Glutaraldehyde-treated Bovine Pericardium: Effects of Lyophilization on Cytotoxicity and Residual Aldehydes

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Abstract: This work assesses the effect of lyophilization on the cytotoxicity and residual aldehyde concentration of glutaraldehyde-treated bovine pericardium (group A), comparing it to conventional glutaraldehyde-treated bovine pericardium (group B). Cytotoxicity was measured by incubating a pericardium sample from each group in saline and assessing the eluant’s influence on cellular growth. Residual aldehydes were measured by HPLC. Although both groups’ eluants exhibited some cytotoxicity, the eluant from group A was less cytotoxic, with a cytotoxicity index (IC50(%)) of 41%. Group B eluants all had marked cytotoxic effects; cell growth was 24.15% of the negative control at the most dilute eluant concentration (6.25%). The mean residual glutaraldehyde level was less in group A than in group B (2.36 ± 0.11 and 9.90 ± 3.70 g/l, respectively; n = 3, P < 0.05), but residual formaldehyde levels did not differ. These results demonstrate that compared with conventional glutaraldehyde-treated bovine pericardium, lyophilized pericardium is less cytotoxic, with fewer glutaraldehyde residues. Key Words: Biocompatibility—Cytotoxicity—Bioprosthesis—Bovine pericardium—Glutaraldehyde.

Glutaraldehyde-treated bovine pericardium (GABP) has been used extensively to construct heart valve bioprostheses, repair patches, and shape conduits. Bovine pericardium (BP) is usually chemically treated with glutaraldehyde (GA) to improve mechanical and immunogenic properties, reduce thrombogenicity, control degradation, and allow for a longer period of storage. Following treatment, the material is usually stored in 4% formaldehyde (FA) solution and thoroughly washed in saline just before implantation. Despite this wash, aldehyde residues remain, rendering the material less biocompatible, and prone to calcification. Aldehyde residues and consequent cytotoxicity are known to affect the durability and biocompatibility of devices built with GABP (1–5). This work assesses the effect of lyophilization of GABP on the aldehyde content and cytotoxicity of the reconstituted material.

MATERIALS AND METHODS

Sample groups
Bovine pericardium patches were treated with GA at 0.56% (8) and stored in 4% FA for no less than 3 months. Two sample sets, comprising three patches each, were used. Patches in group A were thoroughly washed in saline, lyophilized, and reconstituted in saline. Patches in group B were thoroughly washed in saline.

Cytotoxicity
Cytotoxicity was measured by rinsing the sample to be tested in a saline solution and assessing the impact of the eluant on cellular growth, as described by Nakamura et al. (6) and following ISO 10993 standards (7). Sample area-to-eluant ratios of 3.0 cm²/ml and 0.5 cm²/ml in 60 ml of total volume were used. A solution of 0.05% phenol was used as a positive control, while high-density polyethylene
(HDPE) pellets were used as a negative control. Samples from both groups, as well as the positive and negative control substances, were sterilized with gamma rays.

Two hundred Chinese hamster ovary cells (CHO-K1; ATCC, Manassas, VA, U.S.A.) were plated on 60 mm Petri plates. Patches from groups A and B and the negative control were immersed in culture medium RPMI 1640 (GIBCO-BRL, Invitrogen Corporation, Carlsbad, CA, U.S.A.) with 10% fetal calf serum (FCS) plus 1% penicillin-streptomycin, and incubated stationary at 37°C for 48 hr. Eluants were diluted to concentrations of 100%, 50%, 25%, 12.5%, and 6.25% and then transferred to the culture plates. The positive control (0.05% phenol solution) was diluted to the same concentrations. All eluant concentrations were tested in triplicate.

The plates were incubated for 8 days in a humidified 5% CO₂ atmosphere at 37°C, and the resultant cell colonies were counted. The count for the negative control was regarded as 100%. The cytotoxicity index IC₅₀(%) was defined as the eluant concentration that inhibited colony growth to 50% of that in the negative control.

Residual aldehydes
Residual aldehydes in samples from both groups were measured by high performance liquid chromatography (HPLC) with an SCL-10A-VP Chromatograph (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.). Assays were performed over 10 min in a reverse phase C18 column (Pharmacia, Freiburg, Germany) with an H₂O buffer plus acetonitrile gradient between 65% and 80% (T, 25°C; absorption wavelength, 254 nm). Standard curves also were obtained.

To remove excess aldehydes, all pericardium samples were initially washed three times in 50 ml of saline under agitation for 5 min at room temperature. Three samples from group A were subjected to lyophilization and then immersed in saline for 1 week. Three samples from group B were immersed in saline for 1 week. To measure residual aldehyde levels, eluant from each of these six samples was directed through the column with 2,4-dinitrophenylhydrazine (5,9,10).

Statistical analysis
Data are expressed as the mean ± standard deviation. ANOVA was used to compare results of the cytotoxicity assay, and Student’s t test was used to compare residual aldehyde levels between groups. Differences were considered to be significant when P < 0.05.

RESULTS

Cytotoxicity
At a sample area to eluant ratio of 3.0 cm²/ml, all concentrations of the eluants (100%, 50%, 25%, 12.5%, and 6.25%) from each group inhibited colony growth. However, eluant-induced inhibition of cell growth differed between groups at a sample area to eluant ratio of 0.5 cm²/ml (P < 0.05).

The IC₅₀(%) of eluant from group A samples was calculated as 41%. However, no dilution of eluant from group B samples examined allowed colony growth to attain 50% that of the negative control. Figure 1 shows values of colony growth associated with the different eluant concentrations.

Residual glutaraldehyde and formaldehyde levels
Residual GA levels were lower in group A than in group B (2.36 ± 0.11 g/l and 9.90 ± 3.70 g/l, respectively; n = 3, P = 0.024). No difference in FA residue levels was found between groups A and B (0.25 ± 0.01 mg/l and 0.26 ± 0.02 mg/l, respectively; n = 3, P = 0.677).

DISCUSSION
Biological tissues have been widely used to make prosthetic replacements for heart valves and blood vessels as well as to manufacture patches to repair different anatomical structures. They are connective tissue rich, the main component being collagen.

![Graph displaying the cytotoxicity of various dilutions of eluants from lyophilized glutaraldehyde-treated bovine pericardium (group A), conventional glutaraldehyde-treated bovine pericardium (group B), 0.05% phenol (positive control), and high-density polyethylene pellets (negative control). The sample area to eluant ratio was 0.5 cm²/ml, and cytotoxicity is represented as inhibition of colony growth. The arrowhead indicates the value of the cytotoxicity index IC₅₀(%) = 41; *P < 0.05 versus group B.](image)
Among these tissues, BP is one of the most widely employed. For this purpose, BP is chemically treated to improve its mechanical performance and immunogenic properties, reduce thrombogenicity and degradation, preserve sterility, and prolong the allowable storage period.

Ionescu et al. (8) introduced a procedure for preserving and shaping BP that involves fixing the tissue with a 0.5% GA solution. In this circumstance, the molecular structure is permanently changed, with the creation of cross-links between aldehydes and amino groups, thereby increasing tissue stability (11). Valves are customarily stored in buffered 4.0% FA solution, which is typically replaced every 2 years. Products treated in this manner must be thoroughly washed before use to remove the aldehyde residues. Franks (12) has shown that lyophilization may reduce certain volatile or water-soluble components through vacuum sublimation. Thus, our finding of lower residual GA levels in eluant from pericardium subjected to lyophilization after fixation with GA may be explained by GA sublimation during vacuum treatment. The fact that FA residue levels did not similarly decrease with lyophilization suggests that most FA was eliminated during the prewash.

GA also has been implicated in the process of calcification (2,3,13,14), which is frequently responsible for biological valve dysfunction. This has motivated a search for alternative treatments for biological tissue that do not use aldehydes (11,15,16). As residual GA levels were lower in group A, it is likely that lyophilization renders treated pericardium less prone to calcium deposition.

Cytotoxicity testing is a rapid, standardized, and sensitive means of determining whether a material contains significant quantities of biologically harmful extractables, providing evidence of material bio-compatibility (4). The cytotoxicity of residual GA has been extensively studied, with prior reports that residual GA concentrations are capable of causing cell death in graft materials (3). In our study, eluant from both lyophilized and nonlyophilized tissue inhibited cell growth at a sample area to eluant ratio of 3.0 cm²/ml. However, cytotoxicity occurred in a concentration-dependent manner when a sample area to eluant ratio of 0.5 cm²/ml was tested. The patches subjected to lyophilization showed significantly less cytotoxicity; thus, we may assume that lyophilization decreases the cytotoxic potential of these materials. This result is consistent with our finding of lower residual GA levels in the patches from group A.

CONCLUSIONS

We have previously shown (17) that lyophilization does not alter the mechanical properties of GABP. Thus, this process may be useful in improving the characteristics of implantable devices made of GABP, possibly leading to lower calcification rates.

REFERENCES