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The degeneration of biological cardiovascular prostheses under pro-calcific metabolic conditions in a small animal model

Alexander Assmann [a, 1, 2], Kai Zwirnmann [a], Friederike Heidelberg [a], Franziska Schiffer [a], Kim Horstkötter [a], Hiroshi Munakata [a], Felix Gremse [b], Mareike Barth [a], Artur Lichtenberg [a, *], Payam Akhyari [a]

[a] *Department of Cardiovascular Surgery and Research Group for Experimental Surgery, Heinrich Heine University, Medical Faculty, Moorenstrasse 5, Duesseldorf 40225, Germany*
[b] *Department of Experimental Molecular Imaging, RWTH-Aachen University, Medical Faculty, Pauwelsstr. 30, Aachen D-52074, Germany*

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In order to allow for a comparative evaluation of the *in vivo* degeneration of biological and tissue-engineered heart valves and vascular grafts, a small animal model of accelerated cardiovascular calcification is desired. Wistar rats ($n = 102$; 6 groups) were fed *ad libitum* with regular chow and 5 different regimens of pro-calcific diet supplemented with combinations of vitamin D (VD), cholesterol (CH) and dicalcium phosphate (PH). Moreover, cryopreserved ($n = 7$) or detergent-decellularized rat aortic conduit grafts ($n = 6$) were infrarenally implanted in Wistar rats under severely pro-calcific conditions. The follow-up lasted up to 12 weeks. High-dose application of VD (300,000 IU/kg), CH (2%) and PH (1.5%) resulted in elevated serum calcium and cholesterol levels as well as LDL/HDL ratio. It increased the tissue MMP activity visualized by *in situ* zymography and caused significantly aggravated calcification of the native aortic valve as well as the aortic wall as assessed by histology and micro-computed tomography. (Immuno)histology and quantitative real-time PCR revealed chondro-osteogenic cell transformation, lipid deposition, nitrosative stress and low-level inflammation to be involved in the formation of calcific lesions. Despite pro-calcific *in vivo* conditions, decellularization significantly reduced calcification, inflammation and intimal hyperplasia in aortic conduit implants. A well balanced dietary trigger for pathologic metabolic conditions may represent an appropriate mid-term treatment to induce calcifying degeneration of aortic valves as well as vascular structures in the systemic circulation in rats. With respect to experimental investigation focusing on calcifying degeneration of native or prosthetic tissue, this regimen may serve as a valuable tool with a rapid onset and multi-facetted character of cardiovascular degeneration.

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1. Introduction

All currently available heart valve bioprostheses undergo progressive calcifying degeneration that terminally results in implant failure requiring a redo procedure with an elevated risk for the recipient patient [1]. In order to solve this problem, decellularized and tissue-engineered aortic valve implants have become an alternative with promising results in preclinical as well as clinical studies [2–5]. However, central aspects of engineering these prostheses are controversially discussed, e.g. biological versus artificial scaffolds, preoperative *in vitro* cell seeding versus spontaneous implant repopulation *in vivo*, the method of decellularization or the need for surface coating strategies to enhance the biocompatibility of the implants [6].

* Corresponding author. Department of Cardiovascular Surgery, Heinrich Heine University, Medical Faculty, Moorenstrasse 5, D-40225 Duesseldorf, Germany. Tel.: +49 (0) 211 81 18331; fax: +49 (0) 211 81 18333.
E-mail addresses: alexander.assmann@med.uni-duesseldorf.de (A. Assmann), artur.lichtenberg@med.uni-duesseldorf.de (A. Lichtenberg).
[1] Present address: Center for Biomedical Engineering, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 75 Francis St, Boston, MA 02115, USA.
[2] Present address: Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, 65 Landsdowne St, Cambridge, MA 02139, USA.

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In terms of optimizing the properties of tissue-engineered heart valves, it is widely accepted that the prosthesis-surrounding milieu, e.g. recipient-inherent comorbidities and risk factors, may have an impact on the durability and long-term function of biological heart valves, and this impact may be just as crucial as the material characteristics of the prosthesis are. Data from clinical cohorts confirm renal insufficiency and lipid disorders as independent risk factors for the degeneration of bioprosthetic heart valves. Although the complexity of comorbidities found in patients is hardly to mimic by standardized animal models, yet there is a need for animal models in the preclinical graft testing allowing for an accelerated degeneration of cardiovascular implants. Since they enable large-scale comparative studies and because of the availability of a broader range of molecular-biological readout modalities, small animal models are preferred for the examination of fundamental strategies of engineering cardiovascular implants. Recently, we reported on the development and functional optimization of a standardized rat implantation model facilitating the comparative evaluation of the fate of biological and tissue-engineered aortic conduit grafts in the systemic circulation [7–9]. In order to further optimize our model by allowing for implant degeneration within short observation periods, the present study was designed to develop a reliable model of accelerated cardiovascular calcification in rats.

Dietary vitamin D (VD) and cholesterol (CH) supplementation have been reported to enhance the calcium deposition in the arterial vessel wall of rodents [10]. In rabbits, the combination of VD and CH has been observed to induce histological as well as functional signs of aortic valve sclerosis [11,12]. The effect of VD and CH on the calcifying degeneration of rat aortic valves has not been subject to a systematic and in-depth investigation, yet. Moreover, increased phosphate (PH) serum levels are supposed to contribute to the progression of aortic valve sclerosis in humans [13,14].

The primary aim of the present study was to develop a standardized model of accelerated aortic valve as well as arterial calcification in rats by evaluating different chow regimens supplemented with VD, CH and PH [Table 1]. Furthermore, the suitability of this model to examine the degeneration of cardiovascular prostheses was tested *in vivo* by comparing cryopreserved and decellularized aortic conduit implants.

2. Materials and methods

2.1. Animals

Male Wistar ($n = 115$) and Sprague–Dawley ($n = 13$) rats weighing 200–250 g were obtained from the local animal care facility, received chow *ad libitum* and were exposed to constant temperature, humidity and circadian daylight rhythm. All experiments were conducted according to the national animal welfare act, and approved by the state animal care committee (reference number 87-51.04.2010.A068).

2.2. In vivo examination of different dietary regimens

A Wistar rat population of 102 animals was randomly divided into six groups receiving different regimens of pro-calcific diet containing VD, CH and PH [Table 1]. The follow-up periods were 4, 8 and 12 weeks in each group. Body weight and the amount of fed chow were continuously monitored on a weekly base.

Blood was drawn after 4, 8 and 12 weeks, and the serum levels of calcium, phosphate, urea and cholesterol (including HDL and LDL fractions) were measured according to routine procedures employing standard assays for rats by the Institute of Clinical Chemistry and Laboratory Diagnostics, Heinrich Heine University, Medical Faculty, Duesseldorf, Germany.

Global cardiac function as well as relevant parameters for the detection of valvular stenosis of the aortic valve were assessed by transthoracic echocardiography using a Philips HDX11 ultrasonography system equipped with a 15 MHz probe (Philips, Amsterdam, Netherlands). Following parameters were analyzed: Left ventricular enddiastolic and endsystolic diameter (LVEDD, LVESD), left ventricular ejection fraction (LVEF) and transvalvular pressure gradient at the aortic valve (dp_{AV}).

At the end of the follow-up, echocardiography was performed in rats under inhalative anesthetization with isoflurane (2.0–2.5%) and analgesia with intraperitoneal carprofen (5 mg/kg). After combined thoracotomy, laparotomy and systemic heparinization (1000 IU i.v.), the circulatory system was perfused with PBS, and the aortic valve as well as the whole aorta were excised and further processed for histology, immunohistology, *in situ* zymography, quantitative real-time polymerase chain reaction (RT-PCR), and micro-computed tomography (μCT). Moreover, the heart weight was determined after thorough drying.

2.3. In vivo testing of aortic conduit implants

Aortic conduit grafts from Sprague–Dawley rats were either cryopreserved (group CRYO; $n = 7$) or detergent-decellularized (group DC; $n = 6$) and implanted in male Wistar rats ($n = 13$). Fourteen days before the grafting procedure (d-14), all recipient rats underwent an interventional generation of native aortic valve insufficiency (AI). Controlled generation of native aortic valve insufficiency was conducted as recently published [9]. Under general anesthesia and echocardiographic guidance, the aortic valve leaflets were interventionally perforated to induce an AI grade II–III. Animals with AI grade I or grade IV in the postoperative echocardiography assessment were excluded from the study. At day 0, cryopreserved or decellularized aortic conduits were infrarenally implanted into the systemic circulation according to a recently published standardized small animal model [7,9]. Four or 12 weeks after implantation, conduit grafts were explanted and the animals euthanized. The grafts were rinsed with heparinized PBS via abdominal cannulation and further processed for histology, immunohistology, or *in situ* zymography (each with $n = 3$ per group at days 28 and 84). For μCT, the whole native aorta including the implant was excised ($n = 1$). Graft perfusion was controlled by Doppler sonography at implantation and pre-explantation.

All recipients received a pro-calcifying diet (+300,000 IU/kg vitamin D + 2% cholesterol + 1.5% calcium phosphate) from day −14 until the final explantation of the prostheses.

2.4. Graft harvesting and engineering

Rat aortic conduit grafts were explanted and either cryopreserved or decellularized as recently described [7]. Grafts in group CRYO were transferred into conservation medium (Dulbecco's modified Eagle's medium + 10% dimethylsulfoxide +20% fetal calf serum at 5 °C) immediately after preparation. Afterwards, the conduits were frozen to −80 °C under controlled cooling (1 °C per minute in a propan-2-ol box). Four weeks later, the cryopreserved grafts were thawed, washed three times in heparinized PBS and implanted. Prostheses in group DC were decellularized within four cycles (12 h) with 0.5% sodium dodecyl sulfate + 0.5% deoxycholate, followed by 24 h rinsing with distilled water and three further rinsing cycles (24 h) with PBS supplemented with 1% penicillin/streptomycin and 0.05% sodium azide.

2.5. Histology

Explanted tissue was embedded in TissueTek cryo-mounting medium and tissue sections of 4 and 6 μm were prepared (cryostat CM 1950; Leica Biosystems, Wetzlar, Germany). Histological stainings were performed according to previously published protocols, including hematoxylin/eosin (H&E) staining, Movat's pentachrome staining, von Kossa staining, and Oil Red O staining [11]. Images were acquired on a microscope system DM2000 equipped with a digital camera DFC 425C (Leica

Table 1
Chow regimens.

	Group N Normal $n = 19$	Group HIGH High-dose $n = 19$	Group LOW Low-dose $n = 19$	Group I_{-CH} w/o cholesterol $n = 15$	Group II_{-PH} w/o phosphate $n = 15$	Group III_{-VD} w/o vitamin D $n = 15$
Vitamin D	–	300,000 IU/kg	150,000 IU/kg	300,000 IU/kg	300,000 IU/kg	–
Cholesterol	–	2% (w/w)	1% (w/w)	–	2% (w/w)	2% (w/w)
Dicalcium phosphate	–	1.5% (w/w)	0.75% (w/w)	1.5% (w/w)	–	1.5% (w/w)

A male Wistar rat population ($n = 102$) was randomly divided into six groups receiving different regimens of pro-calcific chow supplementation. Group N received normal chow without any supplementation. In each group, the follow-up periods were 4, 8 and 12 weeks ($n = 5$–7 per time point).

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