Pressure ulcers (PUs) markedly affect patients' quality of life, morbidity and mortality, and account for considerable costs to the healthcare system. Approximately 2.5 million PUs are treated each year in acute care facilities alone with an estimated cost of $11 billion.1 The cost of treating a single pressure ulcer ranges from $500 to $40,000 depending on the severity of the wound.1 Although the prevalence of PUs is relatively low in Japan (3.64% calculated as existing cases of pressure ulcer patients divided by the number of all inpatients surveyed in a Japanese national surveillance study2) compared to the United States and European countries, the proportion of severe PUs in Japan is high; the proportions of Stage III and Stage IV PUs based on National Pressure Ulcer Advisory Panel (NPUAP) classification were estimated as 18.8% and 8.1% of all PUs, respectively.2 It has been shown that the high proportion of severe PUs is, at least in part, due to a newly recognized phenomenon, deep tissue injury (DTI).3,4

Previously, it was thought that PUs developed from the superficial skin into the deep tissue; recently, however, a new concept of DTI proposes that PUs can also develop from the deep tissue rising in muscle layers adjacent to bony prominences as a result of sustained loading.3,4 Therefore, DTIs are

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**Hypoxia is Involved in Deep Tissue Injury Formation in a Rat Model**

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**Abstract:** Pressure ulcers that develop from deep tissue are known as deep tissue injuries (DTI). Although several mechanisms, including ischemic hypoxia, are attributed to pressure ulcer formation, the mechanisms involved in DTI formation are still unclear. Previous studies have suggested that hypoxia is involved in DTI in vitro, but it has yet to be determined whether hypoxia is also involved in DTI in vivo. Therefore, this study aimed to investigate whether hypoxia is involved in DTI using a newly established DTI model. Rats were divided into control, low pressure DTI, and high pressure DTI groups. Results of wound healing tests indicated that more severe DTI resulted in prolonged healing time, more severe inflammation and muscle damage, higher levels of exudate creatine phosphokinase, and greater muscle edema. Increased hypoxia was observed in severe DTI—nuclear localization of hypoxia-inducible factor-1α was markedly increased in the high pressure DTI group, while the low pressure group showed more increased cytoplasm localization compared to the control group on day 3. Study results revealed that hypoxia is involved in DTI in vivo.
gaining increasing attention because they are difficult to
detect and also deteriorate rapidly compared to Stage III
and Stage IV PUs despite aggressive and optimal treat-
ment. The NPUAP classifies suspected DTIs as a different
form of PU. The mechanism involved in DTI formation
must be investigated for early initiation of treatment to
prevent deterioration, as described above. Four factors
are hypothesized to be causes of PUs: hypoxia as a result
of ischemia, reperfusion injury, impaired lymphatic
function, and prolonged mechanical deformation of tis-

tue cells. Although the mechanism of DTI might be sim-
ilar to these four factors, the mechanism involved in DTI
remains unclear.

Several studies have shown that hypoxia is one of the
mechanisms involved in DTI. Gawlitza et al11 suggested
the involvement of hypoxia and deformation in DTI in
an in-vitro model using engineered skeletal muscle pro-
duced from murine muscle cells. However, their study
used only bioengineered muscle in the absence of other
skin layers and blood vessels, which are essential factors
in the hypoxic condition. Therefore, it is difficult to con-
clude from this study whether hypoxia is involved in
DTI in vivo.

Linder-Ganz et al12 determined the presence of ischemia in an in-vivo DTI model using thermography. The
researchers resected the upper layer of the skin leav-
ing only the muscle tissue; pressure was then applied to
the muscle, thus rendering this model unrepresentative of
clinical DTI with an intact surface skin. Although ischemia was estimated by temperature, Davy’s13 thermog-
raphy data were highly influenced by environmental
conditions.13 Based on these studies, it is difficult to con-
clude that ischemia is involved in DTI in vivo. A study
using an in-vivo model was also conducted by Stekelenburg et al14 to examine the presence of ischemia in DTI using magnetic resonance imaging (MRI) to deter-
mine deep tissue damage in combination with histologi-
cal analysis to estimate the presence of ischemia. They
suggested that ischemia was involved in DTI in their
model. However, their findings are speculative because
they only examined the morphological changes of the
tissue. Although these two in-vivo studies suggested the
involvement of ischemia, further mechanisms remain
unknown. Taken together, these studies do not fully
explain whether hypoxia is involved in DTI. Therefore,
the present study was conducted to examine the involve-
ment of hypoxia in DTI in vivo. To investigate the pres-
ence of hypoxia, hypoxia-inducible factor-1α (HIF-1α)
was used as a marker.

When hypoxia persists for more than a few minutes,
cells respond by changes in gene expression. HIF-1 sig-
naling is extremely sensitive and is considered to be the
central effector of the cellular response to hypoxic condi-
tions.15–17 HIF-1 is a heterodimer composed of α and β
subunits. HIF-1β is constantly produced and is stably
maintained in hypoxic conditions.18 HIF-1α is also con-
tinuously expressed, but under normoxic conditions, it is
degraded by prolyl hydroxylase and von Hippel-Lindau
protein. In a hypoxic state, HIF-1α becomes stabilized
and translocates from the cytoplasm to the nucleus
where it is dimerized with HIF-1β to form an activated
HIF complex.19 The activated HIF complex then associ-
ates with hypoxia response elements (HREs) in the reg-
ulatory regions of target genes to induce their expres-
sion.20 Zhang et al21 used HIF-1α as a marker of muscle
ischemia in their flap model. HIF-1α expression was
markedly increased in the ischemic wound tissue. An-
other study used HIF-1α to determine the presence of
ischemia after unilateral femoral artery ligation and
showed a profound increase in HIF-1α in the ischemic
groups compared to the control group.22 These studies
reveal that HIF-1α modulates oxygen hemostasis in vivo;
however, the role of HIF-1α in DTI formation has yet to
be investigated.

The present study used a novel DTI animal model
with differing severity of DTI to determine healing time,
histological analysis, elevation of exudate creatine phos-
phokinase (CPK), and muscle edema. The purpose of this
study was to investigate the involvement of hypoxia in a
DTI model using HIF-1α as a marker.

Materials and Methods

Study design. This experimental study consisted of
three groups: control, low pressure DTI, and high pres-
sure DTI. The healing time, histological analysis, exudate
CPK, and muscle edema were assessed to confirm the
DTI characteristics. Furthermore, HIF-1α immunohisto-
chemistry analysis was compared among groups to eval-
uate hypoxic conditions.

Animals. Wistar rats, 12–14 weeks old weighing
285–300 g, were used. The rats were purchased from
Sankyo Labo Service Corporation (Tokyo, Japan), and
maintained under controlled light conditions (12 hours
light, 12 hours dark) with free access to food and water.
The experimental protocols were approved by the
Animal Research Committee of The University of Tokyo.

Wounding procedures. The DTI model was creat-
ed by modifying a previous PU model used by Sugama
et al. (Figure 1). After shaving the skin, to which the pressure loading was applied, it was excoriated using sandpaper to collect exudate. Excoriation was standardized to the level of the dermis (mimicking a Stage/Grade II PU), by scratching the wound site 12-15 times using sandpaper (no. G-P60) for the same time as the control and two experimental groups. Then, two 2-cm incisions (5-cm apart) extending to the subperitoneum were created using a scalpel. A metal plate was inserted subperitoneally under anesthesia with intraperitoneal injection of pentobarbital sodium (30 mg/kg body weight). Pressure was applied by lowering a cylinder with a contact area of 3 cm² for 4 hours. After relieving the pressure, the metal plate was removed and the incisions were sutured. A transparent semi-permeable dressing (Tegaderm HP™, 3M Health Care, Tokyo, Japan) was applied to the wound area and polyurethane foam dressing (Hydrosite™, Smith & Nephew Wound Management KK, Tokyo, Japan) was applied to the incision sites to prevent contamination of the wound by exudate from the incision sites.

A total of 75 rats were divided into three groups: control (25 rats); 1 kg loading (low pressure DTI, 25 rats); and 10 kg loading (high pressure DTI, 25 rats). The rats in the control and experimental groups all underwent the same procedure for skin excoriation, incision, and plate insertion, which remained in place for 4 hours. Pressure loading was not applied in the control group. Eight rats (2 in the control, 3 in the 1 kg, 3 in the 10 kg) died before sampling and were excluded from the study.

Macrosopic examination. Four rats in each group were maintained until wound closure. Macrosopic observations were performed each day. The number of days required for nearly complete wound closure, determined by visual observation, was recorded.

Histological analysis. Three animals in each group were sacrificed with an overdose of pentobarbital sodium on day 1 and day 3; tissue samples of the wounded area were harvested on the same day. Thus, a total of the 18 samples were analyzed for histology and immunostaining. The samples were fixed with 4% paraformaldehyde, dehydrated through a graded series of alcohol and xylene, and embedded in paraffin. The tissue samples were cut into 5-µm thick sections. Sections were deparaffinized in xylene, rehydrated in ethanol, and washed in distilled water. The sections were stained with hematoxylin and eosin to determine inflammation and muscle damage. The sections were observed under a light microscope at x10 magnification.

Measurements of exudate and serum CPK levels. Eleven rats for each group were used for sampling exudates and serum. Exudate samples (30–80 µl) were obtained from the wound sites on day 1 by aspirating the pooled exudates under the transparent film using a syringe. Blood samples (0.2 mL) were obtained from the tail vein on day 1. The exudate and serum samples were centrifuged at 6000 rpm for 10 minutes and stored at -80°C until analysis. The CPK concentrations in the samples were determined enzymatically by a commercial laboratory (SRL Inc., Tokyo, Japan) using the method recommended by The Japan Society of Clinical Chemistry. The CPK activities are expressed as IU/L. Of the 11 rats used for CPK measurement for each group, 4 rats were immediately sacrificed after exudate and blood sampling; the remaining 7 rats were also used on day 3 for sampling tissues and muscles.

Muscle edema. After sacrificing, all layers of the abdominal wall at the wound sites were excised. Deep muscle tissues of the abdominal wall were obtained from 4 rats each from group on day 1 and 3, except in the control group on day 1, where 5 rats were sacrificed. Thus, a total of 25 samples were harvested and analyzed. After taking the muscle tissue samples, the wet weight of the tissue was measured. The muscle tissues were then dried at 60°C in a tissue heating device until the weight was stable. The wet-weight to dry-weight ratio of the wound site and the contralateral site of the muscle tissue, harvested in the same manner, was measured as the muscle edema index. (edema index [EI] = wound site/contralateral site)

Immunohistochemistry analysis of HIF-1α. Preparation of the sections was the same as described above. For histochemistry analysis, endogenous peroxidase activity was inactivated by incubating the sections in 0.3% hydrogen peroxide/methanol for 30 minutes. Antigen retrieval was done by autoclaving the slides in citrate buffer (pH 6.0) for 20 minutes, and cooling down for 30–60 minutes. The slides were then incubated with the mouse monoclonal anti-HIF-1α antibody (Clone H1a”), Sigma, Saint Louis, MO; diluted 1:500) at room temperature for 60 minutes. After washing three times with phosphate buffered saline (PBS) for 5 minutes each, sections were incubated with biotin-conjugated horse anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA; diluted 1:500) for 30 minutes at room temperature. Both antibodies were diluted by PBS containing 1% bovine serum albumin. Immunoreaction was detected using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with DAB substrate.
(Roche, Indianapolis, IN) according to the manufacturer’s instructions. Lastly, the slides were counterstained with Mayer’s hematoxylin for 30 seconds. The specimens were observed under light microscopy at x10 magnification.

Statistical Analysis

Differences between groups were analyzed using one-way ANOVA performed with SPSS software, (SPSS version 13 for Windows, SPSS Inc, Chicago, IL) followed by a Fisher’s least significant difference (LSD) post-hoc test if the ANOVA was significant. Statistical significance was set at $P = 0.05$.

Results

Macroscopic findings after wounding. In the control and 1 kg groups, the color of the excoriated wounds changed from pale to red on days 3–5, and the wound areas were smaller (Figure 2). In contrast, the wounds in the 10 kg group were larger in area with remarkable slough (Figure 2) and a considerable amount of exudate. The mean time for mostly complete healing was longer in the 10 kg group (17.5 ± 7 days) than in the 1 kg (11 ± 1.4 days; $P < 0.05$) and control groups (9.2 ± 0.5 days; $P < 0.01$; Figure 3). However, there was no significant difference between the control and 1 kg groups ($P = 0.185$). Deep or open ulcers were not observed during the experimental period in any of the groups.

Light microscopy findings. The histological findings are shown in Figure 4. The control tissue sections showed normal, multinucleated myofibers with peripheral nuclei and small interstitial spaces. The cross-sectional area of the myofibers was large, homogeneous in size and polygonal in shape (Figures 4A, 4D). On day 1, the wound areas in the 1 kg group showed muscle degeneration, very little muscle necrosis and small interstitial areas (Figure 4B). There was little infiltration of polymorphonuclear...
neutrophils (PMN) and lymphocytes surrounding the necrotic areas (Figure 4B). The compressed area in the 10 kg group showed a marked increase in necrosis, particularly in deep muscle, and extensive infiltration of PMN and lymphocytes in the interstitial areas and internal muscle fibers. Additionally, the interstitial areas in the 10 kg group widened (Figure 4C). On day 3, some myofibers had regenerated in the 1 kg group (Figure 4E), while in 10 kg group, regeneration has not yet occurred (Figure 4F). Inflammation was more clearly seen on day 3 compared to day 1 in the 10 kg group (Figure 4F). Three animals were used from each group for each day, and we can confirm that these findings were repeatedly observed.

**Exudate and serum CPK.** The mean exudate CPK levels in the control, 1 kg, and 10 kg groups on day 1 were 163.3 ± 99.1, 568.8 ± 273.7, and 3503 ± 3113 IU/L, respectively (Figure 5). The exudate CPK level was significantly higher in the 10 kg group compared to the control (P < 0.01) and 1 kg (P < 0.05) groups; however, there was no difference between the control and 1 kg groups (P = 0.456). There was no difference in the serum CPK level among the three groups (data not shown).

**Muscle edema.** The mean muscle edema levels in the control, 1 kg, and 10 kg groups on day 1 were 1.07 ± 0.10, 1.12 ± 0.89, and 1.27 ± 0.06, respectively. On day 1, the mean muscle edema was significantly higher in the 10 kg group compared to the control (P < 0.01) and 1 kg (P < 0.05) groups; however, there was no difference between the control and 1 kg groups (P = 0.410; n = 5 for control; n = 4 for 1 kg and 10 kg groups). On day 3, the mean muscle edema levels in the control, 1 kg, and 10 kg groups were 1.00 ± 0, 1.01 ± 0.02, and 1.39 ± 0.14, respectively. The mean muscle edema level on day 3 was significantly higher in the 10 kg group compared with the control (P < 0.001) and 1 kg (P < 0.001) groups; however, there was no difference between the control and 1 kg groups (P = 0.870; n = 4 for each group).

**HIF-1α immunostaining.** On day 1, immunohistochemical analysis revealed very weak HIF-1α expression in the muscle cells at a basal level in the control group (Figure 6A). The number of HIF-1α positive cells in the muscle and within the interstitial spaces between the muscle fibers was remarkably increased in the 1 kg and 10 kg groups (Figures 6B, C). The intensity of immunostaining was also enhanced in the two experimental groups. Magnified views clearly showed cytoplasm localization of HIF-1α in most of the HIF-1α positive cells in all three groups (insets in Figures 6A–C). Scant nuclear
localization was observed in the 1 kg and 10 kg groups—an indication of mild hypoxia. Localization of the HIF-1α positive cells within the muscle was limited to the area where the pressure was applied.

On day 3, the number of HIF-1α positive cells remained increased in the 1 kg and 10 kg groups compared with the control groups (Figures 6D–F). The staining intensity was slightly stronger in the 1 kg and 10 kg groups than in the control groups. Of note, higher magnification of the section showed that nuclear localization was remarkably increased in the 10 kg group (inset in Figure 6F), indicating that severe hypoxia induced HIF-1α translocation to the nucleus. Nuclear localization was much lower in the control and 1 kg groups on day 3. In addition, the area of the HIF-1α-positive cell localization within the muscle tissue was greater on day 3 than on day 1, and extended beyond the area of pressure application. We used 3 animals for each group for each day, and we confirmed that these findings were commonly observed.

**Discussion**

A novel animal DTI model was developed for this study, and to our knowledge, is the first study to assess the involvement of hypoxia in an *in-vivo* DTI model. This DTI model showed varying macroscopic findings and healing times. The histological analysis revealed that the 1 kg and 10 kg loads induced a differential severity of DTI, as indicated by the differential inflammatory status and muscle tissue damage. This finding suggests that the wound healing process is strongly associated with muscle damage in our model. It has been suggested that DTI initially appears in muscle tissue because of the dense capillary vasculature in skeletal muscles, which is susceptible to obstruction and occlusion by mechanical forces. Therefore, the present study focused on muscle tissue.

The exudate CPK level was also examined to investigate the varying severities of DTI. We have previously reported that the exudate CPK level is a useful marker of DTI. In the present study, the exudate CPK level in the 10 kg group was higher than in 1 kg and control groups, which is consistent with our previous study that exudate CPK levels reflected the status of local wound microenvironment and the severity of the wound. Many experimental and clinical studies have frequently used serum CPK as a marker for muscle injury, when muscle hypox-
ia, ischemia, necrosis and other conditions occur and the myofiber membrane permeability is changed. In such situations, the intracellular CPK leaks into the extracellular space, resulting in elevated CPK levels in the blood. However, there was no significant difference in the serum CPK levels in the present study. This suggests that, as we previously reported, the exudate CPK level is a more sensitive and more clinically promising marker for muscle damage than serum CPK.

The tissue inflammation process increases vascular permeability, resulting in tissue edema. Previous studies by Sato et al. and Quintavalle et al. in clinical settings revealed the presence of hypoechoic areas in deep tissue, indicating the presence of edema during the inflammation process in patients with DTI. In this study, the muscle edema was significantly increased in the 10 kg group compared with the control and 1 kg groups.

The wound healing, histological, exudate CPK and muscle edema findings in this study indicate that the 1 kg and 10 kg loads induced different DTI severity, confirming the versatility of our model. The previous in-vivo DTI model developed by Linder Ganz et al., in which the upper layer of the skin was resected, did not fully represent the clinical situation because the skin of patients suffering from DTI is intact. Another in-vivo DTI model required a very complicated device, making it difficult to reproduce. We consider that our novel DTI model is relatively simple and precise compared with the previous models, and is thus useful for future studies of the pathophysiology and treatment of DTI.

In this study, we successfully showed that hypoxia is involved in DTI formation using HIF-1α. Loading of 10 kg and 1 kg induced a marked increase in cytoplasm localization on day 1. Previous studies have indicated that the increase in cytoplasmic HIF-1α appears to be a result of enhanced protein stabilization and accumulation before nuclear translocation. On day 3, a marked increase in nuclear localization occurred in the 10 kg group, which is consistent with findings that, in severe hypoxia, HIF-1α becomes stabilized and translocates from the cytoplasm to the nucleus. Previous studies have shown similar results of a marked increase in nuclear localization of HIF-1α as a result of ischemic conditions. On the other hand, only a small amount of nuclear localization was observed in the 1 kg group, suggesting that the 1 kg load only induces slight hypoxia. In this study, the control group, which had a normoxic condition, showed very little cytoplasm localization of HIF-1α. This is consistent with the earlier studies showing that HIF-1α is expressed at very low levels in the brain and skeletal muscle in the basal state under normoxic condition, and HIF-1α has a potential role in muscle oxygen homeostasis during normoxia. Interestingly, nuclear localization was more clearly seen on day 3, indicating that the hypoxic condition still remained on day 3. The HIF-1α positive area was further enlarged on day 3 compared with day 1. According to the conventional tissue ischemic–reperfusion injury theory, ischemia will only occur during the loading phase, following by reperfusion after the release of the load. These findings were mostly based on experiments involving vessel ligation, and the hypoxic condition was immediately returned to normoxia after the ligation was released. However, based on our findings, hypoxia was prolonged or even enhanced on day 3, even though reperfusion had already started to occur. Future studies are needed to investigate the mechanisms responsible for this finding.

A limitation of this study is that HIF-1α immunostaining was only conducted on days 1 and 3. In addition, this study only investigated the simultaneous occurrence of hypoxia and DTI under pressure loading, but we cannot explain the causal relationship between hypoxia and DTI.

Conclusion

We established a novel rat model of DTI. The present study is the first study to show the involvement of hypoxia in DTI in vivo through HIF-1α immunohistochemistry. Further studies are needed to investigate the causal relationship between DTI and hypoxia. Other factors that may be involved in DTI formation, including lymphatic deficiency or tissue deformation, need to be investigated to improve our understanding of the mechanism of DTI.

References


