Ischemia-reperfusion injury in chronic pressure ulcer formation: A skin model in the rat

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Most animal models of chronic pressure ulcers were designed to study only the role of ischemic injury in wound formation, often using single applications of constant pressure. The purpose of this study was to develop and characterize a reproducible model of cyclic ischemia-reperfusion injury in the skin of small un-anesthetized animals using clinically relevant pressures and durations. Ischemia-reperfusion injury was created in a 9 cm^2 region of dorsal skin in male rats by periodically compressing skin under a pressure of 50 mm Hg using an implanted metal plate and an overlying magnet. We varied the total number of ischemia-reperfusion cycles, examined the effect of varying the frequency and duration of ischemic insult, and compared ischemia-induced injury to ischemia-reperfusion-induced injury with this model. Tissue injury increased with an increasing number of total ischemia-reperfusion cycles, duration of ischemia, and frequency of ischemia-reperfusion cycles. This model generates reproducible ischemia-reperfusion skin injury as characterized by tissue necrosis, wound thickness, leukocyte infiltration, transcutaneous oxygen tension, and wound blood flow. Using this model, the biological markers of ischemia-reperfusion-induced wound development can be studied and therapeutic interventions can be evaluated in a cost-effective manner. **(WOUND REP REG 2000;8:68–76)**

Ischemia-reperfusion (I/R) injury can be a factor in the formation of chronic skin wounds, including chronic pressure ulcers, venous stasis ulcers, and diabetic foot ulcers.[1–3] Although ischemia has long been studied in the chronic pressure ulcer, I/R has only recently been directly considered in its pathogenesis.[1,2] Studies of I/R injury in various organs of the body, principally the brain, heart, kidney, liver, skin, intestine, and skeletal muscle, suggested that the etiology of I/R injury is distinct from that of injury caused by a single ischemic insult.[4–8] Therefore, chronic skin wounds must be studied from the perspective of I/R to more completely understand their etiology. In the

| I/R | Ischemia-reperfusion |
| TcPO$_2$ | Transcutaneous oxygen tension |

case of pressure ulcers, it is crucial to model their formation using an I/R mechanism.

Most previous animal models of chronic pressure ulcers have been designed to study only the role of ischemia in wound formation. These models use only one application of constant pressure to create the wound and neglect any contribution to wound formation by I/R. Thus, there is a lack of knowledge about chronic skin wounds from the perspective of I/R injury.[1] The few models that incorporate I/R injury into wound formation have two major shortcomings. They generally have used large animals, which reduces the possibility of conducting experiments on a large scale relatively inexpensively, and/or high pressures and a limited number of I/R cycles to create the ulcer in anesthetized animals, which undermines the clinical relevance.

I/R injury has been defined as cellular injury resulting from the reperfusion of blood to a previously is-

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chemic tissue.[9] When a tissue has been depleted of its blood supply for a significant amount of time, the tissue may reduce its metabolism to preserve function.[4] The reperfusion of blood to the nutrient- and oxygen-deprived tissue can result in a cascade of harmful events.[10] Many mechanisms of I/R injury have been proposed for various tissue types. Reperfusion is presumed to produce levels of oxygen-derived free radicals that exceed the capacity of constitutive free-radical scavenging mechanisms, thus causing a cytotoxic effect in the tissue.[9] This can cause inflammation through the activation of endothelial cells to recruit circulating leukocytes.[10] Subsequent pathways of I/R injury have been hypothesized to include endothelial cell swelling associated with the recruitment of monocytes and macrophages, reduction in arteriolar diameter, permeability changes in postcapillary venules due to leukocyte adhesion, and increased flow resistance in the microcirculation.[7,11,12]

Hussein et al. hypothesized that the cyclic application and removal of pressure can cause I/R injury in skin if the pressure is large enough to substantially reduce blood flow throughout its application period.[1] Ischemia is defined in this paper as a reduction of tissue oxygen supply resulting from an 80–100% decrease in blood flow. Relief of pressure and return of blood flow to the ischemic region is termed the reperfusion event.

A number of animal models have been developed to understand the etiology of chronic pressure ulcers with regard to ischemic insult. Most of these models used a mechanical device that applied pressure to the skin of an animal for a specified cycle of ischemia.[13–17] These models used single applications of high pressures to create ulcers in anesthetized animals. These high pressures are not clinically relevant, and the physiological consequences of anesthesia may compromise the true effects of pressure on unanesthetized animals. The conclusions of these studies were limited to the effects of ischemia on pressure ulcer formation and did not consider the impact of the subsequent reperfusion injury. Similarly, previous models that have incorporated a reperfusion cycle into the ulcer formation have limited clinical relevance because they used an anesthetized animal and/or high pressures and short durations of pressure application.[13,18,19]

The purpose of this study was to develop and characterize a reproducible model of a skin wound induced by cyclic I/R in rodents using clinically relevant parameters of pressure and duration. The uniqueness of the model is that the animal is not anesthetized during periods of skin ischemia and reperfusion, and because

rats are used, the model may be useful as a low-cost reproducible test for chronic skin wound therapies. This article describes the pressure ulcer model and its development, characterizes the model based on the data from dose-response experiments, relates I/R injury in skin to both duration and frequency of pressure applications, and compares ischemia-induced injury to I/R induced injury.

MATERIALS AND METHODS

I/R injury was induced by applying and removing a permanent magnet to a dorsal region of rat skin under which a ferromagnetic steel plate was implanted, as diagrammed in Figure 1. The application of the magnet compressed the skin and reduced blood flow, thus causing ischemia, while the removal of the magnet allowed reperfusion of blood to the ischemic skin region. When the magnet was applied to the skin during the ischemic phase of the I/R cycle, it was held to the skin purely by magnetic attraction.

Magnet selection

The magnet used in conjunction with the 4 cm × 2.5 cm × 0.05 cm pressed steel plate was a rectangular ceramic permanent magnet (Magnet Source,

Cross-sectional View
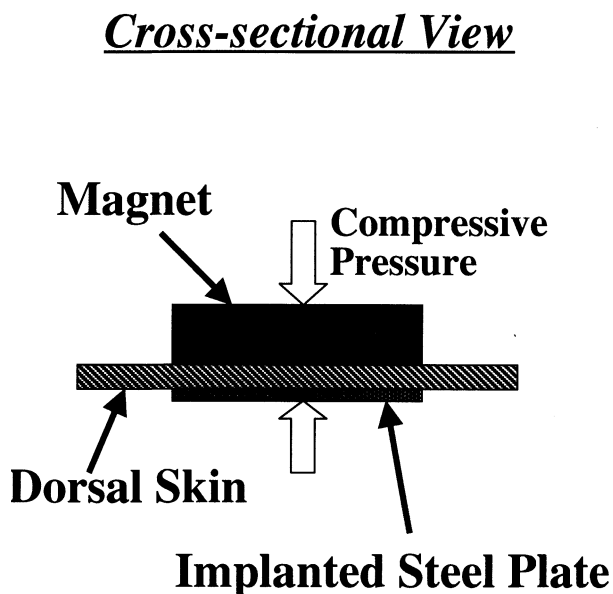
Figure 1. The I/R injury is created by periodically compressing the skin using magnetic force. The ischemia phase of the I/R cycle is accomplished by applying the magnet over a region of dorsal skin under which a ferromagnetic steel plate has been implanted. The resulting compressive pressure of 50 mm Hg is large enough to cause ischemia in the skin. The reperfusion phase of the I/R cycle is achieved by removing the magnet.

Castle Rock, CO) of dimensions 4.0 cm in length, 2.25 cm in width, and 1.0 cm in thickness. The plate weighed 5 g, while the magnet weighed 122 g. The average magnetic field strength over the positive pole was measured on-axis 1 mm from the face of the magnet with a F.W. Bell Model 9500 Gaussmeter (Orlando, FL) to be 1250 Gauss.

The selection of a magnet with this particular magnetic field strength was an integral part of the experimental design. Final magnet selection was based on a desired application pressure of 50 mm Hg, which was chosen to approximate clinically relevant pressure. This pressure was determined by measuring the interface pressure between the trochanter of volunteers and a hospital replacement mattress[20,21]. The calculation for the magnet strength and the assumptions that were made to deduce the applied force and pressure are presented in the Appendix. The selected magnet and steel sheet were tested in a force transducer to verify that 50 mm Hg pressure was applied to the sandwiched skin area by this mechanism.

Steel sheet implantation and testing protocol

All rats underwent the same surgical procedure for implantation of the steel sheet. Each male Sprague-Dawley rat (250 to 300 g, Hilltop, Scottsville, PA) was anesthetized for plate implant surgery with 50 ml/mg of pentobarbitol injected intraperitoneally. A 7 cm × 9 cm rectangle of skin on the rat's back was shaved, depilated with Nair, cleaned with water and detergent, and disinfected with iodophor solution. A 3 cm transverse incision through the full thickness of the skin was made 1 cm below the left shoulder. The autoclaved steel sheet was tunneled under the skin caudal to the incision, where it was held in place by the surrounding fascia. The incision was closed with size 4/0 Polysorb suture (United States Surgical Corporation, Norwalk, CT). No bandages were used. The rats were returned to their cages and allowed to recover from anesthesia for 24 hours, after which the prescribed I/R cycles were initiated.

The contralateral side did not receive an implanted plate and served as a control for each animal. Five rats were allowed to heal for 2 weeks before magnet compressions were initiated to ensure that the initiation of compressions on the day after implantation surgery did not affect the outcome.

An acute test was performed on four anesthetized rats to study the extent of blood flow reduction caused by the magnet application. The blood flow in the dorsal skin with the implanted steel plate was measured with a point probe of a Doppler blood flow monitor (Tran-

sonic Systems, Inc., Ithaca, NY) inserted through a hole in a specially constructed 1250 Gauss magnet. Control readings of blood flow were taken for 0.5 hours before the magnet was applied, during magnet application to the skin surface for 2 hours, and for 0.5 hours after the magnet was removed. The probe was not moved during the entire experiment.

I/R injury procedure

Fifty-two rats were prepared for dorsal surgery as described above. After the 24-hour recovery period, they were subjected to one of the following compression protocols. All of the cycles were administered while the animals were unanesthetized. The animals were fed water and chow and were allowed to roam freely in their cages in a controlled vivarium throughout the compression cycles. Animals were individually housed and cared for under approved guidelines, with a protocol approved by the University of Virginia Animal Research Committee (which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International Public Health Service Animal Assurance).

For Experiment 1, a total of 16 rats were randomly divided into four equal groups. Each group received a different total number of I/R cycles, as illustrated in Table 1. One cycle consisted of 2 hours of ischemia and 0.5 hours of reperfusion. The I/R cycle of 2 hours of ischemia followed by 1 hour of reperfusion is clinically relevant since patients at risk for developing pressure ulcers should be turned at least every two hours.[22]

For each group, a maximum of 5 compression cycles was administered per day followed by a period of 11.5 hours of reperfusion. In the experimental groups, the 5 I/R cycles were conducted for a total of 1, 2, or 3 days. After the specified total number of compression cycles were completed for a group, the magnets were removed for a final 11.5 hours of reperfusion, and the animals were euthanized and their treatment sites analyzed.

In Experiment 2, 32 rats were randomly divided into four equal groups. Group E (Table 1) served as the control group and received the implantation surgery, but did not undergo any I/R cycles. Groups F, G, and H were assigned to a schedule of the prescribed compression cycles and duration times as summarized in Table 1. I/R cycles were applied for five consecutive days. As in Experiment 1, the rats were fed water and chow and were allowed to roam freely in their cages in a controlled vivarium during and between compression cycles. After the specified compression cycles were completed for the day, the magnets were removed

Table 1. I/R cycle protocols for the three experiments

| Experiment | Group designation | I/R Cycle | | Cycles/ day | Number of days | Total Number of I/R | Total hours of ischemia |
		Ischemia	Reperfusion				
#1 (N = 4/group)							
	A			Control: no I/R cycles			
	B	2.0	0.5	5	1	5	10
	C	2.0	0.5	5	2	10	20
	D	2.0	0.5	5	3	15	30
#2 (N = 8/group)	E			Control: no I/R cycles			
	F	1.0	0.5	5	5	25	25
	G	2.0	0.5	5	5	25	50
	H	1.0	0.5	10	5	50	50
#3 (N = 4/group)	I	10.0	0	1	1	1	10
	J	2.0	0.5	5	1	5	10

to allow reperfusion overnight. At the completion of the final ischemia/reperfusion cycle on the fifth day the desired data was collected and the animals were euthanized.

Experiment 3 compared injury induced by ischemia alone to I/R-induced injury. The four rats of Group I received a total of 10 consecutive hours of compression (Table 1). These data were compared to Group J, which received 5 I/R cycles of 2 hours of ischemia/0.5 hours reperfusion for a total of 10 hours of ischemic compression.

Skin blood flow measurement

Skin blood flow of the treatment site and the contralateral untreated skin site was quantitated using a scanning laser Doppler flowmeter (Lisca Perfusion Image Monitor, Lisca, Inc., North Brunswick, NJ). The laser Doppler flowmeter measures to a depth of approximately 300 μm into the skin.[23] Blood flow scans were obtained prior to plate insertion for each rat. The average blood flow was obtained over the treatment area. These images were used for baseline readings. Results in four rats documented that subcutaneous implantation of the metal plate did not alter normal blood flow in the skin. Blood flow scans were also taken at final analysis to determine the alteration in skin blood flow as a consequence of I/R cycles.

Photographic analysis

At analysis, each treatment site was photographed with a digital camera (Kodak DC 120, Rochester, NY). The wound was digitized using computer software and the border of the necrotic area (black eschar) was manually traced. Computer software was used to calculate the necrotic area within the outlined border (Wound-Trak98, Clinically Effective Outcomes, Charleston, SC). The area of the necrotic tissue was calculated as a percentage of total flap area (4.0 cm × 2.25 cm).

Transcutaneous oxygen tension

Animals in experiments 1 and 3 had transcutaneous oxygen tension ($TcPO_2$) determined at the beginning and the end of each experiment. Transcutaneous oxygen was measured with a TCM3 Transcutaneous Combined PO_2/PCO_2 Dual Channel Monitor (Radiometer, Copenhagen, Denmark). This system allowed the simultaneous measurement of transcutaneous oxygen on the treated skin and the untreated skin on the contralateral side of the same animal. The measurement probe was placed in the distal third of the treated site and on the mirror image location in the untreated site.

Histological analysis

Full-thickness biopsies (including the panniculus carnosus muscle layers) for histologic analysis were obtained from each treated site and the corresponding untreated contralateral site. For each treated site, three contiguous samples were obtained: from untreated skin adjacent to treated skin; treated skin that was not necrotic; and treated skin that was necrotic. The samples extended from the outside margin to the center of the treated area. All samples were fixed in neutral buffered formalin (10%), embedded in paraffin, sectioned perpendicular to the skin sur-

face at 4 μm thickness, and stained with hematoxylin and eosin. Skin thickness for each of the three sites was obtained using a stage microscope micrometer. The number of extravasated leukocytes were counted in sections taken from the perimeter of the treatment site. The number of extravasated leukocytes were counted and subsequently averaged in 36 0.05 mm^2 fields of view in 12 sections under 400 × magnification.

Statistical analysis

A one-way analysis of variance test was performed for each of the three experiments using Corel Quattro Pro software. Statistical significance was set at $p < 0.05$. A two-sample Student's t-test assuming unequal variance was performed on each subsequent group in the experiment to determine significant differences within a 95% confidence interval.
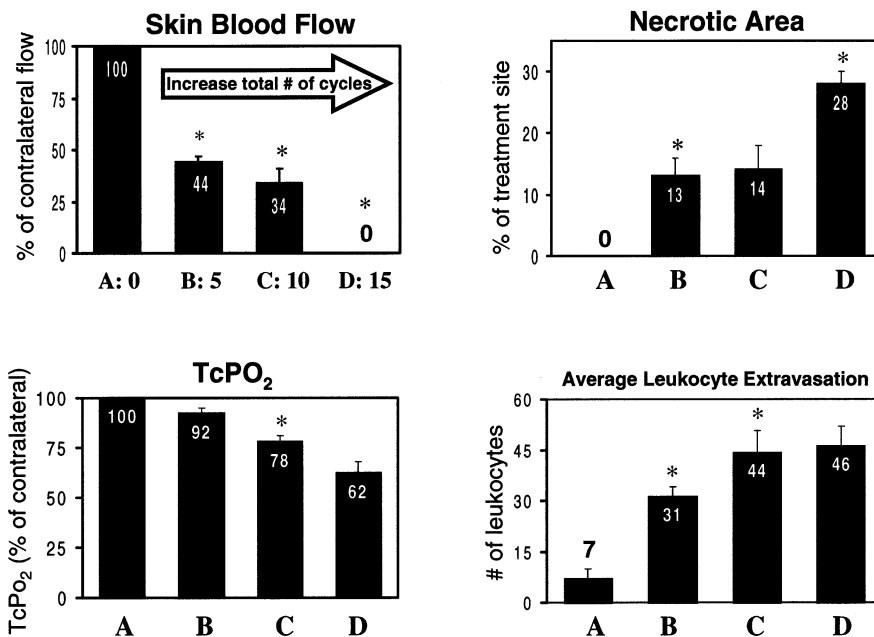
RESULTS

We initially examined the ability of the magnet-plate device to reduce blood flow through the compressed skin. When the magnet was applied to compress the skin, skin blood flow was reduced to an average of 20% of the control value and remained at this reduced level until the magnet was removed (Figure 2). Upon magnet removal, flow returned at least to control levels and some hyperemia was observed during reperfusion. This test indicated that the 1250 Gauss magnet generated a compressive pressure that achieved the desired reduction in blood flow.

Skin Blood Flow
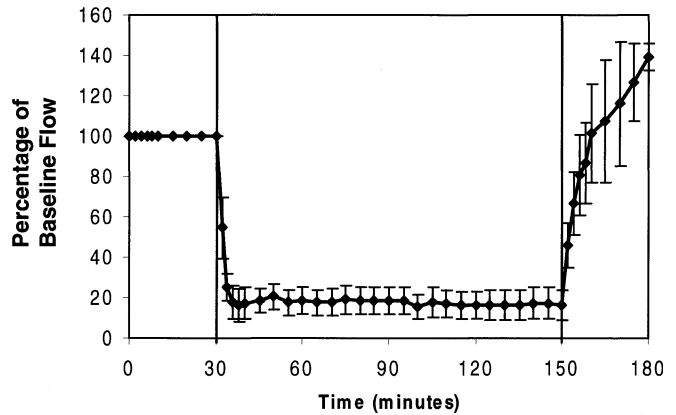
Figure 2. The blood flow reduction capabilities of the magnet/steel sheet device were tested with a point laser Doppler blood flow probe inserted through the magnet. When the magnet was applied to the dorsal skin with implanted steel sheet, the skin blood flow was reduced to 20% of average normal flow. This reduced level is sustained over the 2-hour period during which the magnet was applied. When the magnet was removed, the skin experienced hyperemia over at least a 30 minute period.

As the number of total cycles was increased from 5 to 10 to 15, the degree of tissue damage increased (Figure 3). Increasing tissue damage was characterized by an increased area of tissue necrosis within the treatment site and increased numbers of leukocytes at the treatment site perimeter (Figure 3). Skin blood flow in the treatment site and tissue oxygen

Figure 3. The results from experiment #1 indicate that increasing the total number of I/R cycles decreases skin blood flow and TcPO$_2$, and increases the necrotic area in the treatment site and average leukocyte extravasation. (* indicates statistically significant difference from previous group, $p < 0.05$.)
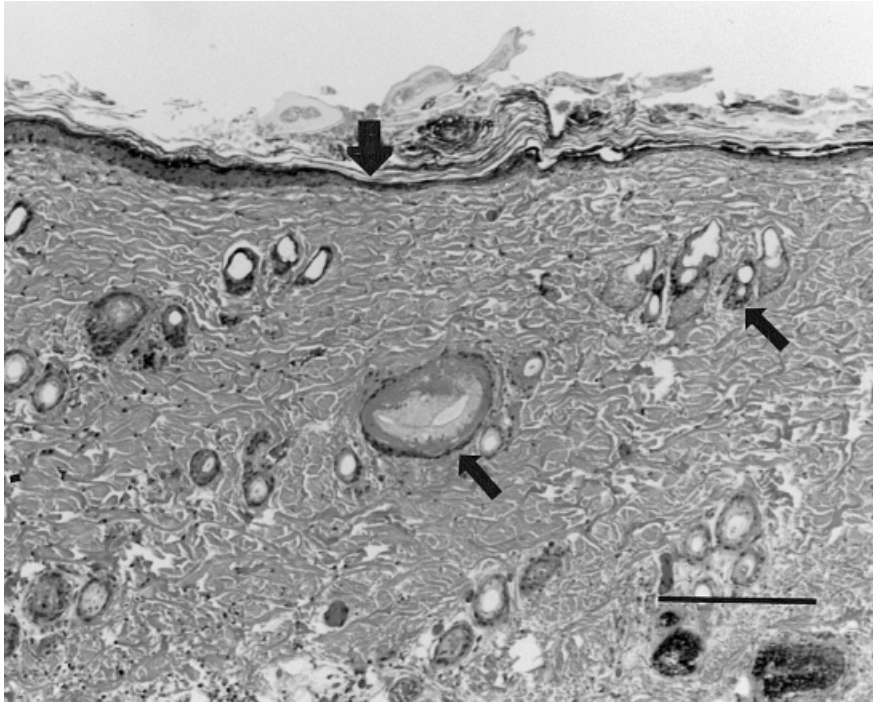
Figure 4. Photomicrograph of treated skin (5 total cycles of 2.0 hours of ischemia followed by 0.5 hours of reperfusion.) Large arrow denotes border between skin adjacent to treated site (left of arrow) and skin subject to ischemia (right of arrow). To the right of the arrow, note necrosis of epidermis and early necrosis of follicular units (small arrows), characterized by more intense eosinophilia of cytoplasm and nuclear pyknosis. Also note absence of crush injury signs (hematoxylin and eosin, scale bar = 200 μm).

measured at a constant anatomical location within the treatment site both decreased as the total number of I/R cycles increased. The skin thickness decreased with increasing total number of I/R cycles (data not reported). Figure 4 shows a photomicrograph of a skin section treated with 5 total cycles of I/R adjacent to an untreated region. The region subjected to the I/R cycles shows signs of early necrosis in the epidermis and follicular units. Furthermore, there is an absence of evidence for crush injury, indicating that the necrosis is a result of I/R injury.

Tissue damage was significantly increased when the duration of ischemia was increased from 1 to 2 hours per cycle (Group F vs. Group G: Figure 5). Furthermore, when the frequency of I/R cycles was increased from 5 to 10 cycles per day, holding the total
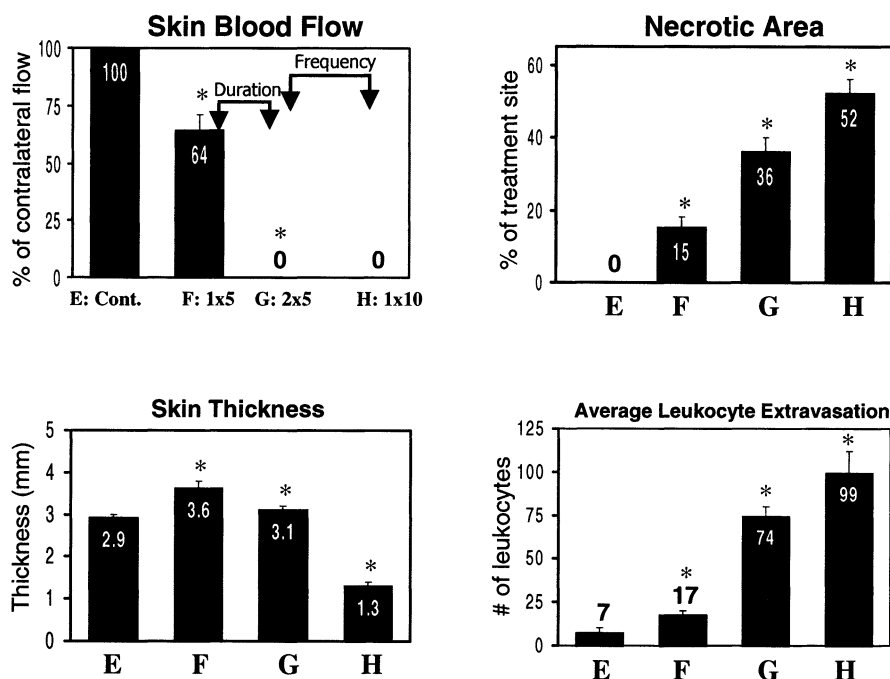
Figure 5. The results from experiment #2 show that increasing duration and frequency of I/R decreases skin blood flow and skin thickness. Increased necrotic area in the treatment site and average leukocyte extravasation were also seen. (* indicates statistically significant difference from previous group, $p < 0.05$.)
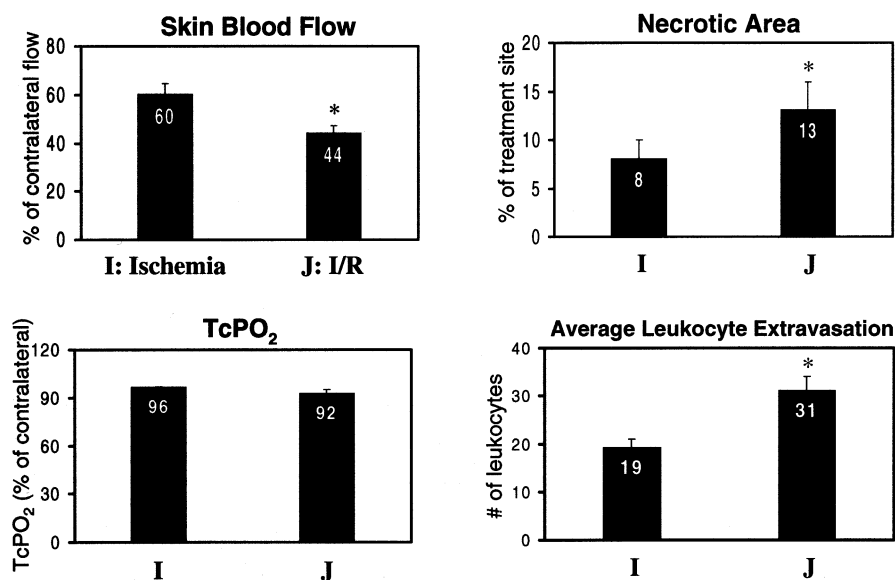
Figure 6. The results from Experiment #3 show that I/R-induced injury decreases skin blood flow and skin thickness, and increases necrotic area in the treatment site and average leukocyte extravasation compared to the ischemia-alone group. (* indicates statistically significant difference from previous group, $p < 0.05$.)

hours of ischemia throughout the entire experiment constant (50 hours), the tissue injury increased: Group G vs. Group H (Figure 5). As with the previous experiment, the increase in tissue damage was marked by an increased area of necrosis and leukocyte extravasation at the treatment site border and decreased skin blood flow and skin thickness. The skin thickness measured at the center of the treatment site also decreased in Groups G and H, but increased in Group F compared to the control, indicating edema in the wound.

The third experiment indicated that 5 I/R cycles that delivered a total of 10 hours of ischemia were more damaging to the skin than one continuous compression that also delivered 10 hours of ischemia (Figure 6). This increase in damage was indicated by an increase in necrotic area and an increase in leukocyte extravasation in the I/R group (Group J) compared to the ischemia-alone group (Group I). The skin thickness at the center of the wound decreased in Group J compared to Group I, though not significantly (data not reported).

DISCUSSION

In this study, a model of reproducible and controlled I/R skin injury was developed and characterized in un-anesthetized rats using applied pressures, pressure durations, and I/R cycles that are clinically relevant to pressure ulcer formation. Clinically, I/R injury is an important component of the etiology of pressure ulcers.[1,2] For the immobile patient, tissue ischemia over bony prominences on which the patient is lying is a persistent problem. With scheduled turning regimens this ischemic tissue is repeatedly exposed to subsequent reperfusion injury.

The clinical relevance of this animal model was supported by the visual changes that occurred in each treatment site which underwent the I/R injury procedure. The skin presented the following sequence of stages that is also seen in the formation of pressure ulcers in human patients: blanchable hyperemia, non-blanchable hyperemia, ecchymosis, and finally tissue necrosis. The skin's progression through this sequence occurred rapidly and was accelerated with increases in total number of I/R cycles, duration of ischemia, and I/R cycle frequency.

The results indicated that the extent of total tissue damage, quantitated by skin thickness, leukocyte extravasation, skin blood flow, $TcPO_2$, and percentage necrotic area, was also attributable to the total number of I/R cycles, duration of ischemia, and I/R cycle frequency. Tissue damage increased with increases in each of these parameters, and when the total extent of ischemia was constant, a greater number of reperfusion events during that period resulted in increased tissue damage.

Furthermore, the results documented that repeated I/R injuries were more damaging to tissue than prolonged ischemia alone. Ten hours of continuous ischemia caused 8% of the treated area to become necrotic, whereas when the 10 total hours of ischemia was comprised of 5 I/R cycles, the area of necrotic tis-

sue increased significantly to 13%. Thus, it was concluded that the reperfusion phase of the I/R cycle is an important component of the total injury produced. Possible hypotheses for the increase in damage due to the reperfusion phase include the presence of toxic oxygen free radicals, inflammation and recruitment of neutrophils and macrophages, and capillary plugging by leukocytes.

The 5 I/R cycle group had decreased skin blood flow and decreased $TcPO_2$ compared to the ischemia-alone group. Furthermore, it was noted that there was a larger disparity in skin blood flow between these groups than in $TcPO_2$ between these two groups. The explanation for this result is that skin blood flow was measured over the entire area that was treated with the I/R cycles, including the necrotic area at the center of the treatment site. This necrotic region, compromising a larger percentage in the 5 I/R group than in the ischemia-alone group, had zero blood flow. These zero values were averaged into the total blood flow, effectively augmenting the difference in average blood flow between the two groups. In contrast, the necrotic area does not contribute to the $TcPO_2$ measurement because the $TcPO_2$ measurement was taken at the distal end of the flap over a region that did not contain any necrotic tissue. The greater decrease in the blood flow metric is a result of the incorporation of the necrotic area (zero blood flow) into the reported value. Thus, there was a larger disparity in skin blood flow between these groups than in $TcPO_2$ between these two groups.

All animals were analyzed within 12 hours of ending the I/R regimens. The amount of tissue injury that would occur with extended recovery time will have to be determined in future studies. The longest experiment in this study lasted for 5 days and a total of 50 I/R cycles, therefore future studies are also needed to examine the long-term effects of I/R injury.

This model has many advantages over past models, including its clinical relevance and ability to be used for large scale inexpensive testing. Because the I/R cycle was applied only to the skin and not to the underlying muscle or bone, the resulting ulcer formed only in the skin. These ulcers are particularly relevant to pressure ulcers seen in the elderly where there is a lack of significant underlying muscle and where the skin is relatively thin. The model was developed in a small animal, and because it produces ulcers that are remarkably repeatable, it can be used as a relatively inexpensive tool to test the efficacy of various treatments, such as growth factors, angiogenic factors, or tissue engineered skin replacements, on a large scale.

A wide range of ulcer stages can also be generated by varying the total number of I/R cycles, durations of ischemia, and cycle frequencies. Using this approach, the model can be used to study different pressures and I/R protocols to prescribe clinical treatments such as patient turning cycles and pressure relief systems. Additionally, physical and biological parameters can be defined as markers of "at-risk tissue." With the knowledge of such markers, patients can be tested for the presence of these markers upon admittance to the hospital and the appropriate precautions can be taken to prevent pressure ulcers from forming. Finally, preventative techniques can be improved by using the model to study skin adaptation to I/R cycles.

This model not only has the potential to improve diagnosis, treatment, and prevention of pressure ulcers, but the ulcers created by the model are large enough to accommodate a subsequent wound, allowing the model to be used to study wound healing in impaired tissue. With this range of possible applications, this model can be used as both a testing tool in an industrial setting and as a means to study the basic etiology of I/R injury in chronic skin wounds.

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APPENDIX

To calculate magnetic field strength from the applied pressure value, Equation 1 is used to obtain the force required to produce the desired pressure over the skin flap area:

$$F = A \times P \quad \text{Equation 1}$$

where F = compressive force, A = area of application, and P = pressure. A compressive force equal to 5.99 N was required to produce 50 mm Hg of pressure over the 9 cm^2 skin flap area.

The magnetic energy, W, stored in the skin-filled gap between the magnet and the plate is given by Equation 2:[24]

$$W = [h \times A \times (B)^2] / (2 \times \mu_o) \quad \text{Equation 2}$$

where h = gap width between the magnet and ferromagnetic steel sheet, i.e., skin thickness (cm), A = surface area of the magnet (9 cm^2), B = magnetic field flux density (Gauss), and μ_o = magnetic permeability of the material within the gap (skin or air = 1.26×10^6 Henrys/m^2).

The resulting compressive force, F, is found by taking the first derivative of magnetic energy (given by Equation 2) with respect to the gap distance:

$$F = [d(W)] / dh = [A \times (B)^2] / (2 \times \mu_o) \quad \text{Equation 3}$$

The magnetic field flux density, B, can then be solved for in terms of A, F, and μ_o to obtain:

$$B = [(F \times 2 \times \mu_o) / A]^{1/2} \quad \text{Equation 4}$$

The desired compressive force, F, equal to 5.99N (given by Equation 1), the area of the magnet, A (a known quantity that is determined by the size of the desired flap), and magnetic permeability, μ_o (a given material property of skin) are substituted into Equation 4. The magnetic field flux density, B, is calculated to equal to 0.1295 Tesla or 1295 Gauss (1 Gauss = 10^{-4} Tesla). A 1250 Gauss magnet was used in this study because of availability. The magnetic flux density of this magnet yields a compressive force of 5.58N according to Equation 3. When this force is applied over the 9 cm^2 area, a pressure of 47 mm Hg is calculated.

The following assumptions were made with regard to the experimental design: 1) Magnetic field strength is homogeneous throughout the surface of the magnet; 2) magnetic permeability of skin is equal to that of air; and 3) the magnet completely covers the skin and underlying steel sheet.