding to concentrations of 39 pM and 41 pM, respectively, in the 500 ml plasma samples. The limit of detection was calculated using a signal-to-noise approach. First, baseline noise was determined from two blank injections as American Society for Testing Materials noise with 30-second intervals; then, on the basis of the response of the lowest concentration calibrator, we determined the concentration corresponding to signal-to-noise of 1.5. Because injection precision and detector stability were quite good, we opted for external calibration using a surrogate rather than the more commonly used internal standard approach (i.e., quantitation by response ratios). An external calibration plot was created for each of the catecholamines and for epinine. A known quantity of epinine was spiked into each sample before analysis, and the concentration of epinine in the finished extracts was determined

Table 1. Sling Training Improves the Overall Recovering Rate of Wound Tissue in Swine

Wound Studies in Swine	Pig Sling Training	Wound Size and Total Wound Generated	Postoperative Healing Time, Days	Re-Epithelialization Rate (Control Group Only)	Recovering Rate of Wound Tissue Reported in the Study
Yang et al., (current report) and Yang, unpublished data, 2021.	Yes, pig training through the whole experiment	2 cm^2 area or 16 mm circular wounds; a total of 68 wounds	15	43.5% from 15 wounds	Overall, 95.6% (65 of 68 wounds); dressing detachment or dosing failure occurred in 3 wounds; no signs of wound infection
Agren et al. (1997)	No	3.14 cm^2 area or 20 mm circular wounds; a total of 54 wounds	10	57% from 12 wounds	Control group, 83.3% (10 of 12 wounds); the wound dressing and treatment failed in one wound, and bacterial infection occurred on the other wound
De Coninck et al. (1996)	No	9 cm^2 area or 3 cm × 3 cm square wounds; a total of 192 wounds	26	100% from eight wounds	Overall, 16.7%; 4 of 24 wounds were selected from each animal for analysis; wound infection was not documented
Singer and McClain (2003)	No	6.25 cm^2 area or 2.5 cm × 2.5 cm square wounds; a total of 40 wounds	14	0% from eight wounds	Overall, 100%; all the wounds were included; no signs of wound infection

by comparison with the calibration plot. This provided the percentage recovery data for each sample, allowing us to account for losses of catecholamines during the sample preparation procedure.

RESULTS

The modified sling training was successfully accomplished on five domestic pigs, and the postoperational restraint time of each pig on the sling was 20–30 minutes daily (Figure 1c and d).

Using this technique, there was a 95.6% success rate for recovering wound tissue of the postoperational treatments. Among the total 68 wounds created on these pigs, only three wounds needed to be excluded from analysis because of dressing detachment or dosing failure. A comparison of four wound studies in swine with similar wounding techniques (full-thickness wounds generated) and with a similar dressing of the occlusive Tegaderm film is shown in Table 1. The numbers of the excluded wounds are generally not reported in the animal wound literature because those may be considered to be experimental failures and may be omitted from analysis. From the wound recovery rates in Table 1, the overall results suggest that the current sling-training method improves the outcomes of successful, postoperative treatments and a high retrieval rate of the wound tissue among the four studies.

Images of the wound placement on the pig dorsum (Figure 2a) and representative wound beds are shown (Figure 2b). Similar to a 65.6% improvement of healing examined in a mouse wound model (Yang et al., 2020), the wound treatment increases re-epithelialization by 40.2% compared with that of the control in swine. Of note, the reported murine wound studies were performed in mice with diabetes that had impaired healing, whereas in this study, a 40% improvement over control healing is observed in healthy pigs without diabetes, with no healing impairment. Plasma catecholamines were measured from blood collected on day 0 before the onset of wounding surgery and on day 15

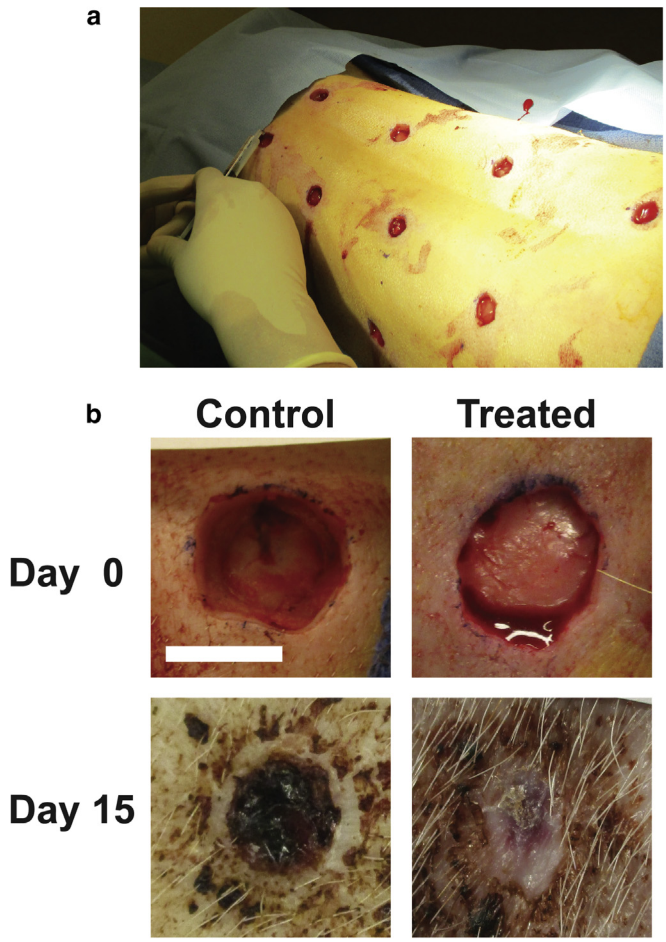

Figure 2. Wound placement on the pig dorsum and representative images of the full-thickness wounds. (**a**) Images of the wound placement on the pig dorsum. Multiple full-thickness, circular wounds (16 mm in diameter, >5 cm apart) were made by surgical blades, and the treatment drugs were applied by a syringe to the wound bed. (**b**) Representative images of wounds on day 0 and day 15 after the course of sling training. Bar = 10 mm.

Table 2. Plasma Catecholamines in Pigs after Sling Lifting, with or without Training

Swine Studies	Sling Training	Condition of Blood Collection	Norepinephrine, (pM)	Epinephrine, (pM)
Yang et al. (current report)	Preoperative training (7–10 sessions, 30 min each)	Day 0 (surgery day); blood was collected under anesthesia by ear vein; immediately after anesthesia induction	543 ± 204	308 ± 94
Yang et al. (current report)	Preoperative and postoperative sling lifting (21–24 sessions, 30 min each)	Day 15 (wound harvest day); blood was collected under anesthesia by ear vein; immediately after anesthesia induction	1,228 ± 1,199	684 ± 132
Braumann et al. (2011)	No	Day 0 (surgery day); blood was collected under anesthesia by arterial catheter; immediately after anesthesia induction	1993.1 (median, range from 508.0 to 4,397.1)	1969.4 (median, range from 420.3 to 4,817.6)
Carlson et al. (1989)	Minimal sling training (1–3 sessions, 30 min each)	Blood was collected by implanted arterial catheter immediately after sling lift	2,778 ± 739	1,719 ± 246
Carlson et al. (1989)	Preoperative and postoperative sling training (5 sessions, >1 h each)	Blood was collected by implanted arterial catheter immediately after sling lift	733 ± 130	486 ± 93

immediately before killing. The published levels of stress catecholamines epinephrine and norepinephrine from the pigs in control groups with similar body weights and handling methods are shown in Table 2. After 15 days of postoperational sling lifting and daily wound manipulation, the catecholamine levels increased relative to those on day 0 but were far below those reported in the literature for untrained or minimally trained, awake or sedated, unconscious pigs (Table 2).

DISCUSSION AND POTENTIAL APPLICATIONS

This method of enriched sling training with positive reinforcement successfully facilitates the postoperational delivery of wound treatment and wound care in this large animal, porcine wound model, without the need for additional anesthesia administration during the course of the experiment (15 days in this study). Plasma catecholamine levels, indicators of stress, were lower than those reported in the literature for sling-trained pigs or for pigs placed in slings for procedures with no previous training (Table 2), indicating lower stress levels using the protocol of enriched training. The elevation of norepinephrine relative to that of epinephrine is expected because baseline levels are physiologically higher and because trauma with peripheral stimulation (as in the case of skin wounding discussed in this paper) releases norepinephrine (Mellor et al., 2002).

Sling-training advantages have been known for some time to reduce animal stress. The foundational low-stress handling techniques such as calm, quiet voices and slow or minimal sudden movements (Lloyd, 2017; The Ohio State University, 2015) and the pressure provided by the swine sling along the pig's belly (Grandin, 1986) relax the pigs, which allows the changing of dressing and daily dosing to occur. In addition, because it has been demonstrated that pigs are able to recognize individual humans using visual, olfactory, and auditory cues (Koba and Tanida, 2001; Lori and Colvin, 2016; Tanida and Nagano, 1998), the same laboratory members worked with the pigs throughout both sling acclimation and postwounding treatments to minimize the inherent stressors associated with the introduction of novel individuals. Improper handling of the pigs prolongs the wound care time and induces a stress response in the pigs, which may reduce weight gains, elevate serum cortisol and catecholamine levels, enlarge adrenal cortex, and reduce cell-mediated immunity in the pigs (Grandin, 1986), which may converge to delay healing and skew experimental results. Signs of pain or distress of the pigs such as continuous vocalization, orbital tightening, persistent peripheral limb motion (Herskin and Di Giminiani, 2017; Viscardi et al., 2017) while in suspension should be assessed carefully and minimized as well.

The modification of the sling-training technique reported in this study adds positive reinforcements and enrichments of food treats, affiliative human contact, and toys. Food treats are an effective method to guide and reward the pigs when the desired behaviors (adaptation to a restraint device) are performed because pigs have a highly developed sense of smell but poor eyesight (Fox et al., 2015). Human interaction and acclimation with the pigs also provide enrichments while they are singly housed during the experimental periods.

This modified sling-training method also eliminates the risks associated with frequent anesthesia and sedative use, such as severe hypotension and acute death (Smith and Swindle, 2008), and allows for wound assessments on fully conscious, acclimated, and cooperative pigs. Data acquisition from a resting state with baseline readouts from unstressed pigs can be achieved. It has a wide range of potential applications in translational medicine, including (i) noninvasive procedures such as examinations and topical treatments of cutaneous wounds, burn lesions, or skin grafts and examinations of surgical incisions or implanted devices; (ii) minimally invasive procedures such as full veterinary physical examinations, including rectal temperature checks, bandaging, peripheral percutaneous venipuncture (such as through-ear vasculature), blood collection from and maintenance of preimplanted vascular access ports, and placement plus maintenance of peripheral intravenous catheters; (iii) data collection from implanted devices on resting animals for metabolic or cardiovascular studies such as electrocardiography (Scheer et al., 2010) and blood pressure measurements; (iv) multimodal drug administration through both parenteral and oral routes for both veterinary and study-related purposes; and (v) an additional benefit, which is improved safety for laboratory personnel, preventing work-related injuries that may occur by lifting and handling the large, heavy pigs. Cost savings accrue from the need for a fewer number of staff and reduced personnel hours with the cooperative pigs.

However, there are limitations to the current method. The initial training period is labor intensive. At least three people are required to lift a pig in a coordinated move. To lower the stress of the pigs, training for the staff is also required in the handling of the pigs. The pigs are sensitive to subtle changes in the environment, such as the color of the personal protective equipment used, the feeding speed and pattern, human gestures and movements, and any loud noise. Therefore, established daily routine steps while training the pigs and the staff is important to provide consistency and mitigate any novel stresses.

In addition, the sling set used in this study was designed to handle pigs weighing up to 100 lbs or 45 kg. The domestic pigs gained weight quickly during the experiment (7−10 lbs or 3−4.5 kg per week), and this factor should be considered if the planned wound observation period will be longer than 2 weeks. Chaffing of the skin over the limbs or skin folds (e.g., axillary or inguinal areas) by the edge of the sling openings may occur in growing pigs and can be mitigated with the use of additional cushioned padding over the edges of the limb egress sites. Finally, some pigs may be more excited and aggressive with continuous vocalization than others when the training staff appear with food treats. The pig-training order could be adjusted to avoid the pigs getting distressed.

In this study, we show that the young, domestic farm pigs can adapt to the wound treatment procedures using the modified sling-training method with positive reinforcement. This method not only decreases the stress of the pigs, reduces the need for anesthesia, improves wound outcomes, decreases personnel costs, but also improves the safety and efficiency of the pig handling for the researchers. With the increasing need for large animal models for translating wound therapeutics to clinical use, this porcine wound protocol can provide a more reliable outcome with fewer stress-mediated pathologies and a streamlined workload for the investigators.

Data availability statement

No large datasets were generated or analyzed during this study. Minimal datasets necessary to interpret and replicate the data in this paper are available on request to the corresponding author.

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AUTHOR CONTRIBUTIONS

Conceptualization: HYY, KGG, BWM; Data Curation: HYY, AG; Formal Analysis: HYY, AG; Funding Acquisition: RRI; Methodology: HYY, KGG, BWM; Supervision: BWM, RRI; Writing - Original Draft Preparation: HYY, KGG, AG; Writing - Review and Editing: BWM, RRI

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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