Decellularized and Secured Porcine Arteries with NaOH-based Process: Proof of Concept

Mathieu van Steenberghe,[1] Thomas Schubert,[1,2,3] Caroline Bouzin,[4] Carlo Caravaggio,[5] Yves Guiot,[6] Daela Xhema,[1] and Pierre Gianello,[1] Brussels and Tournai, Belgium

Background: There is a need for small caliber vascular prosthesis. Synthetic grafts are hindered by thrombogenicity and rapid occlusion. Decellularized matrices could be an alternative. We assessed in vitro and in vivo the biocompatibility of porcine artery treated with a chemical/physical process for decellularization and graft securitization with non/conventional pathogens inactivation.

Methods: Porcine carotid arteries (PCA) were treated. First, biopsies (n = 4/tissue) were performed before/after treatment to assess decellularization (hematoxylin and eosin/-4', 6-diamidino-2-phenylindole/DNA/Miller). Second, 5 rats received an abdominal aortic patch of decellularized PCA (DPCA). Four pigs received subcutaneous DPCA implants (n = 2/pig). Half were explanted at day 15 and half at day 30. Finally, 2 pigs received DPCA (n = 2) and polytetrafluoroethylene prosthesis (n = 1), respectively, as carotid interposition. Implants were removed at day 30. Inflammation (CD3 and CD68 immunostaining) calcifications (von Kossa staining), remodeling (hematoxylin and eosin), and vascular characterization (CD31 and alpha-smooth muscle actin immunofluorescent staining) were investigated.

Results: Ninety-five percentage of decellularization was obtained without structural deterioration. No death occurred. Low inflammatory reaction was found in the 2 models for DPCA. Acquisition of vascular identity was confirmed in the rodent and porcine models. Similarity between native PCA and DPCA was observed after 30 days. In contrast, polytetrafluoroethylene graft showed severe calcifications, higher CD3 reaction, and higher intimal hyperplasia ($P < 0.05$).

Conclusions: The physical and chemical process ensures decellularization of carotid porcine arteries and their in vivo remodeling with the presence of an endothelium and smooth-muscle–like cells as well as a low level of inflammatory cells.

The authors received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

The authors declare no conflicts of interest to disclose.

Ethics approval and consent to participate: Tissue sources/subcutaneous and intravascular porcine studies: Animals were housed according to the guidelines of the French Ministry of Agriculture and Animal Care. All procedures were approved by the Local Ethics Committee for Animal Care of the INRA (Jouy en Josas, France). Intravascular rodent study: Animals were housed according to the guidelines of the Belgian Ministry of Agriculture and Animal Care. All procedures were approved by the Local Ethics Committee for Animal Care of the Université catholique de Louvain.

[1]*Université catholique de Louvain, Secteur des Sciences de la Santé, Institut de Recherche Expérimentale et Clinique, Pôle de Chirurgie Expérimentale et Transplantation (CHEX), Brussels, Belgium.*

[2]*Cliniques Universitaires Saint-Luc, Service d'orthopédie et de Traumatologie de l'appareil Locomoteur, Brussels, Belgium.*

[3]*Cliniques Universitaires Saint-Luc, Unité de Thérapie Cellulaire et Tissulaire de l'appareil Locomoteur, Banque de Tissus, Brussels, Belgium.*

[4]*Université Catholique de Louvain, Secteur des Sciences de la Santé, Institut de Recherche Expérimentale et Clinique, IREC Imaging Platform (2IP), Brussels, Belgium.*

[5]*Centre Hospitalier de Wallonie Picarde (CHwapi), Site Notre-Dame, Service de Chirurgie Vasculaire, Tournai, Belgium.*

[6]*Cliniques Universitaires Saint-Luc, Service d'anatomopathologie, Brussels, Belgium.*

Correspondence to: Mathieu van Steenberghe, Université catholique de Louvain, Secteur des Sciences de la Santé, Institut de Recherche Expérimentale et Clinique, Pôle de Chirurgie Expérimentale et Transplantation (CHEX), Avenue Hippocrate 55-Bte B1.55.04 B-1200 Brussels, Belgium; E-mail: mathieu.vansteenberghe@uclouvain.be

Ann Vasc Surg 2018; 49: 179–190
https://doi.org/10.1016/j.avsg.2017.12.013

Manuscript received: September 21, 2017; manuscript accepted: December 26, 2017; published online: 6 March 2018

INTRODUCTION

Cardiovascular disease is the major cause of mortality in the world. Despite the advances in endovascular therapy, vascular bypass remains the optimal choice for patients requiring long-term revascularization solutions.[1]

The better long-term patency rates of bypass are obtained with autologous vessels, but they have limited availability, may be of poor quality, and their harvesting can cause donor site morbidity.

Cryopreserved nondecellularized allogeneic arterial grafts used in infected area, have a lower patency rate than autologous grafts and have limited availability.[2]

Synthetic vascular grafts have poorer patency rates in small diameter arteries due to thrombosis or intimal hyperplasia or atherosclerosis.[3] Moreover, graft infection is more common in synthetic conduits due to their susceptibility to bacterial colonization.[4,5]

Tissue engineering and decellularized xenogeneic vessels could be an alternative. Similarly to synthetic grafts, decellularized matrices would be readily available. They can provide a microenvironment for supporting cell invasion, growth, and differentiation leading to new viable vessels.[6,7] These prostheses can be directly implanted or after cell seeding.[1,2,4–8]

No standard decellularization treatment exists. Various protocols are proposed. Most popular are those using detergents and/or enzymes.[9–12]

We previously demonstrated enhancement of biocompatibility and vascular remodeling of allogeneic and xenogeneic pericardium with a particular treatment.[13] This decellularization treatment has the advantage to be inactivator for conventional (virus/bacteria) and nonconventional (prion) pathogens,[14,15] and therefore offers graft securitization.

We investigated this treatment as a decellularization process to improve biocompatibility and remodeling of xenogenic small caliber porcine carotid arteries (PCA) for tissue engineering applications. In vitro tests assessed decellularization and histoarchitecture integrity of PCA after treatment. In vivo protocols were conducted to assess tissue biocompatibility and vascular remodeling in rodent and porcine implantation models (Fig. 1).

MATERIAL AND METHODS

In Vitro Study

Sources of matrices. Porcine carotid arteries were procured from Vietnamese pigs of 40 to 45 kilograms carried out at the Centre de Recherche en imagerie Interventionnelle (Cr2i) (the National Institute of Agronomic Research [INRA], Jouy-en-Josas, France.) The study was conducted in accordance with ISO 9001 Quality Management Standards. A premedication of ketamine (1,000 mg) was administered by intramuscular injection. The animals were then intubated and kept under general anesthesia with a mixture of isoflurane (1.5–2%) and oxygen (98–98.5%) throughout the operation. After midline incision in the neck, the 2 carotid arteries were harvested. The tissues were rinsed with NaCl 0.9% and frozen at −80°C just after harvesting.

A synthetic prosthesis (polytetrafluoroethylene [PTFE]) of 5 mm diameter was purchased from Edwards Lifesciences Company.

Preparation of matrices. PCA of 7 cm in length was treated according to a protocol previously described.[13] Briefly, adherent tissues were removed. The protocol consisted in a succession of baths of acetone, ethanol, NaOH, and H_2O_2 and washed. Finally, extracellular matrices (ECMs) were frozen at −80°C and sterilized by gamma irradiation (25 KGy).

Decellularization assessment

Hematoxylin and eosin and DAPI staining. Decellularization process was evaluated first by hematoxylin and eosin staining and 40,6-diamidino-2-phenylindole (4′,6-diamidino-2-phenylindole [DAPI]; 0.2 µg/mL/Sigma-Aldrich) staining on paraformaldehyde (4%)-fixed slices from native and treated ECM from 4 different donors.

For each sample, 5 optical sections were acquired by structured illumination using a Zeiss AxioImager-Apotome system.

DNA quantification. DNA isolation was made with Qiagen, QIAamp kit® DNA Mini Kit (Hilden Deutschland) on equal volume paraffin samples from frozen native tissues and posttreatment tissues from 4 different donors. The DNA concentration was measured by fluorometry with a Nanodrop™ fluorometer (Invitrogen™).

Alpha gal removal. Alpha-galactosyl (M86) for porcine tissues was previously investigated.[13] Briefly, 5 µm sections were cut from paraffin-embedded biopsies from frozen native tissues ($n = 4$) and posttreatment tissues ($n = 4$). After deparaffinization, endogenous peroxidases were inhibited 20 min with 3% hydrogen peroxide in methanol. Primary antibodies (mouse anti-alpha-galactose, clone M86, Enzo Life Sciences) were revealed with Envision systems (Dako) followed by diaminobenzidine (Dako). After counterstaining with hematoxylin, slides were dehydrated and

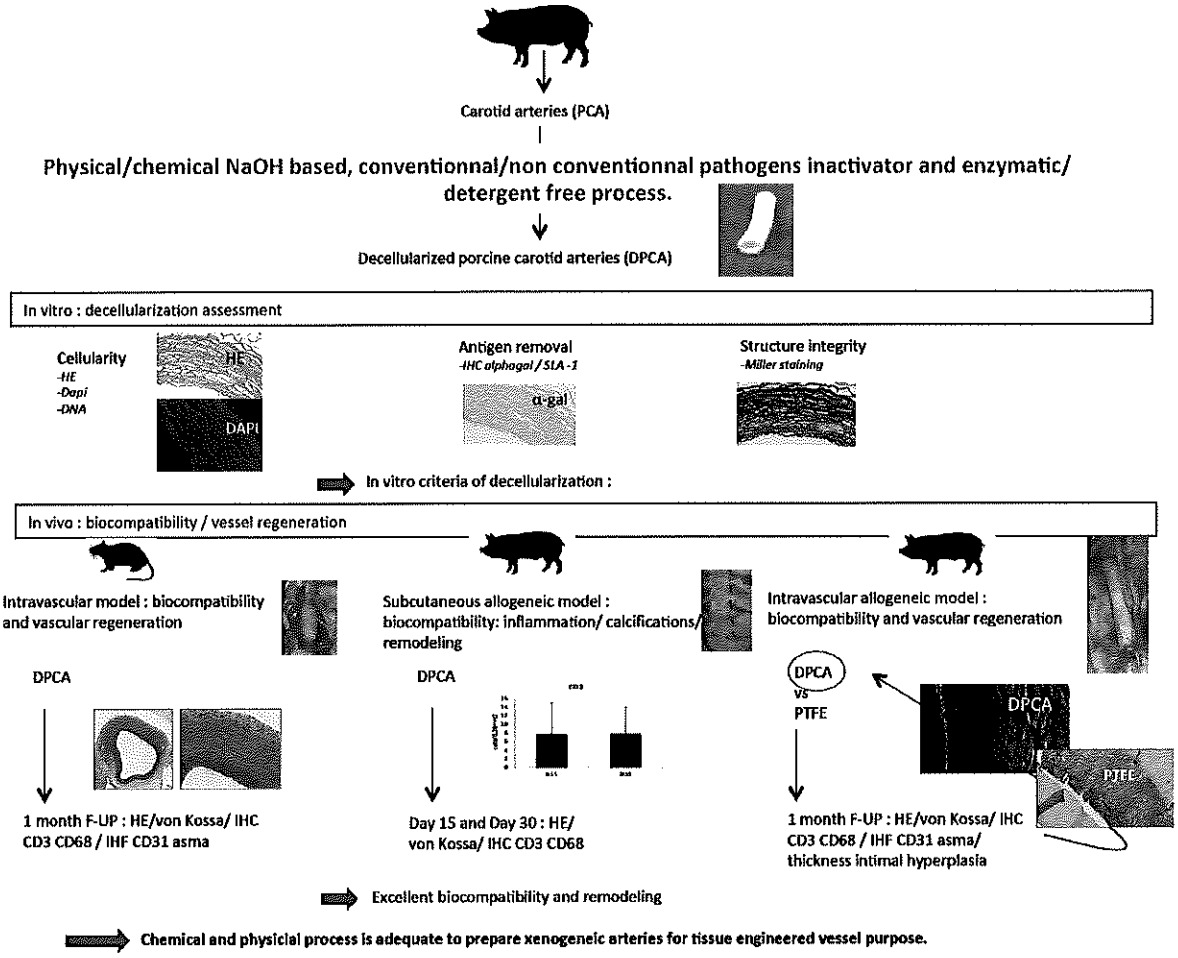


Fig. 1. Carotid porcine arteries were decellularized following a physical/chemical nonenzymatic and nondetergent process. In vitro study investigated major criteria of adequate decellularization (cellularity-antigen removal and structure integrity). Moreover, in vivo study, using 3 different models (vascular rodent/subcutaneous and vascular porcine models) assessed biocompatibility and vascular remodeling of DPCA. DPCA were compared to PTFE grafts in the vascular porcine model. The results allowed to conclude the decellularization and securitization process are suitable to prepare porcine carotid arteries for vessel tissue engineering. IHF, immunohistofluorescence; IHC, immunnohistochemical.

mounted. Stained slides were then digitalized using a SCN400 slide scanner (Leica, Leica Biosystems, Wetzlar, Germany) at a 20× magnification.

Histoarchitecture preservation. Elastic fiber preservation was assessed with Miller staining on paraformaldehyde (4%)-fixed slices from native and treated ECM from 4 different donors.

In Vivo Study

Surgical procedures. Animals were housed according to the guidelines of the Belgian and French Ministries of Agriculture and Animal Care. All procedures were approved by the Local Ethics Committee for Animal Care of the Université catholique de Louvain (UCL) and the INRA (Jouy-en-Josas, France).

Rodent study. Five male Wistar rats of 300 g received a patch of decellularized PCA (DPCA) (1 cm × 0.5 cm) on the abdominal aorta under general anesthesia (intraperitoneal injection of zoletil, xylazine, and ketamine mix). The aorta was clamped after intraperitoneal injection of heparin (100 UI/kg/0.3 mL). The aorta was opened and rinsed with saline. The patch was sutured with a 9.0 running prolene suture as previously

described[13] (Fig. 1). The rats were followed up during 1 month. Taken apart, the peroperative heparin injection, no antiaggregation, or anticoagulation was performed during follow-up. No antibiotics were given. After 1 month, the rats were sacrificed by intracardiac injection of T61 (Intervet) under general anesthesia. The abdominal aorta was harvested, rinsed with saline serum. The aorta was then cut transversely to the middle part of the patch, fixed overnight in 4% formaldehyde and paraffin embedded.

Porcine study. Animals were kept unfed for 24 hr before the operation. A premedication of ketamine (1,000 mg) was administered by intramuscular injection. The animals were then intubated and kept under general anesthesia with a mixture of isoflurane (1.5–2%) and oxygen (98–98.5%) throughout the operation. A physiological follow-up (oximetry, pulse, and heart rate) was provided during the entire operating procedure.

Subcutaneous study. Four Vietnamese pigs of 40 kg each received decellularized tissues bilaterally in the subcutaneous space in the dorsal region. Tissues were harvested after 15 days for 2 pigs and after 30 days for the rest. Tissues were fixed overnight in 4% formaldehyde and paraffin embedded.

Intravascular study. The grafts were implanted in 2 Vietnamese pigs of 40 kgs after general heparinization (100 UI/kg) as carotid interposition under general anesthesia (Fig. 1). Two DPCA (4 mm in ID and 60 mm in length) were implanted in 1 recipient. One segment of 60 mm in length of the PTFE tubular prosthesis (Edwards®) was implanted in another recipient. The pigs received low-molecular-weight heparin prophylaxis (40 mg/day) during 3 days and aspirin (80 mg/day) during 30 days. The animals did not receive antibiotics. The pigs were sacrificed at day 30. Carotid arteries were harvested and rinsed with saline serum. The prosthesis was then sectioned transversely in 4 parts to analyze the proximal, medial, and distal prosthesis segments, fixed in formaldehyde 4% overnight and paraffin embedded.

Histological evaluation. In the rodent model, the central region of the patched aorta was analyzed. The contralateral native aorta was the control.

In the porcine vascular protocol, 3 regions of the prosthesis (proximal/medial and distal) were analyzed. One native artery served as control.

Histology and immunostaining. Hematoxylin and eosin, Masson's trichrome, and von Kossa colorations assessed remodeling/cell infiltration, structure, angiogenesis, and calcifications, respectively.

Immunohistochemistry for CD3 and CD68 was performed using a Ventana Benchmark XT machine (Roche®, USA) to assess inflammatory reaction as previously described.[13]

For immunofluorescent costaining, 5 μm sections were subjected to endogenous peroxidase inhibition 20 min and then to aspecific antigen binding sites blocking 1 hr (PBS with 5% BSA and 0.05% Triton). Rabbit anti-CD31 (polyclonal, Abcam, # ab28364, 1/100 dilution for rat, 1/50 dilution for pig) and Mouse anti-alpha smooth muscle actin (ASMA) (clone 1A4, Dako #M0851, 1/50 dilution for rat; clone 1A4, Abcam #ab7817, 1/100 dilution, for pig) primary antibodies were incubated overnight at 4°C in PBS containing 1% BSA and 0.05% Triton X-100. This was followed by an incubation with AlexaFluor 568 anti-rabbit and AlexaFluor647 anti-mouse (Invitrogen) secondary antibodies, incubated at a 1/1000 dilution for 1 h at room temperature. Nuclei were stained with DAPI, and labeled sections were imaged by epifluorescence with a 5× Plan-Neofluar objective or by structured illumination with a 20× Plan-Apochromat objective using a Zeiss AxioImager microscope equipped with an ApoTome module.

Histomorphometry. In the rodent protocol, CD 3 and CD68 positive cells were counted as previously described[13] in internal/middle and external regions of the patches with a histomorphometrical grid representing a surface of 0.16 mm^2 with 5 regions of interest (ROI) per region (Fig. 2).

In the subcutaneous porcine protocol, 5 ROI were analyzed close to the DPCA. CD3 and CD68 positive cells were counted in these regions within a standard grid representing a surface of 0.16 mm^2.

In the porcine vascular protocol, the diameter of the prosthesis was divided in 3 equal regions. The thickness of these 3 regions was then divided in 3 parts: internal/middle and external (Fig. 2). The inflammatory reaction was principally located in the external part. In this part, 5 ROI were analyzed. CD3 and CD 68 positive cells were counted within a standard grid representing a surface of 0.16 mm^2.

Neointima thicknesses of DPCA and PTFE in the porcine vascular model were measured with the Digital Image Hub program (Leica Biosystems, Dublin, Ireland) on digitalized slides (SCN400 slide scanner, Leica, Wetzlar, Germany). The neointima thicknesses were measured in the section of the 3 parts (proximal/medial/distal) of the explanted grafts. Three measures were done on each slide.

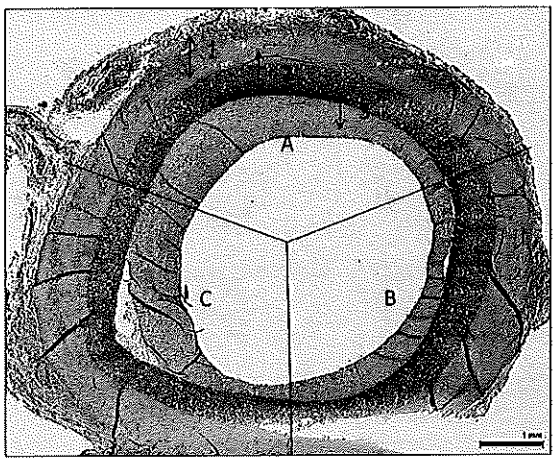


Fig. 2. Segmentation of the vascular histologic slide for histomorphometry analysis. The figure is a histologic sample after hematoxylin and eosin staining of PTFE graft after 1 month in the porcine vascular model (scale bar: 1 mm). In the rodent vascular model, a patch is implanted in the abdominal aorta. The patch corresponds to the zone A. This part is divided into 3 regions: 1: external; 2: middle; and 3: internal. The cell counts were done in these 3 regions with a grid of 0.16 mm^2/5 counts per region. In the porcine vascular model, a tubular graft (DPCA or PTFE) is implanted. Three zones are delimited: A, B, and C. In these 3 zones, cell counts are done in the 3 regions: 1, 2, and 3.

Statistical Analysis

The one-sample Kolmogorov–Smirnov test and QQ-plots were used to ensure the normal distribution of values. Results were expressed as means ± standard deviation or in ratios. The statistical significance of differences between experimental groups was tested by Student's *t*-test or 1-way analysis of variance with a Bonferroni's post hoc test. The statistical tests were carried out with PASW 18. Differences were considered to be significant at $P < 0.05$.

RESULTS

ECM Properties

After treatment, the number of positive fluorescent nuclei per millimeter square of tissue fell to 2%, relative to native tissues, respectively, with 24.58 cells/inch square versus 0.287 cells/inch square.

Immunostaining for alpha-galactosyl antigen showed disappearance of staining after treatment.

Miller and hematoxylin and eosin staining showed preservation of artery histoarchitecture after treatment (Fig. 3A).

DNA quantification showed 95% DNA reduction after treatment (0.7 ± 0.85 vs. 14.16 ± 5.2 microg/microl) (Fig. 3B).

In Vivo

Rodent study. Patches of DPCA had excellent characteristics for surgical purpose on these small arteries. They were easily saturable, and complete hemostasis was concomitant to vessel unclamping. During follow-up, there was no death. After 1 month, we noticed 1 pseudo aneurysm. No thrombosis occurred.

Inflammatory reaction. The inflammatory reaction was significantly located in the external part of the patch in comparison to the middle and internal portions, respectively, for CD3: 5.65 ± 2.37 vs. 0.3 ± 0.57 vs. 1.25 ± 2.33 cells/0.16 mm^2 with $P < 0.05$ and for CD68: 3.25 ± 2.15 vs. 0 and 0 cells/0.16 mm^2 with $P < 0.05$ (Fig. 4A, B).

Patch integration and remodeling. The DPCA was partially recellularized after 1 month (Fig. 4A, C).

A neomedia was present below the graft, which showed the same characteristics than the native aorta with similar thickness (Fig. 4A), with elastic fibers organized in a dense network cellularized with ASMA positive cells and covered by CD31 positive cells (Fig. 4C).

No calcifications were seen with von Kossa staining.

Subcutaneous porcine study. There was a lack of remodeling in the scaffolds themselves with preservation of elastic fibers, highly repopulated by non-CD3 and CD68 cells with signs of revascularization (Fig. 5A1–4.). The inflammatory reaction was essentially CD3 type (Fig. 5A2–4)

The CD3 inflammatory reaction was not statistically different between day 15 and day 30 with respectively 7.6 ± 7.30 cells vs. 7.65 ± 6.23 cells/0.16 mm^2 ($P > 0.05$). CD68 reaction was low at day 30 (<1 cell/0.16 mm^2) and was not statistically different to day 15 with 0.15 ± 0.36 cells/0.16 mm^2 vs. 0.5 ± 0.76 cells/0.16 mm^2 ($P > 0.05$) (Fig. 5A5).

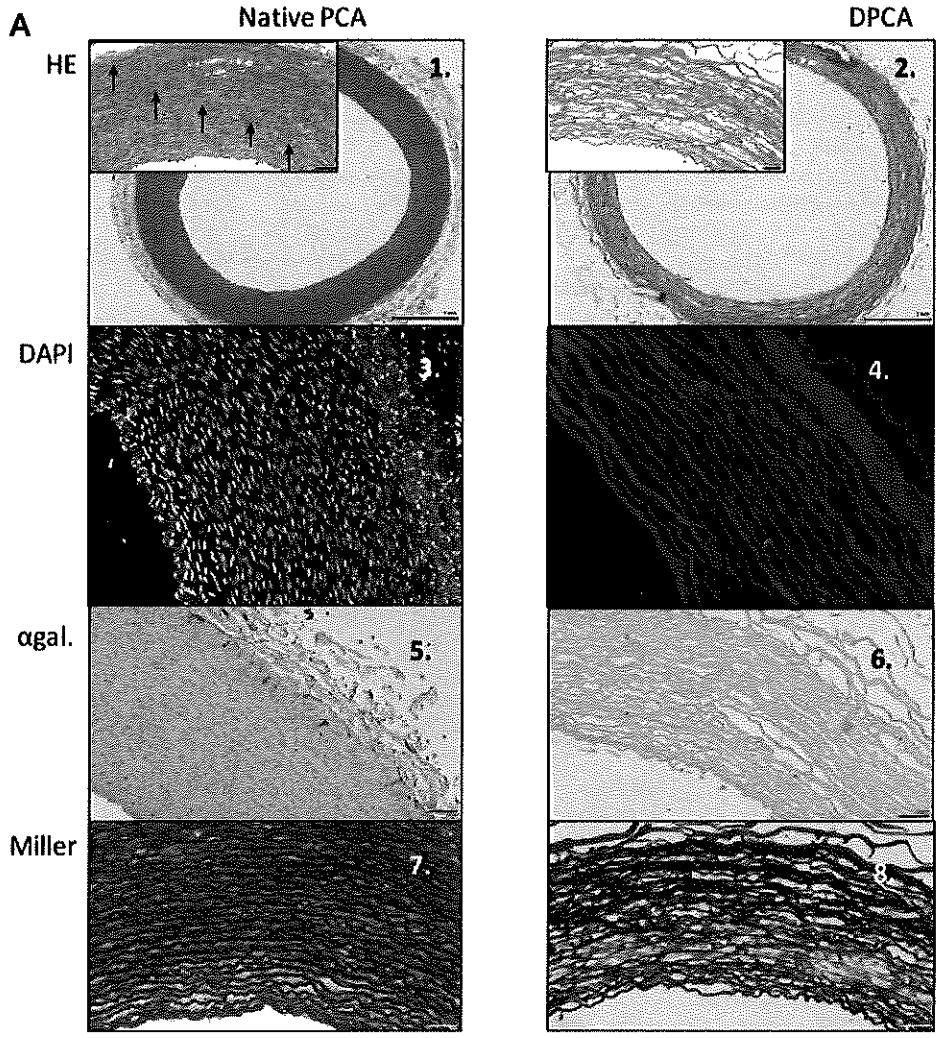
No calcifications were observed.

Preclinical porcine study

Follow-up. No death occurred. DPCA offered better hemostasis in comparison to PTFE graft with complete hemostasis concomitant to vessel unclamping.

No pseudo-aneurysmal or aneurysmal formations or thrombi occurred, and the prostheses remained patent at 1 month.

Inflammatory reaction. No inflammatory cells were seen in the medial and internal part of the reconstructed vessel with the PTFE graft (Fig. 5B1, 2). In the external part, CD3 reaction was significantly higher for PTFE in comparison to DPCA with



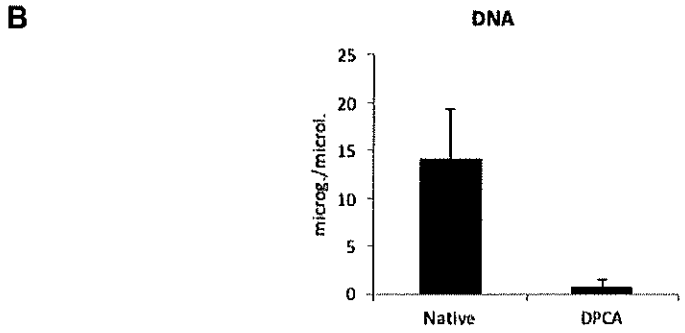


Fig. 3. In vitro study: decellularization assessment of DPCA. **(A)** Paraffin sections of carotid porcine arteries, before (left column) or after (right column) treatment, highlighting cellularity (1 and 2: hematoxylin and eosin staining; 3 and 4: DAPI staining), major porcine antigens (5 and 6: alpha-galactosyl IHC), or structural component such as elastin (7 and 8: Miller staining). *Arrows* indicate nuclei in 1. Scale bar 1 mm (low-magnification pictures) or 100 μm (high-magnification pictures of artery wall). **(B)** DNA quantification in DPCA indicates 95% DNA reduction after treatment.

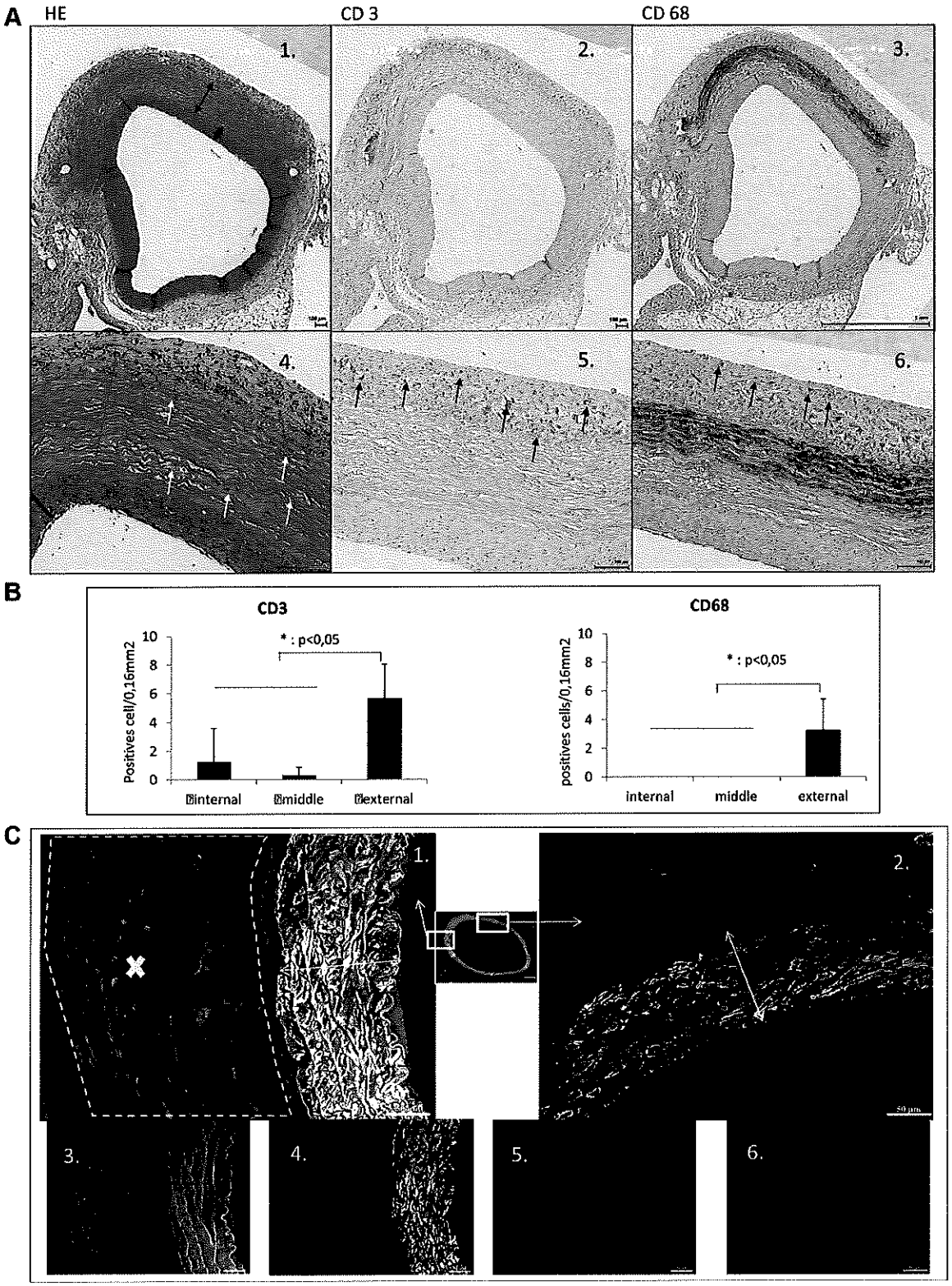


Fig. 4. Inflammation and vascular remodeling with DPCA patch in the rodent. **(A)** Serial sections of the patched aorta stained with hematoxylin and eosin (1 and 4), CD3 (2 and 5), or CD68 (3 and 6) immunostainings. (1–3): whole patched aorta illustrated at low magnification (scale bar: 100 μm [1 and 2] or 1 mm

respectively 23.03 ± 8.79 vs. 18.02 ± 11.87 CD3 positive cells/0.16 mm^2 with $P < 0.05$. Macrophagic reaction was low for both grafts and was statistically higher for DPCA with 4.29 ± 4.43 vs. 0.47 ± 0.74 with $P < 0.05$ suggesting remodeling of DPCA (Fig. 5B3).

Graft integration and remodeling. Hematoxylin and eosin and DAPI staining showed transmural recellularization of the DPCA. ASMA positive cells were detected in the outer half of the prosthesis.

Elastic fibers were still present and well organized. A neointima was present with ASMA cells and with an intraluminal layer of CD31 positive cells (Fig. 6A) in the proximal/medial and distal portions of the prosthesis (Fig. 6A).

In the middle part of the explanted DPCA, the thickness of the neointima was similar to native artery and significantly lower than for PTFE graft (90.3 ± 54.56 vs. 304.52 ± 331.7 μm [$P < 0.05$]) (Fig. 6B).

However, in the distal and proximal parts of the DPCA, near the sutures edges, these thicknesses were significantly higher than in the middle part (90.3 ± 54.56 vs. 615.12 ± 215.71 μm [$P < 0.05$])

No calcifications were detected for DPCA with von Kossa staining while it was positive for PTFE (Fig. 6C).

DISCUSSION

The aim of this study was to determine whether the chemical and physical process can be applied to porcine arteries to improve their biocompatibility for cardiovascular applications. Current biological cardiovascular prostheses are hindered by absence of growth potential and low patency rates. Decellularization of allogeneic or xenogeneic biological scaffolds is an alternative to reduce inflammatory reaction and subsequently calcifications while promoting better long-term patency rates, induced regeneration, and growth potential.[16–18] The main decellularization agent of our treatment is NaOH. The duration of exposure of the tissue to this agent and the combination with other chemicals offer a supplementary property to inactivate conventional (bacteria and virus) and nonconventional (prion) pathogen agents that a conventional detergent process does not offer.[15,19] To our knowledge, this method for vessel decellularization was not yet reported. Indeed, decellularization treatments have to respect a perfect balance between decellularization and maintenance of physical properties. The use of NaOH is considered at risk to denature the tissue.[20] Our in vitro and in vivo results showed that the treatment did not denature the arteries and can ensure this balance. The DPCA were well decellularized, whereas histoarchitecture was maintained with no aneurysm formation in vivo. Another argument is that thinner tissues as porcine or human pericardium or thicker tissues treated with the similar treatment maintained their mechanical properties in vitro and in vivo.[13] Moreover, musculoskeletal allografts processed with the same treatment are routinely implanted in clinical setup and showed mechanical stability.[21]

In a previous study, xenogeneic and allogeneic processed tissues were implanted in a same rodent model and analyzed in a similar way. The results showed that the tissues were significantly less inflammatory than glutaraldehyde-fixed pericardium or than the partially decellularized, clinically used, and well-tolerated human fascia lata.[13] In the actual study, DPCA showed the same characteristics than treated pericardium regarding in vitro decellularization and with comparable inflammatory response in vivo.[13] Following this and considering removal of the major antigen alpha-galactosyl, the DPCA should be well tolerated in human regarding inflammation.[22–24]

These good results in the rodent and in the pig for subcutaneous assessment let us go to a preliminary preclinical evaluation to compare DPCA to the usual PTFE as a long interposition carotid segment that the rodent model does not allow.[25]

[3]); (4–6): focus on patched aorta (DPCA) region at higher magnification (scale bar: 100 μm). *Double arrows* show the limits of the remodeled DPCA (*long arrow*) and the neomedia (*small arrow*) with similar thickness than native. The *yellow arrows* show cell colonization (4) and *black arrows* indicate CD3 cells (5) and CD68 cells (6). **(B)** CD3 and CD68 cell counting in the patched wall. **(C)** CD31 (red) and alpha-SMA (white) immunofluorescent staining of the patched aorta. Autofluorescence of the elastic fibers is visible in green and nuclei, stained with DAPI, in blue. The whole patched aorta is in low magnification between image 1 and image 2. Image 1: higher magnification of the patched aorta: the patch is delineated in yellow and indicated with the *yellow cross*, the neointima is delineated with double *yellow arrow*. Image 2: higher magnification of corresponding native artery. 3–6: Patched region at higher magnification (merge and separated fluorescence channels).

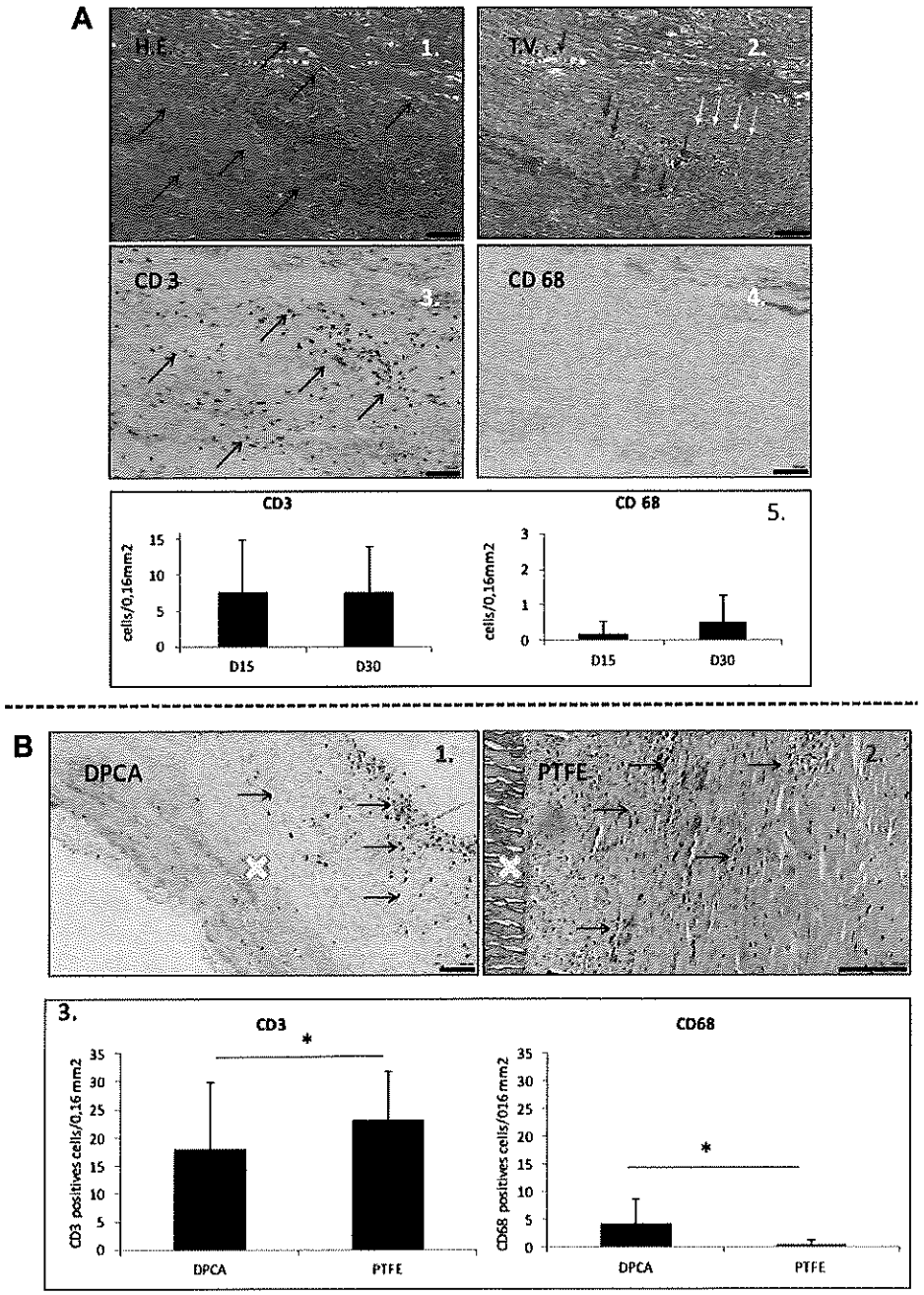
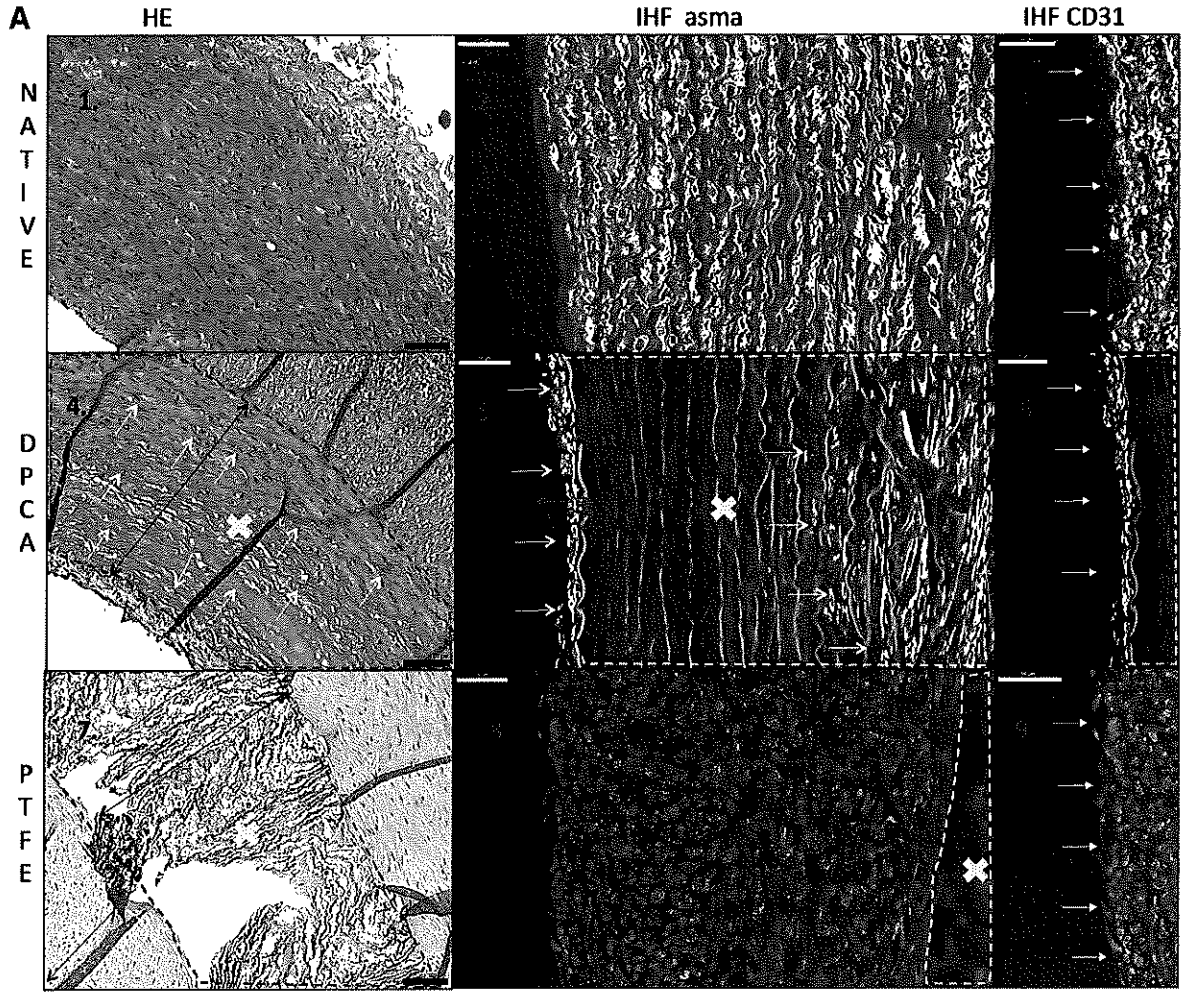
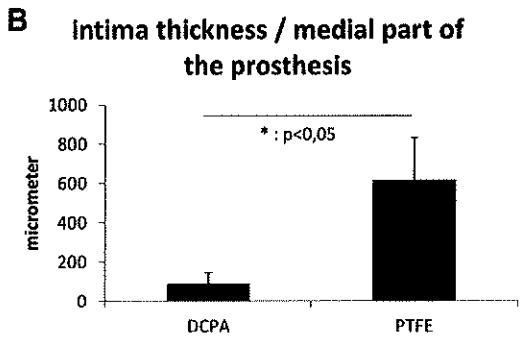


Fig. 5. DPCA inflammation/remodeling in the subcutaneous porcine model **(A)** and DPCA versus PTFE inflammation in the intravascular porcine model **(B)**. **(A)** Histology of DPCA (scale bar:100 micrometer) at day 30 in subcutaneous model showed recellularization of the graft with noninflammatory cells (*black arrows* in [H]E. staining [1]). The elastic fibers were preserved (T.V. staining [2]/*yellow arrows*), angiogenesis occurred (T.V. staining [2]/*red arrows*), and inflammatory reaction was essentially CD3 type (*black arrows* in [3]) and not CD68 (4). The CD3 and CD68 cells counting confirmed this observation. The inflammatory reaction was low at day 15 and 30, and no significant difference between day 15 and day 30 was noticed ($P > 0.05$) (2 graphs (A5)). **(B)** Paraffin sections of CD3 IHC of DPCA (1) and PTFE (2) grafts after 1 month implantation. The positive cells are essentially localized externally to the prosthesis (*black arrows*). The implants are indicated by the *yellow cross* and surrounded by interrupted line. Scale bar, 100 μm. 3: CD3 and CD68 cell counting at day 30 for DPCA and PTFE. ($^*P < 0.05$).





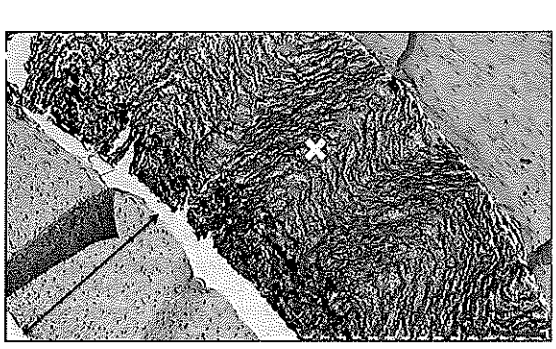


Fig. 6. Vascular remodeling of DPCA versus PTFE in the porcine carotid artery model. **(A)** The panel A presents histologic comparison between Native PCA (first line: 1–3)/DPCA (second line: 4–6) and PTFE (third line: 7–9) explants after hematoxylin and eosin (1, 4, 7)/immunofluorescent alpha-sma (2, 5, 8) and CD31 (3, 6, 9) stainings. Scale bar, 100 μm. The DPCA was more similar to native artery than PTFE graft. The grafts were pointed with a *yellow cross*, a *double blue arrow*, and surrounded by interrupted line. The double red line (in 4–8) showed neointima that was severe for PTFE prosthesis. Moreover, the DPCA was well recellularized (*yellow arrows* in 4) and more specifically recolonized by positive alpha-sma cells (*yellow arrows* in 5). The IHF for CD31 confirmed acquisition of an endothelium for DPCA as well as for PTFE grafts (6 and 9). **(B)** Graph represents the thickness of intimal hyperplasia for PTFE and DPCA grafts (90.3 ± 54.56 vs. 304.52 ± 331.7 μm with $P < 0.05$). **(C)** Paraffin section of PTFE graft explants with von Kossa staining. Scale bar, 100 μm. *Red arrows* indicate von kossa positive staining. The *yellow cross* indicates the PTFE graft surrounded by interrupted blue line and the *double blue arrow* indicates intimal hyperplasia.

In these, after 1 month, the DPCA showed better results in term of inflammation and calcifications than the control PTFE graft, an inert, nonantigenic material thus reporting excellent biocompatibility.

These findings are corroborated with clinical reports. Calcifications of right ventricular outflow tract reconstruction with PTFE conduit, extracardiac PTFE conduit for Fontan completion, PTFE patches or long segment conduits are not uncommon in pediatric care.[5,26,27] PTFE graft calcification is not age related and can concern also adults.[4] Therefore, our prostheses could be advantageous in all the clinical setup and more particularly in children who need viable prostheses. The challenge of decellularized materials is to promote regeneration. Therefore, recellularization of these prostheses and reacquisition of an arterial identity leading to viable prosthesis is also of major concern. Progressive DPCA recellularization with ASMA positive cells without calcium deposits in the porcine model abound in this sense. Moreover, in the long segment the porcine model allowed, recellularization was seen in the center of the prosthesis after a short follow-up. This is a critical point for long segment of non-seeded grafts.[1]

Another crucial point, specifically related to vascular prosthesis, is intimal hyperplasia.[1]

Proliferative intimal hyperplasia can lead to occlusion of the graft as it can be seen for synthetic grafts. In the porcine model, intimal hyperplasia was lower with the DPCA than with the largely used in clinical practice PTFE in the central portion of the graft, and there was no obstruction of the vascular flow after 1 month. The higher thickness of intimal hyperplasia in the proximal regions near the suture can be related to a diameter mismatch between native artery and DPCA due to a continuous sutured line on a high spastic artery.[1] Modification of the surgical management with separated stitches and oblique anastomosis should reduce it.

Our good patency rate is different of a recent study of Cho et al.[8] They showed total occlusion at day 15 of decellularized canine carotid arteries treated with a detergent-based process in a canine carotid artery interposition model. These results are surprising considering (1) a good level of decellularization (1.7% remaining DNA), (2) maintenance of physical properties, and (3) the use of a model which is known to be lower thrombogenic than ours. But, contrary to our study, the animals did not receive antiagregant therapy, which is susceptible to decrease intimal hyperplasia and subsequent thrombosis.[28,29] A comparative study with different decellularization protocols should answer this question.

Otherwise, in their study, as for other authors, they showed also the seeding with cells would lead to better patency rate. The primary end point of our study was to assess DPCA biocompatibility, but a second step could be cell seeding although the DPCA offers excellent results when it is used alone and shows arterial phenotype acquisition after 1 month in our hand.

Last, the preservation method that we used is simple and cost-effective, and DPCA can be easily stored and banked for a long time as musculoskeletal tissues in a tissue bank.[15]

The results in the porcine study are encouraging. Another study with a larger number of samples, with longer follow-up and echodoppler data will confirm long-term patency of the graft, long-term mechanical stability, and remodeling in this preclinical model.[25]

CONCLUSION

The physical and chemical, enzyme/detergent-free process ensures adequate decellularization of PCA. Secured DPCA lead to a poor inflammatory response, can promote vascular cell repopulation and reendothelialization without cell seeding, and show lower intimal hyperplasia and inflammation than PTFE graft in a preliminary study. DPCA is a promising candidate for vessel tissue engineering.

The authors thank Rose-Marie Goebbels, Gwen Beaurin, and Martial Vergauwen for technical assistance; Pascale Segers, Eric Legrand, and Walter Hudders for writing assistance.

REFERENCES

1. Pashneh-Tala S, MacNeil S, Claeyssens F. The tissue-engineered vascular graft-past, present, and future. Tissue Eng Part B Rev 2015;22:68–100.
2. Della Schiava N, Mathevet JL, Boudjelit T, et al. Cryopreserved arterial allografts and ABO and rhesus compatibility. Ann Vasc Surg 2016;33:173–80.
3. Moroni F, Mirabella T. Decellularized matrices for cardiovascular tissue engineering. Am J Stem Cells 2014;3:1–20.
4. Mehta RI, Mukherjee AK, Patterson TD, et al. Pathology of explanted polytetrafluoroethylene vascular grafts. Cardiovasc Pathol 2011;20:213–21.
5. Tomizawa Y, Takanashi Y, Noishiki Y, et al. Evaluation of small caliber vascular prostheses implanted in small children: activated angiogenesis and accelerated calcification. ASAIO J 1998;44:M496–500.
6. Koens MJ, Krasznai AG, Hanssen AE, et al. Vascular replacement using a layered elastin-collagen vascular graft in a porcine model: one week patency versus one month occlusion. Organogenesis 2015;11:105–21.
7. Bergmeister H, Plasenzotti R, Walter I, et al. Decellularized, xenogeneic small-diameter arteries: transition from a

muscular to an elastic phenotype in vivo. J Biomed Mater Res B Appl Biomater 2008;87:95–104.
8. Cho SW, Lim SH, Kim IK, et al. Small-diameter blood vessels engineered with bone marrow-derived cells. Ann Surg 2005;241:506–15.
9. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. Biomaterials 2011;32:3233–43.
10. Pellegata AF, Asnaghi MA, Stefani I, et al. Detergent-enzymatic decellularization of swine blood vessels: insight on mechanical properties for vascular tissue engineering. Biomed Res Int 2013;2013:918753.
11. Zou Y, Zhang Y. Mechanical evaluation of decellularized porcine thoracic aorta. J Surg Res 2012;175:359–68.
12. Ishino N, Fujisato T. Decellularization of porcine carotid by the recipient's serum and evaluation of its biocompatibility using a rat autograft model. J Artif Organs 2015;18:136–42.
13. van Steenberghe M, Schubert T, Guiot Y, et al. Enhanced vascular biocompatibility of decellularized xeno-/allogeneic matrices in a rodent model. Cell Tissue Bank 2017;18: 249–62.
14. Comu O, Schubert T, Libouton X, et al. Particle size influence in an impaction bone grafting model. Comparison of fresh-frozen and freeze-dried allografts. J Biomech 2009;42:2238–42.
15. Fawzi-Grancher S, Goebbels RM, Bigare E, et al. Human tissue allograft processing: impact on in vitro and in vivo biocompatibility. J Mater Sci Mater Med 2009;20:1709–20.
16. Umashankar PR, Mohanan PV, Kumari TV. Glutaraldehyde treatment elicits toxic response compared to decellularization in bovine pericardium. Toxicol Int 2012;19:51–8.
17. Badylak SF. Decellularized allogeneic and xenogeneic tissue as a bioscaffold for regenerative medicine: factors that influence the host response. Ann Biomed Eng 2014;42:1517–27.
18. Cho SW, Kim IK, Kang JM, et al. Evidence for in vivo growth potential and vascular remodeling of tissue-engineered artery. Tissue Eng Part A 2009;15:901–12.
19. WHO. Decontamination methods for transmissible spongiform encephalopathies. Report of a WHO consultation, Geneva, Switzerland, 23–26 March 1999. In: WHO Infection Control Guidelines for Transmissible Spongiform Encephalopathies. WHO/CDS/CSR/APH/2000/3.
20. Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: structure and function. Acta Biomater 2009;5:1–13.
21. Dufrane D, Mourad M, van Steenberghe M, et al. Regeneration of abdominal wall musculofascial defects by a human acellular collagen matrix. Biomaterials 2008;29:2237–48.
22. Wong ML, Griffiths LG. Immunogenicity in xenogeneic scaffold generation: antigen removal vs. decellularization. Acta Biomater 2014;10:1806–16.
23. Wong ML, Wong JL, Vapniarsky N, et al. In vivo xenogeneic scaffold fate is determined by residual antigenicity and extracellular matrix preservation. Biomaterials 2016;92: 1–12.
24. Cravedi P, Farouk S, Angeletti A, et al. Regenerative immunology: the immunological reaction to biomaterials. Transpl Int 2017;30:1199–208.
25. Byrom MJ, Bannon PG, White GH, et al. Animal models for the assessment of novel vascular conduits. J Vasc Surg 2010;52:176–95.
26. Hayabuchi Y, Mori K, Kitagawa T, et al. Polytetrafluoroethylene graft calcification in patients with surgically repaired congenital heart disease: evaluation using multidetector-row computed tomography. Am Heart J 2007;153:806–8.
27. Chong DST, Constantinou J, Davis M, et al. Calcification of a synthetic renovascular graft in a child. EJVES Short Rep 2016;33:13–5.
28. McCann RL, Hagen PO, Fuchs JC. Aspirin and dipyridamole decrease intimal hyperplasia in experimental vein grafts. Ann Surg 1980;191:238–43.
29. Quinones-Baldrich WJ, Ziomek S, Henderson T, et al. Patency and intimal hyperplasia: the effect of aspirin on small arterial anastomosis. Ann Vasc Surg 1988;2:50–6.