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Gamma-radiation of heart valves at 4° C; a comparative study using techniques of histochemistry and electron and light microscopy

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The optimal method of valve sterilization and storage is still not known. Pig aortic valve cusps have been studied using techniques of histochemistry, light microscopy, and electron microscopy, and the effects of gamma-radiation at 4° C have been determined. The effects of freezing, β-propriolactone, and freeze-drying were also studied.

Gamma-radiation at 4° C and storage at this temperature in a polyionic environment for up to six weeks appears to have minimal adverse effects. This technique of sterilization and storage has now been used in our clinical valve programme for over two years with encouraging results.

Biological heart valves can be sterilized and stored in different ways. Controversy persists regarding the optimal method.

Chemical sterilization with either β-propriolactone or ethylene oxide and storage in the freeze-dried state has been advocated (Barratt-Boyes, 1965; Duran and Whitehead, 1966; Longmore, Lockey, Ross, and Pickering, 1966; Pickering, 1966; Sands, Nelson, Mohri, and Merendino, 1967) but the long-term clinical results using valves treated by these methods are unsatisfactory (Smith, 1967; Davies *et al.*, 1968; Barratt-Boyes *et al.*, 1969).

Sterilization by electron beam irradiation was described by Meeker and Gross (1951) and good clinical results have been reported with valves sterilized in this way (Malm, Bowman, Harris and Kowalik, 1967; Karp and Kirklin, 1969). This technique requires the valves to be in the frozen state and is thus unsuitable for use with valves pre-mounted on to prosthetic frames.

Gamma-radiation causes minimal adverse effects on human valve tissue (King, Heimbecker, and Trimble, 1967; Welch, 1969) and valves so treated may undergo fibroblastic infiltration with the formation of new endothelium (Little, 1970; Gibbons and Alladine, 1970; Welch, 1970). Valve sterilization with gamma-radiation at 4° C was therefore studied and the observed changes were compared with those occurring in tissue sterilized with β-propriolactone and stored in the freeze-dried state. The effects of freezing alone have also been studied.

Pig aortic valves were used in these experiments as a standard fresh specimen, five hours after death of a healthy donor, could be obtained regularly. It seems likely that at least the major changes observed in pig valves would also occur in human valves. The latter are not easy to obtain and those from young healthy donors with no degenerative changes are in demand for clinical use.

MATERIALS AND METHODS

All valves used in this study were taken from 25-week-old pigs weighing approximately 200 lb (100 kg). The valves were dissected for processing within five hours of death of the animals and were allocated to the groups shown in the Table.

TABLE

TECHNIQUES OF VALVE PREPARATION STUDIED

A. Controls
(Five hours after donor death)
B. Method I
(B.P.L., snap freezing, freeze-drying)
1. Freezing
2. β-propriolactone (B.P.L.)
3. Freeze-drying
4. B.P.L., snap freezing, freeze-drying
C. Method II
(Gamma-radiation and storage at 4° C)
1. 4° C for 10 days
2. Gamma-radiation at 4° C with storage for up to three months

There were six valves in each subgroup. The control valves were fixed in 10% formol saline five hours after death. All the remaining valves were fixed in 10% formol saline on completion of treatment.

METHOD I

(1) *Freezing* Six valves were individually double packed in heat-sealed 500 gauge polyethylene envelopes and were snap frozen by being plunged into a mixture of acetone and CO_2 at −80° C. The valves were maintained frozen for four hours and were then thawed by immersion in water at 40° C.

(2) *β-Propriolactone* Six valves were immersed in 1% B.P.L. in individual containers and incubated at 37° C for two hours.

(3) *Freeze-drying* Six valves were snap frozen at −80° C and freeze-dried according to the standard method and apparatus described by Dexter (1967).

Six further valves were freeze-dried at a constant temperature of −20° C and a further six valves at a constant temperature of 20° C.

On completion of freeze-drying all tubes containing valves were hermetically sealed in vacuo. After seven days the valves were reconstituted in normal saline.

(4) *Snap-freezing, β-propriolactone, and freeze-drying* This combination of treatments constituted the full technique previously used in our clinical practice for the sterilization and storage of heart valves. The individual treatments were as described under (1), (2), and (3) above.

METHOD II

(1) *4° C for 10 days* Six valves were stored in Hanks' (Oxoid) solution with added penicillin, 100 units/ml, and streptomycin, 0·1 mg/ml, and maintained at 4° C for 10 days.

(2) *Gamma-radiation at 4° C and storage for up to three months* Thirty valves were individually packed in sealed 500 gauge polyethylene envelopes containing 30 ml Hanks' (Oxoid) solution. The valves were then placed in a refrigerator at 4° C before dispatch for irradiation. During transit and irradiation the valves were held in a specially designed vacuum flask. This flask was cooled with and contained ordinary ice and received the valves direct from the refrigerator. By using plastic bag packing, the envelopes containing the valves were prevented from coming into direct contact with the ice, so that at no time did any part of the tissue become frozen. The valves were subjected to 2·5 megarads of gamma-radiation from a spent fuel source. The time taken to deliver this dosage varies between one and three hours, depending on the age of the spent fuel. After irradiation the container remained unopened until its return to the Tissue Bank. Six valves were then immediately placed in 10% formol saline and the remainder were refrigerated at 4° C. Groups of six valves were subsequently removed at one week, three weeks, six weeks, and three months and fixed in 10% formol saline.

TECHNIQUES OF VALVE ASSESSMENT

Light microscopy Valves were fixed in 10% formol saline for one week and were then embedded in paraffin. Sections were cut at 3 μm and stained with (1) haematoxylin and eosin, (2) van Gieson's stain for collagen and Verhoeff's stain for elastic fibrils, and (3) Gordon and Sweet's stain for reticulin. The acid mucopolysaccharide distribution in the extracellular matrix was studied by three techniques: (1) Alcian Blue at pH 1·0 to pH 5·0; (2) Azur A metachromasia at pH 1·0 to pH 5·0; and (3) Hale's colloidal iron.

Electron microscopy Blocks of tissue from each leaflet of each valve were fixed in 6·5% glutaraldehyde, post-fixed in 2% osmium tetroxide solution, embedded in Epon, and trimmed. Quarter-micron sections were stained with methylene blue—basic fuchsin for light microscopy (Aparacio and Marsden, 1969). Ultra-thin sections were prepared from sites identified by this technique and double-stained with lead citrate and uranyl acetate before examination with a Phillips EM100 electron microscope.

RESULTS

LIGHT MICROSCOPY No tinctorial differences were found in paraffin sections stained with any of the methods used. The pattern of fibrillar distribution (collagen, elastin, and reticulin fibres) was similar in control and treated valves. However, severe vacuolation of the matrix was observed in the valves treated with methods involving any form of freeze-drying. Histochemical study of acid mucopolysaccharides did not reveal any major changes. Alcian blue at pH 1·0 stained the matrix in all experiments without any significant variation in intensity. Metachromasia with Azur A was only observed from pH 3·0 upwards in all cases. The colloidal iron technique also gave a strong staining reaction in all subgroups.

ELECTRON MICROSCOPY Owing to the five-hour delay between the death of the animal and formalin fixation, minor ultrastructural abnormalities were present in the control valves. These were observed as chromatin clumping, mitochondrial swelling, and cellular membrane disruption. Only major differences from this post-mortem pattern were recorded as representing alterations induced by a particular treatment schedule.

(a) *Controls* All leaflets showed abundant fibroblasts intermixed with connective tissue fibres and in this respect pig valves differ markedly from human valves, whose fibroblasts are much fewer. The fibroblasts had a typical spindle-shaped form with an elongated nucleus and a regular nuclear outline without coarse clumping of chromatin (Fig. 1). Nucleoli were observed infrequently. The

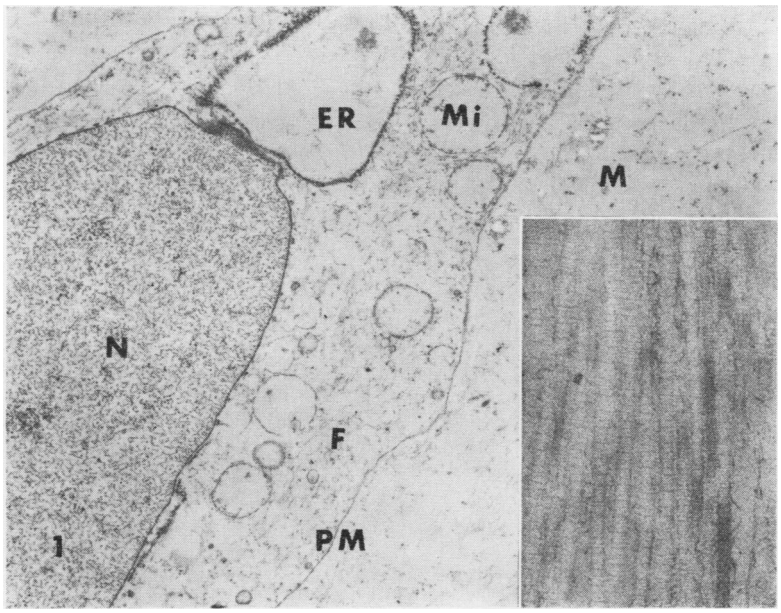

FIG. 1. *Control normal pig aortic valve fixed five hours after death. Electron micrograph shows fibroblast and extracellular matrix (M). The fibroblast nucleus (N) on the left has a regular chromatin and smooth appearance; the cytoplasm is electron-clear, contains fine fibrils (F), swollen endoplasmic reticulum (ER), and swollen mitochondria (Mi), and is limited by intact plasma membrane (PM). The extracellular matrix (M) is electron-clear and in this field shows only few fibrils.* ×12,000. *Inset: same specimen, another field shows at high magnification the typical cross-banding of the collagen fibrils.* ×37,000.

cytoplasm usually had bipolar distribution in relation to the nucleus and terminated in slender processes. Most of the cellular membrane was intact though there were instances of disruption.

The cytoplasmic matrix was electron-clear and contained many loosely packed microfibrils several microns long and 50–70 Å wide (Fig. 1). Cellular organelles appeared swollen but intact, especially the mitochondria and endoplasmic reticulum.

The extracellular matrix was electron-clear and appeared uninterrupted, containing a dense network of collagen, elastin, and reticulin fibres. Reticulin fibres were denser in the subendothelial regions and more thinly distributed towards the depth of the leaflet. Collagen fibres consisted of fibrils with typical cross-banding (Fig. 1 inset).

(b) Valves treated with method I

(1) *Freezing* In general the effects encountered after snap freezing at −80° C followed by rapid thawing were minor differences from those observed in the control valves. A similar picture was seen in those valves stored for one week at −45° C.

The extracellular matrix and connective tissue fibres did not differ from the controls.

Clumping of chromatin was somewhat coarser and more frequent than was seen in the control valves. Swelling of cytoplasmic organelles was present in all specimens in this group.

(2) *β-propriolactone* Collagen fibres were more electron dense and the collagen fibres appeared more closely packed than in the control material. Elastic fibres were unaltered.

There was severe nuclear and cytoplasmic shrinkage with the formation of clear pericellular haloes. Nuclei showed clumping of chromatin with the formation of empty looking areas within the nuclear surface. Cellular organelles were totally destroyed, leaving only membranous remnants.

(3) *Freeze-drying* Severe vacuolation of the extracellular matrix was the common feature of valves treated by any of the freeze-drying methods (Fig. 2). The vacuoles were electron-clear,

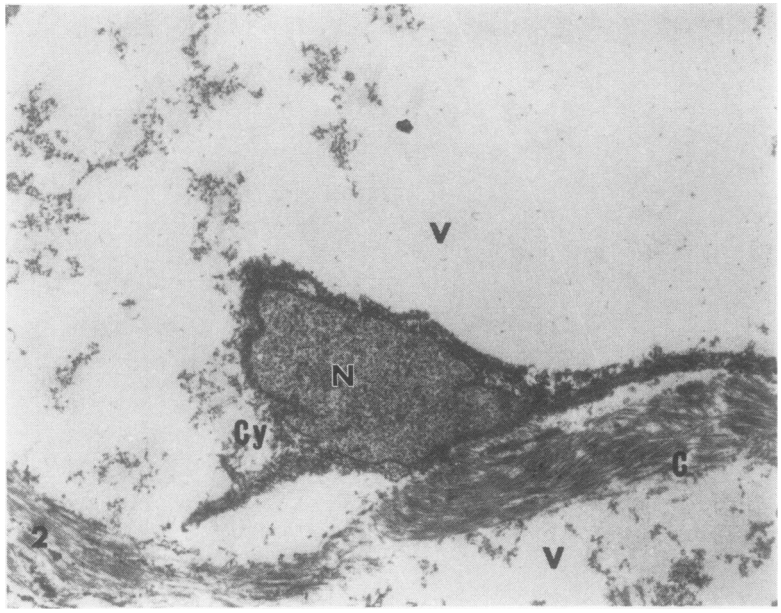

FIG. 2. *Freeze-dried valve. Part of a large extracellular vacuole (V) due to swelling of the matrix surrounds a fibroblast with irregular nucleus (N) and shrunken cytoplasm (Cy). A bundle of collagen fibrils (C) separates the cell from part of another vacuole (V) at the bottom.* ×4,500.

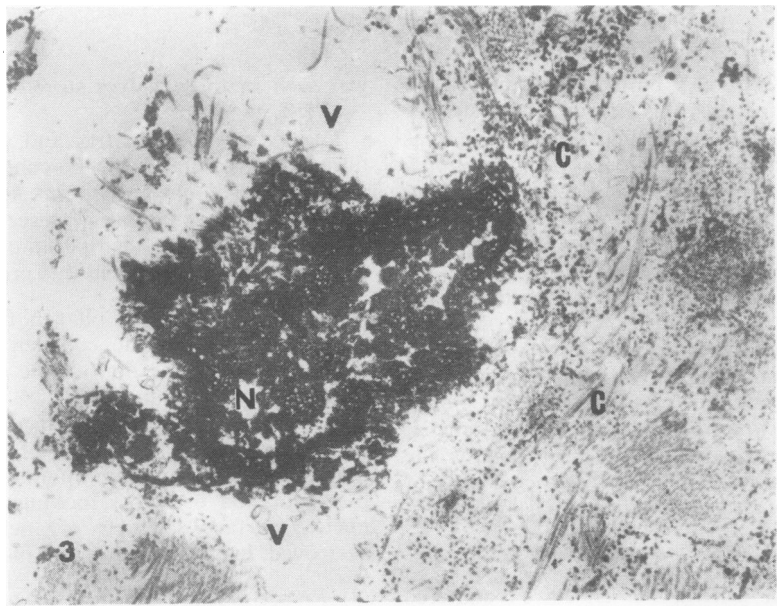

FIG. 3. *Valve treated with B.P.L. and freeze-dried. The cell in the centre of the field is reduced to amorphous nuclear material (N) surrounded by extracellular swelling (V) and disrupted collagen bundles (C).* ×10,000.

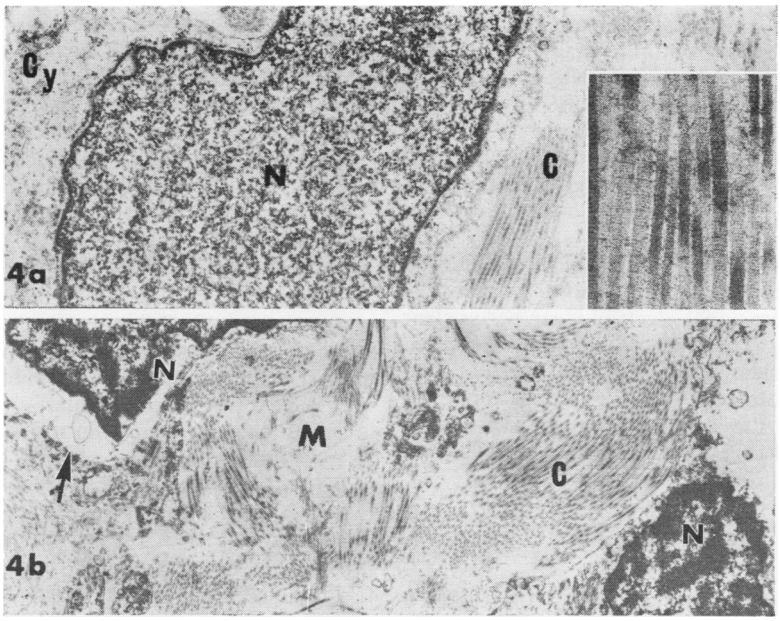

FIG. 4(a). *Valve gamma-radiated with 2·5 megarads at 4°C and stored for six weeks. A fibroblast shows uniformly distributed nuclear chromatin (N) and normal cytoplasm (Cy). There is no extracellular swelling and collagen bundles (C) appear intact.* ×12,000. *Inset: collagen fibrils show normal cross-banding.* ×33,000.

(b). *Same treatment as in* 4(a) *but stored for three months. Two fibroblasts in the field show coarse nuclear chromatin (N) and some perinuclear swelling (at arrow). Extracellular matrix (M) and collagen bundles (C) are unaltered.* ×10,000.

appeared empty, and increased in size towards the depth of the cusp where they measured 500 mμ or more in diameter in extreme cases. They were distributed at random and were either in contact with cellular elements, as in Fig. 2, or appeared isolated in the matrix proper, in many instances separating and disrupting the collagen fibres.

The diameter of the individual collagen fibres was in many cases reduced by 50% compared with the controls. Elastin and reticulin fibres were unaltered. Cellular elements were disrupted and fragmented in many cases.

There was no significant difference between the different methods of freeze-drying studied. However, valves freeze-dried by the standard method showed more and coarser clumping of chromatin and more shrunken cells. Valves treated with the other two methods differed less from the controls with regard to the cellular elements but the degree of vacuolation of the matrix was as marked as in the standard method.

(4) *Combined effects of freezing, B.P.L., and freeze-drying* In this group of valves treated by the complete method I, the effects were a summation of the effects observed in the groups of valves treated with each of the individual procedures. Both the cellular and extracellular changes, already described, were observed.

There was marked cellular shrinkage with condensation of nuclear and cytoplasmic structures leading to the formation of dense amorphous profiles (Fig. 3). There was severe vacuolation of the extracellular matrix, accompanied by separation and disruption of collagen fibres. Elastin and reticulin fibres appeared intact.

(c) Valves treated with method II

(1) *Storage in Hanks' solution at 4° C for 10 days* These valves showed only minor changes from the controls. No connective tissue changes were observed. There was a slight increase in chromatin clumping and swelling of cellular organelles.

(2) *Gamma-radiation with 2·5 megarads at 4° C and storage for up to three months* These valves also showed only minor changes from the controls. There were no extracellular changes in valves

stored for up to six weeks. The collagen fibres did not show any ultrastructural changes from the control valves (Fig. 4a inset). Nuclei preserved their identity, but occasionally there was some swelling of the nuclear membrane gap. The chromatin showed uniformly distributed medium coarse granularity (Fig. 4a). No cellular organelles were identifiable but the plasma membrane was, on the whole, intact (Fig. 4a).

Valves stored for longer than six weeks showed coarser chromatin clumping, more cytoplasmic swelling, and occasional electron-clear perinuclear haloes (Fig. 4b). The extracellular matrix showed more electron-clear areas between the collagen bundles than were seen in valves stored for up to six weeks, but there was no gross vacuolation (Fig. 4b). No changes were observed on collagen, elastin or reticulin fibres.

DISCUSSION

The results of this study can only be interpreted on a morphological basis and further evaluation should include additional physical and biochemical studies and, in due course, long-term clinical results.

The failure to identify chemical changes with the histochemical methods used in this study seems to indicate that the overall changes observed are of a predominantly physical nature. It is possible that there are chemical changes, especially in the acid mucopolysaccharide composition which would be detected only with refined biochemical methods. However, similar studies we have carried out on freeze-dried valves removed from patients have shown marked chemical changes in collagen structure and these presumably occurred after grafting (unpublished data).

Snap freezing at −80° C and storage at −40° C did not cause major changes from the controls, but we did not investigate the effect of storage for longer than seven days. We feel this method of storage may deserve further investigation if combined with a suitable sterilization technique.

Freeze-drying induced severe morphological changes in the tissues treated, confirming the results of others (King *et al.*, 1967 ; Smith, 1967 ; Welch, 1969). Temperatures as low as −100° C have been recommended (Sjöstrand, 1967) during the drying stage in order to prevent the formation of ice crystals believed to be responsible for the large vacuoles found in the cusp matrix. However, this technique preserves tissue only to a depth of a few microns and is not likely to be of practical value with whole tissues. When the honeycomb changes seen with freeze-drying are combined with the shrinkage and cellular damage observed in the valves treated with β-propriolactone alone, it is clear that this combined method is unacceptable for use in valve sterilization and storage.

Valves preserved in Hanks' solution at 4° and sterilized by gamma-radiation were morphologically similar to the normal control untreated material, confirming the work of King and his colleagues (1967) using other techniques of assessment.

Storage at 4° C for up to three months after irradiation appears to accentuate minor changes in cusp tissue including the development of small perinuclear and extracellular clear areas. However, no changes were seen in the collagen, elastin or reticulin fibres. The changes observed become apparent after six weeks' storage. It is not known whether these minor changes are important from the point of view of the clinical use of these valves.

Previous studies have shown that post-mortem shrinkage of valves makes it necessary to keep valves for at least five days after the death of the donor before mounting them on to graft support frames (Dexter, Donnelly, Deverall and Watson, 1972). A series of valves, immersed in Hanks' solution with antibiotics at 4° C for a maximum of 10 days, were, therefore, included in this study. No connective tissue changes were observed in these valves. The optimum time interval between death of the donor and irradiation is not known though it has been suggested that seven days is the maximum period if mechanical strength is not to be significantly impaired (Gibbons and Alladine, 1970).

From a recent series of animal experiments using valves sterilized in the dry state with gamma-radiation, Gunning and Meade (1971) concluded that this method was unsuitable for use in valve preparation. Valves used in the present study were maintained in Hanks' solution throughout and were not allowed to become dry. Therefore, our results cannot be strictly compared with those of Gunning and Meade though it may be concluded that maintenance of the valves in a fluid environment would appear to be of considerable importance.

Valves sterilized with gamma-radiation at 4° C and mounted on to carrier frames with a meticulous technique (Donnelly, Dexter, Deverall and Watson, 1971) have been used for mitral valve replacement at Killingbeck Hospital since November 1970. There has been no evidence of cusp failure to this date.

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