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Reduced hypoxia-related genes in porcine limbs in *ex vivo* hypothermic perfusion versus cold storage

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ARTICLE INFO

Article history:
Received 29 August 2017
Received in revised form
15 April 2018
Accepted 30 May 2018
Available online xxx

Keywords:
Static cold storage
Oxidative metabolism
Glycolytic pathway
Vascularised composite allotransplantation perfusion
Composite tissue allotransplantation
Machine perfusion

ABSTRACT

Background: Ischemia-reperfusion injury remains the major limiting factor for limb replantation and transplantation. Static cold storage (SCS) on ice currently represents the standard mode of preservation but is limited to 6 h of duration. Ex vivo machine perfusion has evolved as a potential alternative to safely extend the duration of ex vivo preservation by providing continuous supply of oxygen and nutrients. This study aims to evaluate underlying molecular mechanisms of both preservation modalities.

Methods: We assessed molecular changes in amputated porcine forelimbs stored on ice at 4°C for 2 h ($n = 2$) and limbs perfused with Perfadex solution at 10°C for 2 h ($n = 3$) or 12 h ($n = 3$) before replantation. Muscle biopsies were examined for histological changes and gene expression levels using H&E staining and a hypoxia-related PCR gene array, respectively.

Results: Histology revealed only minor differences between the ice (SCS) and perfusion groups after 2 h of preservation, with decreased muscle fiber disruption in the perfusion groups compared with the ice (SCS) group. Perfused limbs demonstrated downregulation of genes coding for glycolytic pathways and glucose transporters after 2 h and 12 h when compared with SCS after 2 h. Similarly, genes that induce angiogenesis and those that are activated on DNA damage were downregulated in both perfusion groups as compared with SCS.

Conclusions: Perfusion of porcine limbs resulted in less activation of hypoxia-related gene families when compared with SCS. This may indicate a state more closely resembling physiological conditions during perfusion and potentially limiting ischemic injury. Our study confirms ex vivo perfusion for up to 12 h as a viable alternative for preservation of vascularized composite tissues.

Presentation of this work: Annual conference of the American Society of Reconstructive Transplantation, Chicago, IL, November 3-5, 2016. Harvard Surgery Research Day Symposium, Boston, MA, May 13, 2017.

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0022-4804/$ – see front matter

https://doi.org/10.1016/j.jss.2018.05.067

Introduction

Ischemia-reperfusion injury (IRI) is a major limitation to the applicability of both limb replantation and allotransplantation. Mediated by a cascade of autoimmune and metabolic events, IRI is a life- and limb-threatening response to the re-establishment of blood flow following prolonged ischemia. Hypoxia-induced processes damage cellular and mitochondrial membranes with buildup of reactive oxygen species that can cause cell death on reperfusion. This in turn triggers inflammatory infiltration, and activation of complement and coagulation cascades with vascular thrombosis and subsequent further disruption of normal muscle architecture and function.[1-3] The method and duration of ischemic limb preservation after amputation are thought to be major determinants of the extent of sustained IRI. The current gold standard of preservation entails storing limbs on a bed of ice slurry (approximately 4°C) to reduce metabolic rates and limit subsequent hypoxia-induced cellular changes.[4-6] As such, previous studies describe a maximum allowable limb ischemia time of 4-6 h under these conditions of static cold storage (SCS).[7,8]

Recent efforts to prolong the allowable ischemia time have centered on dynamic storage techniques such as extracorporeal perfusion to maintain continuous delivery of oxygen and nutrients to the tissues. In a previous study, we demonstrated the extension to 12 h of ischemia time via ex vivo hypothermic perfusion with oxygenated acellular fluid in a porcine model of limb amputation with comparable results to conventional cold storage for 4 h.[9] However, the molecular mechanisms underlying the protective role of ex vivo limb perfusion in reducing IRI are yet to be elucidated. Toward this end, the aim of this study is to assess the hypoxia-related gene expression profile in different storage modalities in a porcine model of limb replantation.

Material and methods

Source of data

Healthy female Yorkshire pigs of 35-45 kg about 3 mo of age (Parson's Farm, Hadley, Mass) were used for heterotopic forelimb replantation according to the technique described by Constantinescu *et al.*[10] Samples acquired from these pigs were subjected to molecular analysis. This model was established and approved by the Institutional Animal Use and Care Committee from Harvard Medical School under protocol number 05111.

Experimental design

Animals were anesthetized, and the left forelimb was surgically disarticulated with removal of the scapula. Following this, the isolated limbs were either preserved by SCS or perfusion. After preservation, the limbs were replanted in a 180° heterotopic fashion with anastomoses of the axillary artery and 1-2 veins.

Animals were randomly subjected to either the SCS or the perfusion group. In the SCS group, limbs were stored on ice slurry at 4°C for 4 h. In the perfusion group, limbs were connected to a dedicated ex vivo preservation system through cannulation of the axillary artery and perfused continuously for 12 h with modified Perfadex perfusion solution (XVIVO Perfusion AB, Göteborg, Sweden) at 10°C. Limbs subjected to SCS ($n = 2$) were biopsied at 2 h. Limbs subjected to *ex vivo* perfusion ($n = 3$) were biopsied at 2 h and 12 h. Muscle biopsy specimens were then fixed in formalin for histological analysis or stored in RNAlater (Qiagen, Valencia, CA) for molecular analysis.

Histology

Formalin-fixed biopsy specimens were sectioned and stained with hematoxylin and eosin (H&E). Images were captured with a Nikon DS-Fi1 (Nikon Corporation, Tokyo, Japan) attached to a light microscope (Nikon Eclipse E400; Nikon Corporation). Five deidentified specimens were randomly selected among the cross-sectional biopsies obtained at each time point. The selected specimens were then assessed for damage of muscle fibers by a blinded reviewer. Signs for damaged muscle fibers were defined as necrosis of fibers and cells, hypercontractility, and vacuolization.[11]

Molecular analysis

Biopsy specimens were thawed on ice and later homogenized with TRIzol (Invitrogen Inc, Carlsbad, CA) using a gentleMACS dissociator (Miltenyi Biotec Inc, Cambridge, MA) according to the manufacturer's manual. RNA extraction was performed according to the standard protocol (Invitrogen Inc) and quantified using NanoDrop (Thermo Fisher Scientific, Waltham, MA). RNA was then transcribed to cDNA with the RT^2 First Strand Kit (Qiagen). We used a custom-made porcine hypoxia signaling pathway array—RT^2 Profiler PCR Array (Catalog number: PASS-032Z; Qiagen)—to assess hypoxia-related changes in gene expression levels. A RT^2 SYBR green PCR master mix (Qiagen) was used on a Bio-Rad myIQ cycler (Bio-Rad Laboratories, Hercules, CA). The hypoxia array consisted of 84 genes known to be involved in the hypoxic response, cell differentiation, and metabolism, as well as 12 sequences to control for five housekeeping genes, one genomic DNA contamination, three RT controls, and three positive PCR controls. The cycle number at threshold (35 CT value) was used for calculations of relative amount of mRNA molecules.

Statistical analysis

Statistical analysis was completed using the GeneGlobe Data Analysis Center (Qiagen, http://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/?akamai-feo=off).

The CT value of each target gene was normalized by arithmetic mean of the CT value from average of five housekeeping genes. This value is defined as the ΔCT. Relative