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ABSTRACT

AN *IN VITRO* CYTOTOXICITY STUDY OF GLUTARALDEHYDE AND NO- REACT® TREATED BIOPROSTHETIC HEART VALVES AND THE AORTIC WALL

by
Roger Ongosi

Valvular failure due to calcification and leaflet disruption of artificial bioprostheses is still a major concern in valve replacement surgery. Previous studies have shown that Glutaraldehyde, a chemical used in the treatment of artificial valves promotes calcification. In this investigation glutaraldehyde and No-React® treated tissue samples of pericardium, cusp and the aortic wall were tested for cytocompatibility using live mouse fibroblast cultures. The samples were cut into 3 X 3 mm^2 washed in phosphate buffered saline solution, transferred into cell culture flask containing cells that had been cultured for 24 hours and incubated at 37 °C in 5 % CO_2 . Cell viability was monitored after 24 hours by dye exclusion method. The concentration of glutaraldehyde released from the tissues was monitored by incubating 3 X 3 mm^2 glutaraldehyde treated tissue samples in cell culture media at 37 °C and 5 % CO_2. The media was then analyzed for glutaraldehyde using UV/Visible spectrometer. The toxic levels of glutaraldehyde was monitored by first incubating the cell for 24 hours in cell culture media at 37 °C and 5 % CO_2 and then injecting various know concentrations of standard glutaraldehyde and the viability monitored by use of dye exclusion method.

Experimental results showed that detoxified (No-React®) pericardium and cusp from

Shelhigh Inc. had the highest cytocompatibility as compared to the aortic wall. There was high cell mortality in glutaraldehyde treated tissues and most of the cell die close to the tissue. They also show that glutaraldehyde concentrations less than 10 ppm does not have significant cell mortality. No glutaraldehyde was detected from the tissues tested for its release.

From the results it can be concluded that glutaraldehyde is one, but not the only factor responsible for cell death in fibroblast culture and that the aortic wall is much more difficult to detoxify as compared to pericardium and cusp. Lack of high glutaraldehyde release from incubated tissue and high cell mortality closer to the tissue shows that surface toxicity may play a part in cell mortality.

AN *IN VITRO* CYTOTOXICITY STUDY OF GLUTARALDEHYDE AND NO- REACT® TREATED BIOPROSTHETIC HEART VALVES AND THE AORTIC WALL

by
Roger Ongosi

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AN *IN VITRO* CYTOTOXICITY STUDY OF GLUTARALDEHYDE AND NO- REACT® TREATED BIOPROSTHETIC HEART VALVES AND THE AORTIC WALL

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Thesis Dedicated to my Beloved Family.

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CHAPTER I

INTRODUCTION

1.1 Objective

The objective of this thesis is to try and understand the factors responsible for valvular heart failure by:

- studying the mechanism of cell death in in-vitro fibroblast culture.
- studying the rate of glutaraldehyde release from conventionally treated animal tissues.
- investigating the influence of glutaraldehyde concentrations on the viability of the cells in the culture.
- investigating possible other factors responsible for cell death in the culture media, in the presence of conventionally treated tissue or detoxified tissues.

1.2 Background Information

The heart valves open and close in response to cyclical changes in intra-cardiac and arterial pressures. This directs the cardiac output forward into the pulmonary and systemic circulation without impending flow. Valvular heart disease adversely affects ventricular loading and tends to diminish cardiac output. Obstruction of forward (stenosis) or regurgitation of flow at any of the four heart valves is considered to be valvular heart disease. Malfunction of the valves is much more common on the left side of the heart than the right side.[1] Inadequate performance of the left pump results in pulmonary congestion, a reduced cardiac output and increased volume and pressure in the left atrium

and pulmonary vasculature, Whereas inadequate performance of the right pump leads to systemic congestion, a reduced cardiac output and increased volume and pressure in the right atrium and systemic venous system. This dysfunction commonly leads to heart failure, although compensatory mechanisms will often preserve haemodynamic stability. Surgery has revolutionized the management of valvular heart disease and can produce close to complete haemodynamic correction. The timing of valve surgery being very important. If it is delayed until ventricular dysfunction or pulmonary hypertension has become irreversible, the risks are greater and the results less satisfactory. In most cases surgical correction requires replacement of the valve with a tissue graft or a mechanical prostheses.

Glutaraldehyde is the standard reagent for the modification of fresh bioprosthetic leaflet materials. It reacts effectively with collagen-based biomaterials, cross-linking the molecules via amino groups, and reduces the antigenicity of the materials. Glutaraldehyde preserved bioprosthetic heart valves are widely used to replace the diseased human heart valves. A large proportion of patients receiving these bioprostheses do not require long-term anti-coagulation therapy. However, the long term function and durability of these valves is far from ideal. There are many clinical studies reporting valve failure [2], [3], but very few studies have been made of the different types of failure modes and the influence of valve design and biological processes on the mechanism of failure. For the past three decades the clinical reality of cardiac valve, continued improvement in design and fabrication of mechanical and biological valve prostheses that have led to improved hemodynamics and durability.

The two most important causes of valve failure are reported as calcification and leaflet disruption. The most common being calcification [4]. The No-React(R) anticalcification treatment has been reported as the ideal treatment for bioprostheses and acts to prevent adhesions, thrombosis and calcification in animals. [5]. The precise mechanism of calcification is not known, although Glutaraldehyde has been implicated as a promoter of the calcification process. Previous studies done at UMDNJ cardiovascular laboratory have shown a direct correlation between calcification and cytocompatibility of the different tissues treated with glutaraldehyde only or detoxified with the No-React® process. Cell death in the cytocompatibility tests is thought to be associated to a release of glutaraldehyde from the tissue when introduced into the cell culture. In this study we have investigated the mechanisms of cell death, the rate of glutaraldehyde release from conventionally treated tissues and the influence of glutaraldehyde concentration on the viability of the cells in the culture. Cytocompatibility tests done today on glutaraldehyde treated tissues considers 70 % cell viability of the cells after 24 hours as good, while Shelhigh consider excellent cytocompatibility to have viability of 95 % or close to 95 % or both. Tissue detoxification is considered by Shelhigh to be excellent if close to 100 %, However tissues stored in glutaraldehyde for long periods are found to be more toxic. Studies done with Dr. Gabbay's group have shown that the regular 15 minutes rinsing in the operation room just before implanting a valve are inadequate. The studies have shown that glutaraldehyde continue to leach out for more than 500 hours of saline incubation. Studies done at Shelhigh and outside laboratory reveled that no glutaraldehyde could be detected from detoxified tissue. If detoxified tissue is 100 % cytocompatible and does not release any glutaraldehyde molecules one might conclude that the total cell viability is the

results of no glutaraldehyde leaching to the culture media. In this study we intended to identify if this assumption is correct or may be there is another factor that is responsible for the cell death. If glutaraldehyde is the culprit we intend to study what is the culture concentration that is toxic enough to cause cell death.

CHAPTER 2

CYTOCOMPATIBILITY TESTS

2.1 Apparatus, Materials and Methods

2.1.1 Apparatus and Materials

a. Carbon dioxide Incubator (Napco® controlled automatic water jacketed CO_2 incubator series 6301 from Precision scientific Inc. Chicago, IL USA.)

b. Reverse phase microscope (Olympus model CK2 from Olympus optical co. Ltd Japan.)

c. Surgical tools

d. Autoclave - Amsco Eagle series 3021 gravity.

e. Filter assembly, T-25 cm^2 cell culture flasks, 6-Well cell culture flasks, Bottle top filter (0.2μm pore size)from Corning and supplied Fisher Scientific, Pittsburgh, PA USA.

f. Cell culture media - Dulbecco's modified Eagle media (DMEM), pH = 7.3, Penicillin, Streptomycin, Fetal Bovine Serum, Non-essential Amino acids Phosphate Buffered Saline Water (PBS solution). Erythrocin B dye (red). Sodium bicarbonate ($NaHCO_3$)for cell culture. Are all GIBCO products supplied by Life Technologies, Inc. Gaithersburg, MD USA.

g. Cells -L929 cell line, Live mouse fibroblast cells.

h. 25% Standard glutaraldehyde solution - Baker analyzed reagent for biological applications obtained from J. T. Baker Chemical Co.

i. Glutaraldehyde treated tissues given by Dr. S. Gabbay.

2.1.2.. Preparation of Cell Culture Media

A 3.7g of $NaHCO_3$ was weighed into a 1-liter conical flask and DMEM powder added (whole pack), The pack was rinsed several times with Millipore water into the conical flask. The Millipore water was added to about the liter mark and the contents stirred for 30 minutes gently to allow uniform mixing. The resulting solution was sterilized by filtration through a sterile cellulose acetate membrane under maximum aseptic conditions. To the sterized media 1 % non-essential amino acids, 10 % fetal bovine serum, 10 % antibiotic solution was added. This was ready for use.

2.1.3 Cell Culture

L-929 cell line in vials preserved under liquid nitrogen was pre-warmed by putting it in a water bath at 37 °C with constant shaking, it was then washed with 70 % alcohol and kept in a laminar flow hood where the cells were quickly transferred into the culture flask containing cell culture media under total aseptic conditions. All care was taken to avoid any contaminations. The culture was labeled and incubated in 5 % carbon dioxide at 37 °C. After 48 hours, the cells were subcultured into 6-well culture flasks in 1 ml of fresh media.

2.2. Tissue Sample Preparation and Analysis

The tissue sample to be tested were cut into 3 x 3 mm square under luminar flow hood observing total aseptic conditions and washed for 30 minutes in 3 portions of each 10ml using sterile PBS solution. The tissues were then transferred into a 6-well culture flask containing cells that had been cultured for 24 hours. This was followed by incubation at

37 °C in 5 % carbon dioxide. After 24 hours the viability of the cells was tested by use of the dye exclusion method and observed under a reverse phase microscope. The number of stained/dead and unstained/live was counted and the result computed.

2.2.1 Dye Application

Erythrocin B dye is used because it only stains dead cells. Once the cells die there cell membrane is weakened and the dye is able to go through and stain the cells unlike the live cell where the membrane is still interact. To one well at a time, using a pipette the cell culture media was carefully removed and quickly added a few drops of erythrocin B just enough to cover the bottom of the well. After 10 seconds the dye was taken out using a pipette by tilting the well to the side and avoiding scratching the bottom of the well and any tissue movement. The wells were covered and the cells observed under the reverse phase microscope. With the help of a counter, stained cells (red) were and the unstained ones were counted within the grid. The grid was as close as possible or just about to touch the tissue. This counting was repeated all round the tissue and the average numbers determined.

2.2.2 Determination of the Toxicity Levels of Glutaraldehyde

This analysis was done in order to determine the minimum concentration of glutaraldehyde that can cause cell death. Glutaraldehyde (25 %) was diluted to 1.0 ppm, 5 ppm, 10 ppm, 20 ppm and 50 ppm using cell culture media under total aseptic conditions. 1 ml of 25 % glutaraldehyde solution was diluted to 250 ml using cell culture media. 1ml of fresh culture media was mixed with 1, 5, 10, 20, and 50 µl of this stock solution to give the 1,

5, 10, 20, and 50 ppm solutions respectively. The live mouse fibroblasts cells were cultured in the 25cm^2 cell culture flasks at 37 ^{0}C and 5 % carbon dioxide concentration for 48 hours. The confluenced cells were further sub-cultured into a six well culture flask for 24 hours after which the various glutaraldehyde concentrations prepared above were injected into the wells and the cells further incubated. After a period of 24 hours the % cell viability was monitored using a reverse phase microscope and 0.1 % erythrocin B as the dye.

2.2.3 Calculations

The % Cell viability was calculated from counts obtained for dead(stained) and live (unstained)cells.

% Cell viability = # of unstained cells/Total number of cells X 100

From the results, the level of toxicity was defined.

CHAPTER 3

SPECTROMETRIC ANALYSIS OF GLUTARALDEHYDE CONCENTRATION

3.1 Materials and Methods

3.1.1 Materials

Apparatus

a. UV/Visible Recording Spectrometer Shimadzu UV160, from Shimadzu Scientific Instruments, Inc. Columbia. Maryland, USA.

b. Analytical Balance - Sartorius Handy H51 from Brinkmann Instruments Co. Division of Sybron. Westbury, New York.

c. Analytical Grade Methanol and 2,4-dinitrophenylhydrazine solution obtained from Sigma Chemical Co. MD USA

d. 25% Standard Glutaraldehyde Aqueous Solution Baker analyzed reagent for biological applications, Potassium Hydroxide Pellet - food grade, Concentrated Hydrochloric Acid obtained from J.T. Baker Chemical Co.

e. Phosphate Buffered Saline Water (PBS solution). GIBCO product supplied by Life Technologies, Inc. Gaithersburg, MD USA.

3.1.2. Preparation of Standard Glutaraldehyde Concentrations

200 μl of 25 % standard glutaraldehyde solution was diluted to 50 ml using methanol and this gave 1000 ppm glutaraldehyde solution. 1000 μl of PBS solution was mixed with

5 μl, 10 μl, 15 μl and 20 μl of the 1000 ppm standard glutaraldehyde solution to give 5, 10, 15 and 20 ppm standard glutaraldehyde solutions respectively.

3.1.3 Preparation of 2,4-Dinitrophenylhydrazine Solution

A saturated solution in methanol was prepared using 2,4-dinitrophenylhydrazine. To 10 ml of methanol in vial, 2,4-dinitrophenylhydrazine was added with stirring till saturation. This solution was not used more than a week or two after preparation.

3.1.4 Preparation of Potassium Hydroxide Solution

10 grams of potassium hydroxide was dissolved in 20 ml of distilled water and the solution was made up to 100 ml using methanol. This solution was kept indefinitely.

3.1.5 Glutaraldehyde Analysis

Analysis of the prepared standard glutaraldehyde concentrations was used in plotting of the standard curve. To 1 ml of 5 ppm, 10 ppm, 15 ppm and 20 ppm standard glutaraldehyde solutions in methanol, 1 ml of 2,4-dinitrophenylhydrazine solution was added and followed by one drop of concentrated hydrochloric acid. The vials were stoppered loosely and heated in a water bath at 100 ^{o}C for 5 minutes. After cooling 5 ml of potassium hydroxide solution was added. The almost black solution that resulted rapidly cleared to a characteristic wine-red color. Blank determination was simultaneously prepared using 1 ml of methanol. The absorbance of the resulting solutions were read using a UV/Visible Recording spectrometer. This was done at 480 nm wavelength.

3.1.6 Influence of Glutaraldehyde Concentrations on Cell Death

This was done in-order to investigate the if glutaraldehyde was released from the conventionally glutaraldehyde treated tissue and also the concentration of the glutaraldehyde released. Pericardium tissue samples were used because the conventionally glutaraldehyde treated pericardium will kill the cells. This could therefore show the factors responsible for the cell death.

Glutaraldehyde treated tissue (pericardium) was cut into 3 x 3 mm^2 samples and washed in three changes of phosphate buffered saline water for 30 minutes. This tissues were put in 1 ml of culture media in a 6-well culture flask wells as shown in the figure 3.1 and incubated at 37 ^{0}C and 5 % CO_2 concentration. After 24 hours the following was done. To one patch:

a) One ml from post-incubation culture media was drawn and the concentration of glutaraldehyde analyzed using the UV/Visible Recording Spectrometer as per previously outlined procedures.

b) One ml of post-incubation culture media was drawn and injected in previously cultured confluenced cells and the cell viability monitored using 0.1 % erythrocin B dye and a reverse phase microscope.

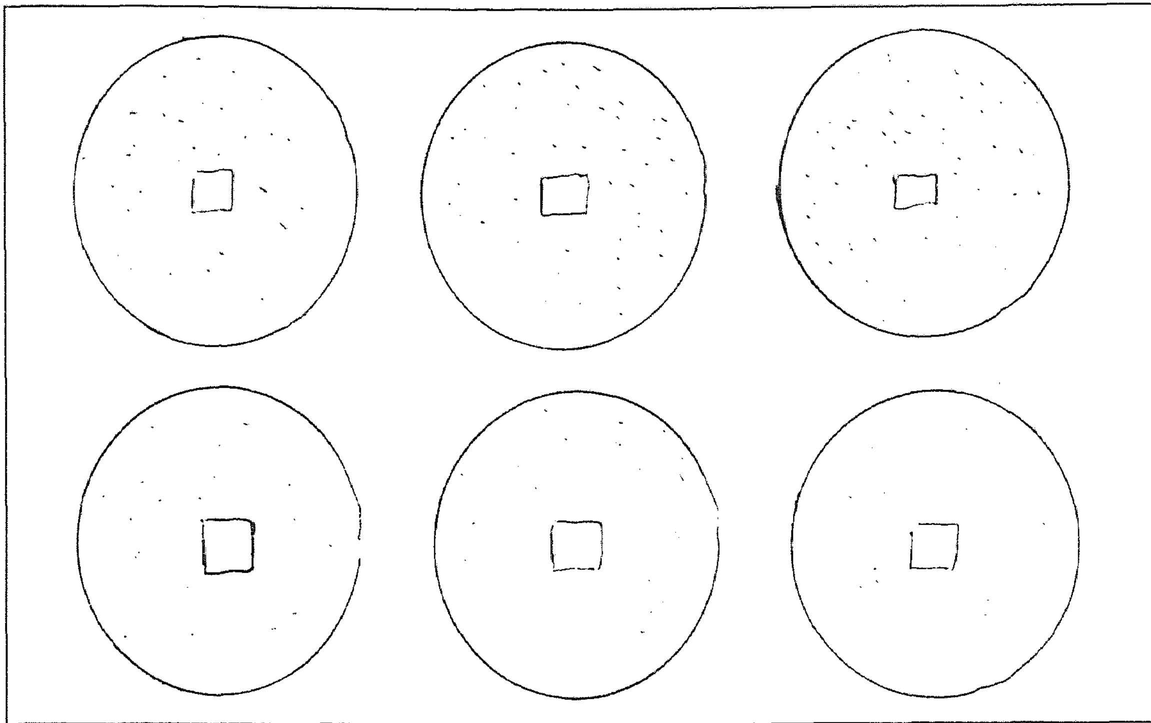

Figure 3.1 Cell Culture flask Showing the Position of the Tissue at the Center

CHAPTER 4

RESULTS

4.1 Cytocompatibility Tests

Glutaraldehyde and No react® treated tissues were tested for cytocompatibility. The conventionally glutaraldehyde treated tissue were used as a control to monitor the effectiveness of the No-React® detoxification process. The detoxified cusps and pericardium from Shelhigh Inc. displayed high cytocompatibility close to or 100 % viability and this is considered excellent as compared to the aortic wall. The In-house detoxified aortic wall although known to be more difficult to detoxify, has been successfully detoxified although the results do not reach 100 % cytocompatibility it reached 93 –96 % cytocompatibility which is considered excellent as well. The aortic wall supplied from outside sources to Shelhigh was found to be difficult to detoxify and showed a viability of 63 %. This was closely followed by the aortic wall from Ionescu-Shiley which was 46 % both lower that the accepted 70 % viability as good. This forms a case for further investigation. The Biocor glutaraldehyde treated aortic wall was the worst and showed 0 % viability while the cusp had 37 % viability and this was the lowest of all the different tissue analyzed (Figure 4.1). The results are an average of several test done in a period of one year as part of the quality control.

4.2 Determination of the Toxicity Levels of Glutaraldehyde

The average cell viability was calculated as shown in figure 4.2. There were no stained/dead cells at 1 ppm and 5 ppm solutions. At 10 ppm glutaraldehyde concentrations there was 5 % mortality of the cells. The viability decreased with increased glutaraldehyde concentrations. However, the decrease in the viability was not drastic as early though. At 50 ppm there was 65 viability. This leaves many questions as to whether glutaraldehyde is the sore source of cytocompatibility.

4.3 Spectrometric Analysis of Glutaraldehyde Concetration

a) A test for aldehydes done of the media incubated with the tissues for 24 hours showed that no glutaraldehyde was released from conventionally glutaraldehyde treated tissues or the concentration was below the detection limit (figure 3). This clearly shows that there might be another factor other than glutaraldehyde that is responsible for the poor cytocompatibility of this tissues. Previous studies also showed that tissues conventionally treated with glutaraldehyde and kept in glutaraldehyde solution had high cell mortality but not necessarily high glutaraldehyde release.

b) When the culture media was drawn and injected into previously cultured confluenced cells no cell dead was observed after 24 hours. This may be due to the absence or low concentration of glutaraldehyde in the media as glutaraldehyde has been shown to reduce cell viability. The concentration of glutaraldehyde has to be close or 10 ppm before significant cell mortality can be observed. The presence of the tissue may play a role in cell death too, because when this used tissue is incubated with fresh cells there is a substantial decrease in cell viability when conventionally treated tissue is put in the culture media.

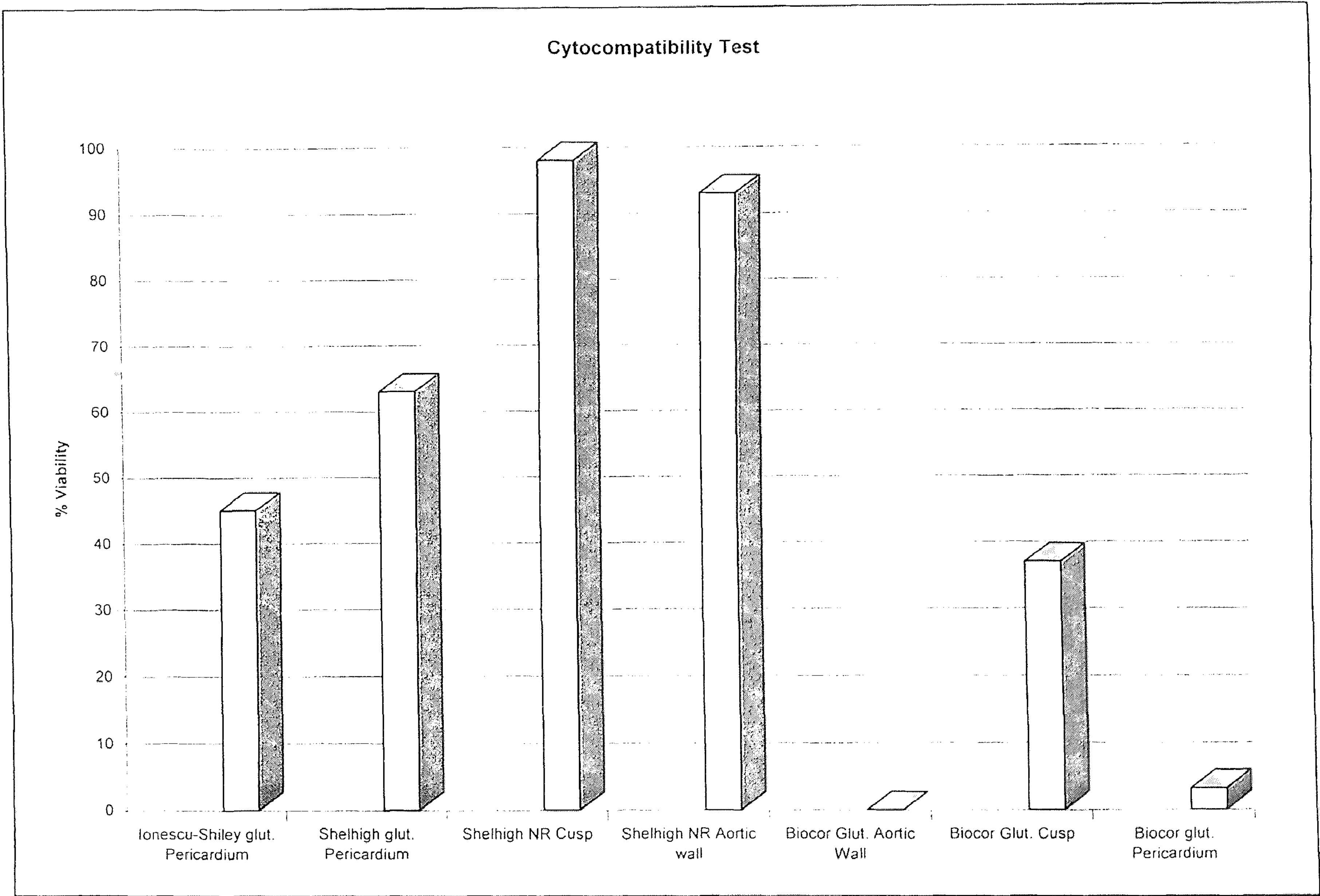

Figure 4.1: Comparison of % Cell Viability of tissues from different manufacturers.

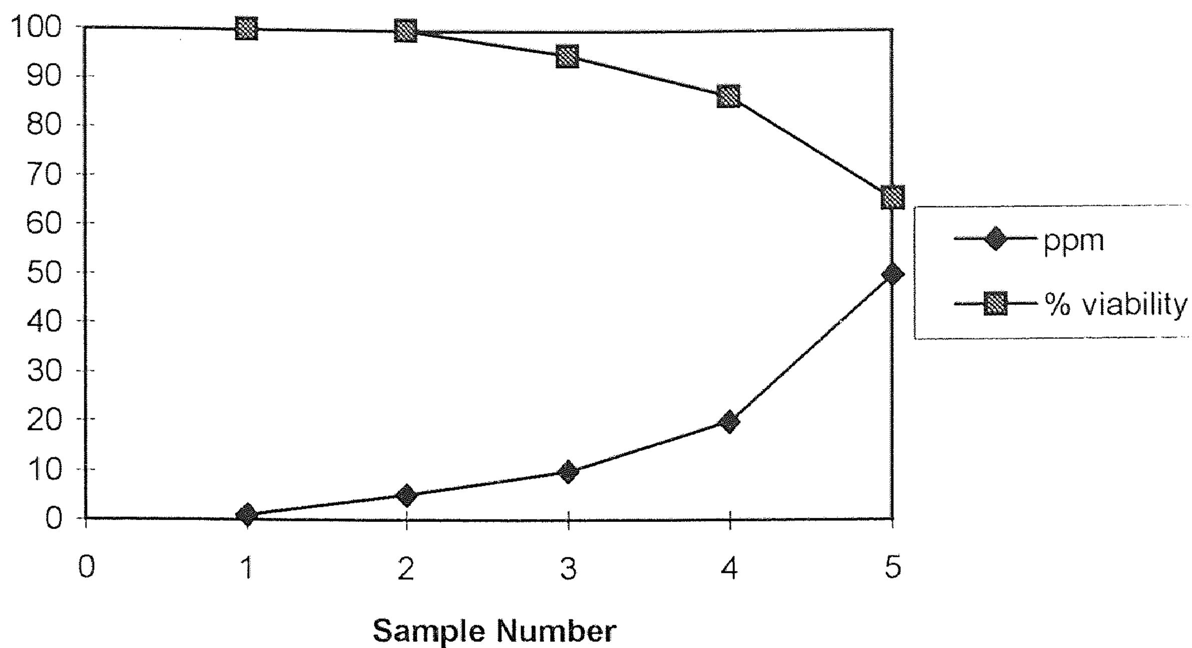

Figure 4.2 Effect of Glutaraldehyde Concetration on Cell Viability

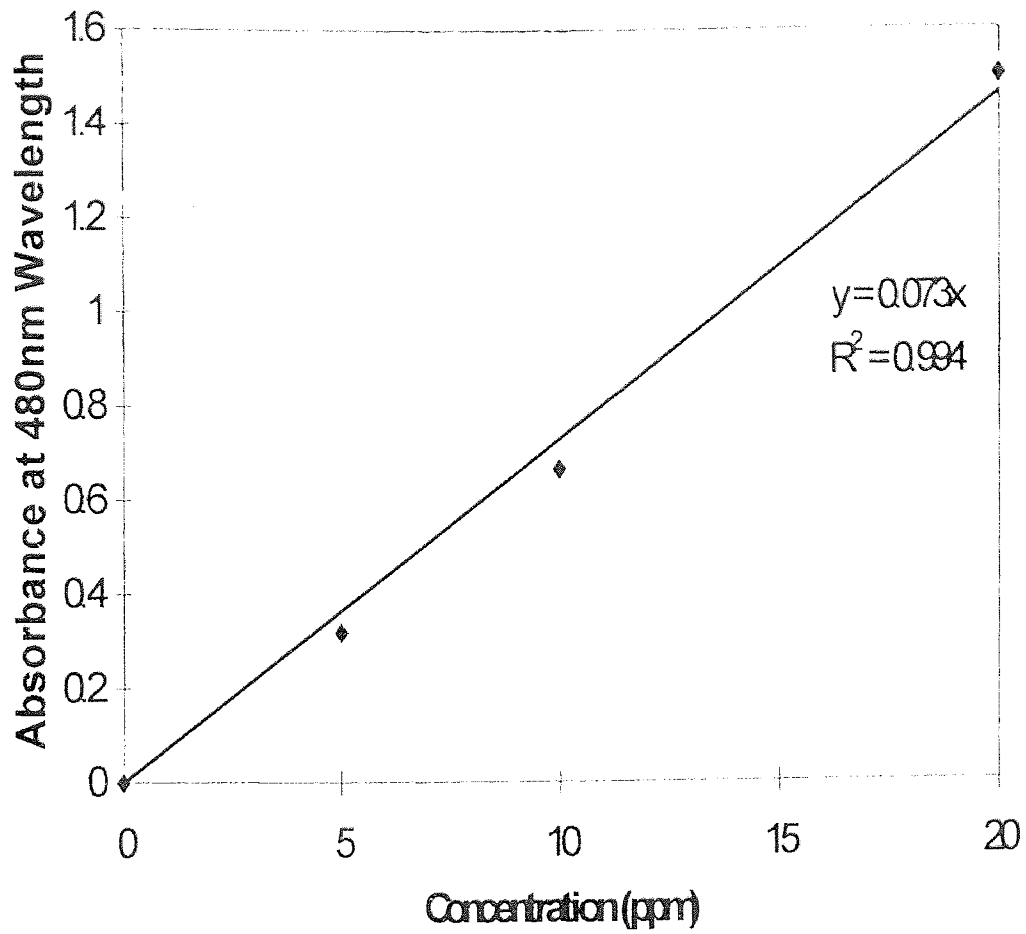

Figure 4.3 Standard Glutaraldehyde Curve Showing Zero Absorbance for Samples Tested

CHAPTER 5

DISCUSSION

Research on calcific degeneration of biological implants in the heart particularly the glutaraldehyde-treated bioprostheses has shown that glutaraldehyde is one of the factors responsible for the calcification. Cytocompatibility studies carried out on glutaraldehyde treated tissue has shown that there is a high cell mortality of the cells close to the tissue as compared to those far away from the tissue. This may be due to the release of glutaraldehyde from the tissues, the concentration being high close to the tissue and/or there might be a surface toxicity leading to a cascade phenomena. The toxicity levels of glutaraldehyde were found to be low. This highly supported the theory of surface toxicity since it was found at a concentration of 5 ppm in the solution of glutaraldehyde do we start seeing signs of toxicity. At 50 ppm there is still a high cell viability and this leads to the conclusion that there must be a additional factor "cytocompatibility factor" which is also responsible for the low cell viability in glutaraldehyde treated bioprostheses. This is a subject for future investigation.

The cytocompatibility of various bioprostheses carried out also showed that glutaraldehyde treated and not-detoxified tissues from biocor had the lowest viability as compared to those from Shelhigh Inc (Table 5.1). The glutaraldehyde-treated detoxified pericardium and cusp from Shelhigh Inc. displayed the highest viability. It is interesting to note that tissue from Biocor conventionally treated and especially aortic wall tissue, was found to be toxic to the level close to 100 % death of the cells in 24 hours. It is well

accepted fact that aortic wall for unclear reasons is more difficult to detoxify or to treat for anticalcification treatment. For example the AOA treatment, seems to work on cusp tissue but not at all on the aortic wall [5]. In the past Shelhigh detoxified tissue for Biocor (Belo Horizonte, Brazil) showed very high cell viability in cell cultures, while cusps and pericardium gave excellent results and aortic wall gave less than adequate results (between 30 – 60 % cell viability). Aortic wall treated at Shelhigh with the conventional method then detoxified resulted in an excellent detoxification (92 – 96 % cell viability) There is no clear explanation as to why the aortic wall of one manufacturer is difficult to detoxify while others can be detoxified more readily. More testing should be done to elucidate the aortic wall behavior as a calcification enhancement.

This investigation clearly showed that the reason for cell death is not necessarily glutaraldehyde release, since we could not detect sufficient amount in the cell culture media. The question is what exactly is the factor responsible for the cell death? It could be a chemical factor of unknown origin, but it could also be that the direct contact of the cells with the tissue that is treated on it's surface has glutaraldehyde molecules causing the cell to die and the dead cells change the pH of the solution (pH measurements done by Dr. Gabbay's group shows that the pH is reduced from 7.4 to 3 or 4) If enough cells on contact with the tissue die, then there will be enough tissue destruction to lower the pH which can cause cell death practically a dominant effect. More studies should be performed to give credence to the theory. It could also be that the detoxification process can also inhibit or clear a chemical factor that might be responsible to the cell death. More research is required to investigate this subject, since it has a direct clinical application. The No-React® detoxification process seem to change the characteristics of the tissue which

result to uniqueness of the tissue, long term clinical follow up and studies are needed to confirm all these interesting in-vitro results.

Table 5.1 The % Cell Viability for Different Animal Tissues Tested

CYTOCOMPATIBILITY STUDIES		
Tissue Sample Lot Number	Sample Description	% Cell Viability
971010 SHP	Pericardium NR BOH	99
971010 SHP	Aortic wall NR-BOH	86
971010 SHP	Cusp NR-BOH	98
971010 SHP	Pericardium NR BOH	98
971010 SHP	Aortic wall NR-BOH	94
971024 -	Pericardium Glut rx BOH	63
YSP-1-0409-10	Ionescu-Shiley 4% glut.	46
971117-Ts	Pericardium NR BOH	96
970925 SHP	Pericardium NR BOH	97.5
971008 SHP	Pericardium NR BOH	97.5
970421 A	Shelhigh Aortic Wall NR BOH	88.3
970807 - SHP	Pericardium Patch	90.5
940901 II	Pericardium OH small	92.8
970929 - A	Aortic wall NR-BOH	93
970929 -A	Cusp No-React	98
970929 -A	Bovine mammary vessel NR BOH	97
970929 -A	Bovine mammary vessel NR BOH	94
970929 SHP	Pericardium NR BOH	99
971010 SHP	Pericardium NR BOH	99
971010 A	Aortic Wall NR BOH	86
971010 A	Cusp NR BOH	98
971010 SHP	Pericardium NR BOH	98
971010 A	Cusp NR BOH	99
971010 A	Aortic wall NR BOH	94
960215 A	Biocor Glut. Aortic wall	0
960215 A	Biocor glut. Cusp	37
960401 II	Biocor Glut. Pericardium	3.2

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