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Mapping the calcification of bovine pericardium in rat model by enhanced micro-computed tomography

Jing Liu [a, 1], Shengping Zhong [a, 1], Hualin Lan [b], Xu Meng [c], Haibo Zhang [c], Yubo Fan [d], Yuxing Wang [d], Chunren Wang [b], Zhaoxu Wang [b, *]

[a] College of Materials Science and Engineering, Chongqing University, Chongqing 400044, China
[b] Testing Department of Biomaterials and Tissue Engineering Products, Chinese National Institutes for Food and Drug Control, Beijing 100050, China
[c] Beijing Heart Lung Blood-Vessel Medical Center, Beijing Anzhen Hospital, Beijing University of Medical Science, Beijing 100029, China
[d] Key Laboratory for Biomechanics and Mechanobiology of Ministry of Education, School of Biological Science and Medical Engineering, Beihang University, Beijing, China

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ABSTRACT

The calcification initiation and progression of bioprosthetic heart valve were investigated in a rat model by enhanced micro-computed tomography, together with histologic study and scanning electron microscope analysis. The implantation data at early stage showed apparent dendritic patterns in the radiographic images for the glutaraldehyde-treated bovine pericardium and this dendritic pattern was verified to be associated with the vessel distribution in the tissue. Histologic study and scanning electron microscope analysis both indicated that the calcium deposits in the pericardium vessels regions were more grievous than those scattered in the collagen fibers in the first two weeks after implantation. Subsequently, calcification spreaded and the entire sample was severely calcified in 60 days.

1. Introduction

Calcification has been one of the most concerned issues for the glutaraldehyde (GA)-treated heart valve bioprostheses since it was first emerged in the 1960s [1–3]. Pathologic calcification has been considered as an important cause of the bioprosthetic valves' failure [4–8]. While the mechanism of the calcification is yet to be fully understood, it has been suggested that the major factors contributing to the mineralization including dead cells, lipids, free carboxyl and aldehyde groups in the porcine aortic cusps or bovine pericardium tissue [7]. For years researchers have made great efforts to develop various treatment processes to retard or minimize the calcification, and among them XenologiX®, ThermaFix®, and AOA® have demonstrated abilities in clinical applications to prevent predominant calcium deposits in the valve cusps [9–11]. The current anti-calcification techniques can be summarized into two categories. The first is to eliminate the cell remnants and lipids [12], and the second is to decrease the amount of the free aldehyde groups [10,11]. In addition some researchers suggested that blocking the free carboxyl groups in the tissue may also restrain the calcium deposition due to the ionic effect [13]. Many alternative cross-linkers has been proposed and evaluated over the decades, although few of them have actually reached clinical applications [14–16].

The methods to investigate the tissue calcification includes detecting the calcium content of the explant by atomic absorption spectrum or inductively coupled plasma (ICP) [7], and viewing the explanted tissue's histology section under light microscope. Stained histology section could pinpoint the calcified sites in a micro scale level while calcium content testing could provide overall information regarding the degree of calcification. Unfortunately neither method could provide information related to the distribution and the patterns of the calcification on a pericardium patch. The previous reported calcium content data only reflected the averaged calcium level in a pericardium patch, without considering the fact that the deposition of the calcium on the patch may well not be uniform. Sometimes stained histology section observation indicates the detailed calcified tissue in the specific sections, but the distribution pattern of calcium deposits in the whole sample is still unknown.

Conventional X-ray photography is often taken for the explanted calcified native valve or artificial tissue valve, to distinguish the heavy calcified region in the valve such as the cusp or the base of the valve [17–19]. Due to the low resolution and two-dimensional feature, no more information could be available.

* Corresponding author. Tel.: +86 010 67095500; fax: +86 010 67020395.
E-mail address: wangzx@nicpbp.org.cn (Z. Wang).
[1] These authors contributed equally to this work.

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In the endeavor to commercialize any anti-calcification technique, it is important to know the mechanism of calcification and the effect of anti-calcification treatment in details. However, the traditional methods could not meet the requirement well.

Micro-computed tomography (micro-CT), which has high resolution and often used for non-destructive analysis of small size sample, is a good tool to investigate the fine structure of samples in a micro scale such as the bone status [20,21]. Recently researchers tested the excised aortic valves by the micro-CT technique. They calculated the volume proportion of the calcified cusps to the total valve and tried to correlate the calcification volume proportion to the severity of aortic stenosis in order to provide an alternative testing method for serious aortic stenosis [22].

In this study, a high resolution micro-CT was employed to investigate the fine calcification distribution and pattern in the explanted bovine pericardium samples. An image of the calcification process in the subcutaneously implanted bovine pericardium tissue was provided. The initiation and propagation of calcium deposits inside the GA-treated tissue was explored, which provides a fresh insight of the calcification mechanism in the bioprosthetic heart valve.

The 3D morphologies of partly calcified and non-calcified samples after subcutaneous implantation are also displayed by a high resolution micro-CT.

2. Materials and methods

2.1. Materials

All the tissue samples were supplied by KinstronBio (Changshu) Co., Ltd. Bovine pericardial tissues were harvested under non-sterile conditions from a local slaughter house, stored in phosphate-buffered saline (PBS, 0.1 M, pH 7.4)) and immediately transported to the laboratory. The adherent fat was removed from the pericardial tissues carefully and then the tissues were thoroughly cleaned by Hanks balanced solutions. Tissue was cross-linked in the 0.4% (w/w) glutaraldehyde phosphate buffer solution, which was prepared from a 25% solution (electron microscopy grade; Sigma–Aldrich Corporation, St. Louis MO, USA).

The anti-calcification process involved cleaning and extraction of cell remnant by surfactant and followed by immobilization of long chain aliphatic acid into the tissue. Sub-optimal samples, which were treated with the same anti-calcification process but with shorter surfactant cleaning time, were also produced for comparison. All tissue samples were cut into pieces (1×1 cm^2) and chemically sterilized prior to subcutaneous implantation.

2.2. Animals and implantation

3-week-old male Wistar rats, each weighing 50–55 g, were used in this study. The rats were supplied by the Laboratory Animal Breeding and Supply of the Chinese National Institute for Food and Drug Control (CNIFDC). The animal study protocol was also reviewed and approved by the Experimental Animal Management Committee of the CNIFDC. The review ensured the compliance with the Regulations for the Administration of Affairs Concerning Experimental Animals published by the Chinese Government. Upon receipt and prior to use, the animals were held for environmental acclimation at the Division of Laboratory Animal Breeding and Supply of CNIFDC for 1 day in temperature- and humidity-controlled animal quarters with a 12 h light/dark cycle. Qualified food was offered daily and tap water was provided *ad libitum*.

The operations were performed under general anesthesia by intravenous injection of sodium pentobarbital (60 mg/kg.weight). The left and right dorsal regions of each rat were then shaved and disinfected prior to receiving implants. Each rat hosted two samples with one on each side. Seventy-two samples were implanted in thirty-six rats. The animals were sacrificed at 7, 14, 21 and 60 days. Nine rats were sacrificed at each time point. Bovine pericardium tissue samples were explanted for further testing. Six samples of each group were collected at every time point. Residual rat tissue was carefully removed from pericardium surface. All the six explanted samples were scanned by Micro-CT. Then each sample was cut into three pieces, one piece for histology study, one for SEM and the third one for calcium content analysis.

2.3. Micro-CT analysis

Bovine pericardium tissue samples were examined by the Micro-CT machine (SkyScan 1076; Skyscan, Aartselaar, Belgium) immediately after explantation. The sample was placed horizontally in the sample chamber with the serosal surface facing downwards. All scans were performed at the 40 kV voltages, 250 uA current, 0.5 mm aluminum filter, and a rotation step of 0.6°. The image was recorded with the pixel size of 9 μm. After scans, the results were reconstructed with the same parameters and viewed with the CTvox software.

2.4. Calcium content assay

The explanted tissue samples after Micro-CT testing was firstly dried at the 80 °C to constant weight, then digested in 70% nitric acid after weighed. The calcium analyses were performed by inductively coupled plasma optical emission machine (ICP-OES, Integra XL, GBC, Australia) in an independent laboratory.

2.5. Histological study

After fixation in 4% neutral buffered formaldehyde solution for 3 days, the implants were embedded in paraffin and sectioned parallel to the serosal layer. Slices of 5 mm thickness were obtained. The sections were processed with hematoxylin and eosin and van Kossa staining and observed under an optical microscope (Leica Microsystems, GMS GmbH, DM6000 B, Germany).

2.6. Scanning electron microscope (SEM) study

The explanted sample squares were unfolded in Tissue Freezing Medium (Leica Surgipath FSC22 frozen section compound), and quenched to −25 °C. The samples were then cryosectioned horizontally till the desire layer of the bovine pericardium tissue was revealed, just as the sampler preparation of the histological observation. After cleaned with saline, samples were stained by osmic acid, dehydrated in graded alcohol series and dried. The dried tissue samples were mounted on an aluminum stub and coated with gold, then observed by scanning electron microscope (Hitachi TM-1000 tabletop microscope).

3. Results

The Micro-CT images of the GA-treated bovine pericardium tissue samples after implantation for 7, 14, 21 and 60 days are shown in Fig. 1. In the radiographs, the bright area corresponds to the calcified tissue while the dark area corresponds to the tissue without apparent calcification. It could be found that longer the tissue implanted, brighter the radiograph images became. The progression of the calcification could be viewed directly from the images.

It's worthwhile to notice that the unique dendritic pattern of the calcified regions appeared in Fig. 1, especially for the samples at the early stage of the implantation. After 7 days' implantation, some bright lines started to emerge, they could be found scattered inside the sample (the Fig. 1(a)) although the majority area remained relatively dark. It should be noted that the lines from dendritic patterns at this time point are still relatively faint. The lines seemed to be oriented in certain patterns. The diameters of the lines were estimated as below 100 μm according to the images. The dendritic pattern became clearer with the extending implantation period, just as shown in Fig. 1b (14 days' implantation) and 1c (21 days' implantation). At this time point, the major and minor branches could be easily recognized by the distribution and shapes. However, after implantation for 60 days, as shown in Fig. 1d, both the branches and the areas between the branches turned brighter. The branches could not be identified as clearly as those showed in the early stages (the images of Fig. 1(a)–(c)). It seemed that calcium phosphates had already propagated to the entire sample tissue.

Some representative cross-section Micro-CT images of the explanted samples tested in Fig. 1 are showed in Fig. 2. The bright areas still correspond to primary calcified tissue. In the image of Fig. 2a, it appeared the calcification occurred predominant near the lower surface in the samples, which represented the serosal surface of the bovine pericardium. Those bright spots were sporadically connected as a bright line parallel to the serosal surface. It seemed that the calcification of the tissue in 7 days predominantly occurred in a layer near the serosal surface. This phenomenon was very popular for the explanted tissue samples after 7 days' implantation.

After 14 days' implantation, as the Micro-CT cross section image shown in Fig. 2b, those discontinuous bright line near the serosal surface became connected, and also became brighter. This could be viewed as the calcification in that tissue layer became intensified.