

Prevention of Porcine Aortic Wall Calcification by Acellularization: Necessity for a Non-Glutaraldehyde-Based Fixation Treatment

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Background and aim of the study: Acellularization prevents cell-mediated calcification of the aortic wall, but the inflammatory response towards the unfixed tissue is problematic. Two additional fixation methods, applied after tissue acellularization, were studied.

Methods: Porcine aortic wall samples were randomized into four groups: (1) Standard fixation with glutaraldehyde (GA); (2) acellularization by a combined method of enzymes (DNase, RNase) and a detergent (Triton X-100); (3) acellularization followed by standard GA fixation; (4) acellularization followed by photo-oxidation. Samples were implanted into the wall of both jugular veins of six juvenile sheep. Tissue was explanted after three months and evaluated by X-radiography, light and electron microscopy, and calcium content (cc) measurement (atomic absorption spectrometry). Auto-fluorescence of elastic fibers was used to identify the relationship between calcific deposits and elastin.

Results: GA-fixed aortic wall samples showed clear mineralization (cc 41.6 ± 17.8 $\mu\text{g}/\text{mg}$), occurring pre-

dominantly at the level of cell remnants, as confirmed by electron- and fluorescence microscopy, locating calcific deposits in between elastic fibers. Acellularized aortic wall fragments were calcified significantly less, but an important (non-infectious) inflammatory response caused elastolysis and subsequent calcification of the elastic fibers (cc 5.6 ± 2.8 $\mu\text{g}/\text{mg}$). Acellularized and GA-fixed fragments revealed important, inhomogeneously spread calcific deposits (cc 24.7 ± 10.0 $\mu\text{g}/\text{mg}$). Photo-oxidized samples remained free from calcification (cc 0.82 ± 1.6 $\mu\text{g}/\text{mg}$).

Conclusion: Acellularization is a promising tool in the prevention of porcine aortic wall calcification, but additional tissue fixation is necessary to prevent structural degeneration. GA fixation after acellularization causes important inhomogeneous tissue mineralization. Photo-oxidation combines optimal tissue fixation with superior anticalcification characteristics.

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Most stentless bioprosthetic valves consist of an intact or modified porcine aortic root, with a relatively important portion of the porcine aortic wall being incorporated into the valve. The basic concept of stentless valve design assigns the possible benefits in stentless valves to the presence of this pliable aortic wall portion. Resemblance to the native aortic root, extensibility of the aortic wall, and preservation of the aortic sinuses are factors which are believed to favor long-

term valve durability (1-3). However, despite current clinical follow up exceeding 10 years, no clear trend has yet emerged in favor of any superior durability for stentless valves (4,5). Important calcification of the aortic wall portion has been described in both clinical and experimental studies (6,7). The biomechanical relevance of the calcification and stiffening of the aortic wall remains unclear, but as most of the possible advantages of stentless valves depend upon wall extensibility, some adverse effects must be anticipated (8).

In a previous study, attention was focused on porcine aortic wall mineralization, and an important cell-mediated calcification process was revealed (9). In addition, a relatively simple but reliable and reproducible model of aortic wall calcification in juvenile sheep was presented. As the foci of early mineralization were localized mainly in dead cells and cell rem-

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nants, the effect of thorough acellularization of the aortic wall was studied. It was shown that although acellularization resulted in an effective anticalcification, an important inflammatory response towards the unfixed tissue caused severe tissue damage. Consequently, an additional tissue fixation seemed necessary in order to protect the acellularized and unfixed tissue from the destructive inflammatory reaction.

In the present study, two possible post-acellularization fixation treatments were compared: standard fixation with glutaraldehyde; and photo-oxidation. The implantation of tissue fragments was carried out in juvenile sheep, utilizing a simplified 'jugular vein' model.

Materials and methods

Animals

Juvenile sheep, aged 6-8 months and bred especially for this purpose, were selected. All animals were cared for by a veterinarian in accordance with the *Guide for the Care and Use of Laboratory Animals* as published by the National Institutes of Health. The study was approved by the Ethical Committee of the Katholieke Universiteit Leuven.

Materials

HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2,2-ethanesulfonic acid), glutaraldehyde (GA; 8%, electron microscopy grade solution), DNase (bovine pancreas), RNase type I-AS (bovine pancreas) and Triton X-100 were obtained from Sigma-Aldrich, Bornem, Belgium. Further products for animal use included ketamine, fentanyl and heparin.

GA-fixed aortic wall tissue (group I)

Fresh porcine aortic roots were collected from a local abattoir. Pieces of aortic wall (2 × 1 cm) were dissected out and thoroughly rinsed in saline. All tissue fragments were treated in the same way and subjected to the fixation procedure within 6 h of the heart being removed. The fixation process consisted of two phases. First, all samples were placed into a 0.6% GA solution in 50 mM HEPES buffer, pH 7.4, at room temperature for 24 h, with continuous shaking. Subsequently, cross-linking was continued in 0.2% GA in the same buffer for an additional seven days. The samples were then stored in 0.2% GA solution until used for implantation. All tissue samples were rinsed thoroughly in saline immediately before implantation into sheep.

Acellularized aortic wall tissue (group II)

Similar fresh porcine aortic wall samples were collected. After removal of the adventitial fat, cellular disruption was induced by rapid freezing and defrosting.

The acellularization solution, in which the tissue samples were kept for 48 h (with continuous shaking), consisted of 20 µg/ml RNase, 0.2 mg/ml DNase and 1% Triton X-100 in phosphate-buffered saline (PBS). The temperature was maintained at 37°C. Afterwards, all samples were extensively rinsed in PBS at 4°C. All tissue samples were rinsed thoroughly in saline immediately before implantation into sheep.

Acellularized and glutaraldehyde-fixed tissue (group III)

Acellularization was performed as described earlier. Following the acellularization process, the samples underwent standard GA-fixation as described above.

Photo-oxidized tissue (group IV)

The photo-oxidized aortic wall tissue fragments were obtained from Photofix[®] valves; these are valves which are stabilized by dye-mediated photo-oxidation following tissue acellularization (10,11).

Implantation into sheep

The sheep (n = 8) were premedicated with ketamine (10-20 mg/kg intramuscular). Anesthesia was induced and maintained with halothane and nitrous oxide. Fentanyl was administered in bolus doses as necessary to maintain anesthesia. Mechanical ventilation was instituted after endotracheal intubation. Penicillin (Albipen LA; 8 ml, intramuscular) was administered immediately prior to the operative procedure.

Jugular vein implants

After shaving, disinfecting and sterile-draping the animal, the jugular vein was surgically exposed. After full heparinization, the vessel was clamped and opened. In each animal, two aortic wall tissue fragments from each of the four groups were implanted into the jugular vein, using both the left and right sides; thus, a total of eight samples was implanted into each sheep (two samples from each group). The tissue fragments were each about 1-2 cm² in size, and implanted as small separate patches into the wall of the vein. Continuous sutures (Prolene 5-0) were used in all cases.

Explantation and tissue analysis

Tissues were explanted at 12 weeks after implantation. Heparin (250 IU/kg) was administered to each animal before being premedicated, anesthetized and sacrificed.

Gross examination and X-radiographic analysis

All explanted samples were grossly inspected and color photographs taken. A radiographic examination was performed in two dimensions under mammogra-

phy conditions to demonstrate possible major calcifications. Following X-radiography, all tissue fragments were divided into portions for histological analysis, electron microscopic examination and measurement of calcium content.

Histology

For histology, samples were fixed in a buffered 6% formaldehyde solution, dehydrated in an ascending series of alcohol, and finally embedded in paraffin. Sections (4 μm thickness) were cut and routinely stained with hematoxylin and eosin, together with Masson's trichrome staining for collagen, elastic Von Giesson staining, phosphotungstic acid-hematoxylin staining for fibrin, and Alcian-blue staining and Von Kossa staining for calcium.

Fluorescence microscopy

Elastic fibers may be visualized by their auto-fluorescence; this technique enabled the exact location of all elastic fibers to be identified on Von Kossa staining. This in turn enabled the relative involvement of elastic fibers and smooth muscle cells to the wall mineralization to be assessed.

Transmission electron microscopy

Guided by the results of the light microscopy, small samples were taken and embedded in Dow epoxy-resin. Semi-thin sections (1 μm thickness) were prepared and stained with toluidine blue. Areas of interest were marked on these sections, after which ultrathin sections were cut and stained with uranyl acetate and lead citrate. The sections were also treated with 2% potassium pyroantimonate to demonstrate the presence of calcium. The grids were examined in a Philips CM10 electron microscope, and the respective photomicrographs taken.

Quantitative calcium determination

Half of each explanted tissue sample was used to measure the calcium content. Tissue samples were lyophilized, pulverized and then heat-desiccated to constant weight. The dried tissue was dissolved in 20% HCl (10 mg dried tissue/ml), and the tissue calcium content then measured using flame atomic absorption spectrometry. Calcium content was expressed as μg per mg tissue dry weight.

Data management and statistical analysis

Normal-probability plots and the Shapiro-Wilks test for normality showed the calcium content data to be non-normally distributed. Consequently, data were expressed as median \pm interquartile range. The Kruskal-Wallis ANOVA test was used for comparison between different groups. When this test yielded a p-value <0.05 , a pairwise comparison between groups was performed using the Mann-Whitney *U*-test, with Bonferoni correction.

Results

All surgical and experimental procedures were uneventful. All animals survived the surgical procedure and remained in a good condition until the planned date of sacrifice (12 weeks post-implantation). There were no infection-associated problems in any of the animals.

Gross examination and X-radiography

On gross examination and palpation, samples from groups I and III (the GA-fixed samples) showed clear signs of calcification, as judged by the stiffness of the retrieved tissue. The samples from groups II and IV all appeared to be soft and pliable.

These findings were confirmed by X-radiographic analysis. Calcification was clearly visible in the GA-fixed aortic wall samples of groups I and III,

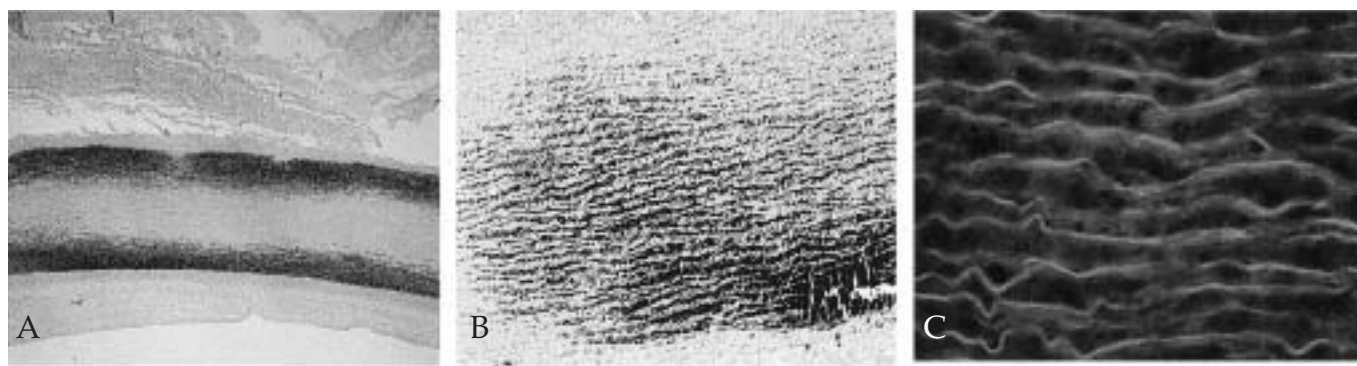


Figure 1: A) Von Kossa staining of an explanted glutaraldehyde-fixed aortic wall fragment showing clear medial calcification. B) Detail showing striped pattern of early calcification. C) Fluorescence microscopy on the Von Kossa staining of an area of early calcification, showing the black dots of calcium precisely in between the well-preserved elastic fibers.

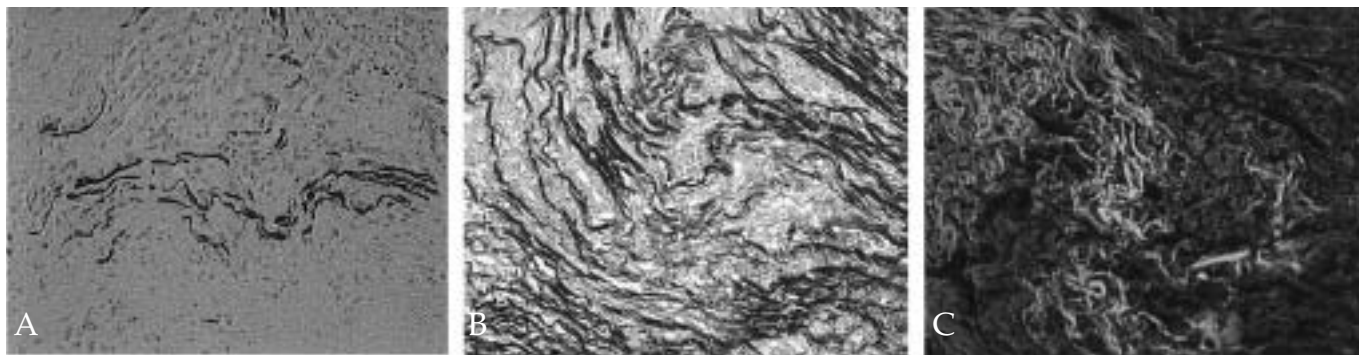


Figure 2: A) Von Kossa staining of an explanted acellular wall sample, showing calcific deposits along fragmented and disorganized elastic fibers. B) Elastica staining showing the important disorganization and destruction of the tissue. C) Fluorescence microscopy of the Von Kossa staining, again showing disorganized elastic fibers.

whereas no major calcification was evident in samples from groups II and IV.

Histology

GA-fixed samples (group I)

These samples showed clear calcification, mainly in the tunica media of the wall fragment (Fig. 1A). The pattern of medial calcification was very similar in all explanted wall samples. Calcium deposition was present in a striped pattern, parallel to the orientation of the elastic lamellae in the aortic wall (Fig. 1B). In areas of early calcification, the mineral deposits were located exclusively in between the elastic lamellae, as seen with fluorescence microscopy (Fig. 1C). It is most likely that smooth muscle cells and their remnants (membranes, cell organelles) formed the main foci of initial mineralization. There was little or no contribution of elastic fibers in areas of early wall calcification. In areas of progressively increasing calcification, the elastic fibers became affected by calcium deposition, they fragmented, and eventually disappeared completely into the calcific aggregate, in which no histological structures could be recognized. A non-infectious inflammatory response was present around the

implanted sample, but the inflammatory cells did not invade the GA-fixed tissue.

Acellularized samples (group II)

All acellularized aortic wall samples showed a completely different biological behavior compared to GA-fixed tissue. Overall, wall calcification was less, but not completely absent. The exact location and pattern of these calcium deposits differed substantially when compared to the GA-fixed aortic wall samples. Calcification was exclusively located at the level of elastic fibers, as seen with Von Kossa staining (Fig. 2A). The elastic fibers showed clear signs of fiber disorganization, fragmentation and lysis, as seen with elastica staining and fluorescence microscopy (Fig. 2B and C). An important, non-infectious inflammatory response was seen at the adventitial side of the implanted tissue, with invasion of lymphocytes and macrophages inside the implanted tissue sample. An invasion by young fibroblasts was also noted.

Acellularized and GA-fixed samples (group III)

The aortic wall samples that underwent acellularization followed by GA-fixation showed a stable and

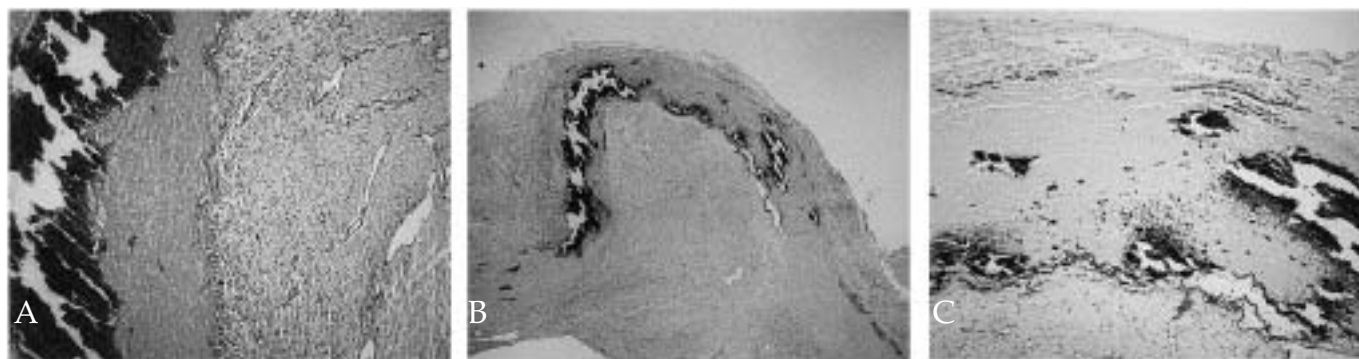


Figure 3: A) Hematoxylin and eosin staining of an explanted acellularized and glutaraldehyde-fixed sample, showing an important foreign-body reaction surrounding, but not invading, the tissue. Important inhomogeneous calcification is also visible. B) Von Kossa staining revealing large calcific deposits. C) Detail of the Von Kossa staining.

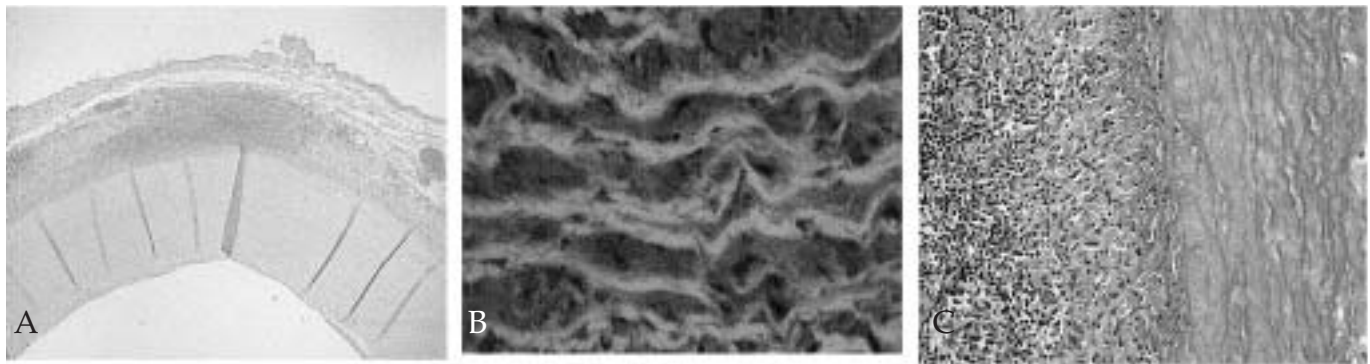


Figure 4: A) Von Kossa staining of an explanted photo-oxidized sample; no calcification is seen. B) Fluorescence microscopy revealing nicely preserved elastic fibers. C) Hematoxylin and eosin staining, showing an important foreign-body reaction surrounding, but not invading, the tissue.

well-preserved tissue texture. An important inflammatory foreign-body reaction, mainly guided by lymphocytes and macrophages, was seen to surround the implanted tissue, but not to invade it (Fig. 3A). However, calcification was clearly present in the form of large calcific aggregates throughout the tissue fragment (Fig. 3B and C). There was no clear histological correlation indicating the origin of these deposits.

Photo-oxidized samples (group IV)

The histological image of these samples completely resembled the result from previous studies in sheep, using Photofix valves (11). No calcification was seen (Fig. 4A), and the global tissue structure and fiber organization was well preserved (Fig. 4B and C). Again, a relatively important inflammatory reaction was noted, though only at the outer borders of the tissue. The inflammatory reaction did not invade the wall fragment at any time (Fig. 4C).

Electron microscopy

Calcification in the GA-fixed aortic wall samples (group I) appeared to commence predominantly in between the elastic fibers, at the level of the smooth muscular cell remnants, thereby confirming the fluorescence microscopy findings. The elastic fibers retained their structural integrity in areas of early mineralization, but as the mineralization proceeded the elastic fibers also became affected and calcified.

In the acellular tissue (group II), inflammatory cells invaded the implanted tissue and caused fragmentation and lysis of the elastic fibers. This elastolytic process appeared to precede the calcification of elastic fibers.

The acellularized and GA-fixed samples (group III) showed inhomogeneous calcification, without any clear pathological correlation. No calcification was seen in the photo-oxidation samples (group IV).

Calcium content

The GA-fixed samples (groups I and III) showed significantly elevated calcium contents (41.6 ± 17.8 and 24.7 ± 10.0 $\mu\text{g}/\text{mg}$, respectively; $p < 0.01$). The acellular wall samples were calcified significantly less than the GA-fixed tissue, but residual calcification of the elastic fibers still led to a slightly elevated calcium content (5.6 ± 3.8 $\mu\text{g}/\text{mg}$). Photo-oxidized samples contained the lowest calcium levels (0.82 ± 1.6 $\mu\text{g}/\text{mg}$).

Discussion

In valve replacement, stentless bioprosthetic valves offer many theoretical advantages such as lower transvalvular pressure gradients and possible enhanced durability, particularly when valves are inserted as a root (1). Many of these possible advantages depend, however, on the flexibility of the valve's aortic wall portion. It has been clearly shown, both clinically and in experimental models, that the porcine aortic wall is prone to calcification that would limit its extensibility (6-8). Previously, a simple and cost-effective sheep model was developed which produced a reliable and representative level of calcification in porcine aortic wall samples (9). Using this model, the effects of aortic wall acellularization, and of two post-acellularization fixation treatments, were studied.

Aortic wall calcification in GA-fixed tissue is mainly initiated at the level of the smooth muscular cells and their cellular remnants. The contribution of elastin is initially limited, but as the aortic wall mineralization progresses the elastic fibers become incorporated into the calcific deposit. Acellularization of the aortic wall tissue prevents this cell-mediated calcification, but a strong inflammatory response invading the tissue causes important tissue damage and disorganization, with ultimate elastolysis and subsequent calcification of elastic fibers. This inflammatory response appears to be a typical, strong foreign-body reaction towards an unfixed tissue fragment. A recent clinical report

appeared to support the present findings in acellular samples (12). In order to prevent this inflammation from invading and destroying the implant, adequate tissue fixation seems necessary. Hence, two different fixation treatments applied after acellularization were compared, namely standard GA fixation and photo-oxidation.

Based on the present results, standard GA fixation after acellularization appears not to be a good option to protect the aortic wall from calcification, although with additional treatment this process can be mitigated (13). GA did prevent the invasion of a destructive inflammatory response into the tissue, but unfortunately severe calcific deposits were noted. By contrast, photo-oxidation of the aortic wall tissue appeared not only to have an efficient anticalcification effect, but also to provide optimal tissue fixation.

In conclusion, acellularization shows promise in the prevention of wall mineralization, but an additional tissue treatment is necessary to protect the implanted tissue from damaging inflammation. In this respect, GA appears not to be an option, given the recurrence of important tissue mineralization. On the other hand, photo-oxidation appears to be a promising tissue treatment.

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Meeting discussion

DR. MICHAEL SACKS (Pittsburgh, Pennsylvania, USA): Did you perform any assays to check if there was any cellular debris, or how well the decellularization process actually worked?

DR. BART MEURIS (Leuven, Belgium): The control of our acellularization procedure was purely by histology, with normal histological staining and some electron microscopy. The acellularization of the aortic wall worked relatively well, but it took time to optimize the treatment because the tissue is relatively thick and it is difficult - and slow - to diffuse the solution into it. It is easier to acellularize a cusp than a piece of aortic wall.

DR. SACKS: I made that comment because it has been suggested, without any histological evidence, that there may be some lipid membrane fragments in the tissue. But some advanced analytical chemistry is needed to see if they are really there.

DR. MEURIS: Yes, I'm sure that's correct.

DR. CARLOS G. DURAN (Missoula, Montana, USA): Why did you choose to place the tissue in a jugular vein and not in an artery? The conditions are different.

DR. MEURIS: In a previous study we did that. We implanted the same samples into the jugular vein and the carotid artery, and there was no significant difference in the level of calcification. This was slightly surprising, but the histological pattern and calcium content values were similar for the aortic wall. Because the carotid artery is relatively small in sheep, it's technically more difficult to implant large samples there - that's why we used the jugular vein model.

DR. DURAN: What type of inflammation did you see?

It's obviously sterile, but what cells were involved?

DR. MEURIS: We checked for infection. It was a non-infectious inflammatory response - a typical foreign-body reaction guided by lymphocytes and macrophages, towards the implanted tissue.

DR. DURAN: Did you study the immunology at all - whether there was a humoral response to this?

DR. MEURIS: No, we did not perform any immunological staining on these samples.