Ischemia-Reperfusion Injury-Induced Histological Changes Affecting Early Stage Pressure Ulcer Development in a Rat Model

Li-ping Jiang, PhD, RN; Qian Tu, RN; Yanyan Wang, RN; and En Zhang, RN

Abstract

Pressure ulcers (PU) are caused by the interplay of multiple factors including skin microcirculation. Ischemia-reperfusion (I/R) injury is considered a significant mechanism in the early stages of pressure ulcer development. The objective of this controlled, single-blinded in vivo study was to create a pressure-induced injury rat animal model and explore the possible mechanism and effects of I/R injury in early stage PU development using clinically relevant amounts of pressure and pressure duration. Forty-eight animals were randomly divided into six groups of eight and a 2.5 cm x 2.5 cm area of the hip was subjected to no pressure (control), ischemia only (I/G - 2 hours of 70 mm Hg pressure), or one of four I/R cycles (70 mm HG of pressure for 2 hours followed by 1, 2, 3, or 4 hours of reperfusion). All I/R cycles were repeated three times. Full-thickness skin samples from the compressed area were harvested for histopathology and femoral artery blood samples obtained to measure serum levels of the following inflammatory mediators: malondialdehyde (MDA), superoxide dismutase (SOD) nitric oxide (NO) and endothelin-1 (ET-1). MDA, NO, and ET-1 levels were significantly higher in the IR than the control (P < 0.01) and ischemia groups (P < 0.05); whereas, SOD activity was significantly lower than in the IG and control groups (P < 0.05). The largest differences were observed in the 2-hour ischemia/3-hour reperfusion group. Biopsy analysis by light-microscopy stain showed no changes in the control, mild changes in the IG, and considerable damage, including leukocyte infiltration, collagen fibrosis, and edema in epidermal, dermal, and muscle tissue from the I/R group. These findings suggest that hypoxic-ischemic tissue injury occurs early following a period of ischemia and that I/R may be an important mechanism in PU development. Although the mechanisms of I/R injury are probably multifactorial and the actions of free radicals may be more complicated in the early stages of PU development in humans, the findings suggest that a minimum of 4 hours pressure relief may be helpful for PU prevention. Research to elucidate these mechanisms and their potential interactive effects to help clinicians develop evidence-based prevention protocols are warranted.

Key Words: pressure ulcer, ischemia-reperfusion, injury

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Potential Conflicts of Interest: none disclosed

A pressure ulcer (PU) is a localized injury to the skin and/or underlying tissue, usually over a bony prominence, as a result of pressure or pressure in combination with shear and/or friction. PU development prolongs hospitalization, increases disability, interferes with patient rehabilitation, and may contribute to patient death. Despite extensive work directed toward PU prevention and treatment, clinical outcomes have not appreciably changed over the past decades. This may be related to the fact that the pathophysiology of PU formation is still incompletely understood.

Early PU studies suggested that impaired or disrupted tissue integrity is the result of prolonged pressure. PU severity

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ranges from erythema of intact skin to tissue destruction involving skin, subcutaneous fat, muscle, and bone. The tissue injury can lead to loss of the stratum corneum, development of hyperemic thrombi in capillaries and venules, and ultimately necrosis and ulceration of the dermis and epidermis. Most animal models of chronic PU were designed to study the role of ischemic injury in wound formation, by, for example, applying cutaneous pressure. In early studies, histopathologic changes were found to occur in tissues subjected to as little as 60 mm Hg for 1 hour because at pressures >35 mm Hg, capillaries will close and ischemic injury occurs. Kosiak deduced an inverse relationship between pressure amount and duration. Daniel et al linked PU development with pressure-induced ischemia through the use of a continuously monitored, computer-controlled, electromechanical pressure applicator. These results indicated that muscle injury occurs first and that muscle tissue is more susceptible to pressure than skin tissue. However, these models employed one-time constant pressure applications that may not accurately reflect how tissue pressure occurs in the clinical setting.

Shayn developed an animal model to study the role of ischemia-reperfusion (I/R) in the PU development using small, unanesthetized animals whose skin was periodically compressed using 50 mm Hg. Jiang hypothesized that the cyclic application and removal of pressure can cause I/R injury in the skin if the pressure is great enough to substantially reduce blood flow throughout its application period.

As result of these early studies, I/R injury now is considered a significant factor in the etiology of PU. After I/R has occurred, an inflammatory response is incited and a mass of inflammatory mediators is released in the ischemic tissue which, in turn, results in the overproduction of cytotoxic oxygen-derived free radicals produced from myocytes such as malondialdehyde (MDA), superoxide dismutase (SOD), endothelin-1 (ET-1), and nitric oxide (NO) changes in blood plasma.

With an I/R injury, tissue and serum MDA levels increase and SOD enzymatic activity decreases. SOD activity is thought to prevent free-radical formation and lessen or inhibit the damage caused by postischemic reperfusion. ET-1 regulates the release of vasoactive substances and stimulates smooth muscle mitogenesis. NO mediates a variety of biological functions, including endothelium-dependent vasodilatation, oxygen radical scavenging, inhibition of platelet aggregation, and reduction of leukocyte-endothelial cell adhesion in the early stage of reperfusion. Administration of exogenous ET-1 and/or NO may help maintain physiological vascular tone, subsequently protecting the endothelium from oxygen-free I/R injury primarily caused by oxygen radicals.

Changes in these mediators can produce further tissue damage. Histological evidence indicates excessive free radicals will mediate tissue injury and ET-1 and NO changes will affect bioactivity on the vascular walls.

Free radicals can generate the lipid peroxidation process. After I/R injury in a rat model, subsequent bleeding was thought to be related to overproduction of reactive oxygen species (ROS), which is involved directly in lipid peroxidation, especially for hydroxyl radicals. I/R injury induces vasospasm through blood vessel contraction or NO depletion. In animal models, levels of MDA and SOD are common markers of oxidative stress and antioxidant status.

Based on a variety of pressure models, it is clear that localized pressure results in the accumulation of metabolic byproducts and cell necrosis. With intermittent pressure, I/R injury will lead to hypoxic-ischemic tissue damage. The objective of this controlled, single-blinded in vivo study was to create a pressure-induced injury animal model and explore the possible mechanism and affects of I/R injury in early stage pressure ulcer development using clinically relevant amounts of pressure and pressure duration.

**Materials and Methods**

**Animal model.** This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Wenzhou Medical College (WMC) and was carried out in compliance with institutional guidelines for care and use of animal models. A total of 48 Wistar rats (24 male, 24 female, body weight 220 g ± 20 g) were obtained from the Laboratory Animal Center of Wenzhou Medical College. Each animal was housed under standard laboratory conditions (12 hours light, 12 hours dark cycles, temperature 2˚ C ± 12˚ C, humidity 60% ± 5%) and maintained on standard laboratory food and water.

An animal model for early stage pressure ulcer development was set up using clinically relevant pressures and durations. Specifically, a protocol of a 2-hour period of ischemia, followed by 1 to 4 hours of reperfusion, is clinically relevant because it is recommended that patients at risk for developing pressure ulcers should be turned at least every 2 hours. This I/R protocol, using 70 mm Hg, previously has been shown in a rat animal model to produce significant skin necrosis with
ulcer formation. After completing three cycles of intermittent pressure, full-thickness skin from the compressed area was harvested for histopathologic and tissues examination.

Experimental groups. The 48 rats were randomly divided into six groups, eight rats per group, one thigh injury per animal. The control (no treatment) and ischemia-only (IG) groups sustained compression for 2 hours. The I/R groups were further divided into four subgroups according to the following reperfusion schedule: 1) 2 hours ischemia plus 1 hour reperfusion (I/R1hG), 2) 2 hours ischemia plus 2 hours reperfusion (I/R2hG); 3) 2 hours ischemia plus 3 hours reperfusion (I/R3hG), and 4) 2 hours ischemia plus 4 hours reperfusion (I/R4hG) (see Table 1).

Animals were anesthetized with subcutaneous injections of 10% chlorohexal (300 mg/Kg body weight). Anesthesia was maintained with isoflurane inhalation (0.4% to 1.0% isoflurane with N₂O/O₂ mixture 1:1) during all procedures. Vital signs (pulse and respiratory rate) were monitored and maintained within the normal physiological range. The anesthetized rats were placed supine in the loading device.

Table 1. Experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of animals</th>
<th>Intervention</th>
<th>Pressure-relief cycle</th>
<th>Number of ischemia/reperfusion cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>No compression</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ischemia</td>
<td>8</td>
<td>Sustained compression for 2 hours</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>I/R1hG</td>
<td>8</td>
<td>Compression 2 hours, relief 1 hour</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>I/R2hG</td>
<td>8</td>
<td>Compression 2 hours, relief 2 hours</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>I/R3hG</td>
<td>8</td>
<td>Compression 2 hours, relief 3 hours</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>I/R4hG</td>
<td>8</td>
<td>Compression 2 hours, relief 4 hours</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

After three cycles of I/R or 2 hours of sustained compression in the non-I/R groups, full-thickness skin samples from the compressed area were harvested and histopathology analysis performed. Tissues were fixed in 10% buffered formalin, embedded in paraffin wax, and then sliced at thickness of 5 µm. The slices were stained with conventional hematoxylin and eosin (H&E) in order to show the changes of microanatomy. At the same time, blood was collected from the femoral artery to measure serum the levels of MDA, NO, and ET-1 and SOD activity in plasma.

Biochemical analyses. Ten (10) mL plasma was put in tubes without anticoagulant and the serum collected. The blood serum was centrifuged at 3,600 rpm for 10 minutes at 4°C and the supernatant was frozen at -70°C. Colorimetric analysis was employed to measure NO, MDA, and SOD activity according to laboratory kit instructions (kits provided by Jiancheng Institute of Bioengineering, Nanjing).

The MDA content was determined spectrophotometrically by measuring the presence of thiobarbituric acid-reactive substances (free fatty acids with lipid peroxidation aldehydic products). Results are expressed as nmol/g.

SOD enzyme activity was determined by the production of H₂O₂ from xanthine by xanthine oxidase and reduction of nitroblue tetrazolium using spectrophotometry. Results are expressed as µ/g.

Tissue nitrate (NO₃) and nitrate (NO₂) levels were used to estimate NO production. Quantification of NO₂ and NO₃ was based on the Griess reaction. Results are shown as µmol/g.

Measurements of ET-1 were performed with 30 µl 10% disodium ethylene diamine tetraacetate (EDTA•Na₂) and 40 µl aprotinin. Radioimmunoassay was employed to assess amounts of ET-1. Results are expressed as pg/mL.

Histological assessment. Biopsied samples were immersed in buffered 10% formalin. Slides were prepared for light microscopy and stained with conventional H&E. Histological changes of the epidermis, dermis, and muscle were evaluated by microscope (Olympus, Japan) and blinded to the animal’s group assignment. The pathological changes of tissue were assessed as ranging from no changes (normal) and mild changes to severe according to integrity of skin structure such as dermal infiltration of inflammatory cells and collagen and capillary and edema formation in the epidermis, dermis, and muscle.

Statistical analysis. Results of one-way analysis of variance (ANOVA) test for statistical significance analysis of variance for MDA, NO, SOD, and ET-1 levels are presented as mean ± standard deviation (SD). Comparisons were tested by Student-Newmann-Keuls test. A P value of <0.05 was considered significant.
statistically significant. SPSS 11.0 (Chicago, IL) was used for data analysis.

Results

Biochemical assays. Average plasma SOD levels ranged from 225.88 ± 25.19 µ/L in the ischemia to 174.45 ± 46.61 in the I/R after 3 hours group (see Figure 1). The difference between the control and ischemia group and all four I/R groups was statistically significant ($P < 0.05$), with the largest difference observed between the I/R3hG and control group ($P < 0.01$).

Mean MDA levels were lowest in the control group (2.03 ± 0.93) and slightly higher in the ischemia group (2.97 ± 1.48). MDA levels in all I/R groups were significantly higher than the control ($P < 0.01$) and ischemia groups ($P < 0.05$) (see Figure 2). However, levels of SOD enzyme and MDA were not significantly different between the IG and control groups.

As with MDA levels, the lowest levels of ET-1 and NO were observed in the control (average 89.22 ± 2.62 and 95.45 ± 17.52, respectively) and the ischemia group (average 91.55 ± 1.23 and 92.04 ± 17.81, respectively) and the highest levels were observed in the 3-hour pressure-relief groups (100.94 ± 3.14 and 155.87 ± 10.67 (see Figures 3 and 4). ET-1 and NO levels were somewhat lower in the 4-hour pressure-relief groups but remained statistically significant compared to the control and ischemia groups ($P < 0.01$).

Histological assessment. Analysis of the biopsies by light-microscopy (H&E) stain showed pressure-induced tissue changes. In the control group, no histological changes were observed (see Figure 5a,b). However, tissue biopsies from the ischemia group showed mild changes (see Figure 5c,d) and severe skin and muscle damage was observed in the group exposed to 2 hours of pressure followed by 1 to 4 hours of reperfusion (see Figure 5e,f).

Discussion

In this PU model, I/R injury and subsequent re-oxygenation of certain hypoxic myocytes was found to result in the release of free radicals. The mechanism underlying the observed higher levels of MDA and NO and lower levels of the SOD enzyme is most likely multifactorial and interdependent and involves hypoxia, inflammatory responses, and free-radical damage. This finding is in accordance with results of flap-transfer studies in a rat model, which showed tissue reperfusion injury, including the presence of free radicals and neutrophils. ET-1 levels were also higher after reperfusion (see Figure 3), a change that may be mediated by intravascular endothelial swelling and ET-1 mediated microvascular constriction.

Interestingly, in this study, the difference in the average levels of biochemical predictors in the plasma of control tissue and tissue subjected to pressure/reperfusion increased with increasing reperfusion time and decreased
One explanation may be that the pressure was applied on skin in the gluteus area, reducing capillary blood flow. Skeletal muscle may be more sensitive to ischemia than skin and exacerbate the potential for tissue necrosis. Using a rat model, Peirce et al reported that soft tissue could bear more pressure loads and for longer duration than muscle. Using laser Doppler flowmetry, Salcido et al measured muscle blood perfusion and their findings suggest that muscle damage correlates with blood ischemia. Two reviews propose that the activation and infiltration of polymorphonuclear cells and concomitant interaction with activated endothelial cells play a key role in reperfusion injury; the current study's histological findings, showing infiltration of polymorphonuclear cells in the dermis and muscle, confirm these observations. It is hoped that results of this study will be help increase understanding of the mechanism behind the development of so-called deep-tissue injury PUs.

**Conclusion**

Using an animal model for pressure ulcer development and clinically relevant periods of pressure/ischemia (2 hours) and pressure-relief/reperfusion (1, 2, 3, and 4 hours), significant differences in the plasma level of inflammatory mediators were found. Levels of MDA, ET-1, and NO were significantly higher in the ischemia group and their findings suggest that muscle fibrosis in dermis in I/R groups after three cycles; F. Severe damage — muscle fibers edematous/not clearly visible. Evidence shows IC infiltration in I/R groups after three cycles.

slightly in the 4-hour reperfusion group. This suggests that most damage occurs during early reperfusion — ie, 1 to 3 hours following pressure relief — whereas, a prolonged period of pressure-relief (>4 hours) may help tissues recover. Houwing et al reported that after 4 hours of I/R, cytotoxic activity of free radicals decreased because deposits of glycogen and ATP were absent and cells could no longer restore them during reperfusion. This was different from previously described PU studies of short-term ischemia wound formation or simple reperfusion cycles. The prolonged release time is advantageous to the degradation of MDA, SOD, and NO.

The current study results indicated that injury occurred in early-stage PUs in the epidermis, dermis, and muscle. I/R injury are probably multifactorial and the actions of free radicals may be more complicated in the early stages of PU development in humans as compared to the rat model. Seemingly, a minimum of 4 hours for the relief of local pressure might be helpful for PU prevention. Further research is needed to elucidate these mechanisms and potential interactive effects.

**Acknowledgment**

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