

Analysis of ischemia-reperfusion injury in a microcirculatory model of pressure ulcers

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The aim of this study was to establish a pressure ulcer model that visualizes the microcirculation, and to examine the participation of ischemia-reperfusion injury in the pathophysiology of pressure ulcers. An original system composed of a new skin fold chamber and compression device allowed loading quantitative vertical stress to the skin. An intravital microscopic technique enabled direct visualization of the microcirculation in the physiological condition and in response to pressure application. To estimate the effect of ischemia-reperfusion injury, animals were divided into two groups: the compression-release group ($n=8$), in which the animals received four cycles of compression-release which consisted of 2 hours of compression followed by 1 hour of pressure release; and the compression alone group ($n=8$) in which the animals underwent continuous compression for 8 hours. Functional capillary density was quantified before the compression procedure and on day 1 (35 hours) after the first evaluation. The cyclic compression-release procedure significantly decreased functional capillary density as compared to continuous compression, indicating that in our experimental setting repetition of ischemia-reperfusion cycle more severely damaged the microcirculation than single prolonged ischemic insult. This finding supports the significant contribution of ischemia-reperfusion injury to the pathophysiology of pressure ulcers at the level of dynamic in vivo microcirculation. (WOUND REP REG 2005;13:209-215)

Although interruption of microcirculation obviously plays a significant role in the pathophysiology of pressure ulcers, the exact mechanisms still remain unclear. Prolonged pressure applied to the skin usually interrupts capillary perfusion, resulting in ischemic soft tissue necrosis or pressure ulcer formation. On the basis of this conventional mechanism, nursing care guidelines for prevention of pressure ulcers have traditionally recommended position changes for bedridden patients according to scheduled regimens to avoid prolonged pressure on the specific anatomical sites. However, clinicians occasionally encounter

FCD	Functional capillary density
FITC	Fluorescein isothiocyanate

development of pressure ulcers in spite of a proper position change regimen and nursing care. Such normal clinical experience suggests that some mechanism(s) other than simple ischemia may participate in the formation of pressure ulcers.

Ischemia-reperfusion injury has become a major concern in many fields of medicine.¹⁻⁵ A number of investigations have shown that impairment of function in various organs is easily attributable to the process of ischemia-reperfusion injury. Ischemia-reperfusion injury has been defined as cellular injury resulting from the reperfusion of blood to a previously ischemic tissue.⁶ When a tissue has been depleted of its blood supply for a significant period of time, the tissue may reduce its metabolism to preserve function. The reperfusion of blood to the nutrient- and oxygen-deprived tissue can result in a cascade of harmful events. Reperfusion produces levels of oxygen-derived free radicals that exceed the capacity of constitutive free radical scavenging mechanisms, thus causing a cytotoxic effect on the tissue.⁶ In addition, many other chemical

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mediators are known to be involved in ischemia-reperfusion injury.^{4,7} Studies of ischemia-reperfusion injury in various organs of the body, principally the brain, heart, kidney, liver, skin, and intestine, have suggested that the etiology of ischemia-reperfusion injury is distinct from that of injury caused by a single ischemic insult.

It is likely that not only ischemic necrosis but also ischemia-reperfusion injury contribute to the formation of pressure ulcers.⁸⁻¹⁰ Therefore, previous studies using experimental animal models have attempted to verify the role of ischemia-reperfusion injury in the development of pressure ulcer. However, from the perspective of modern techniques in the field of microcirculation, they have failed to offer sufficient evidence to understand the *in vivo* microcirculatory response in the development of pressure ulcers.

Microcirculation has been a matter of concern and extensive study in our institution, employing an intravital microscope-video-computer system that allows direct visualization and quantitative analysis of the microcirculatory structure and hemodynamics.^{11,12} In this report, we present an experimental animal model for pressure ulcers using an originally designed skin fold chamber. Under our experimental system, the model visualizes the microcirculatory response following pressure application by a newly developed quantitative pressure delivery device. Using this model, we examined the participation of ischemia-reperfusion injury in the pathophysiology of pressure ulcers.

MATERIALS AND METHODS

Adult male ddY mice (30–40 g) were acclimatized to their holding facility for at least 5 days before the start of the experiment. Animals were treated according to the *Principles of Laboratory Animal Care* (NIH publication 86-23, revised 1985).

Establishment of experimental model

We manufactured an improved version of our original chamber previously reported.¹³ The structure of this chamber (Yasuhisa-koki, Ltd., Tokyo, Japan) is shown in Figure 1A. The new chamber consists of a plastic sheath (inside diameter = 8.0 mm), a pressure tip (outside diameter = 7.5 mm, Figure 1B), and three fixing screws in addition to the former parts. The compression area of the tip is a circular cross section 2 mm in diameter (Figure 1C).

The skin fold chamber was inserted under general anesthesia by flothen (inhalation anesthesia). The dorsal skin was pulled up and fixed to form a skin fold. A square area of one layer of skin (about 7 mm on one side) was removed, and the remaining layer was cov-

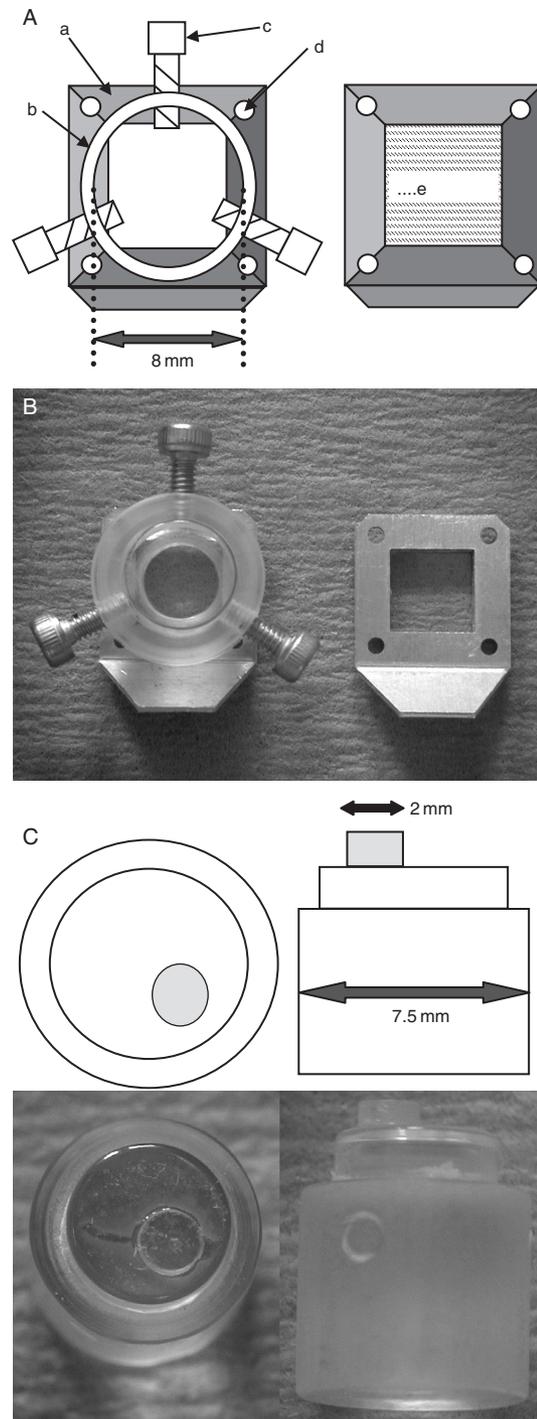


FIGURE 1. Appearance of the microcirculatory analysis chamber. (A) Schematic view of the chamber. The chamber consists of two aluminum frames held together with four polyethylene tubes. One frame is equipped with a plastic sheath and three small screws. The other one is furnished with a glass window for observation. a: aluminum frame; b: plastic sheath; c: fixing screw; d: hole drilled for polyethylene tube to hold two frames together; e: glass window for intravital microscopic observation. (B) Photograph of actual analysis chamber. (C) The compressing region is a 2 mm diameter cylindrical shape. The tip slides in the plastic sheath smoothly in an almost parallel motion to compress the skin perpendicularly.

ered with a cover-glass incorporated in one of the frames of the chamber (Figure 2A). To allow each animal to adjust to the skin fold chamber, mice were used for experiments after more than 48 hours from implantation as a recovery period (Figure 2B). The compressing instrumentation combining a tension gauge and an articulated manipulator could load vertical compressive stress onto a certain area through the original tip. Three screws fixed the compressing tip rigidly and kept it applying pressure at a set point (Figure 3).

The microcirculation in the chamber was inspected with a stereo intravital microscope (SZH10, Olympus Corporation, Tokyo, Japan) coupled with a charge coupled equipped (CCD) color video camera (DXC-107 a, Sony, Tokyo, Japan). To achieve sufficient transillumination of the tissue in the chamber, a 150 W halogen projection lamp (Nikon, Kanagawa, Japan) was used as a light source.

For contrast enhancement of the intravascular space, 0.1 ml of fluorescein isothiocyanate-labeled dextran (FITC-dextran; average molecular weight. 2.5×10^6 , 2.5 mg/100 μ l of physiological saline solution; Sigma-Aldrich, St. Louis, MO) was infused intravenously from the caudal vein. Epi-illumination was achieved with a 100 W mercury lamp through a GFP filter. Microcirculation was imaged by the intravital microscope with a high-gain ICCD camera (C2400-89 V, Hamamatsu Photonics K.K., Shizouka, Japan).

The microcirculatory images were recorded on a hard disk video recorder (*Rec-On*, I-O Data, Ishikawa, Japan) together with time and frame counts (VTG-33, FOR-A, Tokyo, Japan) for later analysis.

Estimation of ischemia-reperfusion injury

Sixteen mice were prepared for chamber implantation. They were randomly divided into two groups and subjected to one of the two following compression protocols. Total duration of compression was equally 8 hours in both groups. The compression pressure was set at 500 mmHg throughout study.

Group 1 was assigned to the compression-release group ($n = 8$). Each mouse received four cycles of compression release. One cycle consisted of 2 hours of compression and 1 hour of release. Through all of the cycles, mice were fed water and chow and were allowed to move freely in their cages in a controlled vivarium during and between compression periods.

Group 2 served as the compression alone group ($n = 8$). The dorsal skin of each mouse was compressed continuously for 8 hours. As in Group 1, animals were unanesthetized during all compressing procedure.

Intravital microscopic images were first recorded before the compression procedure. Reevaluation was performed on day 1 (35 hours) after the initial observation. The functional capillary density (FCD) was meas-

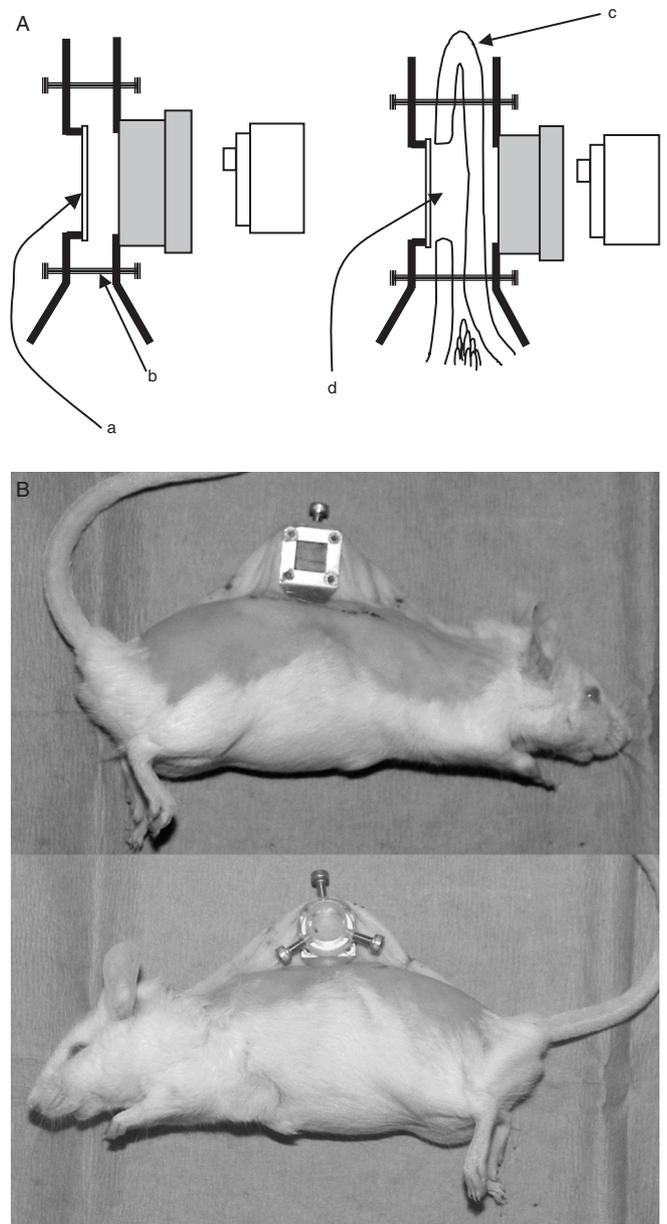


FIGURE 2. Skin fold chamber implantation. (A) Transectional view. The dorsal skin was drawn upward and a square area of one layer of skin was carefully removed to avoid damage to the underlying layer. Frames were implanted so as to sandwich the stretched double layer of the skin and fixed with polyethylene tubes. The remaining layer consists of the epidermis, subcutaneous tissue, and a thin layer of striated skin muscle. a: glass window; b: polyethylene tube was passed through each small hole and heat-melted at both ends to hold the two chamber halves together; c: drawn dorsal skin; d: removed square area of one layer. (B) General appearance of a mouse with the skin fold chamber in place.

ured in each image. The FCD was defined as the total length of capillaries with red cell flow (plasma contrast enhanced with FITC-dextran; Figure 4). It was analyzed using a computer-assisted microcirculation analysis system and was given in mm/mm^2 .

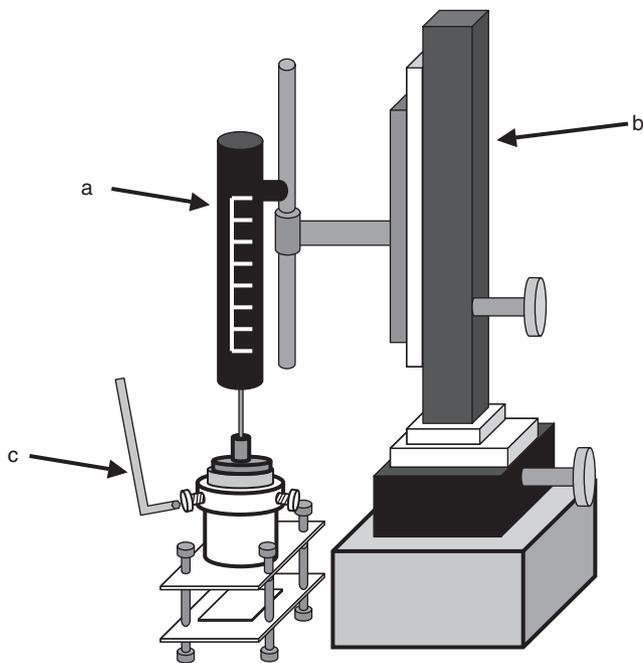


FIGURE 3. Schematic presentation of the apparatus for applying pressure to the skin. Pressure application consists of (a) a tension gauge and (b) an articulated manipulator. This instrument could load a quantitative vertical stress to the dorsal skin. The stress was maintained by tightening three screws with a metal wrench (c).

We defined the ratio of microcirculatory injury by the following equation

$$\text{Ratio of microcirculatory injury (\%)} = \{1 - (\text{FCD}_{\text{post}}/\text{FCD}_{\text{pre}})\} * 100$$

FCD_{pre} = FCD before compression

FCD_{post} = FCD on day 1 after the initial observation

Statistical analysis

FCD data were expressed as mean \pm SD. Differences between groups were compared by an unpaired Student's *t*-test. A difference of $P < 0.05$ was considered significant.

RESULTS

Our new chamber visualized the microcirculation in the skin and skin muscle under the glass window. Equipped with three holding screws, the chamber facilitated the maintenance of ischemia in the skin in the observation window while animals were free from anesthesia and moving without restriction in their cage.

The microscope-video-computer system dynamically visualized both acute and chronic changes of the

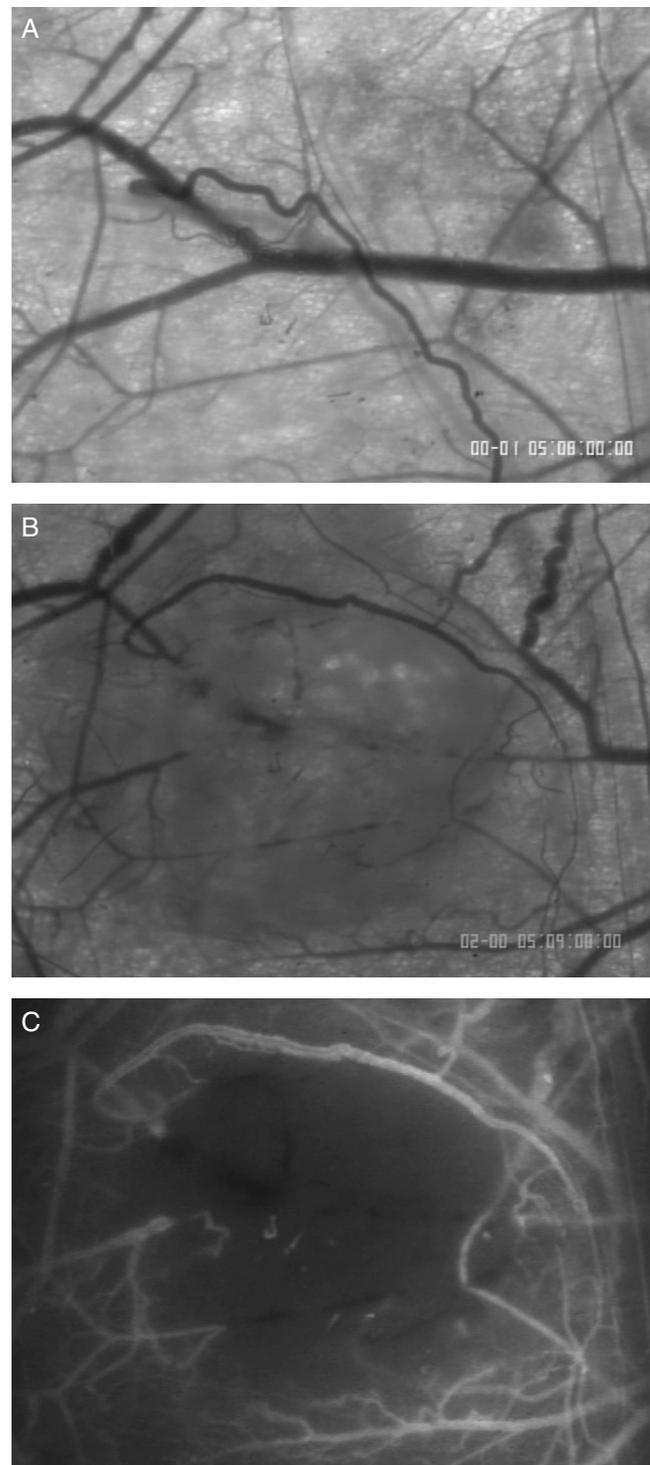


FIGURE 4. Intravital microscopic appearance of the skin under observation. (A) Precompression view of microcirculation. (B) Postcompression view at 1 day after the initial observation. Some vessels without reflux were recognized in the compressed area. (C) Fluorescent microscopy enhanced the plasma contrast with FITC-dextran and clarified the functional capillaries. FCD was calculated by analyzing these images.

microcirculatory structure and hemodynamics both in the physiological condition and in response to pressure

Table 1. Functional capillary densities and the ratios of microcirculatory injury of each group

Group 1	1	2	3	4	5	6	7	8	average
FCD _{PRE} * (mm)	2.874	2.826	3.171	3.354	2.562	2.901	4.118	3.218	3.128
FCD _{POST} (mm)	2.184	2.306	2.684	2.819	1.927	2.069	3.327	1.893	2.401
Ratio of microcirculatory injury (%)	24.0	18.42	15.93	15.93	24.8	18.68	19.21	41.18	23.23
Group 2	1	2	3	4	5	6	7	8	average
FCD _{PRE} (mm)	2.934	3.293	2.411	2.928	3.34	2.732	2.360	3.451	2.931
FCD _{POST} (mm)	2.745	2.901	2.411	2.765	3.218	2.07	2.028	3.451	2.621
Ratio of microcirculatory injury (%)	6.45	11.91	0	5.54	6.64	24.26	14.04	0	10.57

* Unit area for FCD values given in mm.

application. Furthermore FITC and the fluorescent microscope allowed direct visualization of functional capillaries.

In Group 1 (compression release), the ratio of microcirculatory injury was $23.23 \pm 8.53\%$ (Table 1). On the other hand, the ratio in Group 2 (compression alone) was $10.57 \pm 8.20\%$. The difference was highly significant ($P = 0.002694$; Figure 5).

DISCUSSION

Numerous studies dealing with pressure ulcers have regarded prolonged ischemia due to pressure-induced vascular occlusion as the principal etiologic factor for ulcer development.^{8,14,15} In 1959, one of the earliest reports appeared in the article of Kosiak.¹⁴ He applied external pressure over the femoral trochanter and the ischial tuberosity of dogs. His experiment, examining 62 sites in 16 dogs, revealed that either high pressure for a short duration or low pressure for a long duration could produce ischemic ulcers. This finding suggested that in the formation of pressure ulcers, an inverse relationship exists between pressure intensity and duration of pressure application.

Thereafter, most investigators employing animal models for pressure ulcers have designed their studies

to clarify only the role of ischemic injury in the wound formation, often using a single application of constant pressure.^{8,14,15} However, recent advances in medicine have strongly indicated significant involvement of ischemia-reperfusion injury as well as simple ischemic insult in the process of tissue impairment.^{1-5,10} This situation led us to design our present study to show that ischemia-reperfusion injury should be taken into consideration to give a better understanding of the pathophysiology of pressure ulcers.

Several previous studies have examined the microcirculatory disturbance induced by ischemia-reperfusion injury.^{10,16} In terms of modern technology in the field of microcirculation, one of the limitations of these previous investigations was their methodology. Most of them have evaluated histology or traditional parameters of tissue perfusion (e.g., laser Doppler flowmetry and transcutaneous oxygen tension).^{10,17-19} These methods characteristically measure indirect indicators of blood perfusion and do not allow distinct analysis of microcirculatory or hemodynamic mechanisms within individual segments of the microvasculature. Use of these indirect techniques has been criticized for providing only speculation as to the microcirculatory pathophysiology.²⁰ Another drawback of the prior studies is associated with their pressure ulcer models, which used relatively large animals, including rabbits and pigs, that required considerable expense and labor to carry out the experimental protocols.^{15,16,21,22}

The first step of the present study was the establishment of a more sophisticated experimental model providing the following features: a mouse as the subject, which is currently the preferred laboratory animal because of its size, the large variety of lines and disease models available; newly designed skin fold chamber allowing animals to behave freely under no anesthesia or restriction during the compression procedure; chamber and pressure delivery systems that can load and maintain the correct quantitative vertical stress on the microcirculation of the skin and skin muscle; intravital microscopic techniques that enable direct visualization

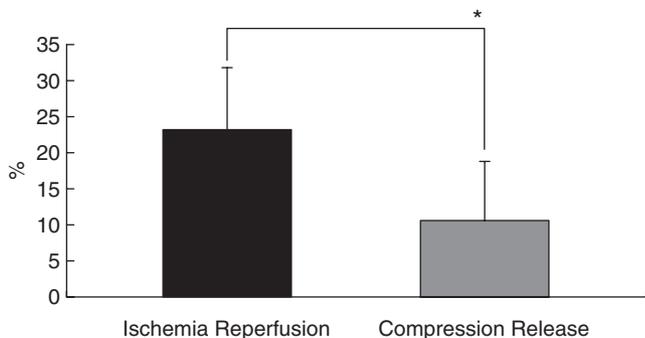


FIGURE 5. The ratio of microcirculatory injury caused by compression release was significantly more severe compared with that caused by prolonged compression ($P = 0.002694$).

and quantitative analysis of both acute and chronic changes of the microcirculatory structure and hemodynamics.

Using our new model we designed an experiment to clarify the role of ischemia-reperfusion injury in pressure ulcer development. To identify the loading pressure intensity appropriate for our experimental system, we had planned a preparatory experiment.²³ In our experimental setting, a pressure of 70 mmHg caused temporal microcirculatory occlusion, but the circulation resumed shortly after pressure application. The similar reopening phenomenon occurred even under pressure of 400 mmHg. This phenomenon may plausibly result from the adaptive response of the skin plasticity and the microcirculatory regulation to disperse and overcome stress loading. Thus, our pilot study had indicated that a pressure of 500 mmHg was necessary to maintain microvascular closure for a certain period of time even though the intensity seemed excessive. Several pieces of data in the literature support the rationale for this seemingly excessive pressure for ulcer development. In 1981, Daniel et al. designed a study involving pressure over the greater trochanter in pigs.¹⁵ In their model, initial tissue destruction was confined to the muscle, with muscle skin damage occurring only at a high pressure for a moderate duration (800 mmHg, 8 hours) or a low pressure for a long duration (200 mmHg, 17 hours). In 2000, Sundin et al. used pigs to study the contribution of ischemia-reperfusion injury to the formation of pressure ulcers.²⁴ In their report, pressure was applied to the scapulae for 210 minutes, and it was relieved for 30 minutes. According to this study, severe damage to the skin and underlying muscle occurred with a pressure of 350 mmHg, whereas only reactive hyperemia occurred with pressures of 100 mmHg.

In 2000, Peirce et al. histologically demonstrated in a rat model that 10 hours of continuous compression caused 8% of the treated area to become necrotic, whereas when the 10 total hours of compression was comprised of 5 compression-release cycles, the area of necrotic tissue increased significantly to 13%.¹⁰ They suggested participation of ischemia-reperfusion injury in pressure ulcer development.

In our current study, we followed a similar protocol comparing 8 hours of continuous compression with four cycles of compression (2 hours) and release (1 hour). The results showed that the cyclic compression-release procedure significantly decreased functional capillary density as compared to continuous compression. It indicated that in our experimental setting, repetition of the ischemia-reperfusion cycle more severely damaged the microcirculation than a single prolonged ischemic insult. Although different conditions, including the duration of ischemia, number of cycles, and pressure intensity, may likely induce different results,

the finding is evidence that there may be a significant contribution of ischemia-reperfusion injury to the pathophysiology of pressure ulcers at the level of dynamic in vivo microcirculation. Using our experimental model, further studies are in progress investigating detailed hemodynamics, chronic responses, and treatment interventions.

Clinically, nurses looking after bedridden patients are encouraged to change their position every 2–3 hours to avoid prolonged pressure on specific anatomical sites. Our experimental protocol aimed to mimic the clinical situation and the results suggested that conventional care alone occasionally fails to prevent pressure ulcers. One of the candidates to reduce this risk is the appropriate use of supporting surfaces that relieve pressure, as recent guidelines have recommended.²⁵ Health care professionals should take these findings into consideration in the management of critically bedridden patients.

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