GENOTOXICITY AND CYTOTOXICITY OF BOVINE PERICARDIUM TREATED WITH GLUTARALDEHYDE AND PRESERVED IN FORMALDEHYDE

A. C. D. Rodas[1], O. Z. Higa[1], M. J.S. Maizato[2], A. A. Leirner[2], R. N.M. Pitombo[3]

[1]Instituto de Pesquisas Energéticas e Nucleares (IPEN), Centro de Biotecnologia Av. Professor Lineu Prestes, 2242, Cidade Universitária, CEP 05508-900, São Paulo, Brasil. Email: arodas@ipen.br

[2] Divisão de Bioengenharia do Instituto do Coração (InCor) HC-FMUSP, São Paulo, Brasil

[3]Faculdade de Ciências Farmacêuticas (FCF-USP), Departamento de Tecnologia Bioquímico-Farmacêutica, São Paulo, Brasil

ABSTRACT

Bovine pericardium is one of the most used biomaterial to manufacture living tissue implants as heart valves. To improve its shelf live the bovine pericardium requires glutaraldehyde fixation and preservants as formaldehyde. The genotoxicity and cytotoxicity tests were used to measure the genome damage and the potential metabolic effect, respectively, by molecules released by a material. The studies were developed according to the guidelines of ISO 10993-5 and OECD-487. The natural biocompatibity of the bovine pericardium could be maintained diminishing the use of preservants which presented genotoxic and/or cytotoxic effects. For this reason, this study was performed to verify the genotoxicity and cytotoxicity effects of the components released from bovine pericardium preserved in formaldehyde. The cytotoxicity test was performed by a colorimetric method for determining the number of viable cells in proliferation. The same extract submitted to the micronucleus assay test showed to be genotoxic.

Key words: bovine pericardium, glutaraldehyde, formaldehyde, cytotoxicity, genotoxicity.

INTRODUCTION

Biological tissues have been widely used to make prosthetic replacement 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. Among these tissues, bovine pericardium is one of the most widely employed. Collagenous tissues obtained from the abattoir, cadaver or patient begins to degradate immediately. Therefore, in the exploitation of tissue as clinical material this deterioration must be arrested and deferred, preferably beyond the recipient's natural life. The aim is to prolong the material's original structural and mechanical integrity and remove or at least neutralize the antigenic properties attributed to these materials. Methods typically concentrate on creating new additional chemicals bonds between the collagen molecules. These supplementary links reinforce the tissue to give a tough and strong but non-viable material that maintains the original shape of the tissue [(1)].

For this purpose, bovine pericardium is chemically treated to improve its mechanical performance and immunogenic properties, reduce thrombogenicity and degradation, preserve sterility, and prolong the allowable storage period [(1, 2, 3)].
Ionescu et al. introduced a procedure for preserving and shaping bovine pericardium that involves fixing the tissue with a 0.5% glutaraldehyde 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 [(1, 2)]. Valves are customarily stored in buffered 4.0% formaldehyde solution, which is typically replaced every 2 years. Products treated in this manner must be thoroughly washed before use to remove the aldehyde residues [(1, 3)].

A drawback of chemical agents is the potential toxic effects a recipient may be exposed to form residues and/or chemicals resulting from a reversal of the cross-links. Unlike chemical cross-linking, these methods do not introduce toxic chemicals into the tissue, but this does not preclude undefined side-effects that may arise due to these processes [(1)].

Glutaraldehyde is the most successful chemical agent and is the only commercially viable process that has received widespread acceptance. Glutaraldehyde was first applied successfully for bioprotheses in the late 1960s by Carpentier et al [(4)]. Since then many variations and conditions have been applied to

optimize its efficiency and, consequently, entrenching xenografts as the tissue of choice. Commercial bioprosthetic heart valves fabricated from porcine aortic valves are usually treated with a low concentration glutaraldehyde solution (typically around 0.5%) for more than 24 hours to ensure optimum fixation. This release glutaraldehyde into the recipient. However, the toxic effects may be minimal or at a level that is tolerable [5, 6].

Formaldehyde, a common preservative for biological tissue, was also considered alongside glutaraldehyde as a candidate fixative. The long-term durability was found to be inferior to glutaraldehyde. However, the use of formaldehyde as a sterilant remains a primary post-treatment step for glutaraldehyde-fixed bioprostheses, with through rinsing being necessary prior to implant to remove residual formaldehyde [2, 5, 6]. For this reason, the present study was performed to verify the genotoxicity and cytotoxicity effects of the components released from bovine pericardium treated with glutaraldehyde and preserved in formaldehyde.

MATERIALS AND METHODS

2.1 Cell culture

For this study it was used Chinese hamster ovary cell line (CHO-k1). They were maintained in RPMI medium supplemented with antibiotics and antimicotic (100 units/mL penicillin, 100 µg/mL streptomycin and 0.025 µg/mL amphotericin), 2mM glutamine, and 10%calf serum, at 37^{o} C in a humidified 5% CO_2 atmosphere until they reached confluence. For subculturing and for experiments, cells were harvested using 0.05% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.4.

2.2 Sample preparation

Bovine Pericardium was treated with 0.56% glutaraldehyde solution for two weeks and stored in 4% formaldehyde solution. The bovine pericardium was cut, packed and sterilized by gamma radiation (25kGy). After sterilization, it was washed in saline solution 0.9% for 3 cycles in intervals of 2 days. At the last wash, all residual saline solution was genteelly removed. Then, the sample was imersed in RPMI culture medium and leaved in an incubator at 37^{o}C for 72 hours to fulfill the extraction condition. The same extract was used to cytotoxicity and genotoxicity tests.

2.3 Cytotoxicity test

The procedure is a colorimetric method which uses a tetrazolium compound MTS for determining the number of viable cells in proliferation [8] .The microplates of 96 wells were prepared with 50µL in quadruplicate of extracts diluted from 6.25 to 100% in RPMI medium. A suspension of CHO-k1 with 6 x 10^4 cell/ml was prepared and 50 µl/well was pipetted in the microplates and incubated for 72 hours at 37^{o}C in a humidified 5% CO_2 atmosphere. Blank and control of the cells were also prepared. The cell viability was measured by adding 20µL of MTS/PMS (20:1) solution and incubated for 2 hours at 37^{o}C in the humidified 5% CO_2 incubator. The microplates were read in a spectrophotometer reader at 495 nm. The test was compared with a negative control of HDPE and a positive control of phenol 0.3% in saline 0.9% solution. The Cytotoxicity Index for 50% of cell viability (CI_{50}) was graphically determined.

2.4 Genotoxicity test

The procedure followed was the *in vitro* micronucleus test as recommended by ISO 10993-3 [9] and OECD-487 [10]. The test was performed in 6 well plates with and without the S9 metabolic activation system. CHO-k1 cells were seeded at a concentration of 2 x 10^4 cell/well and incubated for 24 hours at 37^{o}C in a humidified 5% CO_2 atmosphere. The culture medium were removed from the plates and the cells were treated with the controls and with the bovine pericardium extracts in the concentrations of 5, 1.6 and 0.55% for third wash, defined after the cytotoxicity test.

Assay with S9 metabolic activation

The S9 metabolic activation mixture was added to the plates, which included cyclophosphamide (CPA) and benzopyrene (BZP), as positive control. The cultures were taken to the incubator for a 4 h exposure period. After the treatment period, the culture medium was removed by aspiration and the cells were rinsed with phosphate buffer solution pH 7.4 and replenished with complete medium containing cytochalasin B 3µg / ml. The cultures were then returned to the incubator for additional 20 h of incubation. After this period, the cultures were processed as sub-culture procedure as described below to prepare slides for analysis.

Assay without S9 metabolic activation

Plates containing the pericardium extract in 3 different concentrations and mitomicin C (MMC) and colchicine as control were incubated for 4 h with the cells. After that, cytochalasin B solution was added to the wells at final concentration of 3µg / mL. The cultures were then returned to the incubator for a 20h exposure period. At the end of incubation, the cultures were processed as subculture procedure as described below to prepare slides for analysis.

Slide preparation and analysis

The cell suspension was first washed with 5 mL of saline solution 0.9% (w/v) and after 5 minutes the cells were fixed by addition of 5 mL of methanol/acetic acid (3:1 v/v) for 5 minutes. The cells were treated with the fixative solution for more 3 times and finally resuspended by drawing and expelling with a Pasteur pipette, dropped onto wet clean glass slides on termostatized bath at 65^{o}C for 3 minutes and leaved air-dried. The slides were stained for 15 minutes with Giemsa 5%, rinsed with distilled water, coded and analyzed at optical microscope with 400X of magnification. The mononucleate, binucleate and multinucleate cells were counted by determining the cell proliferation index (CPI) as the followed equation:

$$CPI = \frac{\text{No. mononucleate cells} + 2 \times \text{No. binucleate cells} + 3 \times \text{No. multinucleate cells}}{\text{Total number of cells}}$$

For statistical analyses was used the binomial proportion.

RESULTS AND DISCUSSION

Figure 1 presents the cytotoxicity results. The cytotoxicity test was performed by a colorimetric method for determining the number of viable cells in proliferation. The test is composed with MTS, a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) and the PMS (phenazine methosulphate), an electron coupling reagent. MTS is bioreducted by cells into a formazan product that is soluble in tissue culture medium. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly

proportional to the number of living cells in culture. The graphic representation of the cytotoxicity test is used for the determination of the Cytotoxic Index 50% (CI_{50}) [8].

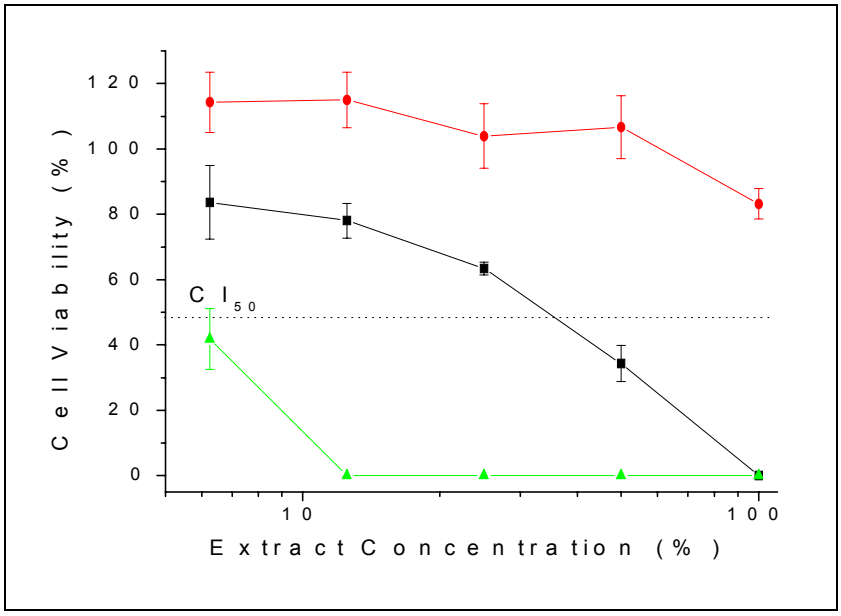

Figure 1. Graphic representation of the cytotoxicity test: Negative control - PEHD(●); Positive control – phenol 0.3% solution(■); bovine pericardium extract after three cycles of cleansing (▲).

Bovine pericardium treated with glutaraldehyde and preserved in formaldehyde presents increase in mechanical properties for manufacture cardiac valves [2]. However, these components present high cytotoxicity level, as demonstrated in Figure 1. The bovine pericardium was extensively washed, but it was not enough to arrive at a satisfactory cytotoxicity level. For this reason, the same extract was used to perform the genotoxic test, which results are presented in Table I and Figure 2.

Table I. Results of the cell proliferation index (CPI ± δ) in the genotoxicity test.

agent	Without S9	With S9
Control (culture medium)	1.914 ± 0.014	1.979 ± 0.013
Extract 5% concentration	1.257 ± 0.012	1.590 ± 0.004
Extract 1.6% concentration	1.936 ± 0.014	2.016 ± 0.020
Extract 0.55% concentration	1.910 ± 0.014	1.963 ± 0.022
Colchicine	1.594 ± 0.007	Non applicable
Mitomicin C (MMC)	1.718 ± 0.014	Non applicable
Cyclophosphamide (CPA)	Non applicable	1.965 ± 0.000
Benzopyrene (BZP)	Non applicable	1.919 ± 0.020

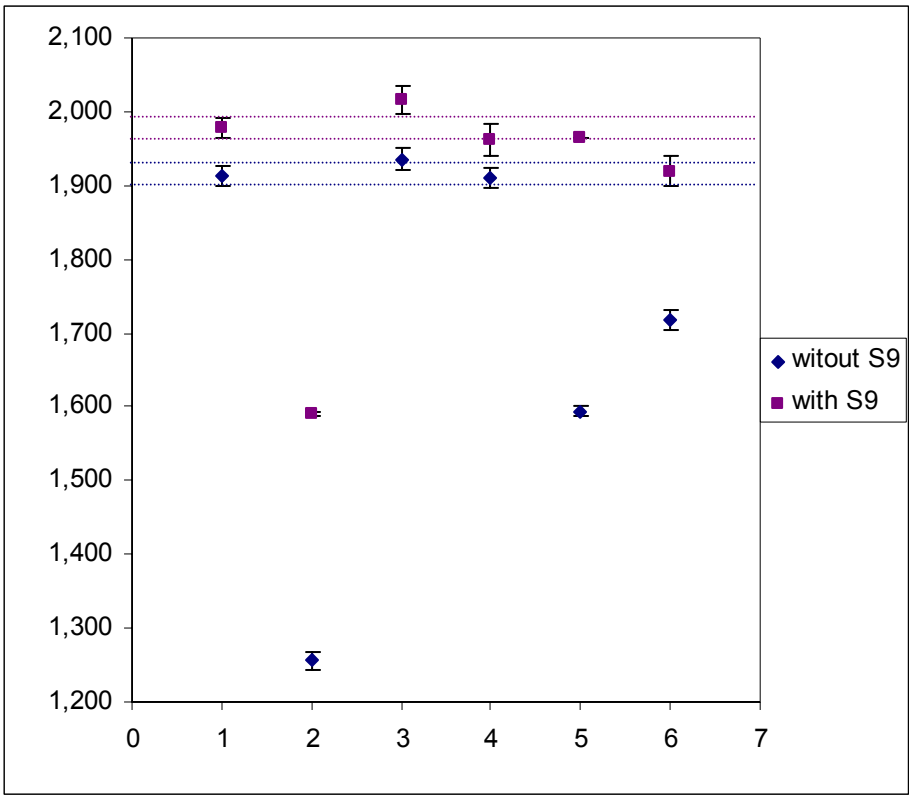

Figure 2. Graphic representation of the genotoxicity test results with the cell proliferation index (CPI ± δ). 1 – culture medium (control); 2 – wash extract 5% concentration; 3 – wash extract 1,6% concentration; 4- wash extract 0.55% concentration; 5 – genotoxic controls (◆) - colchicine; (■) – cyclophosphamide; 6 – genotoxic controls (◆) - mitomicin C; (■) – benzopyrene.

Genotoxicity test should be recommended for biomaterials to verify their potential effect to carry out DNA damage of living cells [9]. For this purpose we choose the *in vitro* micronucleus assay test systems used for detection of chemicals that induce the formation of small membrane-bound DNA fragments such as micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate with the rest of the chromosomes during the anaphase of cell division. The assay thus has the potential to detect the activity of both clastogenic and aneugenic chemicals. Development of the cytokines-block methodology, by adding of the actin polymerization inhibitor cytochalasin B during the targeted mitosis, allows the identification of nuclei that have undergone one division as binucleate. This allows the study of mechanisms of micronucleus induction by combination of the cytokinesis-block method [10].

Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. The most commonly metabolic activation system used is a co-

factor-supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254. Cell cultures are exposed to the test substances both with and without an exogenous source of metabolic activation. After exposure to the test substance, cell cultures are grown for a period sufficient to allow chromosome damage to lead to the formation of micronuclei in interphase cells. Harvested and stained interphase cells are then analyzed microscopically for the presence of micronuclei. Micronuclei should only be scored in those cells that complete nuclear division following exposure to the test chemical. It is important that cell proliferation is demonstrated in both control and treated cells, together with an assessment of cytotoxicity in the treated cells. For the test with S9 we choose cyclophosphamide (CPA) and benzopyrene (BZP) as damage reference; and without S9 we choose colchicine and mitomicin C (MMC) as damage reference [11].

The genotoxic test performed with the extract demonstrate that the residues of the bovine pericardium treated with glutaraldehyde and preserved in formaldehyde disturb the proliferation of the cells as viewed in the Cell Proliferation Index (CPI), but diminishing its concentration, the genotoxicity of the material diminish to secure levels. It suggests that this kind of materials should be extensively washed before it use. It is important to design experiments for *in vivo* applications.

CONCLUSIONS

The materials development to be used as biomaterials requires special care between its manufacture and the final use. The study of the cytotoxicity and genotoxicity are useful to perform changes or add new steps in the procedures. For the bovine pericardium, it was demonstrated that its use could be safer standardizing its cleansing procedure or introducing new procedures to diminish the formaldehyde and the glutaraldehyde released by the material, as for example, the lyophilization of the bovine pericardium treated.

ACKNOWLEDGEMENT

The authors gratefully acknowledge for the financial support of FAPESP by projects number 04/09566-8 and 05/54500-8.

REFERENCES

1. Khor, E. Methods for treatment of collagenous tissues for bioprostheses. ***Biomaterilas***, v.18, p. 95-105, 1997.

2. Maizato, M. J. S., ***Avaliação do pericárdio bovino liofilizado***. 2003, 144p. Tese de Doutorado, UNICAMP, Campinas.

3. Braile, D. M., ***Prótese valvular de pericárdio bovino: desenvolvimento e aplicação clínica em posição mitral***. 1990, 129p.Tese de Doutorado, EPM/SP, São Paulo.

4. Carpentier, A.; Lemaigre, G.; Robert, L.; Carpentier, S.; Dubost, C. Biological factors affecting long-term results of vascular heterografts. ***J Thorac Cardiovasc Surg***, v. 58, p. 467-483, 1969.

5. Maizato, M.J.S.; Higa, O.Z.; Mathor, M.B.; Camillo, M.A. P., Spencer, P.J.; Pitombo, R.N.; Zavaglia, C.A.C.; Leirner, A.A. Glutaraldehyde-treated bovine pericardium: effects of lyophilization on cytotoxicity and residual aldehydes. ***Artificial Organs***, v. 27, n. 8, p. 692-694, 2003.

6. Gendler E, Gendler S, Nimni ME. Toxic reactions evoked by glutaraldehyde-fixed pericardium and cardiac valve tissue bioprosthesis. ***J Biomed Mater Res.,*** v. 18, n. 7, p.727-736, 1984.

7. Eybl E, Griesmacher A, Grimm M, Wolner E. Toxic effects of aldehydes released from fixed pericardium on bovine aortic endothelial cells. ***J Biomed Mater Res.,*** v. 23, n. 11, p. 1355-1365, 1989.

8. Cory, A. H., Owen, T.C., Barltrop, J. A., Cory J.G., “Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture”. ***Cancer Comm.,*** v. 3, p. 207-212, 1991.

9. International Organization For Standardization, ISO 10993-3; Biological testing of medical and dental materials devices – part 3 Tests for genotoxicity and reproductive toxicity. Switzerland. .1992.

10. Organization for Economic Co-operation and Development (2004). OECD Guideline for Testing of Chemicals. No. 487: *In vitro* Micronucleus Test. Available: www.oecd.org/, access March, 29th 2006.

11. Garriottt, M. L.; Phelps, J. B.; Hoffman, W. P., “A protocol for in vitro micronucleus test I. Contributions to development of a protocol suitable for regulatory submission from an examination of 16 chemicals with different mechanisms of action and different levels of activity”, ***Mutation Research,*** v. 517, p. 123-134, 2002.