

A New Storage Solution for Porcine Aortic Valves

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The aim of this study was to assess the calcification tendency of two biovalves manufactured by different fixation techniques and compare their biocompatibility when implanted subcutaneously in rats. Two biological valve types (Intact[®] and Mosaic[®]), stored in either glutaraldehyde or in a solution recently developed in our department, were investigated ultrastructurally and their calcium content was measured following 12 weeks subcutaneous implantation in rats. All valves tested in this study showed a considerable loss of the endothelial cover, as judged by scanning electron microscopy. Independent of fixation conditions, the bioprostheses demonstrated a partial destruction of collagen fibers and a rearrangement of the extracellular matrix. The calcium content of Intact[®] valves was significantly higher than that of Mosaic[®] valves (66 ± 2.6 versus 3.6 ± 0.6 mg/g dry tissue, $p < 0.0001$). Low calcium content of the bioprostheses is considered to result from effective anti-calcification treatment. Ultrastructural changes of prosthetic tissue seem to promote degenerative calcification. The valves stored in the new storage solution exhibited a calcium content which was reduced by approximately 50% compared to those stored in glutaraldehyde. The percentage of reduction in calcification of the valves stored in our newly developed solution is independent of the fixation conditions ($p = 0.886$). The advantage of the new storage solution is based on the fact that rinsing is unnecessary before implantation and, most importantly, a clear reduction in the calcification tendency is achieved. (Ann Thorac Cardiovasc Surg 2007; 13: 102–9)

Key words: new storage solution, biovalves

Introduction

Degenerative calcification of biological prostheses results in adverse effects on long term survival of biovalves.¹ In approximately half of all implanted biovalves, functional problems resulting in hemodynamic deterioration occur within the first 15 years after implantation.² Commonly used biological valves are usually stored in glutaraldehyde solution (GA) following fixation and further processing.³ Even low amounts of glutaraldehyde can produce toxic effects in man, so that biological valves must be washed thoroughly several times before implantation.⁴

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The fact that glutaraldehyde itself promotes calcification of the valves is also well known.⁵ Thus, we have developed a new solution (MM solution) for the storage of biological valves without glutaraldehyde. In this study, two separate experiments were carried out. The aim of this study was (i) to evaluate the biocompatibility of valves stored in this solution after heterotopic implantation and (ii) to compare valves stored in this solution with those conventionally stored in glutaraldehyde with respect to calcium content. The incorporated calcium content of various valve types was measured by atomic absorption spectroscopy following subcutaneous implantation in a rat model.

Materials and Methods

Materials

Two different porcine valve types (n=5 each) that had been subjected to a variety of fixation (with 3.7% glutaraldehyde solution) and anti-calcification methods were ob-

tained from the same manufacturer (Medtronic). Intact[®] valves treated with toluidine blue and Mosaic[®] valves treated with α -amino oleic acid (AOA) were both fixed under zero pressure as obtained from Medtronic Company. For comparison determination of preimplant ultrastructural changes of biological valves, a normal porcine, noncalcified valve and without any treatment was investigated. The finding of this native porcine aortic valve is demonstrated in Fig. 1A.

Methods

Development of a new storage solution (MM solution)

For the storage of porcine valves we have developed a new solution based on the composition of human serum with the following formula: 25 mM NaHCO₃, 11.1 mM glucose, 1.9 mM CaCl₂, 118.2 mM NaCl, 4.7 mM KCl, and 2.6 mM T608. The T 608 is a chelate formation, which is capable of binding to the free positively charged groups in lysines and hydroxylysines as well as the amino groups of collagen molecules, thus effectively hindering calcification. Of the five specimens from each valve, three were first washed three times under sterile conditions in 250 ml 0.9% NaCl solution and thereafter stored for 6 weeks in MM solution. In a set of pilot experiments the highest grade of saturation was observed after 6 weeks culture in MM-Solution. For comparison, two valves were kept in 3.7% glutaraldehyde solution. The following investigations were carried out on each of the five valve specimens.

Scanning electron microscopy

To study the ultrastructure of explanted biological valves, scanning electron microscopy was performed. Specimens from valve leaflets were fixed for 6 h in a solution containing 2.5% glutaraldehyde and 200 mM cacodylate. Samples were dehydrated in an ascending series of alcohol (70–95% alcohol) and dried using a critical point drier. All samples were finally sputtered with gold and palladium in a COOL-sputterer. For electron microscopical observation, the digital scanning microscope DSM 960, Oberkochen, Zeiss, Germany was used.

Transmission electron microscopy

Transmission electron microscopy was performed to reveal the ultrastructure of various biovalves. Specimens from valve leaflets were fixed for 2 h in a solution containing 2% glutaraldehyde and 0.1 M cacodylate buffer at pH 7.3. The samples were then washed in phosphate-

buffered saline and stained for 2 h in cacodylate containing 2% osmium tetroxide. The specimens were dehydrated in a graded series of alcohol and embedded in Araldite. Ultrathin sections were finally stained with 2% methanolic uranyl acetate. The specimens were then examined in a transmission electron microscope, equipped for photo documentation.

Animal model

An animal model was employed to study the extent of calcium loading for each biovalve. Valves rinsed three times in sterile NaCl solution were subcutaneously implanted in 3-week-old female Wistar rats. For surgical procedures, all animals were anaesthetized by intraperitoneal injection of Rampun[®] (15–20 mg/kg per body weight) and Ketanest[®] (75–100 mg/kg). According to the designs of the our study (as described in Experiments 1 and 2) either one incision (Experiment 1) or two incisions (Experiment 2) in the skin of the back parallel to the spinal column were made using aseptic techniques. A piece of 1 cm² taken from a leaflet of a each valve was placed in the subcutaneous pouches. The skin was subsequently sutured to cover the implantation sites. All animals survived the operation and were allowed to recover for 12 weeks. At the end of this period, the animals were sacrificed. Bioprosthetic material was harvested in order to measure the calcium load. All animal procedures were approved by the local Animal Care and Use Committee.

Experiment 1

The aim of the first experiment was to assess the calcification tendency of two types of biovalves (Mosaic[®] and Intact[®]) as a result of different fixation techniques by conservation in two different solutions (glutaraldehyde: GA and new storage solution: MM). Five samples from each type of the biovalves were used in our study. From each valve, one leaflet was left in glutaraldehyde for 6 weeks, while the other leaflet was stored in MM solution for the same time. The third leaflet was reserved for the second experiment. From each leaflet, two pieces were punched out and each piece was implanted in a rat, i.e. each rat received only one implant at a standard location, so as to eliminate the possibility of a reciprocal effect of the specimens or solutions upon each other. Regarding the effect of the solution, this experiment is a paired sample design since the two pre-treatments, GA or MM, are applied to the same valve (albeit to different leaflets). The five different valves from each type of manufacture ($n_1=5$ for Mosaic[®] and $n_2=5$ for Intact[®]) are independent

replications of the trial. The design of this trial is a so-called split-plot repeated measures design. The observations of the two rats for each manufacture type and solution are dependent replications which served to investigate whether the variance of the observations caused by the rats is larger than that caused by the valves. Since two rats were used for each leaflet of a valve, a total of $n=40$ rats was needed in this experiment.

Experiment 2

The third leaflet from each valve was reserved for the second experiment. The leaflets from the Mosaic[®] valves were stored in glutaraldehyde and from Intact[®] valves in MM solution. From each leaflet two implants were punched out and implanted in one of 10 rats. Each rat received two implants, one from a Mosaic[®] valve and one from an Intact[®] valve. In this experiment, a manufacture effect or an effect of the solution could not be investigated since both factors were confounded by the design. Instead it was the aim of this experiment to investigate potential cross-reactions between the different types of valves stored in different solution when implanted in the same rat. If there are no cross-reactions then the results of the Mosaic[®] valves stored in glutaraldehyde in experiment 2 must be comparable to the results of the same valves stored in glutaraldehyde in experiment 1 while the results of the Intact[®] valves stored in MM solution in experiment 2 must be comparable to the results of the same valves stored in MM solution in experiment 1.

Atomic absorption spectrophotometry

Atomic absorption spectrophotometry was performed to measure the calcium content of the subcutaneously implanted valve material. Specimens stored for 4 days in a deep freezer (-70°C) were lyophilized in a vacuum at a cooling temperature of -20°C . The lyophilized samples were then weighed and hydrolyzed in 70% nitric acid to dissolve the tissue. At the end of the reaction, nitric acid was added to the tissue solution to give a final concentration of 5% in a volume of 100 ml. For controls, reagents without bioprosthetic material were prepared. The calcium content of each specimen was determined by means of atomic absorption spectroscopy according to established procedures.

Statistics

The analysis of the data was performed by the statistical software SAS[®] (Version 8.2). The statistical model underlying Experiment 1 is a split-plot repeated measures

design with two crossed fixed factors: manufacture type (Mosaic[®] and Intact[®]) and solution (GA and MM). Valves (nested under the factor manufacture type) and rats (nested under the valves) were considered as random factors. The variances of the data in the different treatment groups turned out to be quite different and, moreover, they were much larger for the Intact[®] valves than for the Mosaic[®] valves for both the solutions GA and MM. To reduce this heteroscedasticity, the data were log-transformed which means that a multiplicative model for the calcification of the valves was considered. Significance of the results was examined by the appropriate F-statistics for main effects and interactions in the split-plot repeated measures design and p -values smaller than 0.05 were considered as significant. For the analysis of experiment 2, two-sided 95%-confidence intervals for the treatment effects of the log-transformed data in the two trial groups were computed and compared with those of the corresponding groups from experiment 1 by transforming the results back to the original scale of the calcium measurements.

Results

Ultrastructure of biovalves stored in a glutaraldehyde solution

In order to study the surface morphology of the two bioprosthetic valves, a scanning electron microscopy was performed. The surface ultrastructure of a normal porcine, noncalcified valve is demonstrated in Fig. 1A. In these samples, an intact endothelial layer covered the surface of the leaflet, and endothelial lesions were not seen. In contrast, in Intact[®] valves surface lesions were the predominant ultrastructural feature. Occasionally, collagen fibers in the extracellular matrix exhibited an irregular shape and were partially destroyed (Fig. 1B). In Mosaic[®] valves, typical endothelial cells were not detectable. Bundles of collagen were the predominant morphological finding at the surface of the valves (Fig. 1C).

Results of transmission electron microscopy

In order to study the ultrastructure of clinically used bioprostheses, a transmission electron microscopy was performed. The structural morphology was poorly preserved, as evidenced by disrupted cell membranes, cellular debris and vacuolation and large spaces in the extracellular matrix. Our results showed that none of the biovalves examined exhibited an intact endothelial cover. Intact[®] valves demonstrated a thin basement membrane showing considerable degenerative destruction of extra-

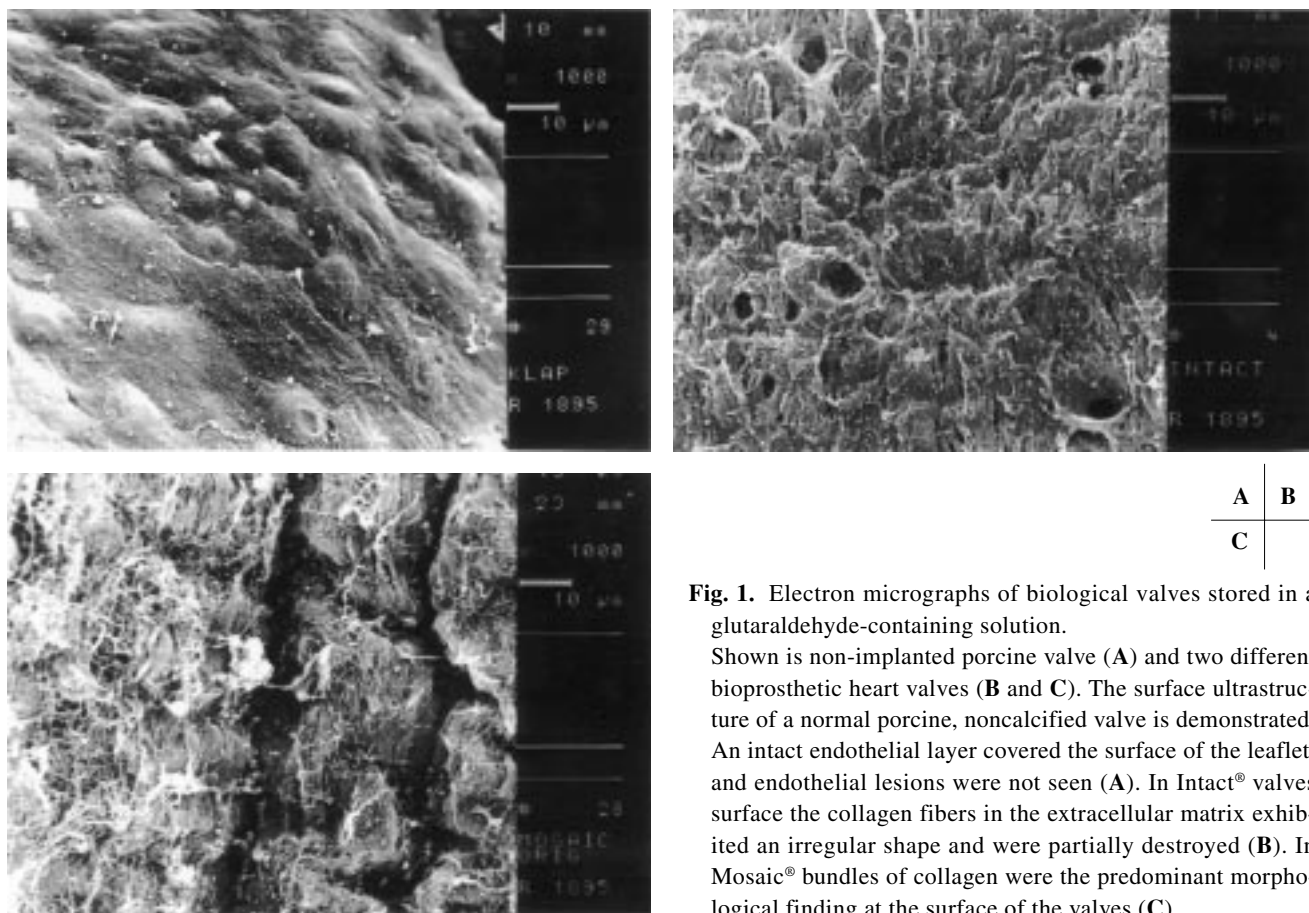


Fig. 1. Electron micrographs of biological valves stored in a glutaraldehyde-containing solution.

Shown is non-implanted porcine valve (A) and two different bioprosthetic heart valves (B and C). The surface ultrastructure of a normal porcine, noncalcified valve is demonstrated. An intact endothelial layer covered the surface of the leaflet, and endothelial lesions were not seen (A). In Intact® valves surface the collagen fibers in the extracellular matrix exhibited an irregular shape and were partially destroyed (B). In Mosaic® bundles of collagen were the predominant morphological finding at the surface of the valves (C).

cellular matrix components (Fig. 2A). Mosaic® valves showed an almost complete loss of endothelial cells, leaving parts of the basement membrane and a few collagen fibers exposed (Fig. 2B).

Ultrastructure of biovalves stored in our new storage solution

In Intact® valves, surface lesions were the predominant ultrastructural feature. Occasionally, collagen fibers in the extracellular matrix exhibited an irregular shape and were partially destroyed. No differences in the ultrastructural features were detected in Intact® valves stored either in glutaraldehyde or the new storage solution. In Mosaic® valves stored in MM solution, endothelial cells were hardly detectable. Bundles of collagen were the predominant ultrastructural finding at the surface of the valves.

Calcium contents of biovalves in experiment 1

The calcium content of the subcutaneously implanted valves varied considerably among the different types of

biological prostheses. The mean calcium content of the various valves stored in glutaraldehyde was 66 ± 2.6 mg/g for the Intact® valves and 3.6 ± 0.6 mg/g for the Mosaic® valves, while the calcium content of the valves stored in the MM solution was 34 ± 3.8 mg/g for the Intact® valves and 1.8 ± 1 mg/g for the Mosaic® valves (Fig. 3). Note that these are the means and standard deviations of the original calcium values not of the log-transformed observations. These original data are only given to describe the outcome of the trial. To assess the significance of the observed results, the log-transformed observations were used for the analysis. Means and standard deviations of the original observations as well as of the log-transformed observations are listed in Table 1 while the results of the repeated measures ANOVA are given in Table 2.

The results in Table 2 show that the calcium content in the Intact® valves is significantly higher than in the Mosaic® valves ($p < 0.0001$) and that the calcium content obtained by storing the valves in solution MM is significantly lower than in solution GA ($p = 0.0007$).

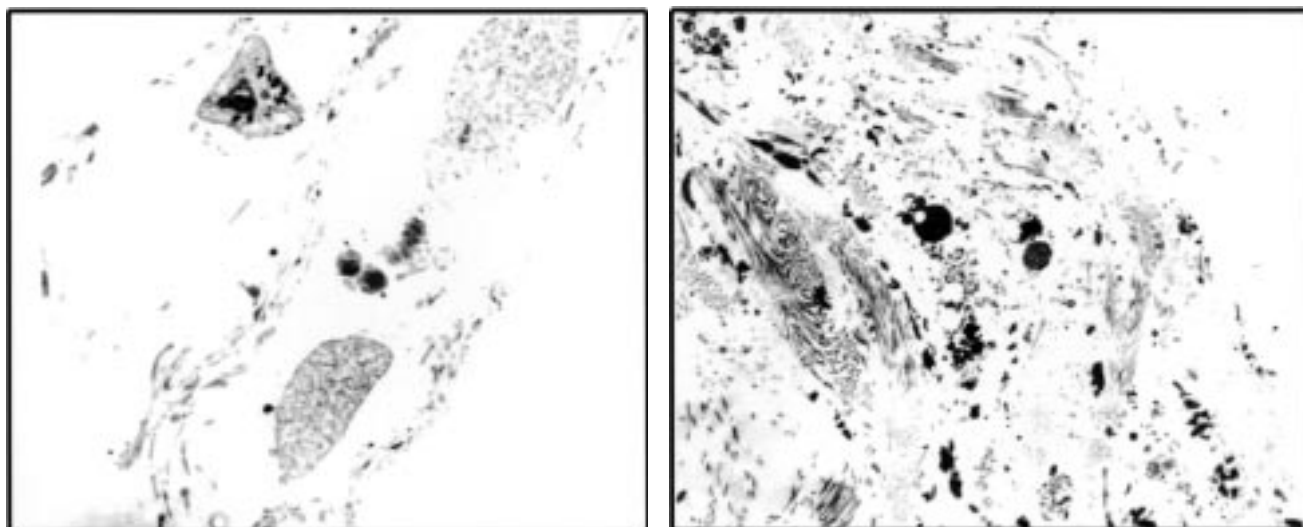


Fig. 2. Transmission electron micrographs of biovalves.

Intact® (A) and Mosaic® (B) lacked a normal endothelial cover. Large spaces in extracellular matrix and cellular debris and vacuolation were visible.

A | B

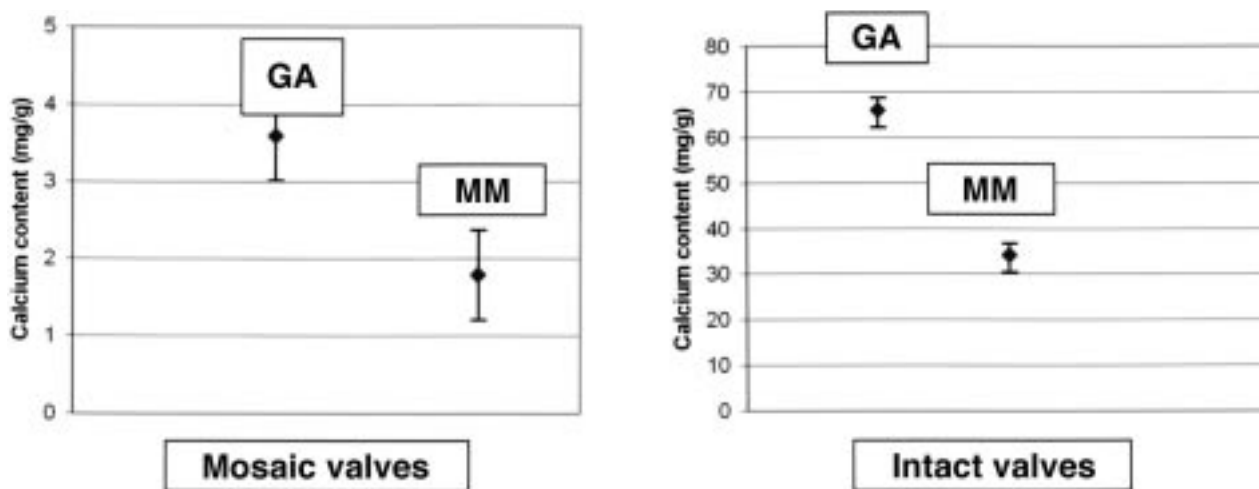


Fig. 3. Observed calcium valves in experiment 1 [means±SD (mg/g)].

Table 1. Calcium content in the different valves in experiment 1

Type of valve	Solution	Calcium (mg/g)	log-Calcium (mg/g)
Mosaic®	GA	3.6±0.6	1.3±0.2
	MMS	1.8±1.0	0.6±0.6
Intact®	GA	66±2.6	4.2±0.04
	MMS	34±3.8	3.5±0.1

Table 2. F-statistics and p-values for experiment 1

Effect	p-value
Manufacture type	<0.0001
Solution	0.0007
Interaction	0.887
Valve	0.87

These effects can be considered as homogeneous for both types of valves since no interaction can be shown ($p=0.87$). Furthermore, no significant random effect of the valves could be shown ($p=0.87$) which means that

the valves are more homogeneous than the rats, i.e. the variation in the observations caused by the different valves is smaller than that caused by the different rats.

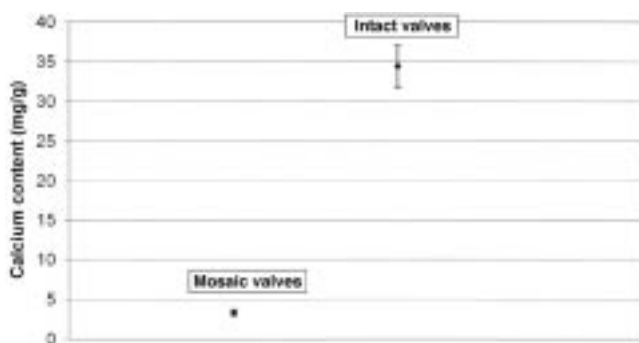


Fig. 4. Observed calcium valves in experiment 2 [means \pm SD (mg/g)]. The mean calcium content of Intact[®] valves and Mosaic[®] valves stored in glutaraldehyde and MM solution (Fig. 1).

Calcium contents of biovalves in experiment 2

In the second experiment, the calcium content of the subcutaneously implanted valves was similar to that in the first experiment. The mean of the calcium content in the Mosaic[®] valves stored in GA solution is 3.3 ± 0.4 with a 95%-confidence interval of (2.9–3.8) and the mean of the calcium content in the Intact[®] valves stored in MM solution is 34.5 ± 2 with a 95%-confidence interval of (31.8–37.3) (Fig. 4). The means and confidence intervals are listed in Table 3.

The confidence intervals for both types of valves are quite similar in experiments 1 and 2 and evidently, there seems to be no cross-reactions between the different types of valves stored in different solutions when implanted in the same rat.

Discussion

In this study we have investigated the biological suitability of a newly developed storage solution for porcine valves. We first carried out an ultrastructural analysis of frequently implanted biological prostheses stored in this solution. Scanning electron microscopy revealed significant changes with respect to the integrity of the extracellular matrix. The data from this investigation showed that the endothelial layers in all the valves investigated in this study were desquamated from the basement membrane, exposing subendothelial collagen fibers as the predominant surface structure. Furthermore, most of the biovalves investigated showed destruction of extracellular matrix. Thus, post mortem ischemia and the handling of the valve, for example, the sewing in of a stent, obviously play just as important a role as the fixation conditions. The biological valves stored in our storage solution showed simi-

Table 3. Calcium contents of the different valves in experiment 2 compared with those in experiment 1

Mosaic [®] valves/GA	Calcium (mg/g)	95%-confidence
Interval		
Experiment 1	3.6	3.1–4.2
Experiment 2	3.3	2.8–3.8
Intact [®] valves/MMS		
Experiment 1	34.1	30.3–38.4
Experiment 2	34.5	31.8–37.3

lar alterations in their ultrastructure. Ferrans et al. reported on a similar observation for post mortem ischemia.⁶⁾ In a study on 55 explanted degenerated bovine pericardial bioprostheses, Nistal et al. found not one with an intact endothelial cover. Using transmission electron microscopy, these authors demonstrated the presence of electron-dense microparticles located in the extracellular space and degeneration of collagen fibers.⁷⁾ Camilleri et al. emphasized the crucial role of the endothelium in pathological processes leading to calcification and degeneration of the biovalves.⁸⁾

Lehner et al. found a direct relationship between spontaneous reendothelialization, biocompatibility and the calcification of biological valves.⁹⁾

In addition to the loss of endothelium, we also found pronounced changes in the collagen fibers in the various biovalves tested. Broom and Thomson showed that high pressure fixation leads to a progressive loss of collagen fibers.¹⁰⁾ The spiral shape of collagen fibers was even partially destroyed by fixation under low pressure. Only fixation under zero pressure guaranteed the preservation of collagen structures. Independent of the fixation methods, collagen fibers exposed to high mechanical stress in paracommissural sites and along the basal line of the leaflets were extensively destroyed.¹¹⁾ Alterations in the three-dimensional structure of collagen lead to rigidity of the tissue, which causes tears in the fibers under mechanical strain.^{12–14)} The importance of tissue preservation is underlined by the fact that calcification of the biological valves usually begins at the sites of disrupted collagen fibers.¹³⁾

The agent most frequently used to cross-link collagen bundles is glutaraldehyde, which causes calcification of the biological valves, so that a variety of alternative agents

have been tested, including formaldehyde, glycerol, epoxy compounds and ethylene glycol diglyceryl ether.¹⁵⁾ However, none of these have been developed for routine use. We have revealed that the degenerative calcification of bioprostheses can be effectively inhibited by either storage in glutaraldehyde-free fixatives or application of anti-calcification treatments. The utility of 2- α amino oleic acid treatment was demonstrated by the low calcium content of Mosaic[®] valves. The high calcium content of Intact[®] valves underlines the ineffectiveness of the toluidine blue treatment to reduce calcification which was also observed in clinical studies.¹⁶⁾

The subcutaneous rat model has been established as a guide for the estimation of the calcification of biovalves under investigation. An extensive search through a large amount of literature does not, however, provide any hint of a standardised implantation method. In addition, whether multiple implants in rats affect the results remains unclear.

The calcification of implanted bioprostheses is determined by (i) host-related factors, (ii) implantation-related factors and (iii) mechanical stress-related factors. The subcutaneous rat model can not reflect in vivo changes in humans. The calcification of bioprosthesis is a particularly serious problem in young pediatric patients, thus the use of young growing Wistar rats in this study as the recipients of such valves is quite appropriate. In the first experiment of the study, we first examined the calcification tendency of two biovalves produced by different methods implanted in different rats, so as to eliminate the possibility of a reciprocal effect of the specimens upon each other. An about 18-fold higher calcium value was found for the Intact[®] valves stored in a glutaraldehyde solution in comparison to the Mosaic[®] prostheses ($p < 0.0001$). In comparison, the calcium content of the valves stored in our specially developed solution was about 50% lower than in the comparable glutaraldehyde-stored group. T608 is a disodium salt of ethylenediaminetetraacetate. Via two pairs of free electrons of the nitrogen and four carboxyl groups, it leads to complexation. Negatively charged carboxyl-groups can be found at the ends of alpha chains of collagen fibers. They are able to bind calcium as well as positively charged lysine endings. T608 induces formation of chelate complexes via collagen, leaving free binding point of calcium. Independent of the fixation and anticalcification techniques used, the active component in our solution, T608 effectively hindering calcification. This effect is caused by the chemical properties of T608 and is not specific to the rat. Since neither the compo-

nents nor the solution itself proved to be toxic for rats, the valves do not need to be rinsed before implantation. Thus, a time-saving of several minutes of myocardial ischemia during heart surgery can be achieved.

In summary, the results of this study underline the importance of tissue preservation of biological valves for the protection against calcification. The newly developed storage solution for biological valves has the ability of rendering rinsing before implantation unnecessary as well as reducing the susceptibility of the valves to accumulate calcium. Clinical studies with valves stored in this solution are now necessary to confirm the results of this study in routine surgical care.

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