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Nano- Coating with Titanium of Glutaraldehyde- Fixed Heart Valve Prostheses Enables a Reduced Immune Response and a Self-Seeding Within Circulation

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1. Introduction

Glutaraldehyd-fixed heart valve prostheses with long durability and function are desirable as a treatment option of heart valve disease. They do not require chronic anticoagulation therapy, however break down quickly in young patients (<5 years) but last for > 15 years in patients older than 70 years. It is generally accepted that the break down of bioprosthetic heart valves is due to calcification occurring because of immunologic and chemical processes.
Thus, new nano- technological methods in combination with chemical and biochemical procedures concerning detoxification and titanization of glutardialdehyde- fixed collagen scaffolds are applied on glutardialdehyde fixed pericardial samples. Nano- technology with titanium using a plasma application under high alternating current, developed elsewhere (GfE Nürnberg, Germany, patent number EP 0 897 997 A1) might decrease the immune response caused by coating and improve endothelialization by detoxification and the biocompatibility of titanium. A plasma-activated surface of collagen allows a titanization under room temperature described later on. Immune response with secondary dystrophic calcification may be why glutaraldehyde fixed xenograft valves fail, especially in young patients. Hypothesis is, detoxification and titanization may reduce immunologic reactions and enable an endothelialization.

2. Detoxification and titanium coating of glutaraldehyde-fixed pericardium

Free aldehyde-groups of glutaraldehyde-fixed pericardium are responsible for its toxicity, which prevents an endothelialization.

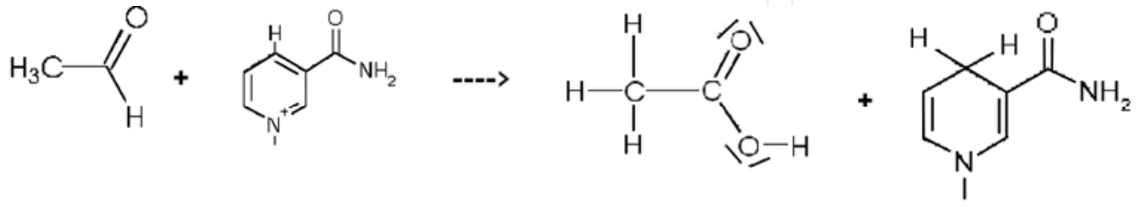
2.1 Detoxification

Platelets of glutaraldehyde- fixed pericardium were treated differently for detoxification. Some were detoxified chemically by incubation with citric acid (CA) of 10%. Others were detoxified enzymatically by aldehyde dehydrogenase and received nano-technological

methods using plasma activation either solely or combined with a treatment of organic bound titanium.

2.2 Determination of free aldehyde groups

A method for the determination of the amount of glutaraldehyde in specimens of glutaraldehyde fixed pericardium has been developed using aldehyde dehydrogenase (ALDH), (Guldner et.al. 2009). In presence of ALDH, aldehydes are oxidated to carbon acids while a reduction equivalent (here: NAD+) is reduced. Aldehyde dehydrogenases have broad substrate specificity. Thus, they process glutardialdehyde as well as other aldehydes



Acetaldehyde + NAD^+ → Acetic acid + NADH

The determination of aldehydes with the ALDH method was performed photometrically. The increase of optical density (extension) by the reduced NADH could be measured at a wave length of 340nm. The amount of NADH being proportional to the original amount of aldehyde, statements on the remaining toxicity of the fixed pericardium could be made. Optimal duration for incubation was inquired.

Enzyme, NAD+ (tablets) and buffer solution from a commercially available kit, originally developed to determine acetaldehyde concentrations in a variety of materials, were used (Acetaldehyde, Enzymatic BioAnalysis; R-Biopharm, Darmstadt, Germany). Platelets of pericardium were incubated in a 24 well plate with 1ml enzyme solution each for 24 hours. Thereafter 100µl from each well were examined photometrically. Native pericardium served as control.

2.3 Chemical Vapor Deposition (CVD) by a titanium containing precursor

Before physical titanization of cardiovascular scaffolds by plasma activated chemical gas deposition (PACVD) it is necessary to extract its water completely, which is feasible in a vacuum by a slow drying process. PACVD is a coating technology (GfE Nürnberg, Germany, patent number EP 0 897 997 A1) where the so called precursor (Tetrakisdimethylamidotitan, Ti $[N(CH_3)_2]_4$) is transferred into the gas phase and brought into the reactor by a carrier gas (nitrogen). The precursor or parts of the precursor react with the substrate creating a resistant layer. Physical plasma however is able to supply the substrate with high energy while the temperature during deposition can kept low. Within this non isotherm plasma with solely high electron temperature and room temperature of the neutrons and ions the electrons can follow a quickly changing electrical field with typical values of a radio frequency (40kHz) low pressure plasma as described elsewhere (Grill,1994; Sivaram S.1995).Thickness of the titanium containing deposit is depend on sputtering time. After PACVD the dehydrated implants came to the same shape in physiological saline solution as before titanization.

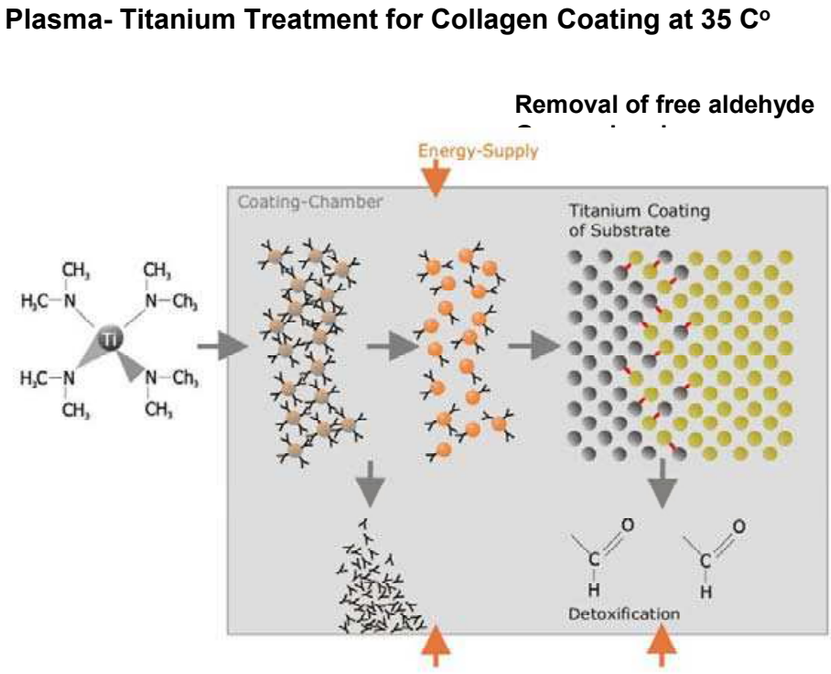


Fig. 1. Scheme of Chemical Vapor Deposition (CVD) with the gasiform titanium bounded organic molecule as precursor at 35 C^0. In a vacuum chamber with energy supply by high alternative current, the precursor is deconstructed and precipitated on the plasma activated collagen surface (Plasma Activated Chemical Vapor Deposition, PACVD) and electron pair bounded to collagen molecules

2.4 Examination of the titanium coat

Deposits composition and binding energy of titanium, carbon, oxygen and nitrogen atoms could become analyzed by X-ray Photoelectron Spectroscopy (XPS). X-ray activated atoms emit electrons from inner electron layers (photo effect) with a distinct kinetic energy being specific for each element. The kinetic energy of the emitted electron diagnoses the element and therefore the composition of elements within the titanic layer. The chemical binding in which an element is, influences the energy levels of its inner electrons. A more electronegative partner binds its electrons tighter on its inner electron layers. That influences the kinetic energy on the emitted photoelectrons during X-ray application. These values in kinetic energy of the emitted photoelectrons enable to determine the chemical binding energy between elements during XPS and so in titanic layers as for oxygen, titanium, nitrogen and carbon (Dag, 1997; Moulderm,1995). The limited intrinsic depth for XPS of about 1 nm requires a removal of substrate in layers using argon ions. The XPS analysis showed a maximal surface atom fraction of 21.1±3 8% titanium atoms within a removal time of about 300 seconds, which translates to a titanium thickness of 30 nm. The presence of oxygen indicated that titanium had been changed by subsequent oxidation into titanium dioxide, with an electron-binding energy for titanium of 463 eV. Two peaks for titanium represented titanium bound carbon and titanium bound with oxygen as titanium oxide. These analyses show an extremely tight electron binding of the titan precursor fragments to collagen. Titanium is non removable bounded to collagen in an extremely thin coating onto collagen with a diameter of about 30 nano-meters.

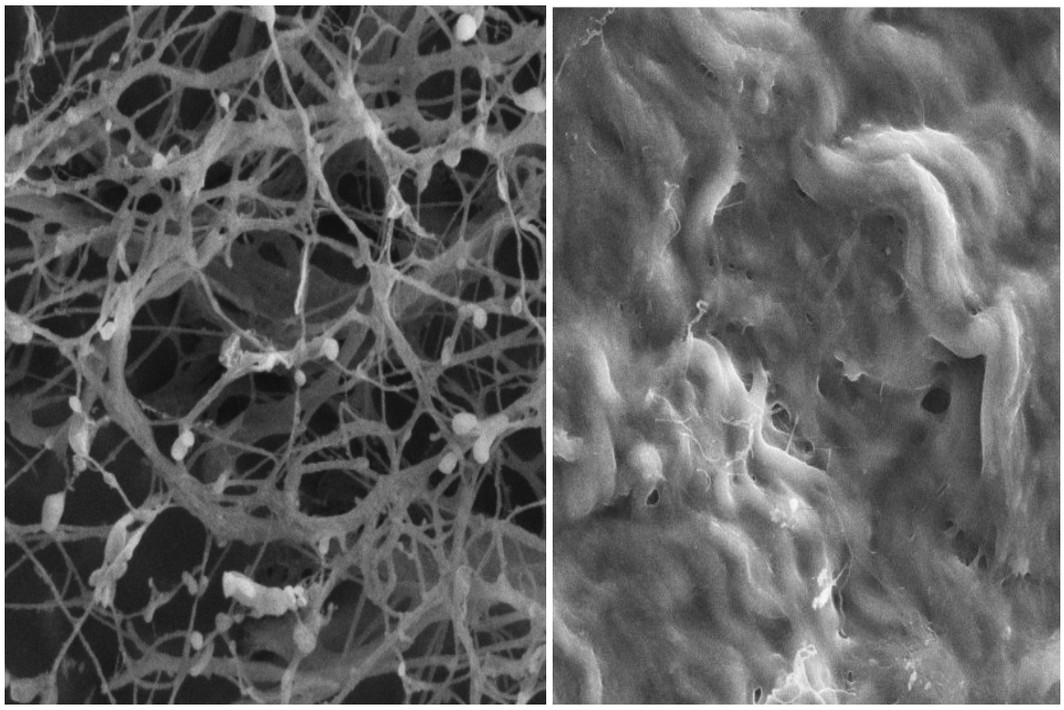


Fig. 2. REM of GA- fixed and dehydrated pericardium with a magnification of 5000X (left), and with a complete covering of the surface by titanium (right)

Scanning electron microskopic images of GA-fixed pericardium show a more filigree net like structure (Fig.2 left) which disappears after titanization (Fig.2 right). These morphologic changes however do not affect the mechanical stability of the pericardium as already investigated (unpublished data). After dehydration and a swelling of the soft collagen, the titanium coat may burst into grooves. This uncomplete surface covering may have important implications on iC3b deposits and PNM attraction as discussed in the following descriptions about the immune response. But nevertheless the decovering of collagene by these grooves is manly dependent on the chosen dehydration procedure.

3. Immunology of glutaraldehyde-fixed bovine pericardium

Glutaraldehyd-fixed heart valve prostheses calcify and fail in a time period lesser than 12 years (Williams et. al.,1982). Some investigators believe that the GA-process leads to calcification and failure of the valves due to chemical processes (Simionescu, 2004). Others are convinced the main break down of GA-fixed valves is caused by an immunologic response (Rocchini et. al.,1981; Williams et.al., 1982; Ueyama et.al, 2002; Rizwan et.al.,2006). They showed in a young animal model that GA-fixed xenograft valves undergo rejection and that inflammation is correlated to calcification (Rizwan et.al., 2006).
Young patients with a strong immune system show a valve break down very quickly (<5) years (Rocchini et. al.,1981; Williams et.al., 1982;). In patients > 70 years with a minor immune response bio-prosthetic valves function well > 10 years (Ueyama et.al, 2002). Xenogenic organ transplantation arises a very aggressive form of humoral and cellular immune rejection with tissue infiltrations by lymphocytes and monocytes (Fischbein et.al., 2000). Whereas the immunologic lymphocytes and monocytes response could become

decreased by de-cellularization or GA-fixation the granulocyte recruitments in de-cellularized xenografts and GA-fixed heart valves were not influenced at all. The residual immuno-stimulatory activity of de-cellularized porcine vascular tissue towards polymorphonuclear (PNM) cells resp. granulocyte recruitments, observed in vitro (Fischbein et. al., 2000; Juthier et.al; 2006), correlates with in vivo findings with de-cellularized human and porcine heart valves (Friedhelm et.al; 2005; Rieder et.al.; 2006; Bastian et.al., 2008). For the evaluation of the immunologic activity of GA- fixed pericardium in vitro blood sampling, plasma preparation and isolation of PNMs are necessary.

3.1 Blood sampling, preparation of plasma, and isolation of PMN

Venous blood was collected from 3 healthy adult volunteers who were not using any medications and who gave informed consent (approved by the ethical committee, Medical University of Vienna). Whole blood was anti-coagulated with 5 IU/ml heparin and centrifuged at 2000 x *g* for 15 min to obtain plasma. Human PMN were retrieved from EDTA-anticoagulated venous blood by lysing 1ml blood with 5ml 0.9% (w/v) ammonium chloride for 15 min at 4°C, followed by centrifugation at 160 x *g* and 4°C for 10 min (Nilsson, 2001). The supernatant was discarded and the cell pellet washed three times with PBS. Cells were subsequently re-suspended in RPMI 1640 medium (BioWhittaker™, Verbiers, Belgium) to a final concentration of 5000/µl and immediately used for the experiments. The cell suspensions comprised 84.4±3.1% PMN, 11.6±2.7% lymphocytes, and 3.5±1.6% monocytes. The platelet contamination was less than 0.1 platelet per PMN. These procedures were necessary for the quantification of IC3b deposits on the blood contacting surface of the GA-fixed pericardium and the granulocyte-matrix adhesion experiments.

3.2 IC3b- deposits

IgG deposits(iC3b) were visualized by an immune staining as described elsewhere (Bastian et. al.; 2008) and shown in figure 4. They are documented as coloured pixels (red). Pixels are counted electronically.

IC3b- deposits within the control group with GA-fixed pericardium were regarded as 100%; in the titanized group IC3b deposits were evaluated as 34%. The authors opinion is, that these preliminary results can become further optimised (further decrease of immunologic response) by an advanced procedure of pericard drying and plasma deposition based on titanium.

3.3 Granulocyte/matrix adhesion experiments

Platelets of titanium coated, glutaraldehyde-fixed bovine pericardium were brought into an immunologic test setting inducing a complement-mediated granulocyte adhesion and activation as described elsewhere (Bastian et. al.; 2008).

Plasma used for pre-treating the tissue samples and granulocytes was obtained from the same donor during each experiment. Tissue specimens (0.5 x 0.5 cm) were either incubated with 20% of heparinized autologous plasma (diluted in PBS containing 0.15 mmol/l Ca^{2+} and 0.5 mmol/l Mg^{2+}) on a rocking platform for 5h each at 37°C, or left untreated. Cryostat sections were incubated with 5,000 PMN/µl for 15 minutes in a humidified incubation chamber at room temperature (RT). This incubation time was chosen since it proved to be optimal for demonstrating PMN adhesion in vitro.

PMN quantification was performed by counting the leucocytes (including 86% PMNs) near the blood contacting surface of the pericard within an areas of 3x3 mm. Five samples from each histologic slice were taken for the evaluation of PNMs.

Nano- coating with titanium remarkably reduced the early immunologic response to GA fixed bovine pericardium in vitro. Thus, the titanium coat is protective against iC3b and immune based PMN attraction in contact with human blood.

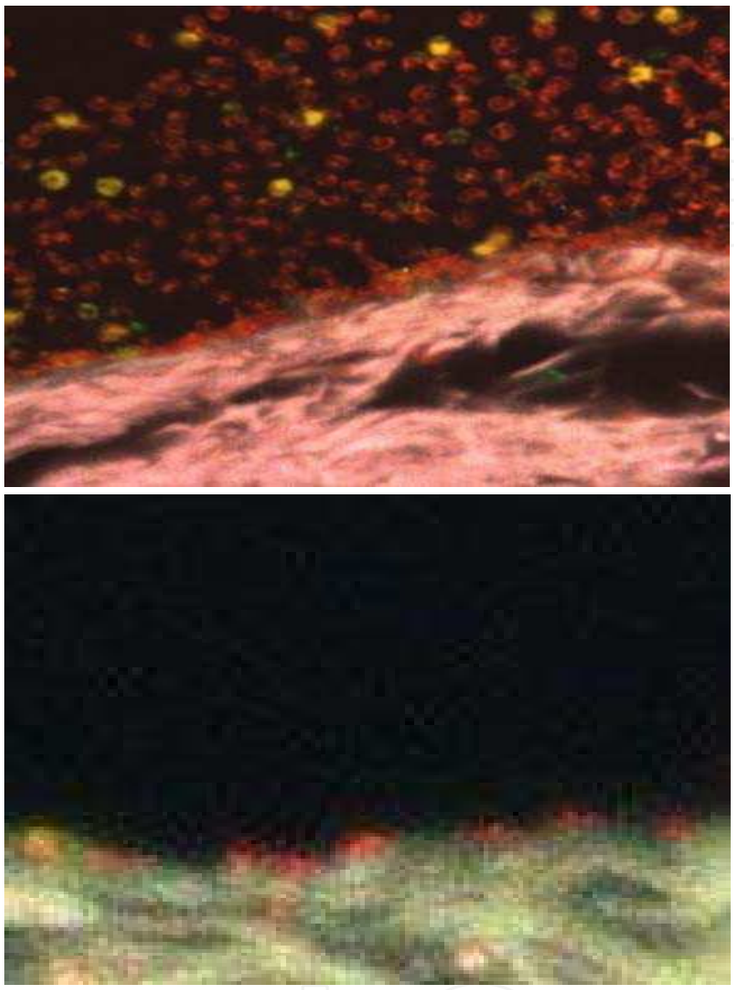


Fig. 3. PNM count in front of the GA-fixed pericard slices were regarded as 100% (top) GA- fixed and titanium coated pericardium however reduced the attraction of PNMs to 6.0% (bottom) as an expression for an extremely reduced immune response

4. Endothelialization

Glutardialdehyde's toxicity however prevents biological coating. Detoxification of glutaraldehyde-fixed scaffolds by amino acids (Fischlein et.al, 1994) and by citric acids (Lehner et.al., 1997; Gulbins et.al., 2003) in vitro were successfully applied for partial detoxification but endothelialization was only possible by pre-seeding with fibroblasts. Such pre-seeded layers on porcine heart valves were stable in a sheep model (Gulbins et.al., 2003,2006). Due to the fibroblasts however, the valves showed a thickening of the leaflets resulting in an impairment of function.

Recent investigations developed detoxification strategies and applied nano- technological methods such as ultrathin titanium surface coating on glutaraldehyde fixed bovine

pericardium as the base material for prosthetic heart valves (Guldner et.al., 2009). Titanization by chemical vapor deposition was feasible at temperatures between 30-35°C (Grill et.al., 1994; Silvaram et.al., 1995; Dag,1995). For endothelial cell adhesion on a titanium surface endothelial cell cultures were performed.

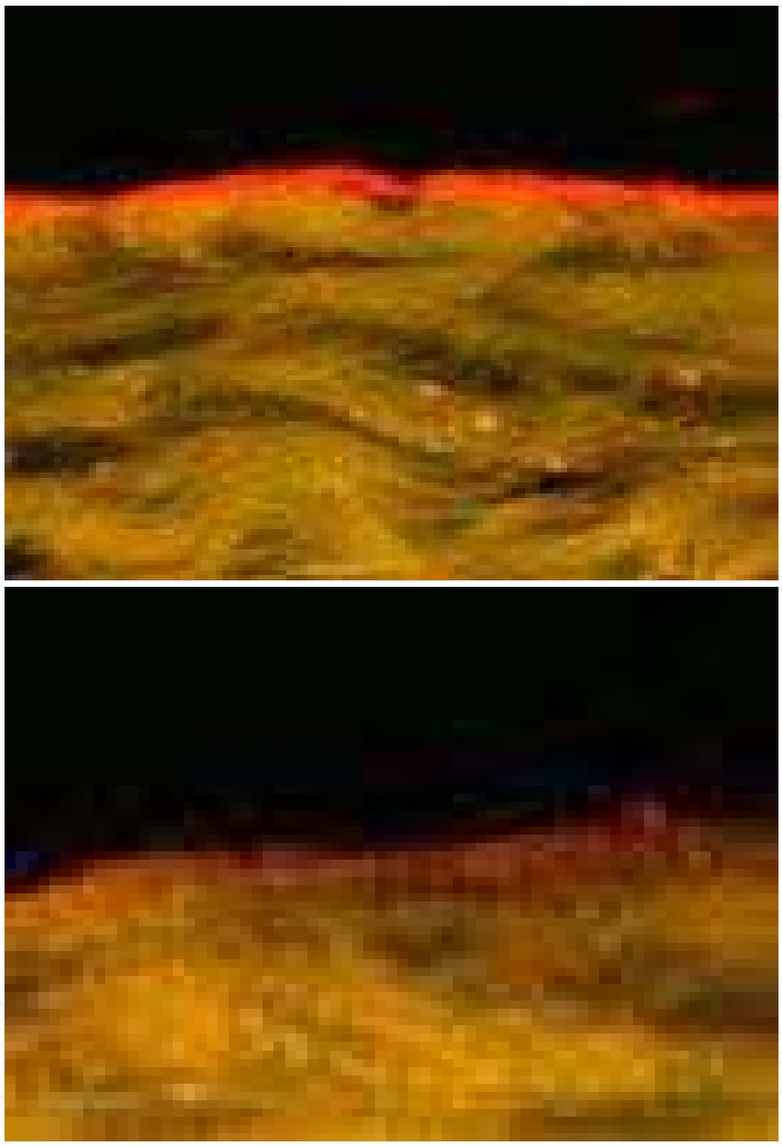


Fig. 4. Immune staining of iC3b deposits on GA-fixed pericardium (top) was control and defined as 100% pixels, titanium coated pericardium platelets showed a count of a decreased number of pixels to 34% (bottom) as an expression of an impressive lesser immune response as to the uncoated GA-fixed pericardium

4.1 Cell culture

Endothelial cells have been isolated from human saphena veins, gained from coronary bypass surgery. Ethical approval was obtained from the Ethical Commission on Research on Humans of the University of Schleswig Holstein, Campus Lübeck, Germany (registration

No.05-097). Patients had been informed before surgery that parts of their veins were to be used for research purposes. The vein pieces were cannulated, rinsed with cell medium then filled with 0.5% dispase and incubated for 20 minutes at 37°C and 5% CO_2. Then solution was then centrifugated at 1200 upm for 5 minutes, the cell pellet was re-suspended in endothelial growth medium (EGM-2; Cambrex, Walkersville, US) and plated on cell culture flasks. For passaging, endothelial cells were trypsinized after reaching confluence, centrifuged at 1200 upm for 5 minutes, then re-suspended in endothelial cell medium and plated out again. Cell counting has been performed by incubating 20µl of the cell suspension with an equal amount of trypan-blue and then counting using a Neubauer chamber.
Pre-treated platelets of glutardialdehyde fixed pericardium were examined by scanning electron microscopy (SEM) after 48 hours of incubation with human endothelial cells. SEM showed malformed non confluent endothelial cells on Citric Acid (10%) only treated pericardium (Figure 5a, n=5). In contrast to that, a completely confluent cell layer of regular human endothelial cells could be observed on pericardial surfaces treated with a combination of Citric Acid (10%) and plasma-titan method (n=5) (Figure 5).Toxicity of pericardium was reduced by combination of procedures using ALDH and plasma-titanium coating (n=12) to 17.3± 2.1% ($p \leq 0.01$).

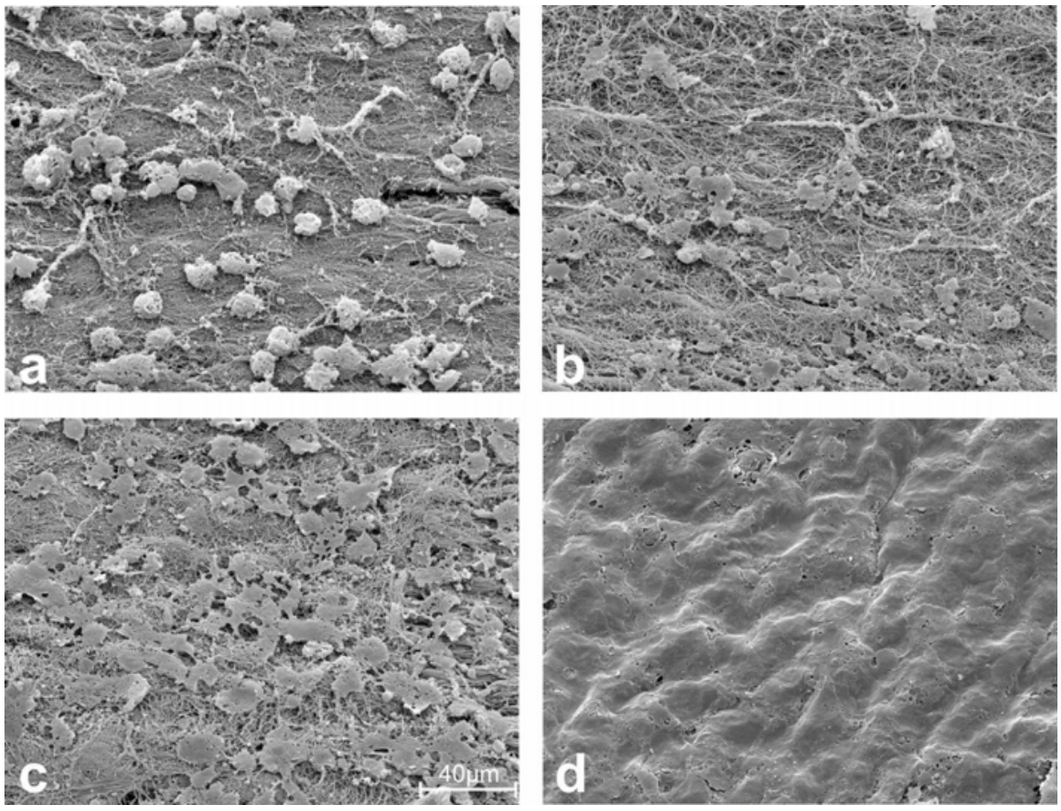


Fig. 5. Scanning electron microscopy of human endothelial cells on glutardialdehyde fixed bovine pericardium shows sporatic cell cadavers (a), treated with citric acid (10%) demonstrates malformed endothelial cells (b) detoxified with citric acid (10%) and ALDH results in non confluent endothelial layers (c) the nano-technological promoted procedure with a detoxification and a titan plasma administration enabled a confluent layer of endothelial cells (d)

Several platelets of pericardium differently detoxified were examined by scanning electron microscopy (SEM) after being seeded in vitro with human endothelial cells. Of each specimen, 10 visual fields were evaluated under 1000x magnification. Both cell morphology and confluence of the cell layer were assessed semi-quantitatively by different examiners.

4.2 Cell adhesion under flow conditions

A flow chamber made of V4A steel and glass was able to incorporate in its bottom six detoxified and endothelialized platelets within V4A steel rings.. It was constructed such, that no significant turbulences happened and that the laminar shear stress of 30 dyn/cm² and flow of 5 L/min was similar to the intra-aortic fluid dynamic.
Adhesion of human endothelial cells on glutaraldehyde fixed pericardial platelets treated by CA 10%, ALDH and a titanium coating was expressed by the vitality of the remaining cells after a flow of 5L/min for 24 hours within a flow chamber.
Vitality of the endothelial cells was measured by the extinction of the MTS-test of 72% (n=6) after a pre-seeding of 15 min and 81% (n=6) after pre-seeding of 60 min (Fig.6).

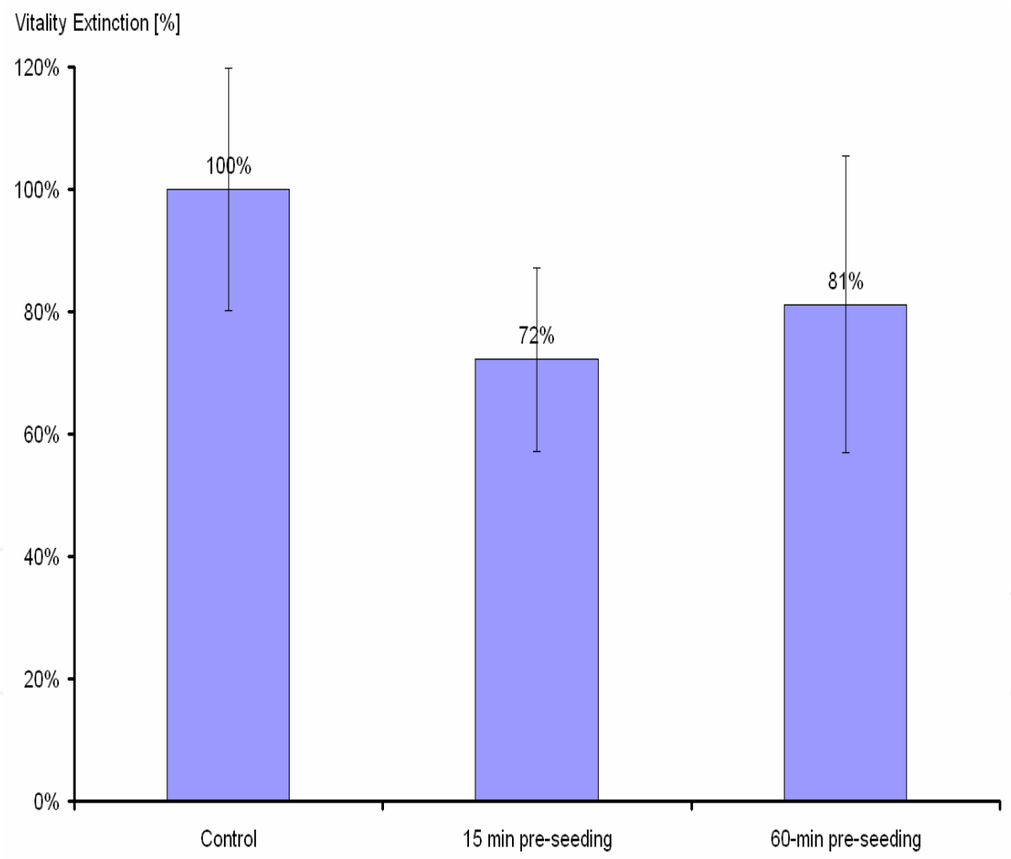


Fig. 6. Extinction of MTS-test for titanium treated (n=6) endothelialized glutaraldehyde-fixed bovine pericardium platelets after in a flow channel of 5L/min. Adhesion of human endothelial cells is 72% after a pre-seeding of 15 min and 81% after pre-seeding of 60 min

This study provides evidence that titanium coating in combination with a CA10% and ALDH treatment removed most of the free aldehyde ligands of glutardialdehyde fixed

pericardium in-vitro and enabled a living and confluent cell layer of human endothelial cells with a high adhesion capacity. This study gave evidence that titanium coating applied to a porcine heart valve may enable a self-seeding with cells within circulation.

4.3 The first self-endothelialized titanium coated glutaraldehyde-fixed heart halve prosthesis within systemic circulation

Implantation of a pre-seeded aortic valve in the aorta descendens position in a sheep model without extra-corporal circulation was already described before (Gulbins et.al., 2006). We used a male Bore goat with a weight of 83 kilograms (ethical accreditation of the Ethics Committees for Animals in Kiel, V 362-72241.122-6) to create a muscular blood pump in aorta descendens position (Fig.7a). We integrated two glutaraldehyde-fixed, titanium coated and detoxified porcine heart valves into the inflow and outflow part of the pumping chamber of this muscular blood pump, the Biomechanical Heart (Guldner et.al., 2001). This skeletal muscle ventricle pumped with a frequency of 20 beats per minute. After 6 months of pumping the valves were removed from the pumping chamber for histological analysis.
Valve leaflets from the two valves were imbedded in paraffin, cut into sections of 4 µm and treated with a hematoxilin-eosin staining . Other sections were treated using polyclonal antibodies against factor VIII (Dako, Hamburg, Germany). After incubation with primary antibody, an anti-rabbit IgG antibody was applied which was conjugated with alkaline phosphatase (SIGMA,Steinheim, Germany). Photographic documentation was performed by using a magnification of 20X.

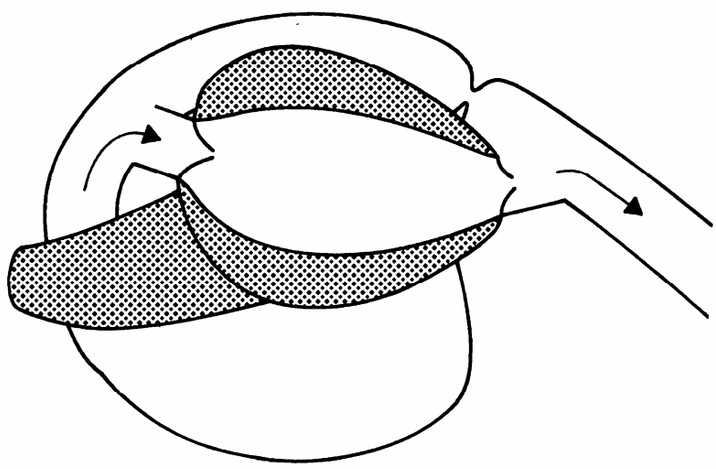


Fig. 7a. Valve's topography within the inflow and outflow part of the pumping chamber of a Biomechanical Heart, a muscular blood pump within aorto -aortic position

Porcine titanized and detoxified aortic valves, having been implanted heterotopic within the systemic circulation of a goat for 6 months, were macroscopically well and showed a normal opening and closure behaviour. Cross-section of a valve leaflet demonstrated a cell-seeding of its blood contacting surface (HE-staining magnification 20X). Immunhistochemic staining against factor VIII proved a complete endothelialization of the total blood contacting surface, labelled with alkaline phosphatase and visualized with immunefluorescense (7b, bottom).

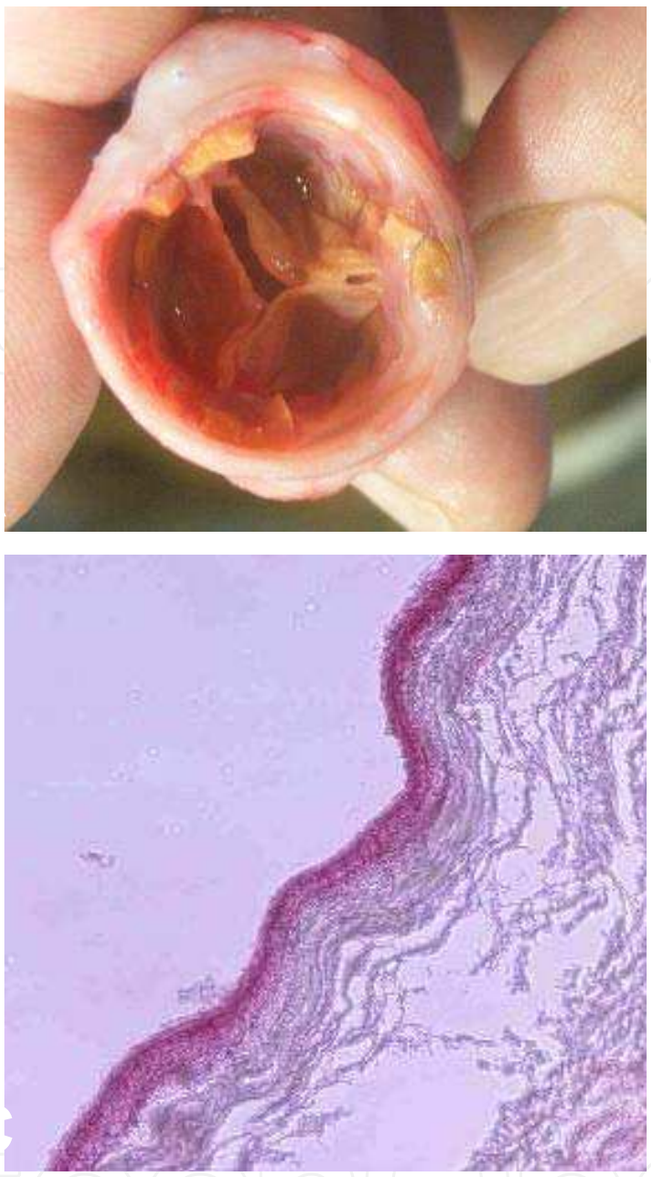


Fig. 7b. Porcine detoxified and titanized aortic valve, having been implanted heterotopic within the aorta of a goat for 6 months (top). Immunhistochemic staining against factor VIII (bottom) proves a complete endothelialization over the total blood contacting surface (bottom)

5. Conclusions

Tissue engineering of heart valves is considered to be a hopeful concept to generate improved substitute bioprostheses. However, because there is yet no realizable blueprint to construct semilunar valves (Sievers, 2007) our aim was to refine conventional, proven bioprostheses using chemical agents as citric acid and aldehyde dehydrogenase (ALDH) for detoxification and innovative nanotechnologies such as plasma deposition with the most biocompatible titanium coating. These procedures have shown to reduce the immune

response to the glutardialdehyde-based cross-linked collagen, probably the main reason why GA- fixed bioprostheses calcify and fail and enable the valve-protecting endothelialization in vitro under static and flow conditions..

Furthermore this new method combining biochemical methods of detoxification with nanotechnology including a titanium coating created the first self-seeded glutaraldehyde-fixed biologic heart valve within circulation under arterial pressure. This technology might open a new field of research for developing new heart valves with improved durability and function. In future big animal studies are necessary to approve this procedure mainly to increase durability and function in biologic glutaraldahyde fixed heart valves over several decades and avoid re-operations caused by valve degeneration

6. Acknowledgment

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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.

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