

# Application of Normobaric Hyperoxygenation to an Ischemic Flap and a Composite Skin Graft

Jun Araki  
Harunosuke Kato  
Kentaro Doi  
Shinichiro Kuno  
Kahori Kinoshita  
Kazuhide Mineda  
Koji Kanayama  
Kotaro Yoshimura

**Background:** Hyperbaric oxygenation has been used for various purposes, but its clinical application is limited due to its pulmonary toxicity. We evaluated the therapeutic value of normobaric hyperoxygenation (NBO) for vascularized and nonvascularized tissue transplantation.

**Methods:** Tissue oxygen partial pressure (PtO<sub>2</sub>) was measured for various organs in mice under inspiratory oxygen of 20%, 60%, or 100%. A rectangular skin flap (1×4 cm) or a composite skin graft (2×2 cm) was made on the back of mice, which were housed under 20% or 60% oxygen for the first 3 days after surgery. Cell survival was also examined in organ culture skin samples.

**Results:** PtO<sub>2</sub> varied among tissues/organs, but increased depending on inspiratory oxygen concentration in all tissues/organs. Although NBO with 100% O<sub>2</sub> was toxic, NBO with 60% O<sub>2</sub> was safe even when used continuously for a long period. NBO did not significantly improve survival of the rectangular skin flap. On the other hand, in the composite skin graft model, the engraftment area increased significantly (52±10 at 20% vs 68±5.1 at 60%) and contraction decreased significantly (42±8.0 at 20% vs 27±5.7 at 60%). Organ culture of a composite skin sample showed significant cell death under lower oxygen concentrations, supporting the data in vivo.

**Conclusions:** The composite graft was maintained until revascularization by plasmatic diffusion from surrounding tissues, in which PtO<sub>2</sub> was improved by NBO. NBO may be an effective adjunct therapy that can be performed readily after nonvascularized tissue grafting. (*Plast Reconstr Surg Glob Open* 2014;2:e152; doi: 10.1097/GOX.0000000000000029; Published online 15 May 2014.)

The concept of oxygen as a therapeutic agent was first introduced in the 1920s by Barach.<sup>1</sup> Since then, the effects of hypoxemia and its reversal with oxygen supplementation have been well-explored, leading to increased use of oxygenation for treating respiratory and/or cardiovascular insufficiency and postsurgery/postanesthesia condi-

tions.<sup>2-4</sup> An extreme therapeutic example is hyperbaric oxygenation (HBO), which typically uses 100% oxygen at 3 atmospheres and has been applied for various purposes, including promoting wound healing for grafting or supporting flap survival.<sup>5,6</sup> HBO, however, can be used clinically only for 60–90 minutes per day due to its pulmonary toxicity.<sup>7</sup> The systemic influences of the strong toxicity and the repeated rebounding effects remain to be determined,

From the Department of Plastic Surgery, University of Tokyo, Tokyo, Japan.

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and the therapeutic benefits of HBO remain controversial, limiting expanded use. The biological roles of oxygen in the skin and wound healing have been examined at the molecular level and its clinical usage were reviewed recently.<sup>8,9</sup>

In this study, we sought to develop a safer and more reliable hyperoxygenation method to improve tissue wound healing using normobaric hyperoxygenation (NBO). We first researched the effects of NBO with various fraction of inspiratory oxygen ( $FiO_2$ ) on the tissue partial pressure of oxygen ( $PtO_2$ ) in various tissues and organs. After establishing an optimized NBO protocol and confirming a beneficial effect on cell survival in skin organ culture, we examined the therapeutic value of postoperative NBO for improving clinical outcomes of an ischemic flap and a composite skin graft.

## MATERIALS AND METHODS

### Oxygen Pressure Measurement

All animal care was in accordance with institutional guidelines. Fifty-five Jcl:ICR mice (males, 7–10 weeks old) were purchased from the Nippon Bio-Supply Center (Tokyo, Japan).  $PtO_2$  in mice ( $n = 3$ ) was measured as described previously.<sup>10</sup> Briefly, it was measured with a modified Clark-type electrode (200  $\mu\text{m}$  in diameter) and an oxygen monitor (Eiko Kagaku, Tokyo, Japan) under minimal anesthesia to prevent substantial cardiovascular or respiratory depression. The oxygen electrode was inserted directly into the abdominal subcutaneous tissue, the inguinal fat pad, the femoral muscles, the liver, and the kidney under oxygen at 20% (room air), 60%, or 100%. The sensor was placed in the tissue for at least 10 minutes until the measured value reaches a plateau. The partial pressures of oxygen in arterial blood ( $PaO_2$ ) and venous blood ( $PvO_2$ ) under oxygen at 20% (room air), 60%, or 100% were also measured with a blood gas analysis apparatus (ABL800 Flex, Radiometer, Tokyo, Japan).

### Mice Models of a Skin Flap and a Composite Skin Graft

After a mouse was anesthetized, with an intraperitoneal injection of pentobarbital, 2 kinds of transplantation models were prepared.

A rectangular skin flap was used as a vascularized but partly ischemic tissue transplantation model, as described previously.<sup>11</sup> In this model, a cranial-based rectangular skin flap (1×4 cm;  $n = 10$ ), including the subcutaneous tissue, was elevated on the back. After placing a thin synthetic film (Asahikasei, Tokyo, Japan) to prevent revascularization from the

underlying wound bed, the skin flap was sutured orthotopically using 6-0 nylon.

A composite skin graft was used as a nonvascularized tissue transplantation model. In this model, a square-shaped composite skin (2×2 cm,  $n = 10$ ) including the subcutaneous tissue was excised from the back and was immediately sutured orthotopically with 6-0 nylon, followed by a gauze dressing.

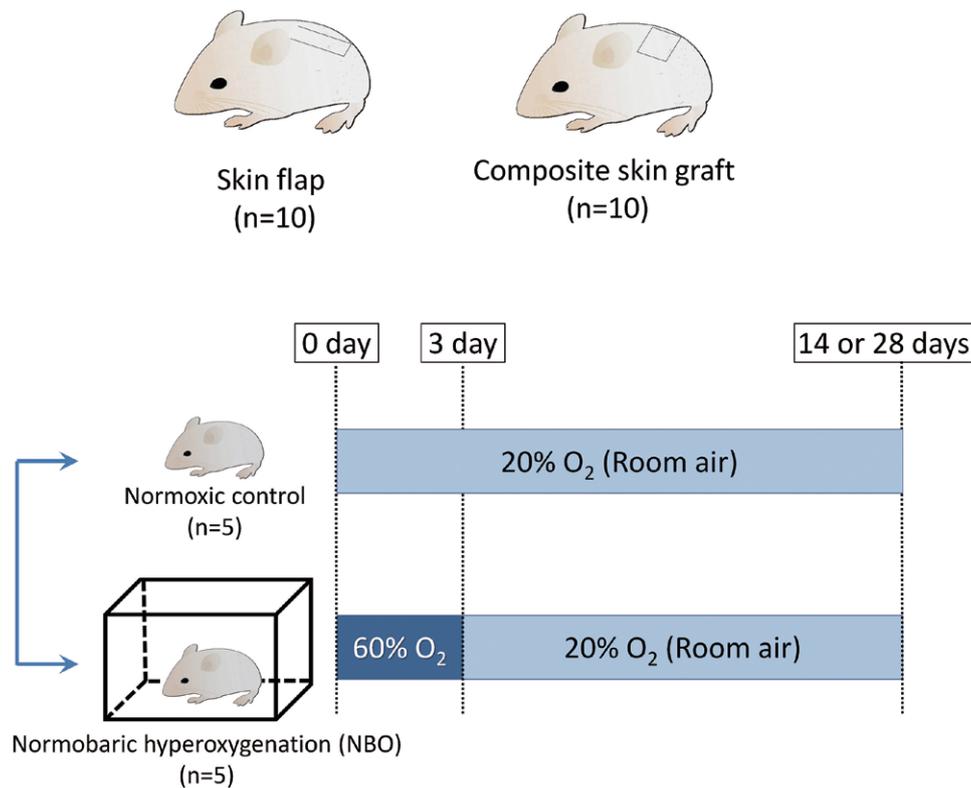
The animals of both models were divided into 2 groups, control and NBO. The control animals were housed under room air (20% oxygen), whereas NBO group animals were kept under NBO (normobaric 60% oxygen and 40% air) for the first 3 days after surgery and under room air for the remaining period. The animals were given free access to food and water. Experimental procedures are summarized in Figure 1 and Supplemental Figure S1. [See **Supplemental Digital Content 1**, which demonstrates animal models of a skin flap and a composite skin graft. (A) A quadrant skin flap was elevated on the back of mice and sutured to the original position. (B) A composite skin was excised on the back of mice and sutured to the original position. The tie-over dressing was performed and removed at 1 week, <http://links.lww.com/PRSGO/A31>.]

### Measurement of Tissue Viability

Surgical sites were photographed at 1, 2, and 4 weeks after surgery. Areas of survival, contraction, and necrosis (or eschar formation) were measured. Each area was color-coded manually in yellow, white, and black (or gray) with graphics software (Photoshop CS6, Adobe Systems, San Jose, Calif.), followed by measuring the pixel number of each area.

### Cell Viability in Skin Organ Culture

Small round skin fragments (6 mm in diameter) were harvested from the back of mice with a disposable punch biopsy (Kai Industries, Gifu, Japan). Skin samples were incubated in Dulbecco's modified Eagle medium containing 4% hyaluronate and 5% fetal bovine serum for 6 hours under 1% (hypoxia), 6% (normoxia), or 20% (hyperoxia) oxygen ( $n = 3$  for each group). After washing with phosphate-buffered saline, they were incubated with Hoechst33342 (Dojindo, Kumamoto, Japan) and propidium iodide (PI; Sigma-Aldrich, St. Louis, Mo.) at 37°C for 30 minutes. The stained samples were evaluated under a TCS SP2 confocal microscope system (Leica, Heerbrugg, Switzerland). Ten serial images of the dermis were obtained at 3- $\mu\text{m}$  intervals and were processed to produce a surface-rendered, 30- $\mu\text{m}$ -thick, three-dimensional image. Numbers of all nuclei (Hoechst-positive) and dead nuclei (Hoechst- and PI-positive) were counted.



**Fig. 1.** Normoxic control and NBO application in mouse model of a rectangular skin flap and a composite skin graft. Using 20 ICR mice, a skin flap model ( $n = 10$ ) and a composite skin graft model (a skin graft containing subcutaneous tissue;  $n = 10$ ) were prepared (above) and divided into 2 groups: the normoxic control and NBO therapy groups (below). In the NBO group, normobaric hyperoxygenation with 60% O<sub>2</sub> was applied for the first 3 days after surgery.

### Statistical Analysis

Results are described as means  $\pm$  standard deviations. Comparisons between the 2 groups were performed using the unpaired Student's *t* test. Comparisons of more than 2 groups were made by analysis of variance with the Bonferroni correction. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### Oxygen Tension of Blood and Tissues Influenced by NBO

PaO<sub>2</sub> and PvO<sub>2</sub> measured in this study were  $115 \pm 27$  mm Hg and  $47 \pm 10$  mm Hg, suggesting that oxygen tension is very similar between mice and humans. It was also found that PtO<sub>2</sub> varied considerably among organs: PtO<sub>2</sub> (mm Hg) was  $50 \pm 9.3$  in inguinal fat pad,  $28 \pm 14$  in femoral muscle,  $28 \pm 16$  in liver,  $25 \pm 16$  in kidney, and  $43 \pm 9.5$  in subcutaneous tissue (Fig. 2).

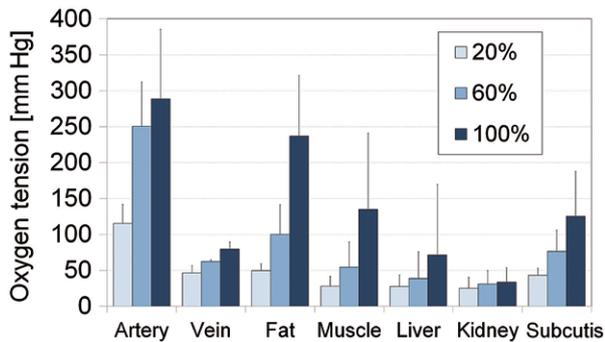
PaO<sub>2</sub> was increased to 217% and 250% by application of 60% (3 times that of room air) and 100% (5 times) oxygen, respectively, while PvO<sub>2</sub> was 134% (60% O<sub>2</sub>) or 171% (100% O<sub>2</sub>). NBO using high FiO<sub>2</sub> (60%

or 100% oxygen) increased PtO<sub>2</sub> in all organs/tissues tested, although the increase in PtO<sub>2</sub> was not proportional to the increase of FiO<sub>2</sub>, as seen in PaO<sub>2</sub> and PvO<sub>2</sub>. The increased percentage of PtO<sub>2</sub> under 60%/100% oxygen was 201%/475% in inguinal fat, 195%/484% in femoral muscle, 141%/259% in liver, 125%/136% in kidney, and 178%/290% in subcutaneous tissue, respectively (Fig. 2). These results supported previous studies showing PtO<sub>2</sub> of the skin and soft tissues.<sup>12–14</sup>

Treatment with normobaric 100% oxygen killed all mice in 2 days, although 100% oxygen at 2–3 atmospheres absolute is generally used in clinical HBO. We also found that long-term (several weeks) continuous application of normobaric 60% oxygen was not toxic and could be used as a continuous NBO therapy.

### NBO Therapy for the Rectangular Skin Flap

The skin flap was evaluated at 1 week after surgery when the necrotic demarcation was definite. In the normoxic control group (FiO<sub>2</sub> = 20% for 7 days), skin flap areas of contraction, engraftment, and necrosis were  $30\% \pm 4.3\%$ ,  $18\% \pm 4.9\%$ , and  $51\% \pm 6.5\%$  of the original flap size, respectively. On the



**Fig. 2.** Oxygen pressure of blood and organs under 3 different inspired oxygen concentrations. NBO (normobaric 60% or 100% oxygen) was applied to mice and compared with room air.  $\text{PaO}_2$ ,  $\text{PvO}_2$ , and  $\text{PtO}_2$  of all tested organs changed depending on the inspired oxygen concentrations, although the increase in  $\text{PtO}_2$  was not always proportional to that of the inspired oxygen concentration.

other hand, in the NBO group ( $\text{FiO}_2 = 60\%$  for the first 3 days and 20% for the next 4 days), flap areas of contraction, engraftment, and necrosis were  $26\% \pm 6.6\%$ ,  $20\% \pm 4.9\%$ , and  $54\% \pm 2.4\%$  of the original flap size, respectively (Fig. 3). Normobaric 60% oxygenation therapy did not significantly improve the survival of the rectangle skin flap.

#### NBO Therapy for the Composite Skin Graft

The composite skin graft was evaluated at 2 and 4 weeks after surgery, but the eschar remained on the graft and disturbed precise evaluation of contraction, engraftment, and necrosis (Fig. 4A). Thus, we evaluated the composite skin graft only at 4 weeks by which time all eschars had disappeared and wound contraction was complete. In the control group ( $\text{FiO}_2 = 20\%$  for 7 days), the areas of contraction, engraftment, and necrosis were  $42\% \pm 8.0\%$ ,  $52\% \pm 10\%$ , and  $6.1\% \pm 4.3\%$  of the original graft size, respectively. In the NBO group ( $\text{FiO}_2 = 60\%$  for the first 3 days and 20% for the next 25 days), the areas of contraction, engraftment, and necrosis were  $27\% \pm 5.7\%$ ,  $68\% \pm 5.2\%$ , and  $4.7\% \pm 3.1\%$  of the original graft size, respectively (Fig. 4B). The 3-day NBO therapy significantly increased the engraftment area ( $P = 0.023$ ) and decreased the contraction area ( $P = 0.014$ ).

#### Organ-cultured Skin Viability under Hypoxia, Normoxia, and Hyperoxia

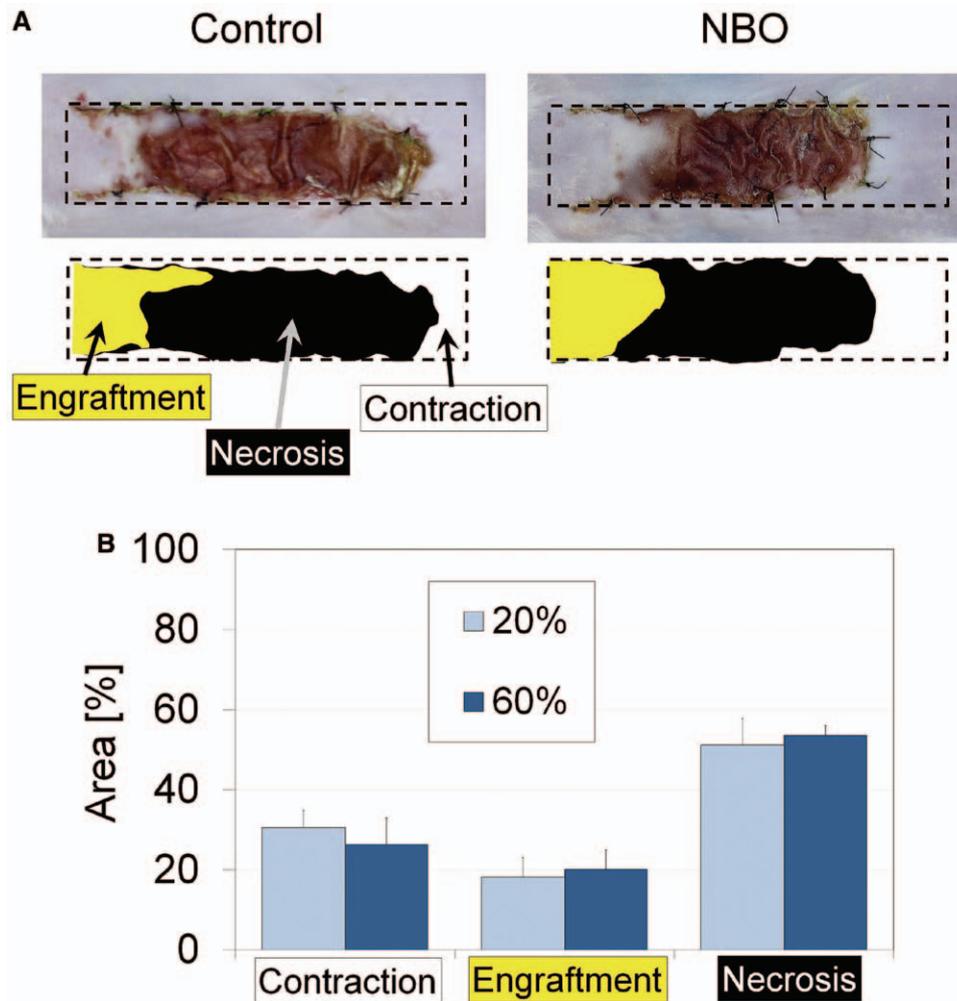
Skin samples were cultured in medium under 1% (8 mm Hg; hypoxia), 6% (46 mm Hg; normoxia), and 20% (152 mm Hg; hyperoxia) oxygen. As the  $\text{PtO}_2$  of skin that we measured in mice was around 40–50 mm Hg, culture under 6%  $\text{O}_2$  corresponded approximately with normoxic condition for the

skin sample in organ culture. Nuclei of dead cells were stained with PI, whereas all nuclei (both viable and dead cells) were stained with Hoechst33342 [Supplemental Fig. S2A, see **Supplemental Digital Content 2**, which demonstrates cell death assay for organ-cultured skin samples. (A) Skin samples were organ-cultured under various oxygen tensions: 1% (8 mm Hg; hypoxia), 6% (46 mm Hg; normoxia), and 20% (152 mm Hg; hyperoxia). All nuclei were stained with Hoechst33342 (blue), whereas nuclei of dead cells were stained also with PI (red). The control is a noncultured skin sample, <http://links.lww.com/PRSGO/A31>. The number of dead cells in skin samples increased after organ culture. The proportion of dead cells was significantly higher under hypoxia than under normoxia or hyperoxia [Supplemental Fig. S2B, see **Supplemental Digital Content 2**, which demonstrates cell death assay for organ-cultured skin samples. (B) Dead cells increased significantly when cultured under hypoxic conditions, suggesting that  $\text{PtO}_2$  of the surrounding recipient tissue may substantially affect the acute damage of the grafted tissue, <http://links.lww.com/PRSGO/A31>. These results supported the in vivo results showing that nonvascularized tissue damage was affected by the oxygen tension of the microenvironment.

## DISCUSSION

This study revealed that systemic NBO elevated  $\text{PtO}_2$  in all tissues/organs and that 3-day postoperative NBO therapy using 60% oxygen improved engraftment significantly of a nonvascularized composite skin graft, but not of a vascularized skin flap. Tissues/organs with vascularization, such as a skin flap, a microsurgical free flap, and a transplanted kidney, are maintained by blood flow from the nutrient pedicles. On the other hand, tissues/organs without vascularization, such as a skin graft or fat graft, are maintained only by plasmatic diffusion from the surrounding vascularized tissue until revascularization occurs.

In our previous studies,<sup>15–17</sup> we found that the first 3 ischemic days are the critical period for determining whether the tissue will survive or die. Under severe ischemic or hypoxic (or even other stress) conditions, almost all differentiated functioning cells will die, but tissue-resident stem/progenitor cells can stay alive up to 3 days even without oxygen.<sup>17</sup> The resident stem/progenitor cells are even temporarily activated by tissue damage. If revascularization is achieved within the 3 days, the tissue may be partially repaired by activated and recruited stem/progenitor cells, but the tissue will finally necrotize in cases when the microenviron-



**Fig. 3.** NBO therapy for skin flap engraftment. A, NBO therapy using normobaric 60% oxygen was applied for the first 3 days after elevation of a skin flap and compared with the control at 1 week. The flap areas of contraction, engraftment, and necrosis were measured and are colored in white, yellow, and black, respectively. B, The areas of contraction, engraftment, and necrosis were not significantly different between the control and NBO groups.

ment does not improve within 3 days. This is the reason why we used NBO therapy only for the first 3 days in this study.

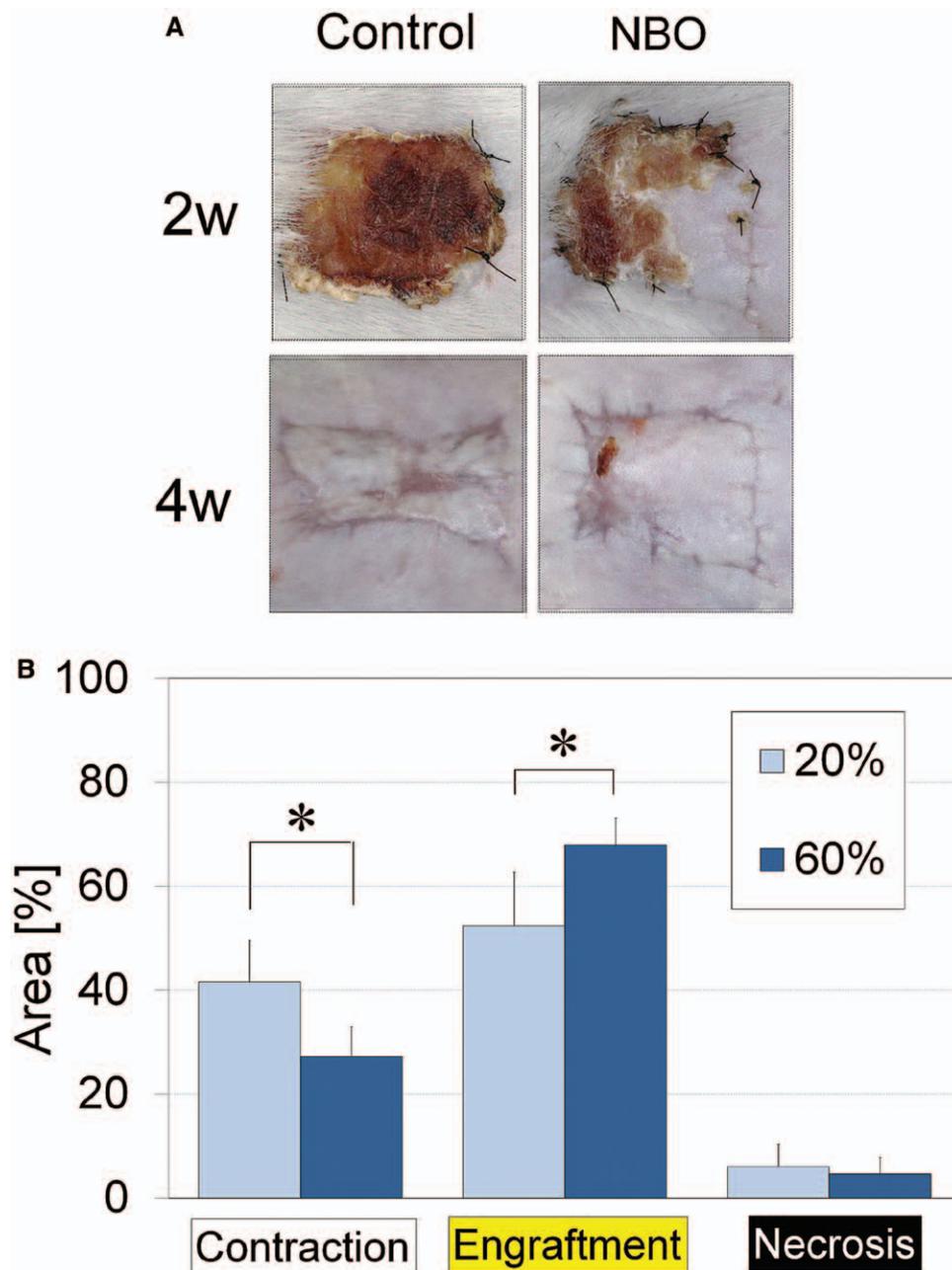
There are many reports on oxygen delivery to tissues/organs.<sup>18,19,20</sup> The oxygen content in a fluid (blood) of one deciliter [ $C(O_2)$ ] is expressed by the following equation<sup>19,20</sup>:

$$C(O_2) [(O_2)\text{mL} / (\text{fluid})\text{dL}] \\ = P(O_2) \times 0.0225 + \text{Hb} \times S(O_2) \times 1.306,$$

where  $P(O_2)$  is the mean oxygen partial pressure (15.4 kPa in arterial blood under 20%  $\text{Fi}O_2$ ), Hb is the hemoglobin concentration (g/dL), and  $S(O_2)$  is the oxygen saturation (generally close to 1).

As indicated in this equation, hemoglobin in red blood cells usually contains most (~99%) of the ox-

xygen in blood, while the rest (the plasma portion) contains only about 1%. Under conditions where  $S(O_2)$  is already about 1, applying hyperoxygenation with 60%  $O_2$  will increase  $P(O_2)$  (up to 3 times) alone and the increase in  $C(O_2)$  is only up to 3%. Thus, this would suggest that the NBO does not provide much benefit to a vascularized flap. On the other hand, a nonvascularized skin fragment has no circulating blood and is maintained only by plasmatic diffusion from surrounding vascularized tissue. The oxygen content of the diffusing plasma, which does not contain hemoglobin, is proportional to  $P(O_2)$ , as indicated by the equation. Thus, it seems to be theoretically reasonable that increased  $P(O_2)$  as a result of NBO will have a significant influence on the nonvascularized composite skin graft survival, but not on the elevated skin flap.



**Fig. 4.** NBO therapy for composite skin graft engraftment. A, NBO therapy using normobaric 60% oxygen was applied for the first 3 days after grafting of a composite skin and compared with the controls at 2 and 4 weeks. B, The areas of contraction, engraftment, and necrosis at 4 weeks were measured. The contraction area decreased significantly and the engraftment area increased significantly by NBO therapy.

Oxygen delivery to the tissues and wound was extensively studied by Hunt and Hopt and their colleagues. Their works cover a variety of physical factors and therapeutic modalities to influence with perfusion and tissue oxygen tension in relation to wound healing and postoperative infection in surgical patients.<sup>12,14,21,22</sup> Although there are a number of adjunct therapies for increasing the survival of vascularized flaps, including vasodilators, antioxidant

drugs,<sup>23</sup> precursor mobilization,<sup>24</sup> hematopoiesis,<sup>24,25</sup> and cooling,<sup>26</sup> we have no reliable supportive treatments for nonvascularized tissue grafts. As shown in this study, systemic NBO therapy with 60% O<sub>2</sub> is a safe therapy and may bring significant benefits to graft survival through improving PtO<sub>2</sub> surrounding the recipient tissue. In clinical settings, systemic NBO therapy can be used readily with a simple oxygen mask or tent and may be a practical therapy to

boost engraftment of many kinds of nonvascularized tissue grafting. In addition, local NBO therapies may also be beneficial for closed wounds on which negative pressure wound therapy has no effect.<sup>27</sup> Although oxygen needs attention in terms of handling,<sup>7,28</sup> it may be useful in diverse ways in the field of plastic surgery.

**Kotaro Yoshimura, MD**

Department of Plastic Surgery

University of Tokyo

7-3-1 Hongo, Bunkyo-Ku

Tokyo 113-8655, Japan

E-mail: kotaro-yoshimura@umin.ac.jp

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