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LYOPHILIZATION OF TREATED BOVINE PERICARDIUM AND CYTOTOXIC EVALUATION

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Abstract. *In this work is presented the cytotoxic evaluation of bovine pericardium membrane treated with glutaraldehyde and preserved in formaldehyde, that was divided in two groups part was submitted to lyophilization and part was not. The toxic effect of the membranes on cells was evaluated by cytotoxicity test, a quantitative method of colony suppression using Chinese Hamster Ovary (CHO) cells in contact with diluted extract of the membranes. The results showed that both treated pericardium lyophilized or not are cytotoxic. However the cytotoxicity presented by the lyophilized membranes is strongly lower, showing that the lyophilization process contributes for the improvement of the biocompatibility of treated bovine pericardium membranes.*

Keywords: *Lyophilization, Biomaterial, Cytotoxicity, Bovine Pericardium.*

1. INTRODUCTION

The bovine pericardium have been used as a biomaterial for many years, in the manufacture of artificial heart valve or vascular grafts. After the conventional treatment with glutaraldehyde, the bovine pericardium products were preserved in formaldehyde to maintain the sterility (*Ionescu, 1977*).

Freeze drying is widely used in food industry, biological and pharmaceutical products to obtain dried product. Lyophilization process consists of separation of liquid water from a wet solid product or from a solution or dispersion of given concentration in the form of solid phase, ice, and its subsequent removal by sublimation, leaving the solutes or substrates in their anhydrous, or almost anhydrous states (*Franks, 1998*).

Lyophilization of products such as biological tissue can improve the sterilization process, storage, transportation and manipulation, and decrease antigenic activity (*Occhiogrosso, 1987*).

The purpose of this work is to see the improvement in the biocompatibility of the bovine pericardium membranes due to the lyophilization process.

2. AIM

Evaluation of the toxicity on cultured cells by a cytotoxicity test, a quantitative method of colony suppression essay using Chinese Hamster Ovary (CHO) cells in contact with diluted extract of treated pericardium membranes with was submitted to the lyophilizatin process comparing to the ones not lyophilized.

3. MATERIALS AND METHODS

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In the present work, fresh bovine pericardium membranes collected in the slaughter houses following standardized methodology for valve prostheses (Ionescu, 1977), had been submitted to treatment with 0.56% glutaraldehyde solution (GA) during 10 days. They were washed with physiologic solution and transfered to 4% formaldehyde solution (FA) to maintain sterility. Part of these membranes had been washed and reconstituted in physiologic solution, freezing in liquid nitrogen and lyophilized by 24 hours using a tray type freeze dryer.

The cytotoxicity assay was carried out in extracts of the materials according to Nakamura *et al* (*Nakamura, 1989*) and ISO 10993 - part 5. The amount of the tested material was in a ratio of 0.5 cm^2/ml of the extraction vehicle. The materials used in the cytotoxicity assay were: (1) bovine pericardium membrane treated with glutaraldehyde and preserved in formaldehyde (BP) and (2) bovine pericardium membrane treated with glutaraldehyde and preserved in formaldehyde that was reconstituted in physiologic solution and lyophilized (BPL). The two samples had been sterilized by 25 kGy gamma-irradiation.

Before the addition of the extraction vehicle, the membranes of bovine pericardium had been reconstituted with physiologic solution, using mechanical stirring. Saline solution was changed 5 times (500 ml of physiologic solution was used for each sample). To each one thus reconstituted samples, 60 ml of RPMI-FCS (RMI 1640 medium supplement with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin solution) was added. These samples were incubated stationary at 37°C for 48 hours. After incubation the supernatants were filtered and the extracts solution were gradually diluted with RPMI-FCS getting extracts with concentrations of 50, 25 12.5 and 6.25% in volume.

Chinese Hamster Ovary cell (CHO) were obtained from American Type Culture Collection bank. CHO cells were grown in RPMI-FCS in a plastic tissue culture flask, at 37°C in a humidified 5% CO_2 air incubator. After a confluent monolayer propagation, the culture medium was removed and the cell were washed with calcium and magnesium free phosphate saline buffer (PBS-CMF). For detachment of the cells from culture tissue flask the cells were treated with 0.25% trypsin solution. After trypsinization the cell were transferred to a screw capped plastic centrifuge tube and washed twice with PBS-CMF. The cells were re-suspended in RPMI-FCS and adjusted to give 100 cells/ml. Two milliliters of this cell suspension was seeded to each 60 mm diameter assay culture dish and incubated for 4 hours at 37°C for adhesion of the cells. The culture medium was removed and replaced with 5 ml of fresh RPMI-FCS in the control plates, and by undiluted (100%) and successively diluted extracts (50, 25, 12.5 and 6.25 %) in culture dishes with the adhered cells. All concentrations were tested in triplicate. The culture dishes were incubated for 8 days in a humidified 5% CO_2 atmosphere at 37°C for the formation of cell colonies. After that the culture medium was removed from dishes and colonies were fixed and colored with 2% rhodamine B in 4% formaldehyde. The number of visible colonies on each dish was counted using Digital Colonies Counter and compared with number of colonies in CHO control dish.

The cytotoxic potential could be quantitatively expressed by $IC_{50\%}$ which is the concentration of the extract necessary to kill half of the cell population or the extract concentration that suppressed colony formation to 50% of the control value.

The 0.05% phenol solution and high density polyethylene were used as positive and negative control, respectively.

4. RESULTS

The semi-logarithmic graphic (Fig.1) shows the results of cytotoxic tendency of the evaluated materials. The cytotoxicity index ($IC_{50\%}$) was also determined in the graphic, with expresses the cytotoxic potencial giving the concentration of the extract necessary to kill half

of the cell population or the extract concentration with suppresses colony formation to fifty percent of the control value.

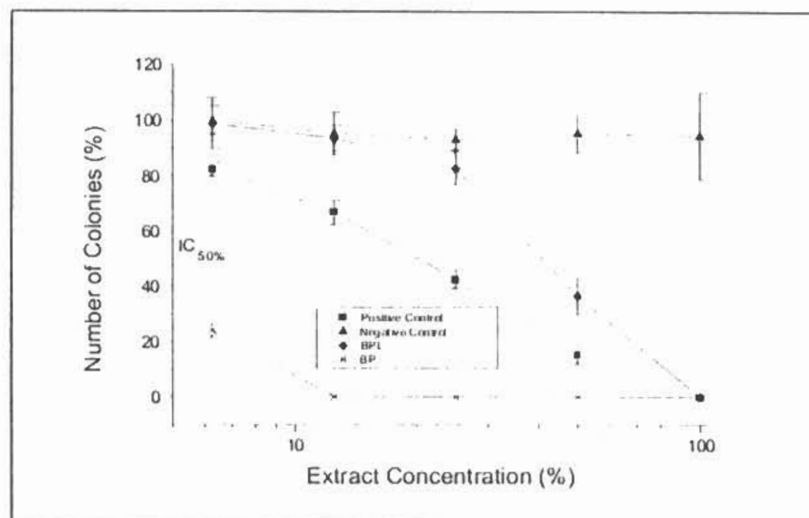

Figure 1: Colony supression curve of treated bovine pericardium

5. DISCUSSIONS AND CONCLUSIONS

The results of the cytotoxicity assay, showed that BP and BPL possess both a cytotoxic potential. The extract of the BP, only allowed the growing of some colonies at the dilution of 6.25%, revealing to be very toxic probably due to the aldehydes residuals leaching from the membrane. By the other hand, a higher number of colonies had grown in the BPL extracts, with suppressed the colonies formation to fifty percent at the extract concentration of 40% ($IC_{50\%}$=40). However the comparison of the citotoxic effect indicated that the lyophilization process diminishes the residual aldehydes in the membranes improving the biocompatibility of the treated bovine pericardium.

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