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Research Paper

Recellularization of Decellularized Venous Grafts Using Peripheral Blood: A Critical Evaluation

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ABSTRACT

Vascular disease is a major cause of death worldwide, and the growing need for replacement vessels is not fully met by autologous grafts or completely synthetic alternatives. Tissue engineering has emerged as a compelling strategy for the creation of blood vessels for reconstructive surgeries.

One promising method to obtain a suitable vessel scaffold is decellularization of donor vascular tissue followed by recellularization with autologous cells. To prevent thrombosis of vascular grafts, a confluent and functional autologous endothelium is required, and researchers are still looking for the optimal cell source and recellularization procedure.

Recellularization of a decellularized scaffold with only a small volume of whole blood was recently put forward as a feasible option. Here we show that, in contrast to the published results, this method fails to re-endothelialize decellularized veins. Only occasional nucleated cells were seen on the luminal surface of the scaffolds. Instead, we saw fibrin threads, platelets and scattered erythrocytes. Molecular remnants of the endothelial cells were still attached to the scaffold, which explains in part why earlier results were misinterpreted.

Decellularized vascular tissues may still be the best scaffolds available for vascular tissue engineering. However, for the establishment of an adequate autologous endothelial lining, methods other than exposure to autologous whole blood need to be developed.

1. Introduction

Cardiovascular disease is the leading cause of death globally [1,2]. A common treatment for patients with advanced vascular disease is the use of vascular grafts to replace or bypass damaged or obstructed vessels [3]. Autologous vessel grafts remain the gold standard, but not all patients have sufficient or healthy autologous veins for vascular grafting [4]. Synthetic alternatives like Dacron and polytetrafluorethylene are being used with relative success for some applications needing large diameter (>6 mm) grafts, but for smaller diameter applications, synthetic grafts tend to suffer unacceptably high failure rates [5–7]. In addition, for cases requiring more than just a new conduit, like reconstructive vein surgery where valve function is essential, these alternatives are not optimal [8,9].

One technique utilized to make a vascular scaffold is to decellularize allogeneic vascular tissues. Decellularization refers to the removal of antigenic cellular material from tissue [10]. The starting point can be native tissue, or extracellular matrix (ECM) produced from cells ex vivo [11]. By using decellularized natural matrices, one can take advantage of the intrinsic properties of the tissue, including ECM composition, biocompatibility, shape and mechanical properties [12,13]. The decellularization process may involve a variety of chemical agents, solvents and enzymes, and must balance the task of removing all cellular material with the aim of preserving composition, biological activity and mechanical integrity of the remaining ECM [12]. Inadequate decellularization could potentially cause immune reactions and graft failure, while an aggressive decellularization process may remove essential ECM components, thus altering the mechanical properties of the tissue [14,15].

One challenge of using decellularized vessels might be limited recellularization in vivo, caused by the dense ECM of the vessel wall or chemical damage to the ECM in the decellularization process [16,17]. The limited success of current commercially available decellularized grafts has been, in part, explained with their lack of cellularity on implantation [18,19]. A viable endothelium is important to suppress thrombosis of smaller caliber vessel grafts, especially crucial for decellularized grafts with their exposed collagen luminal wall surface [20]. This makes a successful recellularization step essential, and a number of different cell sources and strategies have been employed [13,21]. The ideal cell source would be one that is readily available in sufficient

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amounts, can be obtained by a minimally invasive procedure, and that would willingly settle in the graft.

In 2014, Olausson et al. [22] reported two pediatric cases involving clinical transplantation of tissue engineered decellularized allogeneic veins. The veins were reported to be recellularized following exposure to 25 mL of autologous peripheral whole blood. It is known that normal human adult peripheral blood contains a small number of circulating endothelial cells (ECs), and cell cultures from blood have demonstrated endothelial outgrowth [23]. Using cells obtained from a simple blood sample for recellularization, this whole blood procedure would sidestep the time-consuming processes of harvest, isolation and expansion, and would present a promising and available approach.

The number of circulating ECs from peripheral blood has previously been estimated to four cells per mL of blood, or even less [24–26]. If the estimated 100 ECs from 25 mL blood could fully endothelialize a graft luminal surface of at least 10 cm^2 in <10 days, that would indicate a great expansive potential of these cells. This would contradict published evidence, and would suggest that there may be other cells present in blood which acquire an endothelial phenotype and contribute to the formation of neointima of a vessel graft.

Since the theoretical background on the prevalence of blood ECs does not fully integrate with the positive results on blood re-endothelialization published in the Olausson et al. study [22], we decided to re-evaluate the procedure with respect to both the quantity and the quality of the re-endothelializing cells. Of note, although these are new experiments, the procedure and the laboratory performing the de-cellularization and the re-endothelialization were the same as in the re-evaluated study.

2. Methods

2.1. Harvest of Vein Segments

The Norwegian Institutional Review Boards and Ethics Committees approved all research protocols. All methods were performed in accordance with relevant guidelines and regulations.

All veins used for decellularization and recellularization were harvested at Oslo University Hospital (OUH), Norway. Informed consent had been obtained. Donors were free from infectious disease. Segments from the femoral vein ($N = 9$), about 5–8 cm in length, were harvested from two adult human cadavers by using vascular surgical techniques, with careful ligation of all side branches. The segments were flushed thoroughly with saline to remove all blood. Vein samples were stored in individual labelled containers filled with PBS supplemented with antibiotics (0.5% penicillin, 0.5% streptomycin, and 0.5% amphotericin B), and kept at 2–8 °C for all transportation events. Shipping time between Oslo and Gothenburg never exceeded 24 h. Samples from two cadaver veins were kept at OUH for analysis.

Native control veins were obtained from remaining saphenous vein segments after bypass operations with the informed consent of patients. Vein samples were stored in PBS supplemented with antibiotics (0.5% penicillin, 0.5% streptomycin, and 0.5% amphotericin B) at 4 °C for maximum 24 h awaiting further processing.

2.2. Decellularization

The decellularization process was carried out by NovaHep, as described in Olausson et al. [22] Minor changes were introduced in a 2015 publication by the same group [9], considered improvements to the procedure. These are mentioned specifically in the following text. The improvements were used also in the current study. The segments were washed for 72 h in distilled water, and decellularized by 9–-14 cycles of sequential incubation in three different decellularization solutions (7 cycles in Olausson et al. [22]). The solutions used were 1% Triton, 1% tri-*n*-butyl phosphate (TnBP), and 4 mg/L DNAse.

All steps were performed at 37 °C with agitation and continuous perfusion with decellularization solution. The segments were immersed in each solution for 4 h per cycle (3 h in Olausson et al. [22]), and rinsed in distilled water between each chemical incubation. All solutions used for decellularization contained 0.5% penicillin, 0.5% streptomycin, and 0.5% amphotericin B.

After completion of the decellularization process, the segments were washed in PBS for 48 h, sterilized in EtOH and frozen at −80 °C. The complete decellularization process lasted 10 days. Samples for characterization were shipped to OUH after the sterilization step.

2.3. Recellularization

The recellularization was conducted by NovaHep, using a patented procedure developed in the lab of Sumitran-Holgersson at Sahlgrenska University Hospital [22]. Peripheral venous blood for recellularization was collected from three healthy donors aged 48, 52 and 63 in sterile heparin-coated Vacutainer tubes and transported to the laboratory within 2 h. The entire recellularization process was carried out under sterile conditions at 37 °C. Before recellularization, the veins were perfused with heparin at a concentration of 50 IU/mL in phosphate buffered saline (PBS) for 2 h. The heparin was drained off, and whole blood was immediately perfused for 48 h at 2 mL/min speed. 25 mL blood was used for recellularization of each sample.

After 48 h the blood was drained off, and the vein was rinsed with PBS containing 1% penicillin-streptomycin-amphotericin until all traces of blood were completely removed. The vein was subsequently perfused for 4 days with EC medium, and the vein segments were immersed in EC medium throughout the incubation period. Complete endothelial medium was prepared with MCDB131 (Life Technologies, Stockholm, Sweden) basal medium supplemented with 10% heat-inactivated human AB serum (Life Technologies), 1% glutamine, 1% penicillin-streptomycin-amphotericin, and EGM-2 SingleQuot kit (CC4176; Lonza). The recellularization method originally included an additional 4 day incubation step with perfusion of the graft with smooth muscle cell medium. This step was later abandoned, as Olausson et al. did not find that it significantly affected either the endothelialization or the proliferation of ECs in the graft [22]. In line with this, the vessels were not perfused with smooth muscle cell medium for the present study.

After recellularization, the segments were washed in PBS and shipped to OUH for further analysis. Upon arrival in Oslo, all segments were immediately rinsed in PBS and processed for downstream analysis.

2.4. DNA Quantification

Vein samples were freeze dried and DNA was extracted using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions.

2.5. Histology

Vein samples were fixed in 4% paraformaldehyde overnight. The samples were dehydrated in gradients of ethanol and xylene, and embedded in paraffin according to standard protocol. Further, blocks were cut and mounted on SuperFrost Plus Adhesion Slides. 6 μm sections were deparaffinized and stained with hematoxylin and eosin (H&E) using standard laboratory technique. Sections were imaged in an Olympus BX51 microscope.

2.6. Immunohistochemistry

Vein samples were embedded in Frozen Section Medium (Richard-Allan Scientific Neg 50, Thermo Scientific) and frozen in dry ice-cooled isopenthane. Frozen tissue blocks were stored at —80C. The samples

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