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Evaluation of Early Markers of Ischemia-reperfusion Injury and Preservation Solutions in a Modified Hindlimb Model of Vascularized Composite Allotransplantation

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Background. Ischemia-reperfusion injury plays an important role in vascularized composite allotransplantation (VCA). Currently, there is no ideal preservation solution for VCA. In this study, we investigated the effects of 4 different preservation solutions on different tissues within an allogeneic hindlimb rat model. **Methods.** Sprague Dawley rat hindlimbs were flushed and placed at 4°C for 6 h in heparinized saline, histidine-tryptophan-ketoglutarate, University of Wisconsin (UW), and Perfadex and heterotopically transplanted for ease of ambulation. Apoptosis, necrosis, and the extracellular matrix of the tissues within the allograft were analyzed 2 h posttransplantation using immunohistochemistry, terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) assay, and enzyme-linked immunoassay. **Results.** Higher expression of cleaved caspase 3, a significant increase of high-mobility group box 1 and TUNEL-positive apoptotic cells were observed in the muscle and vessels preserved with heparinized saline compared with UW and Perfadex following reperfusion. Higher expression of TUNEL-positive apoptotic cells was observed in the skin at 12 h of ischemia and in the nerve following reperfusion with histidine-tryptophan-ketoglutarate as a preservation solution. **Conclusions.** Our data suggest that UW and Perfadex are preferred solutions in VCA. The vessels within the allografts appear to be very susceptible, with laminins and CD31 playing a role in ischemia-reperfusion injury.

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The data that support the findings of this study will be made openly available. Repository name and reference number will be made available.

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INTRODUCTION

Vascularized composite allotransplantation (VCA) is considered a life-enhancing therapeutic option and can be beneficial for patients with facial disfigurement and limb amputation.[1] Despite its importance, there is need for further research as most of the current data and protocols are derived from solid organ transplantation. Ischemia is clinically an inevitable factor following procurement. Different tissues within these allografts are not affected to the same extent by the loss of blood supply with some being more vulnerable than others.[2,3] The role of ischemia-reperfusion injury (IRI) in VCA is often underestimated, which can determine graft survival, function, and rejection.

During ischemia followed by reperfusion, production of reactive oxygen species and release of damage associated molecular patterns lead to induction of cell injury, apoptosis, chemotaxis, and increase in leukocyte activation.[4-6] An effective preservation solution is necessary to mitigate the harmful effects of ischemia between organ procurement and transplantation.[7] Various preservation solutions have been used in solid organ transplantation and in experimental VCA[8]; however, in clinical VCA, the application of heparinized saline, histidine-tryptophan-ketoglutarate (HTK), University of Wisconsin (UW), and Institute Georges Lopez-1 has only been reported.[9,10] Although an ideal preservation solution can play a pivotal role in attenuating IRI, there is no agreement on the

use of an optimal preservation solution globally in VCA.[11,12] Furthermore, there is lack of high-powered clinical study to rationale the use of 1 preservation solution over another.[11,12]

Moreover, most animal studies comparing different preservation solutions in VCA were performed in isograft and syngeneic models with absence of alloreactivity, making these studies difficult to translate clinically.[13-17] The events surrounding IRI contribute to the stimulation of recipient T cells to the transplanted organ leading to T-cell activation.[4-6,18,19] These are considered obstacles for inducing tolerance in organ allotransplantation therefore leading to graft dysfunction and acute rejection.[18-23]

In this study, a heterotopic allogeneic rat hindlimb model of VCA was utilized to test our hypothesis that various preservation solutions (heparinized saline, HTK, UW, and Perfadex) will have different effects on early markers IRI. We evaluate each of the components within the allograft including skin, muscle, vessels, nerve, and bone. Furthermore, our study focuses on the vascular integrity within these allotransplants.

MATERIALS AND METHODS

Animals

Male Sprague Dawley, Wistar, and inbred Lewis rats (400–450 g; Envigo, Indianapolis, IN) were used in this study. The animal use protocol was approved by the Animal Care Committee at the Toronto General Hospital Research Institute, University Health Network (Toronto, ON, Canada) and was performed in compliance with relevant guidelines. All animals received humane care.

Experimental Design

The experimental groups and design are summarized in Table 1. Experimental group I serves as validation of the animal model. They represent proof of concept experiments looking at graft viability and rejection long-term following transplantation in both syngeneic and allogeneic models. Inbred Lewis rats served as both recipients and donors for the syngeneic transplantation. Sprague Dawley rats served as donors and Wistar rats served as recipients for the allogeneic transplantation (because of size match and availability). In experimental group I, a heterotopic hindlimb transplantation was performed *immediately* after procurement of donor limbs with minimal ischemia time in both syngeneic (n = 3) and allogeneic groups (n = 3). Minimal ischemia time refers to 45 min to 1 h, which is the time required to perform the transplantation in the recipient. The animals were not immunosuppressed in the allogeneic group. Animal survival and flap viability was assessed over a 7-d survival period in the syngeneic group. Signs of rejection were assessed within the flap in allogeneic group by clinical examination and histology on skin biopsies.

Experimental group II represent proof of concept experiments to examine markers of IRI in a syngeneic and allogeneic model following reperfusion after a 6-h ischemia period. Particularly, these experiments represent the importance of using an allogeneic model to study IRI. The effects of IRI were assessed in a separate set of animals. Syngeneic (n = 3) and allogeneic animals (n = 3) limbs underwent an ischemic period of 6 h. The limbs were flushed with heparinized saline (n = 3) through the femoral artery and then wrapped in gauze with saline and placed at 4°C for 6 h. These limbs were heterotopically transplanted (as described below) into the recipients and the animals were euthanized after 2 h. Experiment groups I and II serve as proof of concept for graft viability of our model and differences between syngeneic and allogeneic animals.

Experimental group III serves to test our hypothesis where we compared the effects of different preservation solutions on IRI. *These studies were conducted in an allogeneic model only*. In the reperfusion after 6 h of ischemia group (reperfusion group), the hindlimbs were procured from donor animals (n = 12) and divided into 4 groups. The limbs were flushed with heparinized saline (n = 3), heparinized HTK (HTK; Custodiol HTK; Methapharm, Brantford, Ontario) (n = 3), heparinized UW (Belzer solution, Viaspan, Bridge to Life, Columbia, SC) (n = 3), and heparinized Perfadex (Xvivo Perfusion, Göteborg, Sweden) (n = 3) through the femoral artery, then wrapped in gauze with the respective preservation solution and placed at 4°C for 6 h. These limbs were heterotopically transplanted (as described later) into the recipients. The animals were euthanized following 2 h of reperfusion. In the ischemia only groups, the contralateral hindlimbs of donor rats were procured,

TABLE 1.

Experimental groups and design

Experimental group I	**Rationale:** proof of concept experiments to validate animal model; looking at graft viability and rejection long-term following transplantation	BOTH syngeneic AND allogeneic transplantation (n = 3 for each group); 6 animals total	Minimal ischemia time 7-d survival Data represented in Figure S2, SDC, http://links.lww.com/TXD/A383
Experimental group II	**Rationale:** proof of concept experiments to examine differences in ischemia markers in a syngeneic and allogeneic model following reperfusion; shows importance of using an allogeneic model to study VCA	BOTH syngeneic AND allogeneic transplantation (n = 3 for each group) 6 animals total	6-h ischemia time Transplantation studies where grafts are reperfused 2-h survival Heparinized saline ONLY used as preservation solution Analysis of markers of IRI Data represented in Figure S3, SDC, http://links.lww.com/TXD/A383
Experimental group III	**Rationale:** to evaluate the effect of preservation solutions on ischemia-reperfusion injury in an allogeneic model	Allogeneic transplantation ONLY (n = 3 for each group) 36 animals total	Groups: 6-h ischemia time (n = 12); 12-h ischemia time (n = 12); reperfusion following 6 h of ischemia (n = 12) Preservation solutions: heparinized saline (n = 9); HTK (n = 9); UW (n = 9); Perfadex (n = 9) 2-h survival for reperfusion group Analysis of markers of IRI Data represented in Figures 1–8; Figures S4 and S5, SDC, http://links.lww.com/TXD/A383

HTK, histidine-tryptophan-ketoglutarate; IRI, ischemia-reperfusion injury; UW, University of Wisconsin; VCA, vascularized composite allotransplantation.

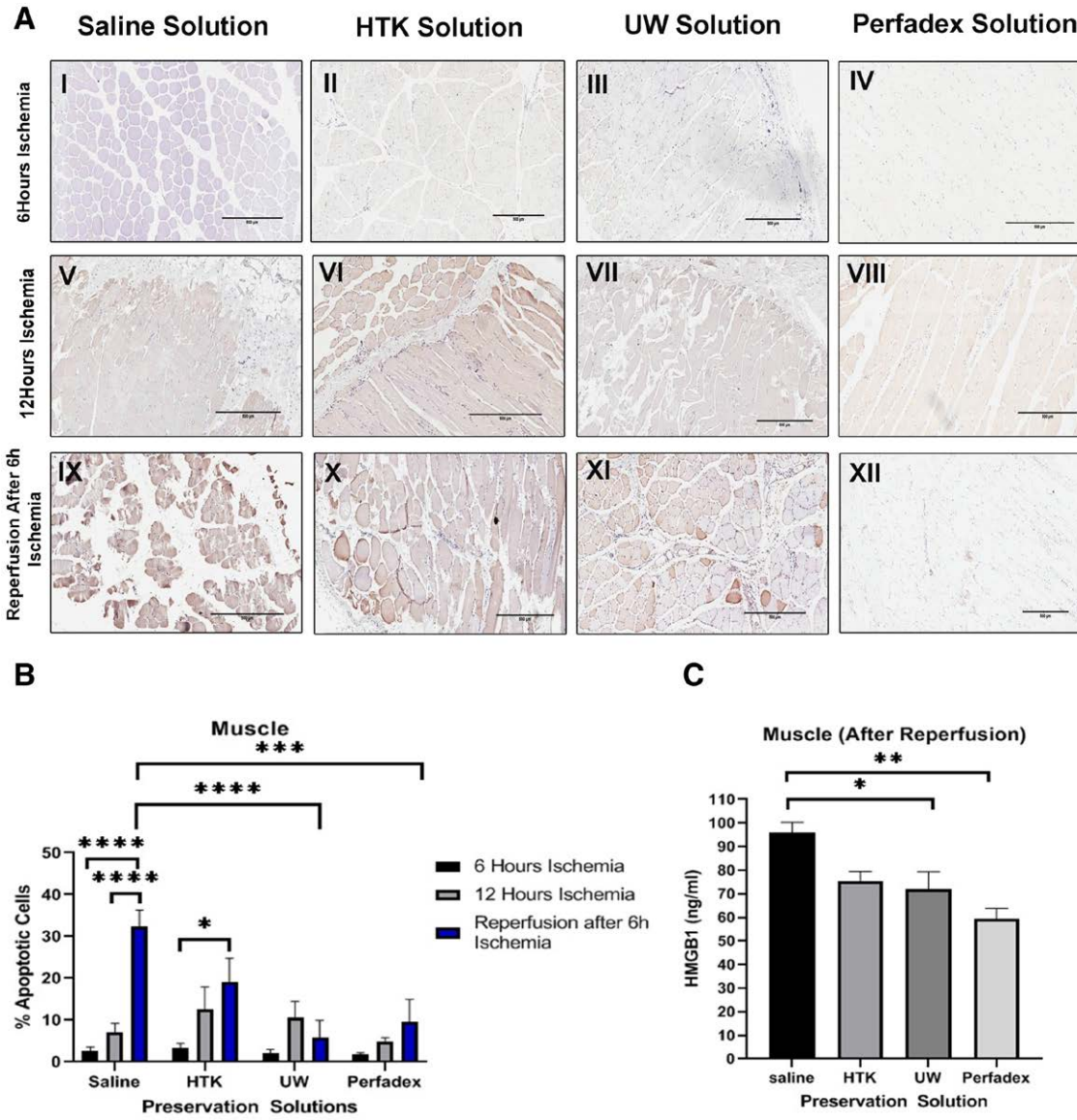

FIGURE 1. A, Expression of CC3 in muscle. The expression of CC3 was not observed in the 6 h ischemia group (I–IV). There is a higher expression of CC3 in all 12 h ischemia groups (V–VIII), especially with HTK (VI). There is also an increased expression of CC3 following reperfusion (IX–XII) in muscle preserved with heparinized saline (IX) with little expression with Perfadex (XII), n = 3 per group. B, Quantification of TUNEL-positive apoptotic cells in muscle. There is a significant increase in the percentage of apoptotic cells following reperfusion with heparinized saline compared with 6 h and 12 h of ischemia. There is also a significant increase in the percentage of apoptotic cells following reperfusion with HTK compared with 6 h of ischemia. The percentage of apoptotic cells is significantly higher in muscle preserved with heparinized saline compared with UW and Perfadex following reperfusion. All data represent a mean ± SEM; n = 3 per group; $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ as determined by a 2-way ANOVA with Tukey's multiple comparisons test. C, HMGB-1 expression in muscle following reperfusion. Muscle preserved with heparinized saline solution demonstrates significantly higher levels of HMGB-1 compared with UW and Perfadex following reperfusion. All data represent a mean ± SEM; n = 3 per group; $^{*}P < 0.05$ and $^{**}P < 0.01$ as determined by a 1-way ANOVA. ANOVA, analysis of variance; CC3, cleaved caspase 3; HMGB-1, high-mobility group box 1; HTK, histidine-tryptophan-ketoglutarate; TUNEL, terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick-end labeling; UW, University of Wisconsin.

flushed with different preservation solutions through the femoral artery and placed at 4°C for 6 (n = 12) or 12 h (n = 12).

Surgical Procedure

The rats were induced with 5% isoflurane and then maintained in 1.5%–2% isoflurane using inhalation anesthetic machine.

Donor Surgery

The surgery was performed as previously described.[18,19,24] Briefly, the skin was incised medially from the ankle to the groin and the muscles, femur and tibia were cut proximal to the knee joint and above the fibular bifurcation. The femoral artery and vein were dissected, skeletonized, and ligated proximally.

Recipient Surgery

Two linear incisions were made in the gluteal area. The flap was trimmed to proper size ensuring that the animal can move without any discomfort and pain. A subcutaneous tunnel was created from the inguinal area toward the gluteal area and the flap was passed through the tunnel and skin was

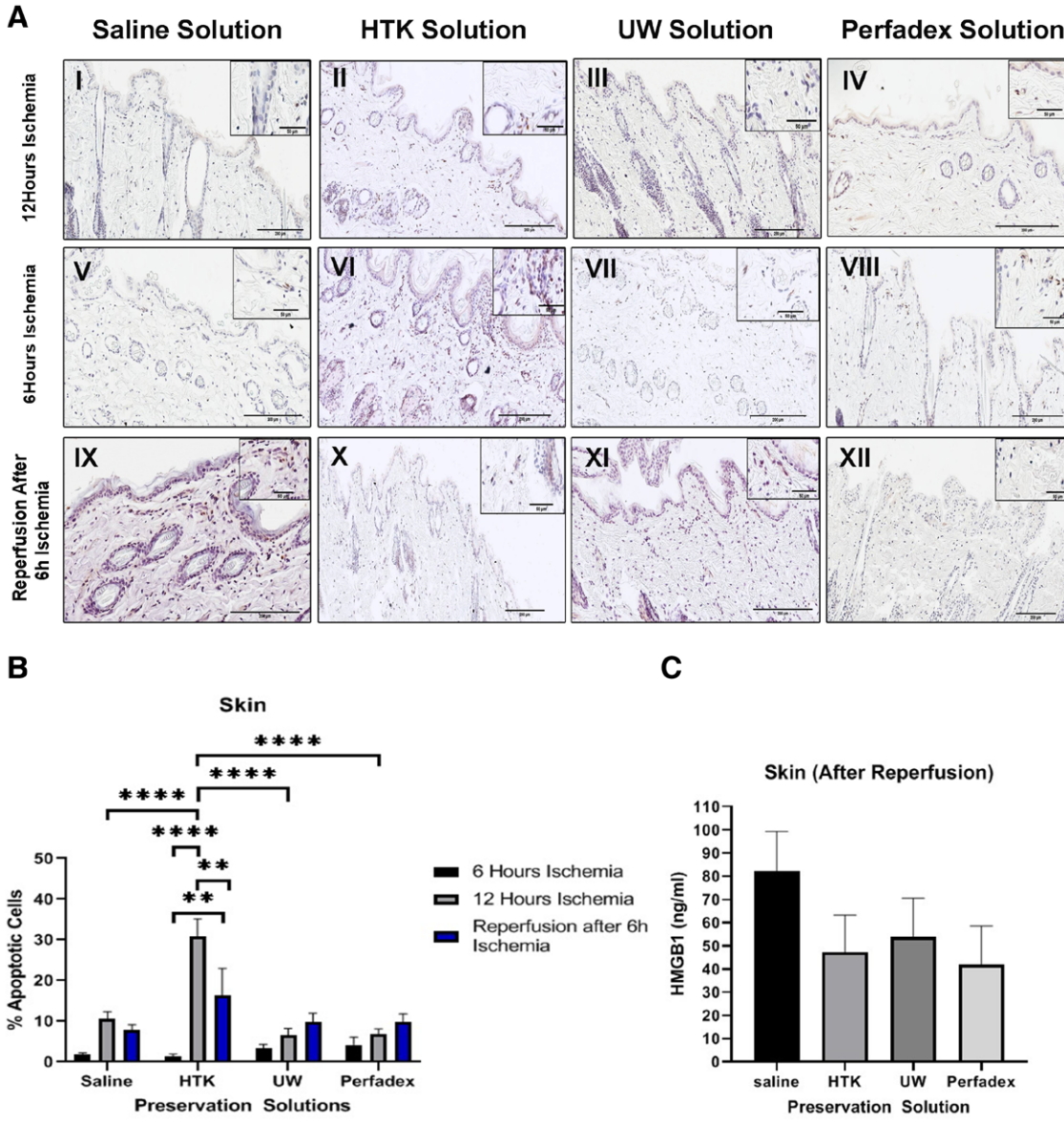

FIGURE 2. A, Expression of CC3 in skin. There is no difference in the expression of CC3 in skin preserved with saline, HTK, UW, and Perfadex solutions following 6 h of ischemia (I–IV), 12 h of ischemia (V–VIII), and reperfusion (IX–XII); n = 3 per group. B, Quantification of TUNEL-positive apoptotic cells in skin. There is a significant increase in the percentage of apoptotic cells in skin preserved with HTK after 12 h of ischemia compared with 6 h of ischemia and reperfusion groups. There is a significant increase in the percentage of apoptotic cells in skin preserved with HTK solution compared with saline, UW, Perfadex at 12 h of ischemia. All data represent a mean ± SEM; n = 3 per group; $^{**}P < 0.01$ and $^{****}P < 0.0001$ as determined by a 2-way ANOVA with Tukey's multiple comparisons test. C, HMGB-1 expression in skin following reperfusion. There is no significant difference in HMGB-1 expression in skin following reperfusion regardless of preservation solution. All data represent a mean ± SEM; n = 3 per group, as determined by a 1-way ANOVA. ANOVA, analysis of variance; CC3, cleaved caspase 3; HMGB-1, high-mobility group box 1; HTK, histidine-tryptophan-ketoglutarate; TUNEL, terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick-end labeling; UW, University of Wisconsin.

sutured in the recipient's gluteal region using 4-0 Prolene. The donor femoral artery and vein were anastomosed using 10-0 nylon sutures to the recipient's femoral vessels using microsurgical technique. The native hindlimb of the recipient was adequately perfused by the proximal deep perforating vessel and the recipient femoral nerve was left intact. The limbs were allowed to reperfuse for a total of 2 h, after which the rats were euthanized and the flaps were procured for assessment.

Histology and Immunohistochemistry

The components within the graft (skin, muscle, nerve, vessels, and bone) were procured separately and were fixed in 10% phosphate-buffered formalin for 24 h and paraffin embedded. The samples were sectioned in 5-μm–thick slides for hematoxylin and eosin and immunohistochemical staining. Specifically, the paraffin samples were cleared and rehydrated through a series of xylene and ethanol and stained with hematoxylin (Sigma-Aldrich, St. Louis, MO) and eosin (Thermo Fisher Scientific, Cheshire, United Kingdom). Presence of cleaved caspase 3 (CC3), laminin, and CD31 was evaluated using VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, CA) for antirabbit cleaved caspase 3 (1:1000, Asp175, Cell Signaling Technology, Beverly, MA), antirabbit laminin (1:1000, Ab11575, Abcam, Cambridge, MA), and antirabbit CD31 (1:1000, NB100-2284, Novus Biologicals, Littleton, CO). Briefly, proteinase K (Dako, Carpinteria, CA) was utilized for antigen retrieval and

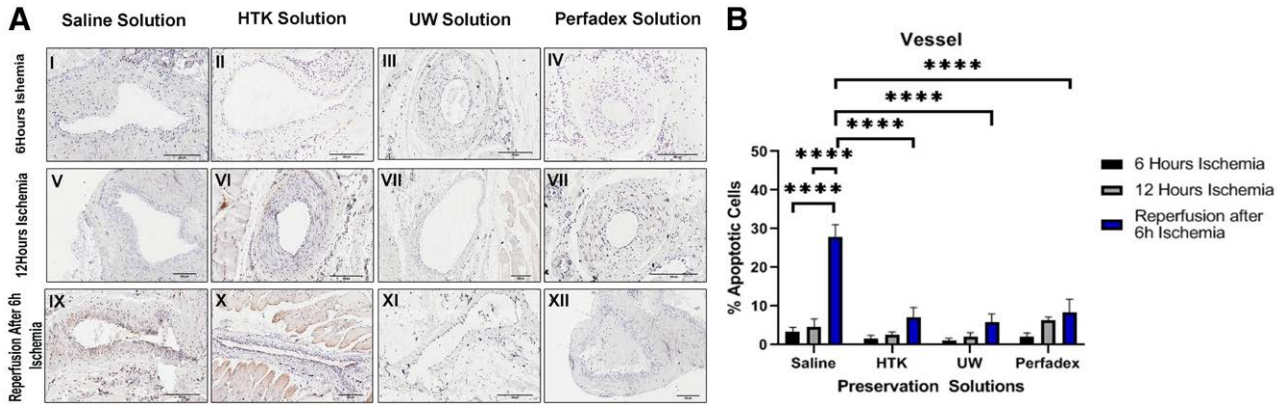

FIGURE 3. A, Expression of CC3 in vessels. There is no difference in the expression of CC3 in skin preserved with any preservation solutions following 6 h of ischemia (I–IV) and 12 h of ischemia (V–VIII). There is a higher expression of CC3 in vessels preserved with heparinized saline (IX) and HTK solutions (X) after reperfusion, n = 3 per group. B, Quantification of TUNEL-positive apoptotic cells in vessels. The percentage of apoptotic cells was significantly higher in the reperfusion compared with 6-h and 12-h ischemia groups preserved with heparinized saline. A significant increase in the percentage of apoptotic cells was also observed when heparinized saline was used as a preservation compared with HTK, UW and Perfadex in the reperfusion group. All data represent a mean ± SEM; n = 3 per group; ****$P < 0.0001$ as determined by a 2-way ANOVA with Tukey's multiple comparisons test. ANOVA, analysis of variance; CC3, cleaved caspase 3; HTK, histidine-tryptophan-ketoglutarate; TUNEL, terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick-end labeling; UW, University of Wisconsin.

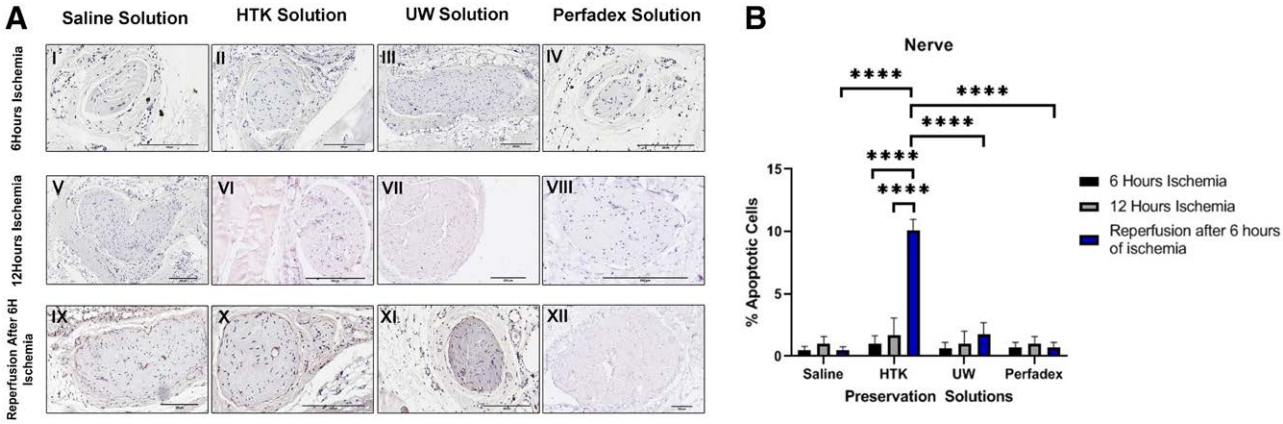

FIGURE 4. A, Expression of CC3 in nerve. There is no difference in the expression of CC3 in the nerve preserved with either solution or any of the groups, n = 3 per group. B, Quantification of TUNEL-positive apoptotic cells in nerve. There is a significant increase in the percentage of apoptotic cells following reperfusion compared with 12 and 6 h ischemia in the nerve preserved with HTK solution. The is also a significant increase in the percentage of apoptotic cells with HTK solution compared with other solutions following reperfusion. All data are mean ± SEM; n = 3 per group; ****$P < 0.0001$ as determined by a 2-way ANOVA with Tukey's multiple comparisons test. ANOVA, anaylsis of variance; CC3, cleaved caspase 3; HTK, histidine-tryptophan-ketoglutarate; TUNEL, terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick-end labeling.

nonspecific binding was blocked using normal horse serum. For the negative control, unspecific rabbit immunoglobulin G was utilized at the same dilution as the primary antibody (Figure S1, SDC, http://links.lww.com/TXD/A383). Slides were incubated with biotinylated secondary antibody for 30 min followed by 30-min incubation with RTU VECTASTATIN ABC Reagent. Then 30 µL of ImmPACT DAB Reagent was diluted in 1 mL of ImmPACT DAB Dilutent (Vector Laboratories, Burlingame, CA) and applied to the slides. They were counterstained with Harris Hematoxylin solution (Sigma-Aldrich, St. Louis, MO) and dehydrated and cleared in ethanol and xylene, respectively.

TUNEL Staining

To assess apoptosis, terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) assay was performed with In Situ Cell Death Detection Kit, TMR red (Roche, Mannheim, Germany). The slides were incubated in a humidified chamber for 20 min at 37°C and covered in proteinase K. The TUNEL reaction mixture was added to each slide. Samples were incubated for 60 min at 37°C, incubated with Hoechst (Molecular Probes Inc, Eugene, OR) for 5 min at room temperature, and mounted with ProLong Gold Antifade Mountant (Life Technologies, Carlsbad, CA). Negative controls received only Label Solution and positive controls were incubated in 3 U/mL DNase I (Invitrogen, Carlsbad, CA) solution in 1 mg/mL BSA, 340 mmol/L NaCl, and 50 mmol/L Tris, pH 7.6, for 10 min at room temperature before the labeling procedure.

Sample Preparation for Enzyme-linked Immunoassay

Skin and muscle were snap-frozen by immersing into liquid nitrogen. Twenty milligram tissue was added to microtube containing 300 µL of ice-cold lysis buffer and agitated for 2 h at 4°C. The samples were then centrifuged at 16 000 *g* for 20 min at 4°C. The pellet was discarded, and the supernatant was collected. Total protein concentration across samples was normalized using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instruction.

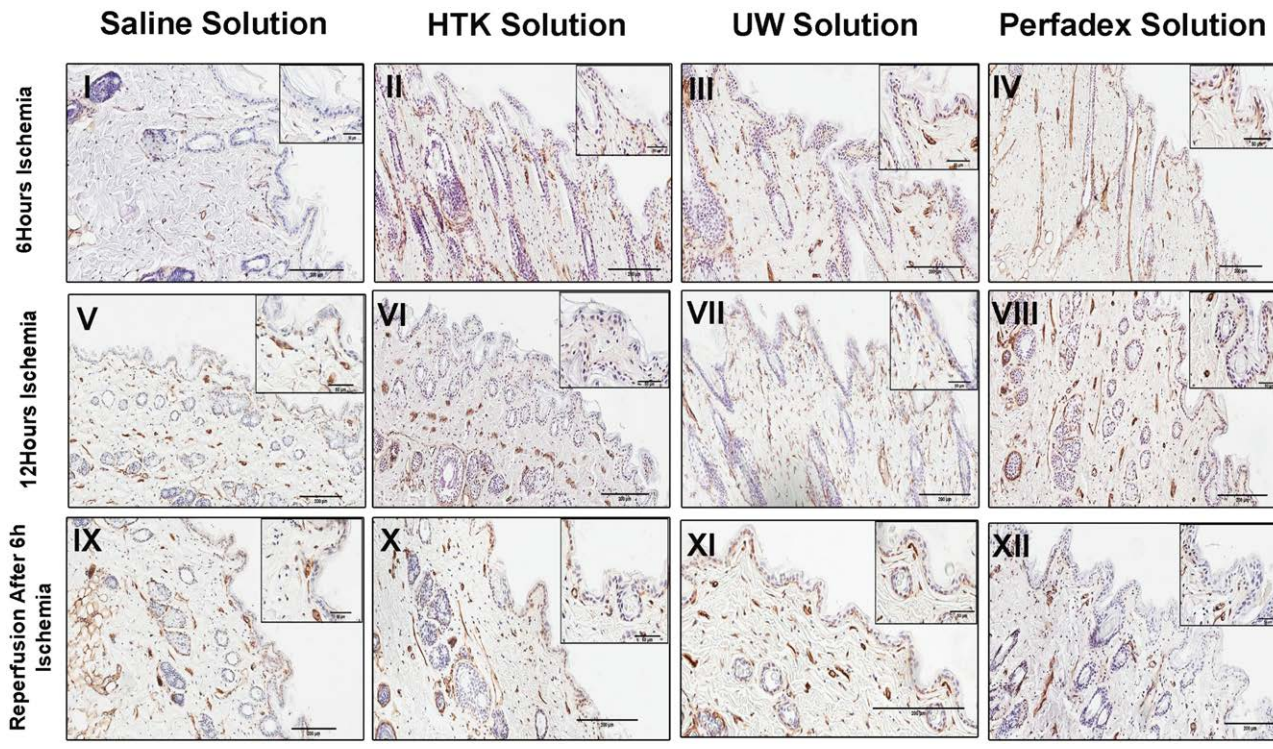

FIGURE 5. Expression of laminin in skin. The expression of laminin is reduced and disrupted in all ischemia groups and with all preservation solutions, n = 3 per group.

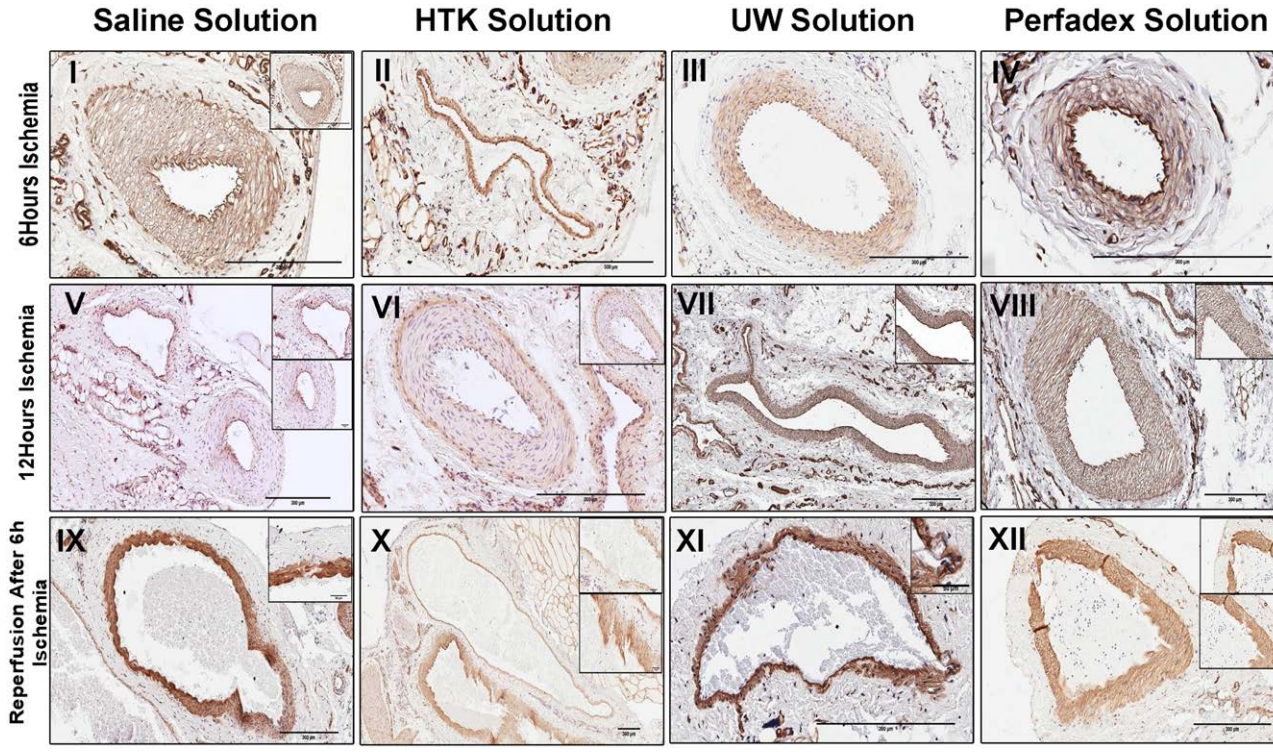

FIGURE 6. Expression of laminin in vessels. Laminin expression was preserved following 6 h of ischemia (I–IV). Laminin expression decreased in samples preserved with saline (V-insert) and HTK (VI-insert) solutions at 12 h of ischemia group. Disruption of laminin was observed in all vessels in the reperfusion group (IX–XII) regardless of preservation solution; n = 3 per group.

High-mobility Group Box 1 Enzyme-linked Immunoassay

Levels of high-mobility group box 1 (HMGB-1) were evaluated in duplicates using Rat HMGB-1 ELISA Kit (MyBioSource, San Diego, CA) according to manufacturer's protocol.

Image Analysis

Using HALO image analysis software (Indica Labs), the number of apoptotic cells were quantified in TUNEL samples and were analyzed as percentage of apoptotic cells compared with total cells.

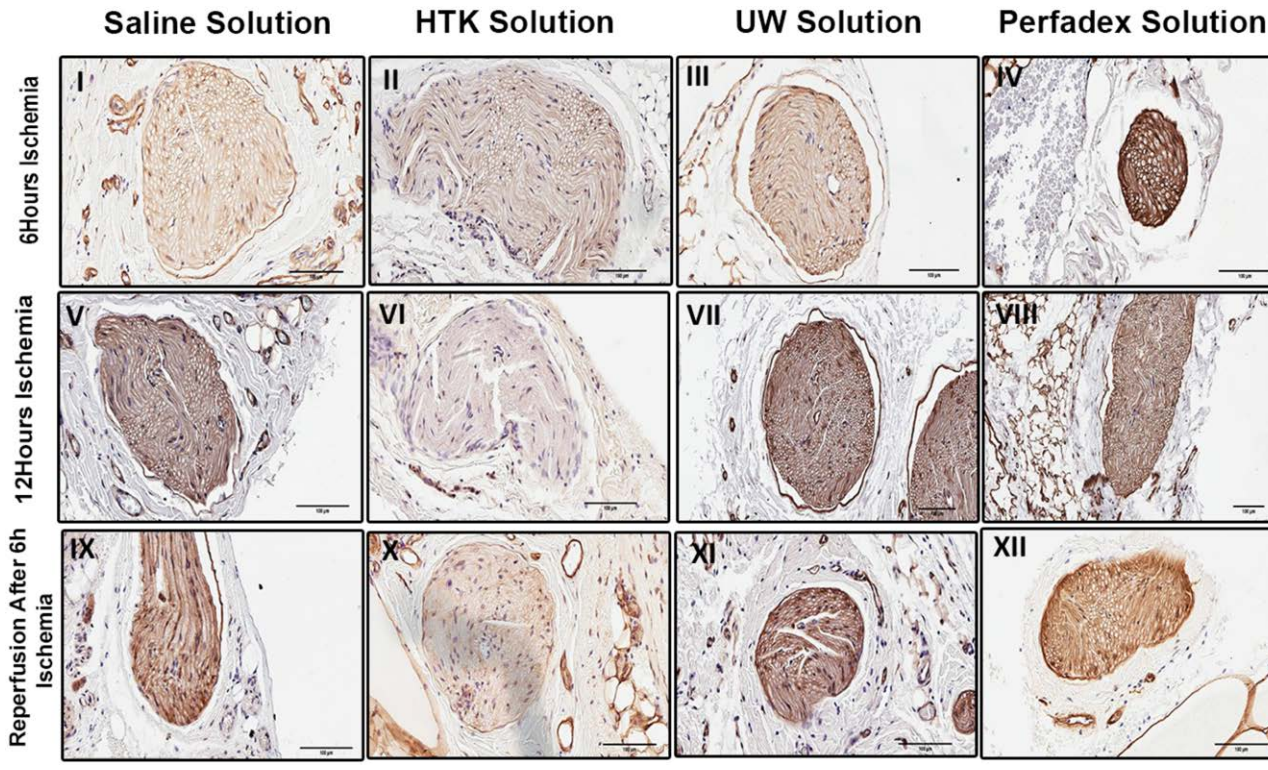

FIGURE 7. Expression of laminin in nerve. Laminin expression was preserved following 6 h of ischemia (I–IV). The expression decreased in samples preserved with HTK solutions at 12 h of ischemia (VI) and following reperfusion (X); n = 3 per group.

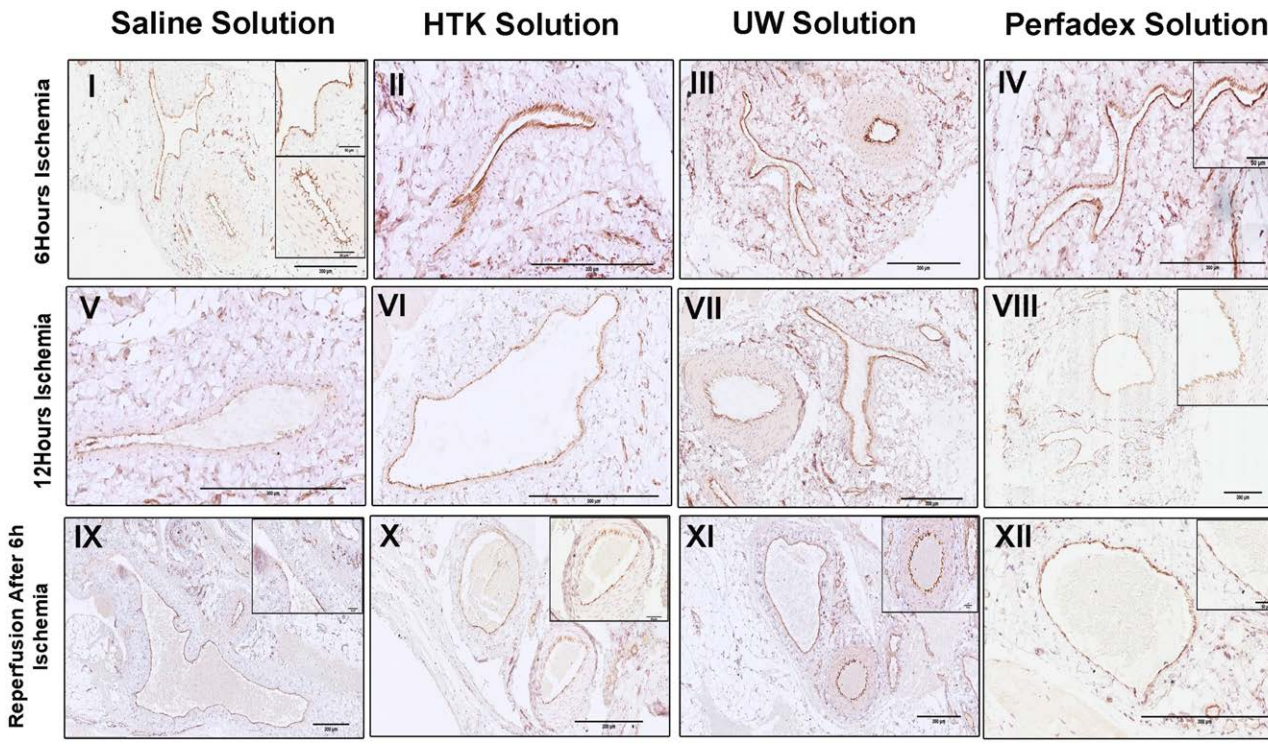

FIGURE 8. Expression of CD31 in vessels. CD31 expression was maintained within the vessels at 6 h of ischemia (I–IV). A decreased and nonhomogeneous expression of CD31 was observed at 12 h of ischemia (V–VIII) and following reperfusion (IX–XII); n = 3 per group.

Statistical Analysis

A power analysis was performed to determine group size. Analysis of variance was used to compare differences between and within groups using GraphPad Prism 8 (GraphPad Software, San Diego, CA). A $P < 0.05$ was considered statistically significant. Correction of multiple comparisons were performed according to Tukey's post hoc test.

RESULTS

Hindlimb Viability and Animal Survival in the Syngeneic Group With Eventual Graft Loss in the Allogeneic Group

All animals in the syngeneic group survived and were clinically well until the study endpoint (postoperative day 7). Hindlimb viability was present until endpoint as assessed by histology (Figure S2A, I, II, SDC, http://links.lww.com/TXD/A383), skin color and capillary refill (Figure S2A, III, SDC, http://links.lww.com/TXD/A383). In the allogeneic group, the hindlimbs showed minor signs of rejection (skin rash) at postoperative day 3, which led to necrosis of the limbs and fever at postoperative day 5 (Figure S2B, III, SDC, http://links.lww.com/TXD/A383) and confirmed by histology (Figure S2B, I, II, SDC, http://links.lww.com/TXD/A383).

Increase in TUNEL-positive Cells in Muscle and Vessels With Disruption of Laminin in the Allogeneic Group Compared With the Syngeneic Group Following Reperfusion

CC3 expression was higher in the muscle in the allogeneic group in comparison to the syngeneic group (Figure S3A, I–II, SDC, http://links.lww.com/TXD/A383). Laminin disruption within the vessels was also only observed in the allogeneic group (Figure S3B, I–II, SDC, http://links.lww.com/TXD/A383). The percentage of TUNEL-positive cells in muscle (32.4% ± 3.81%) and vessels (27.8% ± 3.3%) was significantly higher in the allogeneic group compared with the syngeneic group following reperfusion (muscle 8.30% ± 0.74%; vessels 2.5% ± 0.2% [Figure S3B, I–IV, SDC, http://links.lww.com/TXD/A383]) when ischemia time is minimal.

Increase in the Expression of CC3, TUNEL-positive Cells, and Expression of HMGB-1 Within the Muscle in Allotransplants Preserved With Heparinized Saline

There was an increase in the expression of CC3 in muscle in the 12 h ischemia group (Figure 1A, V-VIII) and following reperfusion (Figure 1A, IX–XII) compared with the 6 h ischemia group. CC3 expression was also higher in allografts preserved with heparinized saline (Figure 1A, IX) and HTK (Figure 1A, X) solutions in the reperfusion group. In muscle preserved with Perfadex, the expression of CC3 was reduced in the reperfusion group (Figure 1A, XII). A significant increase in the percentage of TUNEL-positive apoptotic cells was observed in the muscle following reperfusion compared with the ischemia groups when heparinized saline (32.45% ± 3.8%) or HTK (19% ± 5.87%) was used as a preservation solution (Figure 1B). There was also a significant increase in HMGB-1 concentration in muscle following reperfusion when saline solution (95.91 ± 4.31 ng/mL) was used as a preservation solution compared with UW (72.07 ± 7.23 ng/mL) and Perfadex (59.29 ± 4.54 ng/mL) (Figure 1C). Figure S1 (SDC, http://links.lww.com/TXD/A383) corresponds to isotype negative controls on Figure 1, V–XII.

Increase in TUNEL-positive Cells Within the Skin in Allotransplants Preserved With HTK

There was no difference in the expression of CC3 observed in the skin within allografts preserved with any of the solutions (Figure 2A, I–XII). There was a significant increase in the percentage of TUNEL-positive apoptotic cells within the skin allografts preserved with HTK solution following 12 h ischemia (30.93% ± 4.25%) compared with 6 h ischemia (1.28% ± 0.68%) and reperfusion (16.35% ± 6.64%) groups (Figure 2B). There was no change observed in the levels of HMGB-1 in skin preserved with different preservation solutions following reperfusion (Figure 2C).

Increase in the Expression of CC3 and TUNEL-positive Cells Within the Vessels in Allotransplants Preserved With Heparinized Saline

The expression of CC3 was higher following reperfusion in allografts preserved with heparinized saline (Figure 3A, IX) and HTK (Figure 3A, X) solutions. A significant increase in the percentage of TUNEL-positive apoptotic cells was also observed when heparinized saline (27.8% ± 3.3%) was used in comparison to HTK (7% ± 2.64%), UW (5.83% ± 2.09%), and Perfadex (8.33% ± 3.53%) in the reperfusion group (Figure 3B).

Increase in TUNEL-positive Cells Within the Nerve in Allotransplants Preserved With HTK

There was no difference in CC3 expression within the nerve (Figure 4A, I–XII). The percentage of TUNEL-positive apoptotic cells was significantly higher in nerve samples preserved with HTK (10.06% ± 0.92%) compared with samples preserved with UW (1.76% ± 0.94%), Perfadex (0.73% ± 0.27%), and saline (0.51% ± 0.26%) solutions in the reperfusion group (Figure 4B).

No Difference in Ischemia or Apoptosis Markers Within the Bone in Allotransplants

Expression of CC3 did not change in different groups using different preservation solutions (Figure S4A, I–XII, SDC, http://links.lww.com/TXD/A383). There was no change in the percentage of TUNEL-positive apoptotic cells using different preservation solutions (Figure S4B, SDC, http://links.lww.com/TXD/A383).

Laminin Expression Decreases in Vessels Following Reperfusion in Allotransplants Preserved With All Solutions

As a marker of tissue integrity, laminin expression was evaluated in all tissues. Laminin expression in skin was partially preserved with UW, HTK, and Perfadex solutions (Figure 5). Laminin expression in muscle was maintained with all preservation solutions (Figure S5, SDC, http://links.lww.com/TXD/A383). Laminin expression appears unchanged at 6 h of ischemia (Figure 6, I-IV) yet disrupted and nonhomogenous within the vessel walls at 12 h of ischemia with saline (Figure 6, V) and HTK (Figure 6, VI). UW and Perfadex solutions appear to be protective at 12 h ischemia (Figure 6, VII–VIII). Laminin expression within vessels appears to be particularly disrupted after reperfusion (Figure 6, IX–XII). Nerves stained with laminin showed absent or reduced expression with HTK following 12 h of ischemia (Figure 7, VII) and after reperfusion (Figure 7, X).

Disruption of CD31 Expression Following Reperfusion in Allotransplants Preserved With All Solutions

CD31 expression was maintained in vessels following 6 h of ischemia (Figure 8, I–IV). In contrast, nonhomogenous expression of CD31 was observed and vascular endothelial integrity

was not preserved following 12 h of ischemia (Figure 8, V–VIII) and following reperfusion (Figure 8, IX–XII).

DISCUSSION

Ischemia-reperfusion injury plays a critical role in transplantation and can determine graft and patient survival.[25,26] Preventing or reducing IRI can be critical in the context of VCA since it comprises different tissues with various immunological properties making induction of tolerance more difficult than in solid organ transplantation.[27,28] We have identified major limitations in the VCA literature, which this study begins to address. Many VCA studies have been performed in an autologous or syngeneic model[13-17]; there have been no studies evaluating the early phase of IRI, with no comprehensive review of different preservation solutions and their effect on each of the tissues.[12] Moreover, the mechanisms by which IRI leads to vascular dysfunction continues to be an interesting area of study.

In this study, we have shown the importance of using an allogeneic model. The markers of necrosis and apoptosis following reperfusion were significantly increased in the allogeneic group in comparison to the syngeneic. Although this concept was well known in the solid organ literature,[29-32] it was poorly established in VCA studies.[13-17] Our work focused on an allogeneic model to study IRI in VCA.

Ischemia remains a critical and inevitable factor during procurement and shorter ischemia times are not always possible. Dynamic storage techniques and machine perfusion could improve IRI, however, only static cold storage is currently being performed in clinical VCA. The effects of ischemia in VCA have been reported to increase proinflammatory cytokines as early as a few minutes following reestablishment of blood flow.[33-35] Although some studies have looked at the association between longer ischemia and acute rejection rate in VCA,[3,34,35] their only conclusion was that shorter ischemia time is preferable. Moreover, many studies mistakenly attributed late signs of flap viability to IRI rather than technical failure or acute rejection.[3] Evaluating the early phase of reperfusion can be critical for establishment of therapeutic options and preservation solutions mitigating IRI.[36,37]

The extent of tissue damage within our transplant model was evaluated following a clinically relevant ischemic period of 6 h through markers of apoptosis and necrosis. Our study demonstrated an increase in Caspase 3, TUNEL-positive apoptotic cells and HMGB-1 concentration as early as 2 h after reperfusion with heparinized saline. Our data also supports that muscle is particularly susceptible to ischemia.[38] Furthermore, our results indicate the protective effects of UW and Perfadex solutions in comparison to heparinized saline. Heparinized saline continues to be one of the most utilized perfusate in VCA.[16,17] We believe these results are largely because of its acidity (pH 5.4), which can exacerbate ischemia due to anerobic metabolism.[39,40] Moreover, HTK solution also led to a higher number of apoptotic cells in both skin and muscle, which is consistent with evidence.[41] We understand that there are also caspase independent mechanisms of cell death not explored in this study.

Perfadex solution has been used in limb replantation,[42] it has not been studied in an allogeneic transplantation model and could represent an ideal solution. It is mainly used in lung transplantation.[43] Interestingly, our study showed that Perfadex resulted in less apoptotic and necrotic cells in muscle. This can be attributed to its unique formulation of electrolytes and additives that serve as a closer mimicker of plasma, containing an ionic electrolyte profile that closely aligns with normal physiological levels.[43-45] Furthermore, the presence of additives in Perfadex such as dextran serves as a colloid that has been demonstrated to protect the microvasculature against IRI and minimize pathological leukocyte-endothelial interaction that can injure the endothelium.[44,45]

The extracellular matrix works as a supportive system for the preservation of tissue integrity, regulates cell function and modulates cell behavior through the release of growth factors.[46] In our study, we evaluated laminin as an extracellular matrix protein, which is found particularly in the basement membrane and can be susceptible to ischemia.[47-49] We have shown that as early as 2 h of reperfusion following ischemia, laminin gets degraded within the vessels. Furthermore, our results demonstrate a tendency towards degradation of laminin in skin and vessels regardless of the type of preservation solution.

In the field of VCA, vascular dysfunction is considered an obstacle contributing to perfusion deficits, delayed healing, proinflammatory conditions and rejection events.[24] Microvascular dysfunction is mostly involved in static cold storage and leads to early pathological processes during IRI[50] however the mechanisms are largely unknown. We suspect that since vessels are the first tissues exposed to reperfusion, they are susceptible to a higher degree of IRI.[51,52] In this study, we show that following reperfusion, there is a derangement and decreased expression of CD31 within the vascular endothelium. Absence of CD31 has been shown to increase the susceptibility of murine endothelial cells to apoptosis,[53,54] which could explain the higher number of apoptotic cells in vessels following reperfusion in our VCA model.

In summary, we have shown, in an allogeneic model of VCA, that early markers of IRI are significantly increased with heparinized saline solution in muscle and vessels. The percentage of apoptotic cells is increased with HTK in skin at 12 h of ischemia and in nerve following reperfusion. There is no difference in markers in bone regardless of preservation solution. Overall, the preferred preservation solutions for components within our allografts appear to be UW and Perfadex. The vessels within the allografts also appear to be particularly susceptible, with laminins and CD31 playing a key role in IRI.

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