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Muscle Is a Target for Preservation in a Rat Limb Replantation Model

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Background: Ischemia exceeding 6 hours makes clinical limb replantation difficult and places the patient at risk of functional deficit or limb loss. We investigated the preservation of muscle function and morphology with solutions in rat hindlimb in vivo and in vitro.

Methods: Quadriceps femoris muscles from luciferase transgenic rats were preserved for 24 hours at 4°C in extracellular-type trehalose containing Kyoto (ETK), University of Wisconsin (UW), or lactated Ringer's (LR) solution (control). Muscle luminescence was measured with a bioimaging system. Amputated limbs of Lewis rats preserved with ETK, UW, or LR for 6 or 24 hours at 4°C were transplanted orthotopically. At week 8, terminal latency and amplitude were measured in the tibialis anterior muscle. The muscles were also analyzed histologically.

Results: Isolated muscles preserved in ETK or UW had significantly higher luminescence than did muscles immersed in LR ($P < 0.05$). In the 6-hour-preserved limb transplantation model, although the 3 groups had almost the same terminal latency, electrical amplitude was significantly lower in the LR group. Histologically, muscles preserved with LR showed the most atrophic changes. In the 24-hour-preserved model, the survival rate of the LR group was 37.5% in contrast to 80% in the ETK and UW groups. Electrical signals were not detected in the LR group owing to severe muscle atrophy and fibrosis. The ETK and UW groups showed good muscle function electrophysiologically.

Conclusions: Preservation solutions can protect muscle function and morphology in ischemia–reperfusion limbs and improve recipient survival rates after transplantation of long-term-preserved limbs. *(Plast Reconstr Surg Glob Open 2013;1:e70; doi: 10.1097/GOX.0000000000000017; Published online 15 November 2013.)*

More than 50 human hand transplantations have been performed in centers in Europe, United States, Asia, and Australia.[1] Composite tissue transplantation in the face of major histocompatibility requires heavy use of immunosuppressants or donor-specific bone-marrow-cell-based treatment.[2,3] By contrast, limb replantation needs no immunosuppressive therapy, although the time to surgery from injury greatly influences limb survival and function.[4,5] Muscle tissues are often destroyed by traumatic limb amputation. Prolonged muscle ischemia causes irreversible necrosis, resulting in complications such as muscle atrophy and functional loss. Because ischemic muscular tissues degenerate

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irreversibly within 6 to 8 hours,[6,7] ischemic time is critical in limb replantation. The Mangled Extremity Severity Score, which is used clinically as an objective criterion for amputation prediction, doubles when limb ischemia time exceeds 6 hours.[8,9] With attempts at reperfusion more than 8 hours after ischemic injury, replantation often fails. Moreover, anaerobic metabolic products or potassium from necrotic tissue can cause acute renal failure and fatal shock.[10]

Although preservation solutions are among the items essential for organ storage in transplantation, they have not been routinely used to preserve amputated extremities. There have been many preservation studies of cardiac and smooth muscle because heart or vessel allotransplantation techniques are advanced. By contrast, because skeletal muscle is not generally transplanted, there have not been many reports on the preservation of skeletal muscle. Some experimental studies have shown the beneficial effects of existing solid-organ preservation solutions on muscle tissues.[11–13] These studies have evaluated the viability of muscles mainly by observing transplanted graft condition, adenosine triphosphate (ATP) levels, rate of pathological degeneration, or strength of electrically induced contraction of isolated muscles. However, few studies have assessed functional recovery after transplantation of preserved limbs.[14]

Recent advances in biological imaging have improved our understanding of cellular and molecular biological events in living animals. Our group has created and developed inbred (Lewis; LEW) rats with molecular tags of green fluorescent protein from jellyfish (*Aequorea victoria*) and luciferase from fireflies (*Photinus pyralis*) as tools for organ transplantation, regeneration medicine, and plastic and reconstructive surgery.[15–17] Here, we used optical imaging techniques to evaluate the viability of muscle isolated from firefly-luciferase-expressing LEW transgenic (Luc-LEW Tg) rats after storage of the muscle for up to 24 hours in different solutions in vitro. Moreover, to evaluate functional and morphological recovery of the muscles in preserved limbs, we performed isogenic limb transplantation between LEW rats after limb preservation in each preservation solution for 6 or 24 hours at 4°C.

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METHODS

Rats

Male LEW rats 10–12 weeks old were purchased from Nihon SLC (Hamamatsu, Japan). Luc-LEW Tg rats (10–12 weeks old, male) had been established in our laboratory, as described previously.[16] All surgical procedures were performed under anesthesia with intraperitoneal injection of sodium pentobarbital (40 mg/kg). Anesthesia was maintained by additional injections (15 mg/kg) as required. All rats were euthanized by means of an anesthetic overdose injection at final evaluation. All experiments were performed in accordance with the Jichi Medical School Guide for Laboratory Animals.

Preservation of Muscles Isolated from Luc-LEW Tg Rats

The quadriceps femoris muscles from Luc-LEW Tg rats were cut to a columnar shape (diameter 8 mm, height 3 mm, and weight 0.5 g). The muscle pieces ($n = 16$, each group; total 48 pieces harvested from 4 Luc-LEW Tg rats) were placed in multiwell tissue culture plates, covered with extracellular-type trehalose containing Kyoto solution (ETK, Otsuka Pharmaceutical Factory, Tokyo, Japan) or University of Wisconsin solution (UW, DuPont Merck Pharmaceutical), and kept at 4°C. Lactated Ringer's solution (LR, Otsuka Pharmaceutical Factory) was used as a control. An in vivo bioimaging system (Xenogen, Alameda, Calif.) was used to visualize luciferase expression. D-Luciferin (Wako, Tokyo, Japan) was added to the muscle samples 0, 1.5, 3, 4.5, 6, 9, 12, and 24 hours after the start of preservation to detect photons emitted from the luciferase-expressing cells.

Histological Examination of Isolated Muscles

To evaluate muscle morphology, the quadriceps femoris muscles of LEW rats (10–12 weeks old, male) were harvested and then preserved with ETK, UW, or LR solution for 3, 6, 12, or 24 hours at 4°C ($n = 5$ each group). After preservation they were fixed with 10% formalin and embedded in paraffin. Histological specimens (3 μm thick) were examined after hematoxylin and eosin staining. The central portion of the vastus intermedius muscle in transverse section was evaluated randomly in 10 fields (×200) accord-

ing to the following 2 items: (1) the percentage of damaged muscle fibers (hypercontracted or rounded) and (2) the occurrence of interstitial edema (**Supplemental Fig. 1**, **Supplemental Digital Content 1**, shows (A) cross-section of quadriceps femoris muscle (hematoxylin and eosin staining). The central portion of the vastus intermedius muscle (rectangular frame) was observed histologically. High-magnification view of area in the rectangular frame, ***http://links.lww.com/PRSGO/A14)***. The numbers of hypercontracted fibers and rounded fibers were counted, as was the total number of fibers in each field. The area of interstitial edema as a percentage of the total area was measured in each field.[18,19] These items were evaluated by using an image analysis system (ImageJ-1.44p, National Institutes of Health, Bethesda, Md.).

Transplantation of Preserved Limbs

Donor LEW rat hindlimbs were amputated at midthigh level and placed immediately (without perfusion) into sterile plastic cases filled with ETK, UW, or LR ($n = 5$, each group), where they were kept for 6 hours at 4°C. To evaluate whether preservation solution could have a longer-term protective effect on the amputated limb, other donor limbs were preserved with ETK ($n = 5$), UW ($n = 5$), or LR ($n = 8$) for 24 hours at 4°C. After preservation, the donor limbs were transplanted orthotopically to recipient LEW rats. We added an immediate transplantation model as a control ($n = 4$).

Surgical Procedure

Microsurgical techniques were used, as described previously.[20] Briefly, the graft was fixed with an 18-gauge needle used as an intramedullary rod. The femoral artery and vein were anastomosed with 10-0 nylon under an operating microscope, and the sciatic nerve was sutured with 10-0 nylon. Muscles and skin were approximated with 4-0 nylon. After the operation, neither systemic anticoagulants nor antibiotics were administered. The mean operating time was 60 minutes. Blood samples from the external jugular vein were obtained 60 minutes after surgery for analysis of serum levels of creatine phosphokinase (CPK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatinine (Cr), blood urea nitrogen (BUN), and potassium (K).

Electrophysiological Analysis

Electrophysiological recordings were made 8 weeks after surgery by using a method previously reported.[14] Under anesthesia, the skin and muscles of the transplanted limb were incised and the sciatic nerve was exposed. Evoked electromyography was recorded with needle electrodes. The sciatic nerve was stimulated with the cathode placed 10 mm proximal to the sciatic nerve suture (0.2-mA monophasic pulse at 1 Hz; pulse duration, 1 ms). The recording electrode was attached to the belly of the tibialis anterior muscle. The distance between the stimulating and recording sites was standardized at 30 mm. Both motor latencies and amplitudes were recorded.

Histological Analysis of Muscles in Replanted Limbs

After the electrophysiological analysis, the tibialis anterior muscle was harvested as a single unit, fixed with 10% formalin, and embedded in paraffin. Histological specimens (3 μm thick) were examined after hematoxylin and eosin staining and Masson's trichrome staining; the latter was used to identify fibrous tissues. We evaluated the diameter of the muscle fibers and the percentage of fibrous area. One hundred randomly selected cross-sectional diameters of muscle cells were measured, and the blue collagenous areas after Masson's trichrome staining were measured in 10 random 200× fields per animal. These items were evaluated by using ImageJ-1.44p.

Statistical Analysis

SPSS version 17 software (SPSS, Chicago, Ill.) was used for all statistical analyses. Data were expressed as mean ± 1 SD. Data were compared between 2 groups by using Student's t test. Data were compared among 3 groups by using one-way analysis of variance followed by Turkey's multiple comparison test. Differences with values of $P < 0.05$ were considered statistically significant.

RESULTS

Viability of Isolated Muscles Preserved with ETK, UW, or LR and Assessed by Using an In Vivo Bioimaging System

Isolated muscles preserved in ETK and UW maintained higher luminescence at all observation times than muscles immersed in LR as a control (Fig. 1A). Muscles in the LR group rapidly lost their viability—by only 1.5 hours after the start of preservation. By contrast, the ETK group, especially, maintained a luminescence flux of more than 60% of normal for as long as 12 hours (Fig. 1B).

Histological Analysis of Isolated Muscles

Histologically, damaged muscle fibers and interstitial edema were detected at high rates in the LR group at 3 hours. The percentages of damaged muscle fibers were significantly lower in the ETK group than in the LR group at all observation times ($P < 0.05$) (Fig. 2A). Moreover, the percentages of interstitial edema in the ETK and UW groups were

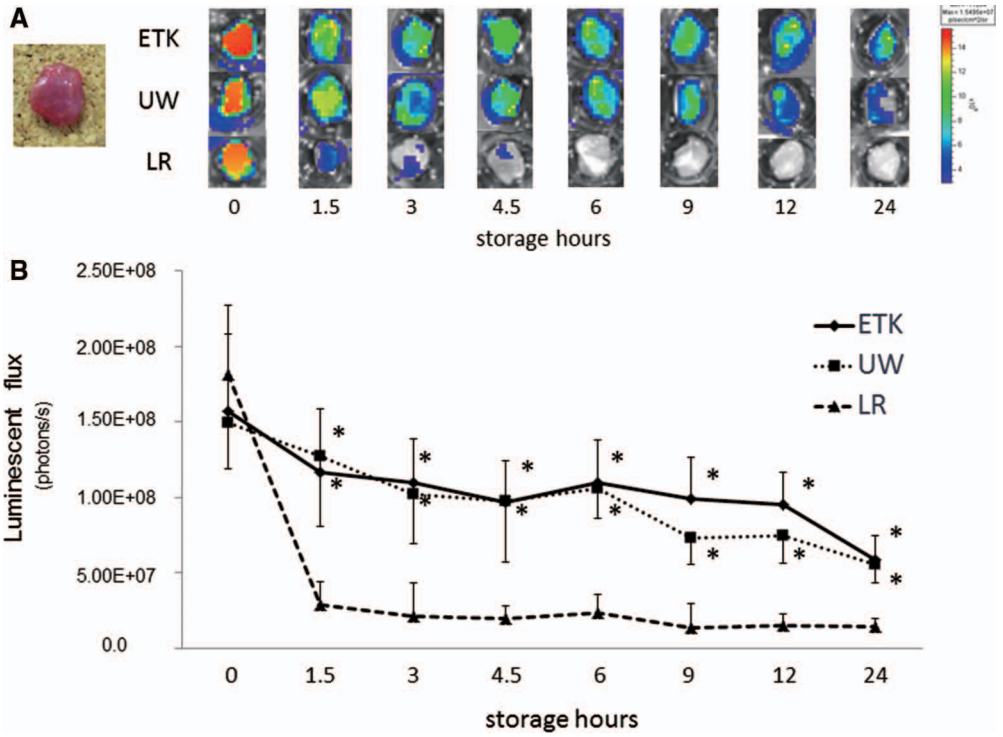

Fig. 1. A, Luc-Tg rat muscle cut to a uniform columnar shape (diameter 8 mm, height 3 mm, and weight 2 g) and muscles emitting bioluminescence in the in vivo bioimaging system. Muscles preserved in either ETK or UW maintained their luminescence, whereas the luminescence of the muscles preserved in LR diminished rapidly. B, Time course of photon signals from muscles stored in each solution. Photon signals from muscles preserved in either ETK or UW stayed high, whereas those from muscles preserved in LR declined rapidly. Error bars = SD (n = 16). *$P < 0.05$ vs LR.

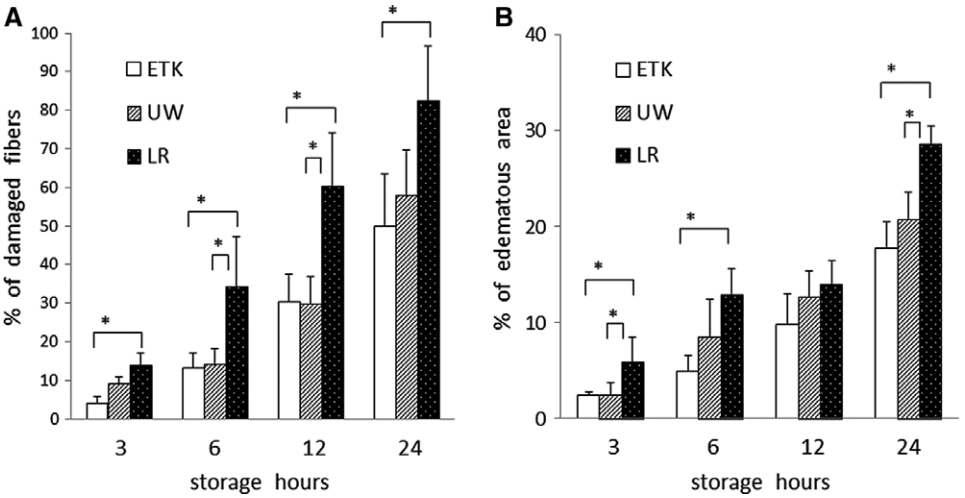

Fig. 2. The percentages of damaged fibers (A) and edematous area (B) 3, 6, 12, and 24 h after the start of preservation in each solution. The percentage of damaged fibers was significantly lower in muscles preserved in ETK than in those preserved in LR at all observation times. At 3 and 24 h, the area of interstitial edema was significantly smaller in muscles preserved in either ETK or UW than in those preserved in LR. Error bars = SD. *$P < 0.05$.

significantly lower than that in the LR group at 3 and 24 hours ($P < 0.05$) (Fig. 2B; **Supplemental Fig. 2**, **Supplemental Digital Content 1**, which shows magnetic resonance image of quadriceps femoris muscles preserved with ETK or LR for 24 h and hematoxylin-and-eosin-stained sections of muscles subjected to magnetic resonance imaging, ***http://links.lww.com/PRSGO/A15***).

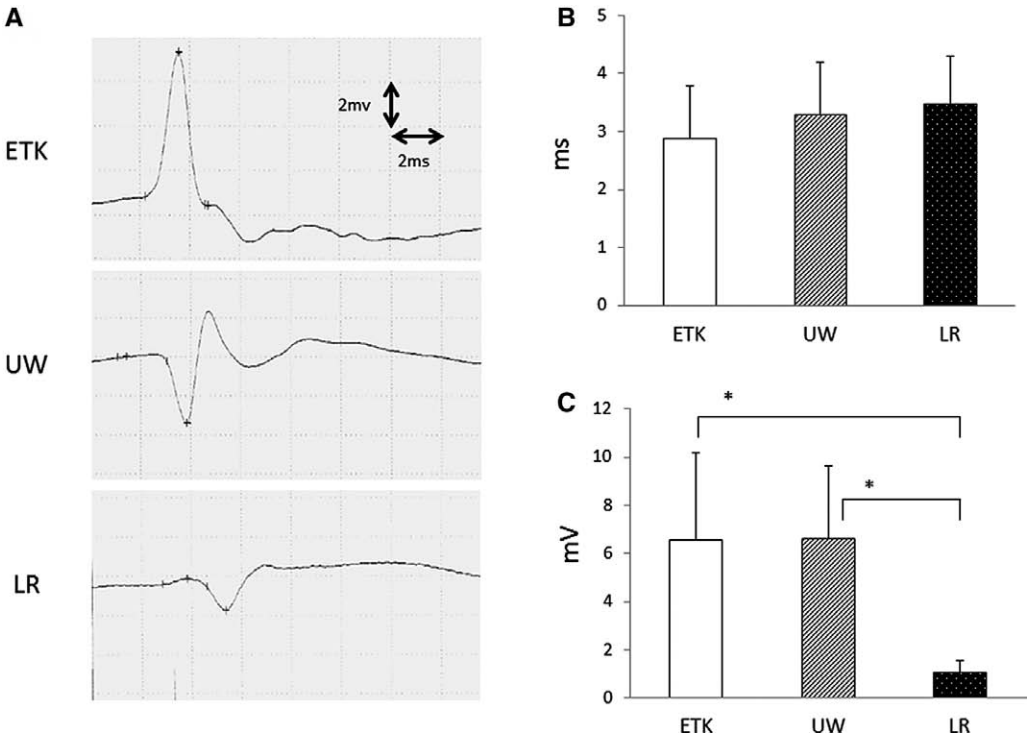

Fig. 3. Results of electrophysiological assessment of limbs preserved for 6 h. A, Representative wave forms of the 3 groups. One vertical division is 2 mV and one horizontal division is 2 ms. Latency duration (B) and amplitude (C) recorded from the tibialis anterior muscle by stimulating the sciatic nerve. Although there was no significant difference in latency time between the 3 groups, amplitude was significantly lower in the LR group than in the ETK or UW group. Error bars = SD. *P < 0.05.

Transplantation of Limbs Preserved for 6 hours

Survival rates in the 3 groups in the 6-hour preservation and transplantation model were all 100% for at least 8 weeks after the transplantation. All rats were killed at 8 weeks for electrophysiological and histological analysis. Blood samples were obtained 60 minutes after the end of surgery for analysis of serum CPK, AST, ALT, LDH, Cr, BUN, and K levels. The serum CPK level in the LR group was significantly higher than those in the ETK and UW groups (ETK, 5603±1032 IU/L; UW, 6591±2149 IU/L; LR, 17,147±8989 IU/L; $P < 0.05$), and the serum K level in the UW group was significantly higher than that in the ETK group (ETK, 4.7±0.29 mEq/L; UW, 5.4±0.36 mEq/L; LR, 4.8±0.45 mEq/L; $P < 0.05$). There were no significant differences in serum AST, ALT, LDH, Cr, and BUN levels (data not shown).

We examined the wave forms in the 3 groups (Fig. 3A). There were no significant differences in latency time among the 3 groups (Fig. 3B). By contrast, the amplitude in the LR group was significantly lower than those in the ETK and UW groups ($P < 0.05$) (Fig. 3C).

Representative cross-sections of the tibialis anterior muscle in limbs preserved with ETK, UW, or LR for 6 hours are shown in Figure 4. Morphology of the muscle fibers was maintained in the 3 groups. There were no significant differences in the percentages of fibrous area, as shown by Masson's trichrome staining, between the 3 groups (ETK, 2.9% ± 1.0%; UW, 3.1% ± 1.4%; LR, 4.1% ± 1.4%). By contrast, the diameter of muscle cells was significantly smaller in the LR group than in the ETK group (ETK, 39.6±8.0 μm; UW, 35.5±5.5 μm; LR, 32.7±6.1 μm; $P < 0.05$). A significant difference was observed between the LR and ETK groups in terms of the wet weight of the tibialis anterior muscle isolated as a mass (ETK, 0.66±0.10 g; UW, 0.53±0.09 g; LR, 0.49±0.04 g; $P < 0.05$).

Transplantation of Limbs Preserved for a Long Time (24 hours)

Survival rates at 8 weeks in the (immediate transplantation) control, ETK, UW, and LR groups in the 24-hour preservation and transplantation model were 100%, 80%, 80%, and 37.5%, respectively (Table 1). All surviving rats were killed at 8 weeks after transplantation for analysis. We then examined the relationship between survival at 8 weeks and the values of CPK and K 60 minutes after surgery. Serum CPK levels were significantly higher in the LR group than in the other groups ($P < 0.05$) (Fig. 5A). Five out of 6 rats that had serum CPK levels > 13,000

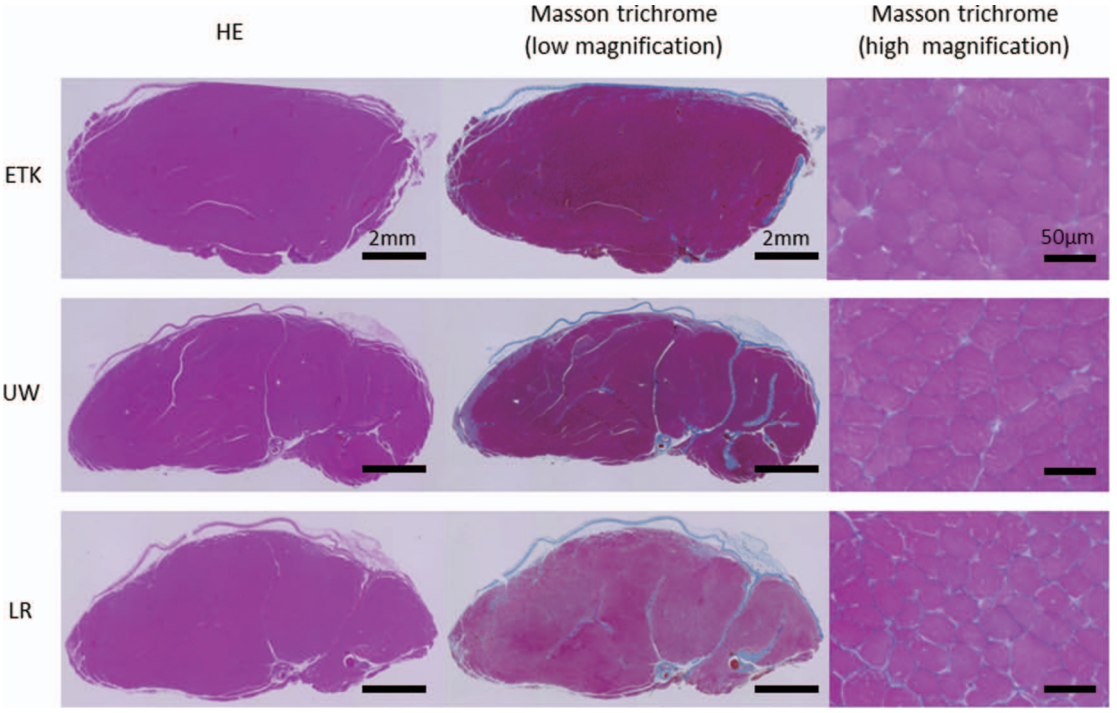

Fig. 4. Hematoxylin and eosin and Masson's trichrome (low and high magnification) staining of cross-sections of tibialis anterior muscle 8 wk after transplantation. Muscle fiber structure was maintained nearly equally in all 3 groups.

Table 1. Survival Times in the 24-Hour-Preserved Limb Transplantation Model

	n	Host Survival Periods (d)*	Survival Rate (%)
Control	4	≥56, ≥56, ≥56, ≥56	100
ETK	5	5, ≥56, ≥56, ≥56, ≥56	80
UW	5	3, ≥56, ≥56, ≥56, ≥56	80
LR	8	3, 3, 3, 3, 4, ≥56, ≥56, ≥56	37.5

*At 56 d, all remaining rats were killed for electrophysiological and histological examination.

IU/L 60 minutes after transplantation were dead by 4 days (Table 1). K levels were significantly higher in the LR group than in the no-preservation control and ETK groups ($P < 0.05$) (Fig. 5B). There were no significant differences in serum AST, ALT, LDH, Cr, and BUN levels (data not shown).

We examined wave forms in the 4 groups (Fig. 6A). We could not detect electrical signals in any of the 3 remaining rats in the LR group. Terminal latency was significantly shorter in the (immediate transplantation) control group than in the other 2 groups ($P < 0.05$) and was significantly longer in UW than ETK ($P < 0.05$). Amplitude was significantly higher in the control group than in the UW and ETK groups ($P < 0.05$) (Figs. 6B, C). In the ETK and UW groups, the terminal latency and amplitude of the muscles in 24-hour-preserved limbs were almost the same as those in 6-hour-preserved limbs (Figs. 3 and 6).

Representative cross-sections of tibialis anterior muscle in limbs preserved with ETK, UW, or LR for 24 hours are shown in Figure 7. The fibrous area of the LR group (stained blue with Masson's trichrome) was much greater than that in the other groups.

The percentage of fibrous area was significantly greater in the LR group than in the ETK, UW, and the control groups (ETK, 6.5% ± 1.4%; UW, 8.5% ± 2.6%; LR, 29.4% ± 2.0%; control, 4.0% ± 0.4%; $P < 0.05$); this was in contrast to the results in the 6-hour preservation model (Fig. 6A). The diameters of the muscle cells were significantly smaller in the LR group than in the other 3 groups (ETK, 33.7 ± 5.7 μm; UW, 31.2 ± 3.6 μm; LR, 22.9 ± 6.1 μm; control, 40.4 ± 5.4; $P < 0.05$). No significant differences were observed in the wet weight of the tibialis anterior muscle between the (immediate transplantation) control and the ETK and UW groups (control, 0.67 ± 0.16 g; ETK, 0.51 ± 0.09 g; UW, 0.42 ± 0.13 g; LR, 0.23 ± 0.06 g).

DISCUSSION

The current method of preserving amputated limbs involves simply wrapping them with saline-moistened gauze and then cooling them on ice.[21] This method is basically similar to Allen's,[22] which was reported more than 70 years ago. Novel methods of preserving muscle tissues have not been developed for a long time.

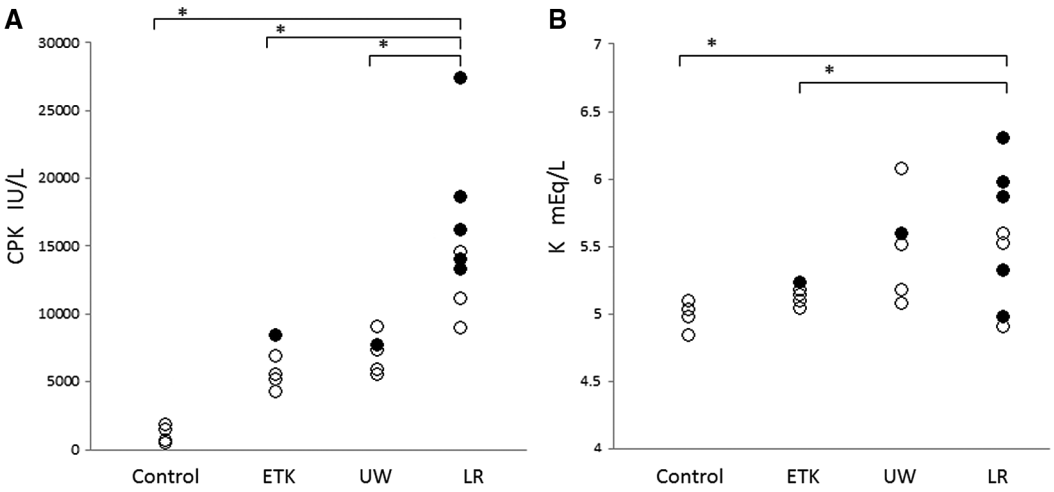

Fig. 5. Recipients' serum levels of CPK (A) and K (B) at 60 min after transplantation (24-hour-preserved model). Numerical values are plotted. White circles, survival; black circles, death. Serum CPK levels of 5 of the mice that later died were more than 13,000 IU/L at 60 min after transplantation. K levels were significantly higher in the LR group than in the (immediate transplantation) control and ETK groups. *$P < 0.05$.

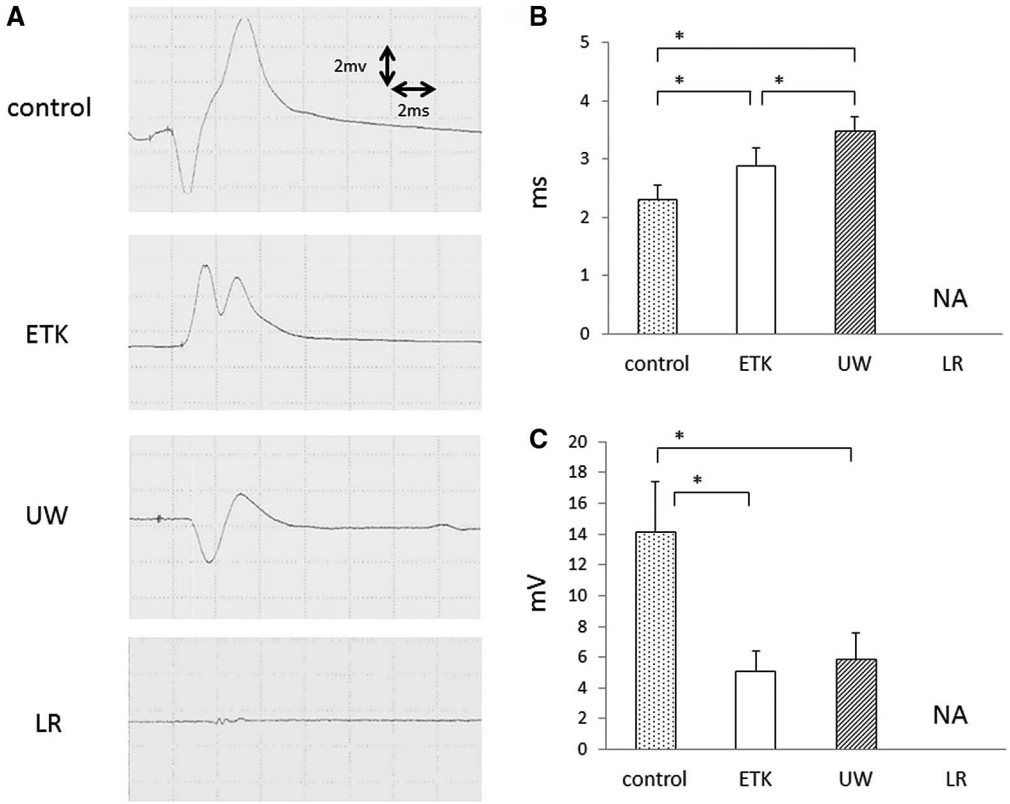

Fig. 6. Results of electrophysiological assessment of limbs preserved for 24 h. A, Representative wave forms of the 4 groups. One vertical division is 2 mV and one horizontal division is 2 ms. Latency time (B) and amplitude (C) recorded from the tibialis anterior muscle by stimulating the sciatic nerve. Electrical signals were not detected in any of the 3 rats remaining in the LR group. In the ETK and UW groups, the terminal latency and amplitude of the muscles in the 24-hour-preserved limbs were almost the same as those in the 6-hour-preserved limb. Error bars = SD. *$P < 0.05$. NA indicates no assessment.

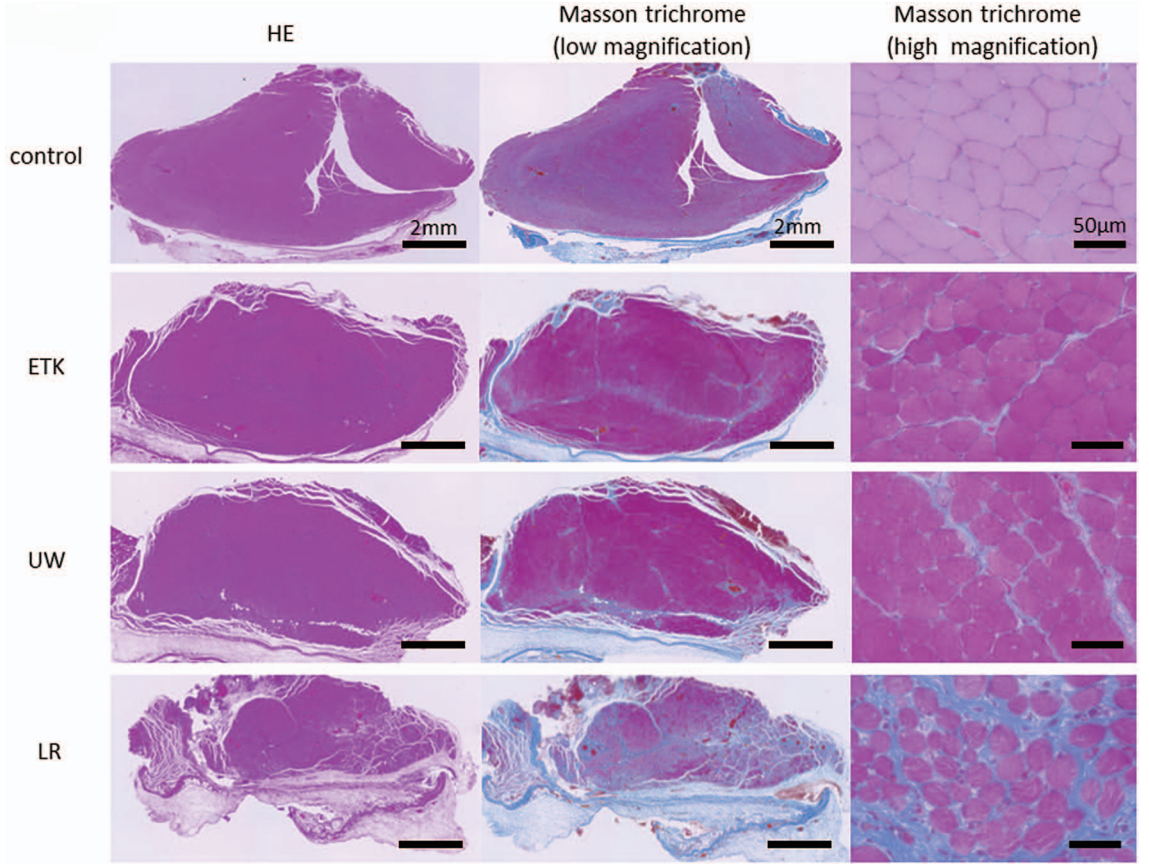

Fig. 7. Hematoxylin and eosin and Masson's trichrome (low and high magnification) stains in cross-sections of the tibialis anterior muscle 8 wk after transplantation. The fibrous area of the LR group (stained blue with Masson's trichrome) was much greater than that in the other groups.

There have been several animal experiments on muscle preservation and composite tissue transplantation. They include the use of organ preservation solutions, determination of optimum preservation temperatures, and evaluation of the effects of tissue perfusion before transplantation.[13,23–25] These experimental studies have indicated the usefulness of various preservation solutions in limb replantation, but to date there is no preservation solution used routinely in clinical practice for preserving amputated limbs. Meanwhile, in human hand transplantation, the graft is simply irrigated with UW solution before transplantation.[2,26]

Perfusion of preservation solution is used routinely in human solid-organ transplantation. However, the effect of perfusion on skeletal muscle is still controversial.[24] In addition, traumatic amputation of limbs and fingers often happens in the field (eg, factory and farm) rather than in a hospital. At these locations, soaking the injured tissue in preservation solution is regarded as the best option, given the likely lack of appropriate facilities and equipment to perfuse the tissue. For this reason, we used the simple soaked-tissue model in our study.

Here, we used ETK and UW to preserve muscle. ETK is an extracellular-type solution, whereas UW is an intracellular-type solution (**Supplemental Table 1**, **Supplemental Digital Content 1**, ***http://links.lww.com/PRSGO/A16***). Our data showed that the serum K level after transplantation was higher in the UW group than in the ETK group. Although there were no differences between the ETK and UW groups in terms of survival rate, hyperkalemia may represent a risk for replantation toxemia shock.[10,27,28] Therefore, extracellular solution, with its lower K level, may be preferable to intracellular solution for preserving muscle tissues. ETK solution has another advantage: its ingredients are chemically stable at room temperature for 3 years.[29] On the other hand, UW solution needs to be kept cold and used within a short period due to oxidization of glutathione in UW.[30] Therefore, ETK solution has an advantage in the case of unexpected amputation accidents, because it means that the ETK can be kept for long periods in an ambulance or primary care clinic.

Currently, replantation is defined as successful not only when there is vascular circulation to the

amputated limb but also when limb function is restored. Some animal experiments have evaluated functional recovery of ischemia–reperfusion limbs. Song et al[31] reported good functional recovery of rat limb allografts after the immediate limb transplantation by using a cutaneous reaction test, walking track analysis, and electrophysiological evaluation. Tsuji et al[14] performed an electrophysiological study after rat limb transplantation in several ischemia periods and found that the distal motor latencies of the sciatic nerve at 3 weeks increased with increasing limb ischemia time. Here, we used an electrophysiological study to demonstrate that preservation solutions improved limb functional recovery after transplantation. The results of this method may depend on not only muscle function recovery but also sciatic nerve regeneration. Nevertheless, our electrophysiological outcomes seemed to parallel the degree of muscle atrophic and fibrous change.

In our in vitro study, we used luciferase transgenic rats to trace muscle viability. In this system, the degree of preservation is evaluated by assessing the amount of ATP, which depends on the intensity of the photons emitted by the tissues. We previously demonstrated a relationship between photon intensity and cell viability.[32] The degree of irreversible muscle damage is correlated with the amount of ATP lost during ischemia.[7] Depletion of ATP means that cell activity stops; as a result, the cell cannot maintain homeostasis. Because the Na^+/K^+ transporter relies strongly on the use of ATP for energy, loss of ATP causes cell swelling owing to inflow of sodium ions into cells.[33] Measuring the amount of ATP is therefore a general way of assessing the degradation of preserved tissue.[34]

Serum CPK levels were significantly higher in the LR group than in the other groups ($P < 0.05$) (Fig. 5A). Moreover, K levels were significantly higher in the LR group than in the no-preservation control and ETK groups ($P < 0.05$) (Fig. 5B). In light of our data, we hypothesize that the progressive ischemic change of the muscles in the LR group led to replantation toxemia, resulting in renal dysfunction and cardiac failure. By contrast, ETK and UW solutions may protect muscles from ischemia, increasing survival among rats that received muscle grafts that had been preserved in ETK or UW solution.

CONCLUSION

We verified the effect of preservation solutions in a rat replantation model. We showed that ETK and UW organ preservation solutions could protect muscle function and morphology in transplanted limbs and improve recipient survival rates following transplantation of long-term-preserved limbs.

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