STERILIZATION AND PRESERVATION FOR AORTIC-VALVE TRANSPLANTATION

PROEFSCHRIFT

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aan Melle en Tisca

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Chapter I

GENERAL INTRODUCTION

Recent advances in modern technology, expertise and surgical enterprise have contributed to the solution of may problems in the field of cardiovascular and thoracic surgery. Among these the surgical treatment of heart-valve dysfunction by valve replacement has become possible (Murray, 1956; Ross, 1962; Starr and Edwards, 1961; Barratt-Boyes, 1964). Today transplantation of valves is an accepted surgical treatment next to corrective valve surgery and conservative therapy in the management of heart-valve disease. Heart-valve replacement becomes a necessity in case of congenital or aquired stenosis or insufficiency, if conservative treatment cannot prevent a progression of the lesions. In the decision for valve replacement the degree of invalidity experienced by the patient is a major factor together with myocardial performance. No valve currently available for valve replacement fulfils the criteria for an ideal valve.

For valve replacement a choice must be made between prosthetic and biological material. Biological valves offer the advantages of central flow characteristics, which tend to streamline the bloodflow into the aorta thereby decreasing turbulence and energy dissipation. This is adventageous regarding trauma to blood cell elements which occurs especially after implantation of prosthetic valves resulting in hemolytic anemia and possibly thromboembolism Although the development of the cloth-covered prosthesis (Braunwald and Bonchek, 1967) drastically reduced the complication of thromboembolism, the lifelong use of anticoagulants is still required. Malfunctioning of prosthetic valves due to cloth-wear, thrombosis, endocarditis and perivalvular

leakage has still frequently been described (Herr, et al. 1968; Rees, et al. 1970; Sheam, F.C. et al. 1971; Isom, et al. 1972; Sutherland, 1973; Bonchek and Starr, 1975).

Biological valves are superior over prosthetic valves with respect to flow characteristics, and total absence of thromboembolism. As a choice for biological valves xenogenic, autogenic and allogenic material can be selected. Disadvantages of biological valves compared to prostheses are: the time consuming procedure for valve procurement including sterilization and preservation of the valves with their inherent effects on the structure of these valves, the more complicated surgical technique and in several instances valve failures due to tissue degeneration which were observed in long-term follow-up studies (Gonzalez - Lavin and Ross, 1970 + 1971; Barratt-Boyes, 1969; Gonzalez - Lavin, et al. 1972; Wallace, et al., 1974).

By the use of autogenic material valve sterilization and preservation with their inherent problems can be avoided. Valves reconstructed from fascia lata were introduced by Senning (1967) and modification of this technique by Ionescu, et al., 1970 stimulated its use at a larger scale. The long-term results of valve transplantation with fascia lata as reported by Rothlin, et al., 1973 and Ionescu, et al., 1974 show, however, that these valves when positioned in the aorta are not superior to allografts. In case of transplantation at the mitral or tricuspidal position the results were even worse due to cusp retraction and endocarditis. Autotransplantation of the pulmonic valve into the subcoronarary position was introduced by Lower, et al., 1960, 1960[a], 1961 and clinically applicated by Ross (1967). This technique involves the implantation of the patient's own pulmonic valve into the aortic or mitral position and subsequent replacement of the exicised pulmonic valve by an allograft. Results reported by Somerville, et al., 1972, with a mean follow-up period of about three years showed good results with no valve failure, haemolysis or thromboembolism whereas a non

progressive aortic regurgitation was observed in 20% of the patients. The operation mortality in this rather complicated procedure, however, is relatively high (Ross, 1967; Sommerville, et al., 1972).

Transplantation of valves from other species has the advantage that different numbers and sizes of the valves can be made available. Long-term studies with xenografts derived from pigs and preserved in formaldehyde (O'Brien, 1970 and Angell, et al., 1973) or a mercury containing solution (Binet, et al., 1968) show a rising failure rate due to thinning and rupture of the cusps after initial good results. Xenografts preserved by glutaraldehyde (Carpentier, et al., 1969) which also stabilizes the collagen fibers and at the same time reduces valve antigenicity offered promising initial results but again long-term studies show graft failures due to collagen degeneration Carpentier, et al., 1974). Reis, et al., 1971 applied the same method but he attached the xenograft to a flexible stent which so far gave good short-terms results (Zuhdi, et al., 1974).

In valve transplantation with biological material, aortic valve allografts have been used most frequently. Both for aortic allografts and for xenografts sterilization and preservation methods are required to enable storage of a wide variety of valves with respect to their orifice diameter. This is necessary for an optimal match in orifice diameter between acceptor and donor. Determination of this parameter can not be carried out before actual surgery and hence a large collection of valves with different sizes must be available at the time of operation. Ross reported the successful clinical transplantation of an aortic allograft in the subcorornary position in 1962. This report was followed by work of Barratt-Boyes (1964) and later by others (Bigelow, et al., 1964; Hoeksema, et al., 1967; Malm, et al., 1967 and Stinson, et al., 1968). The long-term results obtained after these aortic allograft transplantations, however showed an increasing number of valve failures mainly due to calcification avulsion and rupture of the leaflets (Gonzalez-Lavin, et al., 1971; Kerwin, et al., 1962; Davies, et

al., 1965; Barratt-Boyes, et al., 1969; Gonzalez-Lavin and Ross, 1970 and 1971; Wallace, et al., 1974). Another major problem which became apparent was a fairly high incidence (40% of the patients) of postoperative aortic regurgitation (Gonzalez - Lavin and Ross, 1970; Wallace, et al., 1974). Although transplantation of the aortic valve allograft is still frequently performed resulting in a major improvement of life expectancy, the problems mentioned above have not yet been solved. Calcification, avulsion and rupture of the leaflets may well be due to degeneration of the transplanted valve and this in turn might be related to the sterilization and preservation procedures that are being used.

It was the main purpose of the work described in this thesis to investigate the effects of various sterilization and preservation procedures on the structure and function of the cellular and intercellular components of the aortic valve.

The aortic valve is composed of a fairly high number of fibroblasts embedded in a matrix of collagenic and elastic fibers together with acid mucopolysaccharides and glycoproteins. The molecular structure and specific properties of these various intercellular components enable the valve to perform its characteristic function requiring a combination of regular movement, absorption of pressure, resistance to strain, and elasticity. As the intercellular components of the valve are produced by the tissue fibroblasts it is clear that indirectly the maintenance of these cells is a prerequisite for long-term valve function. For a long-term maintenance of valve integrity it seems therefore required that the valve transplant either contains its own fibroblasts or is repopulated by cells from acceptor tissue . In chapter II we describe the effects of existing sterilization and preservation procedures on the structure of the human valve fibroblasts and the intercellular matrix using light microscopic and electron microscopic studies. Furthermore the effect of one of the preservation procedures on valve function was tested by measurement of the stress-strain characteristics

of human aortic wall. After these procedures degenerative changes in the valve fibroblasts were observed and also structural and functional deterioration of the intercellular matrix was found.

In chapter III we describe the investigations for the role of the fibroblast in collagen production. This was performed by autoradiografic techniques and by scintillation counting of rat aortic valves after labelling with ^{3}H-proline; analyses after ^{3}H-methionine labelling were used as controls for non specific protein synthesis in the valve. Also, collagen fibers have been isolated from the intercellular matrix, their composition and some aspects of their turn over have been analyzed. Evidence has been obtained for an active role of the valve fibroblast in collagen production which in the normal valve will balance the slow but continuous degradation of intercellular collagen. Similar processes are likely to occur for the other matrix components as well. Long-term maintenance of aortic valve integrity and function can therefore not be expected in a-cellular valves.

In chapter IV we describe a new preservation method using controlled freezing and storage at -196°C which guarantees a 90-95% survival of valve fibroblasts. If preceeded by sterilization this preservation procedure allows collection and indefinite storage of human valves obtained from autopsies with an average survival of about 80% of the fibroblasts and the maintenance of valve structure and function. As a consequence of the avascularity of the valve it is likely that no strong rejection will follow after transplantation of valves containing a high percentage of viable cells. The procedure developed therefore could be considered for clinical application in heart valve surgery.

SUMMARY

Transplantation of one of the heart-valve systems is indicated in case of congenital or acquired stenosis or because of functional insufficiency that cannot be treated by conservative means. The advantage of prosthetic valves is that they are easily available and the insertion technique is relatively simple. The disadvantage is the excentric blood flow causing fairly frequent complications like haemolitic anemia and possibly thrombosis. By transplantation of human valve material (allografts) these complications can be avoided. A disadvantage of the use of allografts however is the necessity of long-term storage of human valves. The required diameter of the valve orifice can only be determined at the time of operation and hence human valves of different sizes obtained at autopsies must be available. Transplantations of these allografts showed a number of complications (rupture, calcifications) which could be related to the procedures of sterilization and preservation.

The experimental work described in this thesis was performed to investigate the effect of currently used sterilization and preservation methods on the structure and function of human valves and to develop a more valid procedure.

In chapter 2, the influence of γ-irradiation as a sterilization method and of different freezing, freeze-drying, and fixation methods used as preservation procedures was investigated. In all instances a loss of cell viability throughout the heart-valve and structural changes of the intercellular matrix i.e. collagen were observed by light microscopic and electronmicroscopic studies. Determination of stress-strain characteristics of pieces of human aortic wall showed a significant loss of elasticity after preservation with glutaraldehyde according to Carpentier.

Chapter 3 describes the results of a number of experiments on the interrelation between the fibroblasts of the aortic valve and the intercellular matrix which is directly responsible for the valve function. Biochemical analysis and autoradiographic studies at the light microscopic level after labelling with radioactive proline and methionine demonstrated for rat aortic valves that fibroblasts are actively involved in the production of collagen in the intercellular matrix. These results made it unlikely that successful long-term results can be obtained after transplantation of a-cellular valves. This implies that sterilization and preservation procedures are required which do not affect the viability of the valve fibroblasts and which do not change the structural and functional integrity of the intercellular matrix.

In chapter 4 a new preservation method is described which seems to fulfil these criteria. After autopsy the diameter of the valve orifice is measured and sterilization is carried out by immersion of the valve during 24 hours in a solution of antibiotics according to the group of Ross. This procedure was found to be valid with respect to the growth of anaerobic and aerobic bacteria, yeasts and fungi. After sterilization the valve is incubated during one minute in Ham's F 10 nutrient medium containing 10%v/v dimethylsulfoxide and 10% calfserum. Subsequently the valve is frozen in liquid nitrogen with a speed of $1^{\circ}C$ per minute down to $-100^{\circ}C$, followed by snap-freezing to $-196^{\circ}C$. Unlimited storage of the valve is now possible at this temperature. At the moment of transplantation the aortic valve with the diameter needed is rapidly thawned in a waterbath of $40^{\circ}C$ during a period of about 4 minutes. Subsequently the nutrient medium is changed 3 times with media containing a descreasing concentration of dimethylsulfoxide to remove this cryoprotective agent. Autoradiografic studies of canine and human valves performed after in vitro incorporation of tritiated proline showed that about 80% of the fibroblasts maintain their viability after this sterilization and preservation procedure. About 14% of the cell loss is due to the sterilization and 6% is the result of the freezing process. The remaining valve fibroblasts were found to be capable of protein synthesis and hence continuous production

of intercellular matrix components will occur after preservation. Electron-microscopic investigations of isolated collagenic fibrils did not reveal any ultra structural alterations as a result of preservation. Finally, no significant changes in the stress-strain characteristics were found in valves which had been preserved by the method developed.

The results described in this thesis indicate that from a cell-biological point of view the currently used methods cannot be expected to yield a satisfactory long-term function of the transplanted heart-valve. Preservation by controlled freezing as has already been used for other tissues in principle offers the possibility of maintaining both cell viability and the structural and functional integrity of the intercellular matrix and hence of valve function. Transplantation of viable allografts has the inherent disadvantage of a possible immunological reaction by the acceptor. Heart-valve tissue is however a-vascular and hence a very mild immune reaction may be expected as is the case after transplantation of other a-vascular tissues like cartilage and cornea. When this aspect has been sufficiently investigated there seems to be no objections against the clinical application of the preservation method developed.

SAMENVATTING

Transplantatie is aangewezen in gevallen van aangeboren of verworven stenose of insufficiëntie van één of de hartklep systemen. Het gebruik van prothesen heeft het voordeel van gemakkelijke beschikbaarheid en een relatief eenvoudige operatie techniek; het nadeel is dat er geen centrale doorstroming van het bloed mogelijk is waardoor betrekkelijk frequente complicaties zoals thrombose en haemolytisch anemiën optreden. Ten aanzien van dit laatste levert transplantatie van menselijk klepmateriaal (allografts) betere resultaten. Het nadeel hiervan is echter dat materiaal verkregen bij autopsie gedurende langere tijd moet kunnen worden opgeslagen teneinde bij operatie, kleppen van de vereiste afmetingen ter beschikking te hebben. Het doel van het experimentele werk beschreven in dit proefschrift was na te gaan in hoeverre de gebruikelijke sterilisatie en preservatie methoden eventueel nadelige invloed hebben op de structuur en functie van de klep en om een betere procedure uit te werken. In hoofdstuk 2 is het effect van bestraling als sterilisatie procedure en van verschillende vries- vriesdroog- en fixatiemethoden als preservatie procedure onderzocht. Lichtmicroscopische en electronenmicroscopische analyse toonde aan dat al deze prodedures leiden tot celdood van de fibroblast in de hartklep en structurele veranderingen van de intercellulaire matrix oa.het collageen. Door middel van een belastingstest werd ook vastgesteld dat de elasticiteit van de aorta wand aanzienlijk verminderd was na preservatie met glutaraldehyde volgens carpentier.

In hoofdstuk 3 werd in een aantal dierexperimenten de interrelatie tussen fibroblasten van de aortaklep en de intercellulaire matrix onderzocht. Labeling experimenten met radioaktief gemerkt proline en methionine gevolgd

door autoradiografische en biochemische analyse toonden aan dat de fibroblasten in de klep aktief betrokken zijn bij de produktie van collageen Omdat collageen één der belangrijkste bestanddelen van de matrix is dankzij welke de klep zijn specifieke functies kan uitvoeren is het onwaarschijnlijk dat op de lange termijn bevredigdende resultaten worden verkregen na transplantatie met a-cellulaire kleppen. Tijdens sterilisatie en preservatie dienen dus zowel de levensvatbaarheid van de fibroblast als de structurele en functionele integriteit van de intercellulaire matrix behouden te blijven.

In hooofdstuk 4 wordt een nieuwe methode beschreven welke aan deze eisen lijkt te voldoen. Na sterilisatie door middel van 24 uur immersie van de klep in een antibiotica oplossing volgens de groep van Ross (Lockey, et al., 1972) welke procedure betrouwbaar was ten aanzien van de groei van anaerobe en aerobe bacteriën, gisten en schimmels, wordt de klep gedurende één minuut gelncubeerd in Ham's F 10 kweek medium dat 10% v/v dimethylsulfoxide en 10% kalverserum bevat. Vervolgens wordt met behulp van vloeibare stikstof de klep met een snelheid van $1^{\circ}C$ per minuut ingevroren tot $-100^{\circ}C$, waarna zeer snelle bevriezing tot $-196^{\circ}C$ volgt (vloeibare stikstof). Bij deze temperatuur kan de klep onbeperkte tijd bewaard worden. Op het moment van transplantatie wordt de aorta klep met de gewenste afmetingen snel ontdooid in een waterbad van $40^{\circ}C$ waarvoor een tijdsduur van ongeveer 4 minuten nodig is. Hierna wordt het medium een drietal keren ververst met media welke een dalende concentratie aan dimethylsulfoxide bevat teneinde dit cryoprotectivum uit te spoelen.

Door middel van ^{3}H-proline incorporatie gevolgd door autoradiografie is aangetoond dat na deze sterilisatie en preservatie methode ongeveer 80% van de oorspronkelijke aanwezige fibroblasten levensvatvaar blijven. Bovendien blijken deze cellen tot eiwitsynthese in staat. Electronenmicroscopisch onderzoek van gelsoleerde collageen vezels toonde aan dat de preservatie geen veranderingen teweeg brengt in de ultrastructuur van deze vezels. Tenslotte werd met behulp van een belastingsproef gevonden dat de gepreserveerde

kleppen vrijwel normale functionele eigenschappen vertoonden.

De resultaten van het in dit proefschrift beschreven onderzoek tonen aan dat de oorspronkelijk gebruikte sterilisatie en preservatie methoden op celbiologische grond niet geschikt zijn om een langdurig goed functioneren van getransplanteerde hartkleppen te waarborgen. Preservatie door middel van gecontrolleerde invriezing zoals ook voor andere weefsels reeds gebruikt, biedt in principe wel de mogelijkheid tot instandhouding van al die cellulaire processen welke nodig zijn voor het handhaven van structuur en functie van de hartklep na transplantatie. Het mogelijke bezwaar van het gebruik van vitale allografts is de immunologische afweer van de gastheer. Omdat echter de hartklep niet gevasculariseerd is valt te verwachten dat eventuele afweerreacties mild zullen zijn evenals dat het geval is gebleken bij transplantaties van andere a-vasculaire weefsels zoals kraakbeen en cornea.

Wanneer dit laatste aspect voldoende experimenteel is onderzocht lijken er geen bezwaren meer te bestaan de hier ontwikkelde preservatiemethode klinisch toe te passen.

NAWOORD

Curriculum vitae:
Eindexamen H.B.S. B 1966. Studie Geneeskunde te Rotterdam (doctoraal examen 1972). Vanaf 1969 als student assistent, waarna sinds 1972 als wetenschappelijk medewerker, verbonden aan de afdeling Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam; waar het in dit proefschrift beschreven onderzoek werd uitgevoerd.

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Chapter II

EFFECT OF STERILIZATION AND PRESERVATION PROCEDURES ON AORTIC-VALVE STRUCTURE AND FUNCTION

INTRODUCTION

Early experimental attemps to use an aortic valve allograft were performed by Lam, et al. (1952) and Brewin, (1956). These authors used fresh viable dog valves as a transplant into the descending aorta of the recipient animal, but unless the animal had marked and permanent insufficiency of its own valve, the graft would suffer from failure and shrivelling of its cusps.

Murray, (1956) reported one of the first clinical applications of transplantation of aortic valve-segments. He transplanted fresh viable valve segments into the descending aorta with good long-term results. (Murray, 1960; Bigelow, et al., 1964). Ross, (1962) and Barratt-Boyes, (1964) pioneered with the implantation in man of allograft aortic valves in the subcoronary position. The availability of an implantation technique, the dramatic relief of symptoms and the absence of thromboembolic complications led to a general interest in the aortic valve allograft.

The routine use of aortic valve grafts necessitated the availability of a larger number of allografts with different orifice diameters to enable an optimal match to the acceptors aortic diameter. The latter can only be measured during surgery, and consequently methods of sterilization and preservation of valve allografts had to be developed. For sterilization a number of different methods were used: ethylene oxide (Ross, 1962), beta-propiolactone (Barratt-Boyes, 1965a; Barratt-Boyes, et al., 1965b), and γ-irradiation with 2.5 megarads at $-56^{\circ}C$ which was introduced by Harris, et al. (1968). Preservation of the grafts was carried out by lyophilyzation or dry storage at low temperatures. Combined sterilization and

preservation was performed with buffered 4% formaldehyde (O'Brien, 1967) and with 0.6% buffered glutaraldehyde. (Carpentier, et al., 1969). Patients with a valve transplant preserved by these methods all showed a fairly high incidence, about 40%, of diastolic murmers due to cusp-thickening and shortening. (Gonzalez-Lavin, 1970). The incidence of 10% cusp rupture observed in transplants sterilized with beta-propiolactone and preserved by freeze-drying (Barratt Boyes, et al., 1969) is similar to the figure obtained by Gonzalez-Lavin, et al. (1972), who used ethylene oxide as sterilization method and freeze-drying for valve storage. Beta-propiolactone sterilization followed by storage in Hank's solution or dry storage at -80°C of valve grafts showed a 5% to 10% cusp rupture incidence after transplantation. (Barratt-Boyes, et al., 1969; Barnes, et al., 1970). A comparative study of grafts sterilized either by beta-propiolactone or γ-irradiation and stored in Hank's solution or dry at -80°C showed no significant difference in late valve failure, in both instances about 20% failure after an average follow up of 42 months (Wallace, et al., 1974). Combined sterilization and preservation with 4% buffered formaldehyde of allo- and xenografts resulted in a high incidence of graft failures (20%); partly due to endocarditis within a two year follow-up period. (Ionescu, et al., 1972; Angell, et al., 1973). Sterilization and storage using glutaraldehyde was introduced by Carpentier (1969) as part of a technique for xenograft conditioning necessary to reduce valve antigenicity. Within a six year follow-up period the incidence of valve failure was about 10% which was partly due to collagen denaturation. (Carpentier, et al., 1974).

The valve failures found in different follow-up studies may be the result of alterations in valve structure and function caused by the various methods of sterilization and preservation. In our opinion the maintenance of the structural integrity of cellular and intercellular structures in the heart valve are prerequisites for an optimal function. The normal heart valve consists of various cell types, and of an intercellular compartment which is mainly produced by the most common cell in the heart valve, the fibroblast. The function of the heart valve is likely to depend mainly on the characteristics of the main components

of the intercellular compartment, i.e. the collagen and elastic fibers. The purpose of the next paragraphs is to describe the effects of the various sterilization and preservation methods on the structural components of the valve. The light microscopic structure of normal heart valves using several histological staining techniques will be described as well as some electronmicroscopic studies of the cellular and intercellular compartment. The effect of several sterilization and preservation methods as used until recently in most centres for thoracic and cardiovascular surgery will be tested. The effect on the (sub)-microscopic structure of sterilization by 2.5 megarads γ-irradiation and preservation by lyophillization with subsequent storage at $-40^{\circ}C$, dry storage at low temperatures after slow freezing and preservation in Hank's solution or in glutaraldehyde will be investigated. The effect of the last procedure has been tested by measuring the stress-strain characteristics of strips of aortic-wall.

MATERIALS AND METHODS

A Ultra Structural examination of normal aortic valves.

Human aortic valves were obtained from post-mortem examinations and they were investigated with the following methods.

a Light microscopy

Valves were fixed in neutral formaldehyde 4% during 24 hours, rinsed, embedded in paraplast 56° - $57^{\circ}C$ and 5 um sections were cut. Hematoxylin-eosin staining was used for the study of the structure. Azan staining was used for the identification of collagenic fibrils and orcein staining for the localization of the elastic fibers. The exact procedures for the various staining methods can be found in a textbook like that by Romeis (1968).

b Electron microscopy

For the study of the ultrastructure of the fibroblasts small aortic heartvalve fragments were fixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) (Millonig, 1961), for 60 min at 4°C; after dehydration through a series of different concentrations of ethanol, the tissue was embedded in Epon 812. Ultra thin sections were cut on a LKB III microtome, "stained" with Millonig's lead tartrate for 5 min and the preparations were examined with a Zeiss GS electron microscope.

The ultrastructure of the collagenic microfibrils was investigated according to the method of Olsen, 1963. Small pieces of valve tissue were thoroughly homogenized in destillated water using a steel morter. The homogenate was mixed on a glass-slide with phosphotunstic acid (2% in destillated water, adjusted to pH 5.0 with NaOH) and stained for 15 minutes on a copper grid. These preparations were examined with the electron microscope. The banding pattern of the microfibrils was studied by measurements of at least one thousand periods on various electron micrographs. Only straight parts of collagen fibrils were selected for measurements.

B Ultra Structural examination of sterilized and preserved aortic valves.

For each sterilization and preservation method tested, two aortic valve systems were each divided in six equal parts as illustrated in Fig. 2-1. Sterilization by γ-irradiation at -56°C with a dosage of 2.5 Megarads was carried out in the Reactor Nuclear Centre, Petten, The Netherlands, to investigate its effect on the collagen microfibrils. Three control pieces were processed immediately after valve obtainment, according to the previously described method for examination of the collagen microfibrils. After a three weeks preservation period at -20°C, γ-irradiation at the Nuclear Centre Petten took place. At this time six valve pieces were irradiated at -56°C and another three valve pieces were processed without γ-irradiation and used as preservation controls. The various categories of

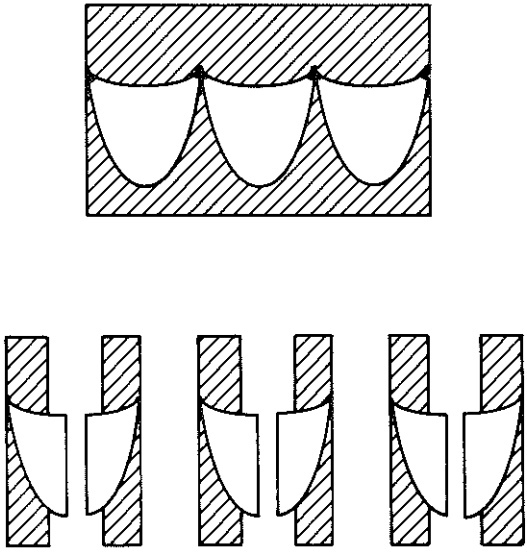


Fig. 2-1. Illustration of the dissection of an aortic-valve system into six equal parts for further processing.

valve fragments were examined with the electron microscope and the distance between the iron bandings of the collagenic microfibrils was measured.

The effect of preservation was tested with histological staining procedures. Two of the twelve pieces of human aortic valve were used as controls; the other ten pieces were preserved for six months by either one of the following methods:

a Preservation in Hank's solution at $4^{\circ}C$

b Quick freezing and lyophillization with subsequent storage at $-40^{\circ}C$ in vacuo

c Slow freezing and storage at low temperature ($-40^{\circ}C$)

All tissues were fixed in 4% formaldehyde and paraffin sections (5 um) of control and preserved valve pieces were studied after haematoxylin and eosin, azan and orcein staining.

C Stress - strain measurements.

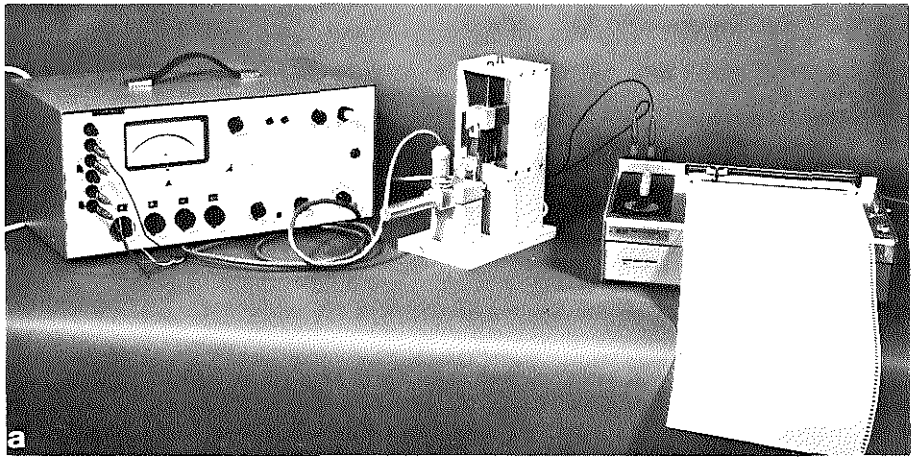
Human thoracic aortas were excised within 24 hours after death. The tissue was preserved in saline solution until the time of testing. Each aorta was opened and cut in the circumferèntial direction with a blademodel, resulting in strips of 3.5 cm length, 1.0 cm width at the ends and 0.6 cm width in the middle part. Control strips and strips to be preserved were taken from adjacent locations. The strips were placed on a rifling bench (Fig. 2-2.) with a cross-head speed of 1.0 mm per minute connected, via a transducer/strainindicator, to a writer with a chart speed of 1.0 cm per minute, yielding a magnification of 10x. The initial length was adjusted to 2.4 cm without buckling or crimping of the tissue.

Material was conditioned according to the method of Carpentier, et al., 1969; to reduce valve antigenicity and stabilize the collagen fibers. This is achieved by removal of soluble proteins, mucopolysaccharides and structural glycoproteins followed by storage in 0.6% phosphate buffered glutaraldehyde at 4°C for two weeks. Controle and conditioned aortic-wall strips were tested for the initial expansion at low stress, stress and elongation at transition and at rupture (tensile strength). A number of 20 aortic-wall strips were used, taken from five different thoracic aortas. To make comparison with the stress-strain characteristics of human aortic-valve leaflets possible (Clark, 1973), the influence of uncontrolled freezing, as performed by Clark on valve leaflet-strips, was tested on aorta-strips cut in the radial and circumferential direction.

RESULTS

1. The effect of sterilization and preservation on the light microscopic structure of the human aortic valve.

The normal human aortic valve consists of three leaflets composed of endocardial folds, covered by endothelium and reinforced with a middle layer of dense connective tissue. This connective tissue contains an abundant number of fibroblasts embedded in a matrix of collagenic and elastic fibers. Capillaries



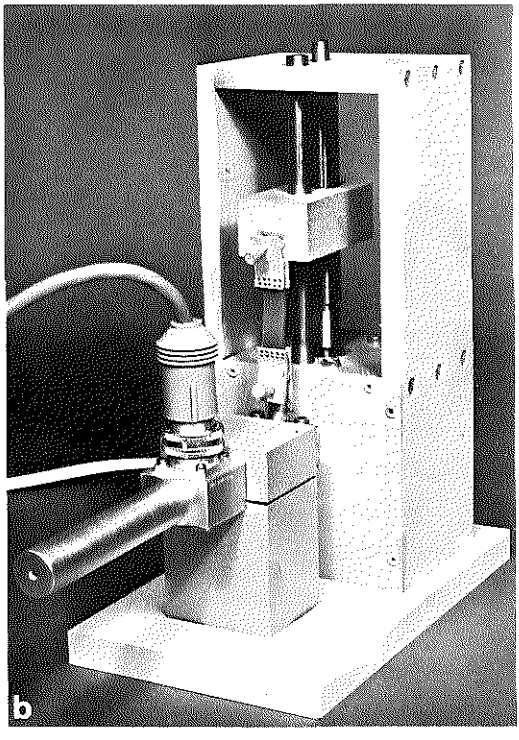


Fig. 2-2. a) Apparatus for stress-strain testing of strips of aortic-wall.
b) Detail of rifling bench with a silicone strip clamped between the jaws.

may be present at the bases of the leaflets, but do not extend into the valves proper. The collagen fibers are concentrated at the aortic side of the valves whereas the elastic fibers are concentrated at the ventricular side (Fig. 2-3.). Towards its free margin the connective tissue layer of the leaflets becomes thickened to form a nodule particularly near the middle of the leaflet.

Preservation methods tested, like storage in Hank's solution at $4^{\circ}C$, lyophillization with subsequent storage at $-40^{\circ}C$ in vacuo and dry storage at low temperatures ($-80^{\circ}C$ and $-40^{\circ}C$), result either in a completely a-cellular aortic valve system with swollen and disrupted collagenic and elastic fibers or in valves which structure is severely damaged by ice-crystal formation (Fig. 2-4.). In all instances no viable cells remain and the novo synthesis of intercellular components is therefore no longer possible in these valves. Also the intercellular components already present are likely to be seriously damaged by disruption and vacuolization.

2. Electron microscopy of the aortic valve and the effect of sterilization.

The electron microscopic studies of the fibroblast in the human aortic valve showed that many of these cells have a reasonably well developed rough endoplasmatic reticulum (Fig. 2-5.) and a considerable number of vesicles surrounded by a membrane (Fig. 2-6.). This suggests that these cells are actively involved in the synthesis and secretion of macromolecules. One of the components produced by these fibroblasts, the collagenic fibrils are located around the cell (Fig. 2-5.). A high number of fibroblasts containing rough endoplasmatic reticulum are located at the cusp-aorta junction and in the cusp-nodules. The number of mitochondria in the fibroblasts was relatively low which may be indicative for a relatively low oxygen consumption and in turn might be related to the poor or absent vascularization of the heart valve.

3. Electronmicroscopy of the collagenic microfibrils.

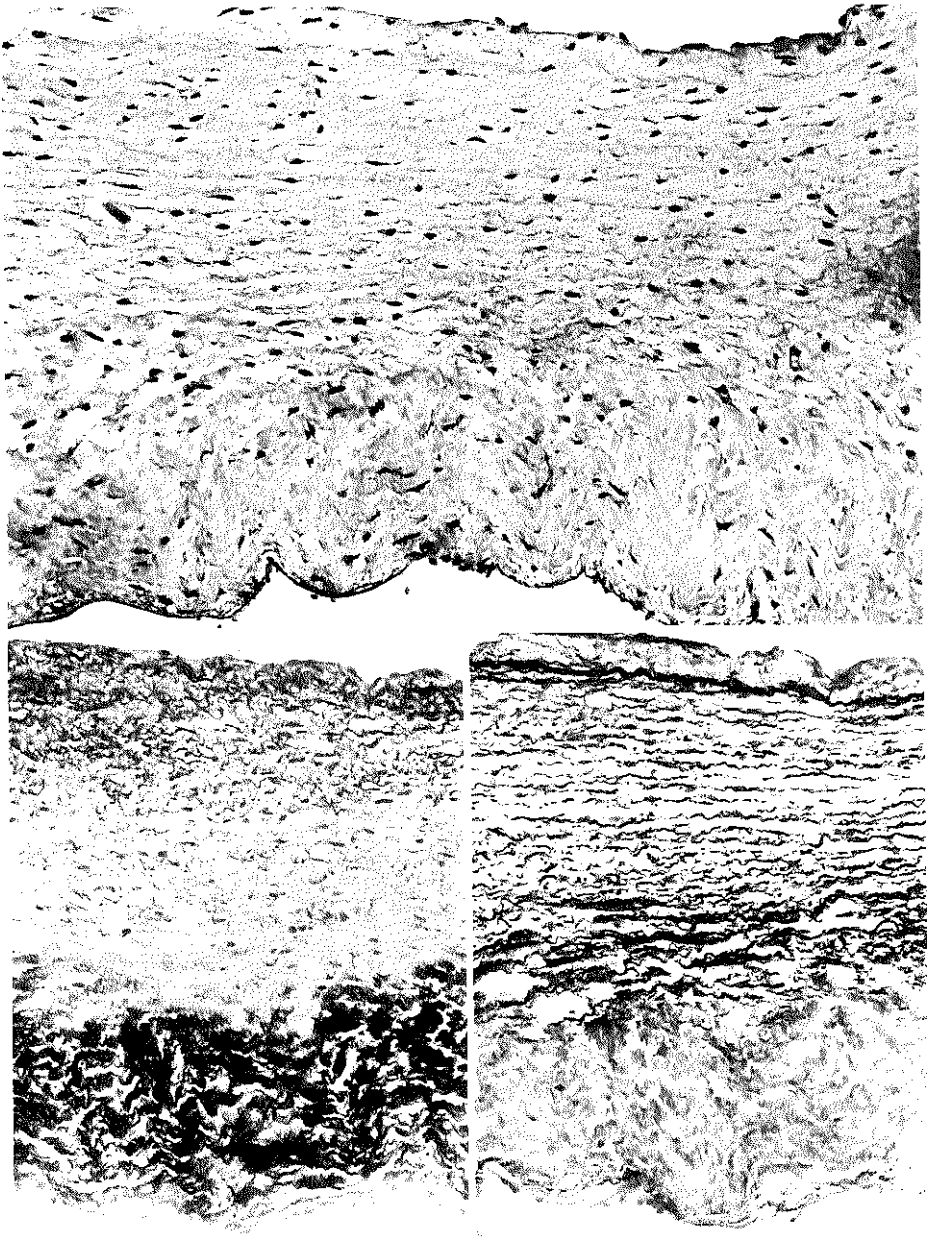
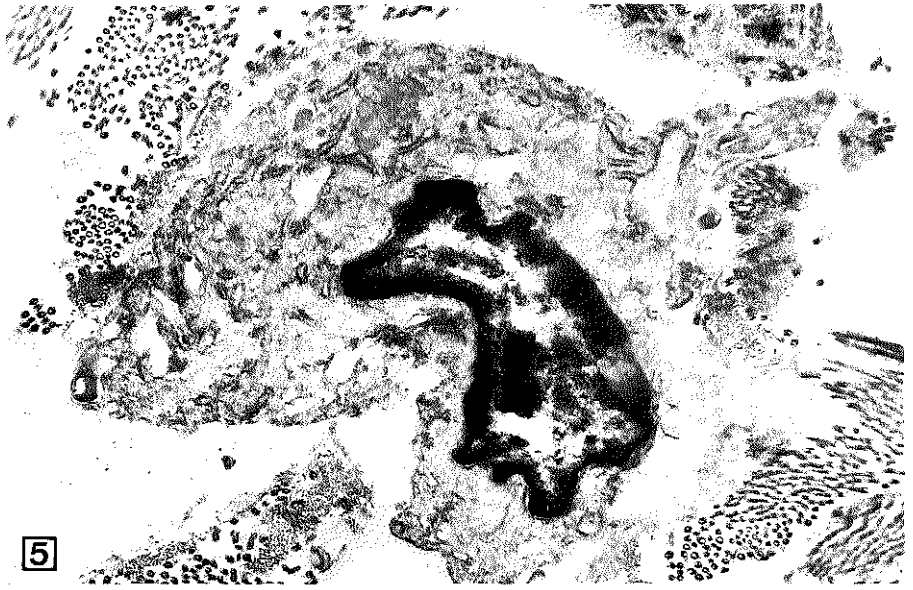


Fig. 2-3. Top: A medium-power photomicrograph of a longitudinal section of the human aortic valve (H-E stain). Note the fibroblasts located in paralel rows between the collagenic and elastic fibers. Lower left: A photomicrograph of a longitudinal section, taken at the same magnification showing the distribution of the collagenic fibers (Azan staining). Lower right: A photomicrograph of a longitudinal section, taken at the same magnification, showing the distribution of the elastic fibers (Orcein staining).



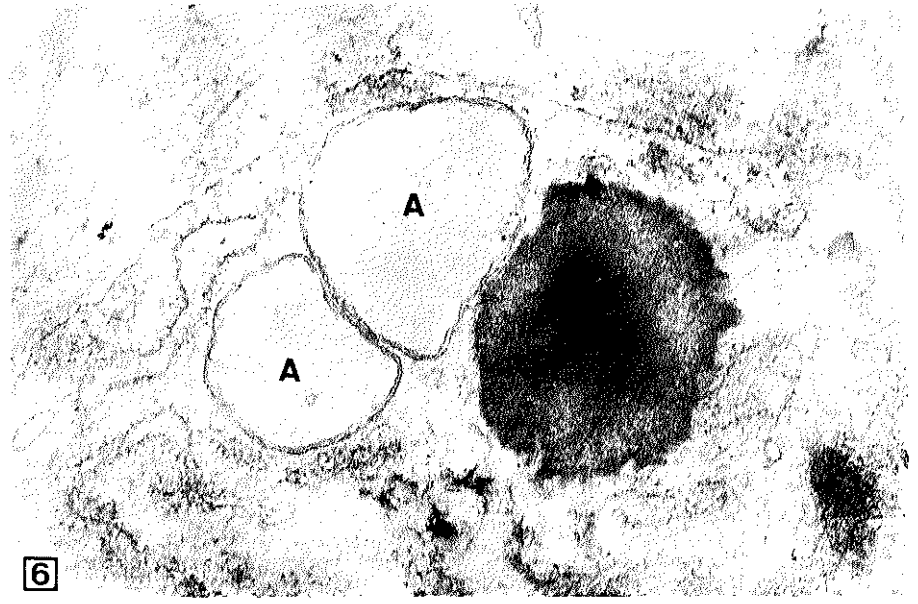


Fig. 2-5. Electronmicrograph (X 7,000) of an aortic-valve fibroblast. Note the strands of rough-surfaced endoplasmic recticulum, and the cross-sectioned collagenic fibers in the extracellular compartment.

Fig. 2-6. Electronmicrograph (X 15,000). Note the membranated vesicles (A) and a lysosome in the cytoplasm of an aortic valve fibroblast.

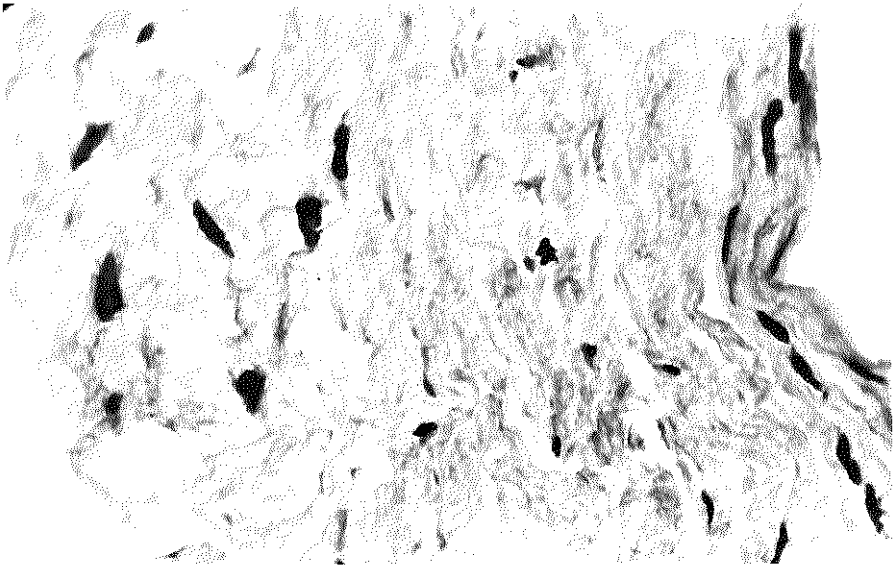


Fig. 2-4. High-power photomicrograph of a section of a human aortic valve preserved by lyophillization with subsequent storage at -40°C for six months. Note the vacuolization of the matrix in the lower part and the pyknotic fibroblasts in the middle part.

The periodicity length of the iron banding and its variation in normal collagen fibrils of human aortic valve was measured and the average valve was 640 Å ± 10 Å (Fig. 2-7.).

Fig. 2-7. Electronmicrograph (final magnification 39,000X) of an isolated collagenic microfibril.

Human aortic valves were stored at -20°C for three weeks and subsequently sterilized by γ - irradiation (2.5 Megarads at -56°C). The periodicity length of collagenic microfibrils was measured in controls, (three pieces), after preservation (three pieces) and after preservation followed by irradiation (six

pieces). The results from these measurements are presented in table 2 - 1.

control valves		640 Å	± 10 Å
(no storage)	n = 3000		
preserved valves		642 Å	± 9 Å
(3 weeks at -20°C)	n = 3000		
γ-irradiated valves		661 Å	± 10 Å
2.5 Megarads after preservation			
(3 weeks at -20°C)	n = 6000		

Table 2-1. Mean values and standard deviations in Å of the periodicity lenght of isolated collagenic fibrils.

The difference between control valves and those after three weeks preservation at -20°C was not found to be significant. However the difference caused by γ-irradiation of the valves is significant. When displayed in a histogram (Fig. 2-8.) the periodicity lengths of the control valves and those after

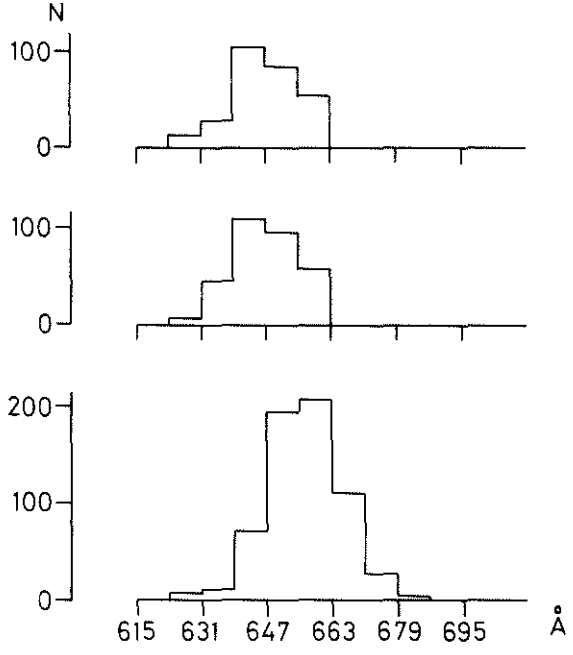


Fig. 2-8. Histogram of the frequency of collagenic microfibril periodicity lengths in Å. Note the overlap in periodicity length of control valves (top) and those after 3 weeks preservation at -20°C (middle). The periodicity lengths after preservation followed by γ-irradiation (bottom) are significantly larger.

preservation show an almost complete overlap. After irradiation 28% of the periodicity lengths are larger than the maximal value of the controls, i.e. larger than 663 Å. There is no difference in the classes below 623 Å which means that the larger standard deviation observed after irradiation is solely due to the occurrence of larger periodicity lengths.

4. Stress - strain measurements of strips of aortic wall.

Stress-strain characteristics of thoracic aortic-wall strips conditioned by the method of Carpentier (1969) were determined according to the procedure used by Clark (1973).

The human aortic-wall strips are characterized (Fig. 2-9.) by an initial

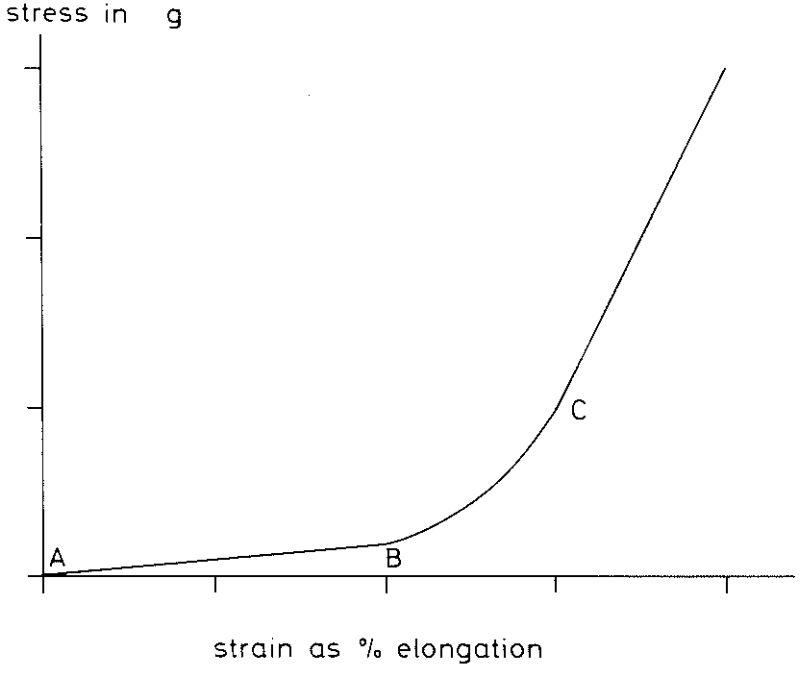


Fig. 2-9. Typical stress-strain behaviour of human aortic-wall tissue.

elongation at low stress (A – B). With additional stress the modulus of elasticity, i.e. stress (g) divided by strain (per cent elongation), changes abruptly (B) and the material stiffens (C). At 50 to 150 per cent elongation, depending on the condition of the aorta, the strips become thinner and rupture at 600 to 1250 9 stress. Clark (1973) has investigated the effect of uncontrolled freezing (to -70°C) on the stress-strain behaviour of the strips of aortic valve leaflets. He found a marked loss of elasticity in strips taken in the radial direction of the valve. We have determined the stress-strain behaviour of strips of aortic-wall taken both in the radial and in the circumferential direction. The results obtained after uncontrolled freezing to -70°C followed by rapid thawing are illustrated in Table 2 - 2.

THE EFFECT OF UNCONTROLLED FREEZING ON STRESS-STRAIN CHARACTERISTICS OF STRIPS OF AORTIC-WALL

Parameter	Fresh		Uncontrolled frozen	
	Circumferential	Radial	Circumferential	Radial
	$\bar{x}$ S.D.	$\bar{x}$ S.D.	$\bar{x}$ S.D.	$\bar{x}$ S.D.
Initial expansion (per cent)	36 ± 7	20 ± 6	24 ± 5	19 ± 6
Elongation at transition (per cent)	62 ± 11	34 ± 7	38 ± 4	30 ± 6
Stress at transition (g)	276 ± 88	317 ± 89	189 ± 38	247 ± 66
Elongation at rupture (per cent)	88 ± 17	46 ± 13	61 ± 20	41 ± 13
Stress at rupture (g)	752 ±225	723 ±172	782 ±211	661 ±159

From these data plotted in Fig. 2-10 it is clear that fresh human aorta is far

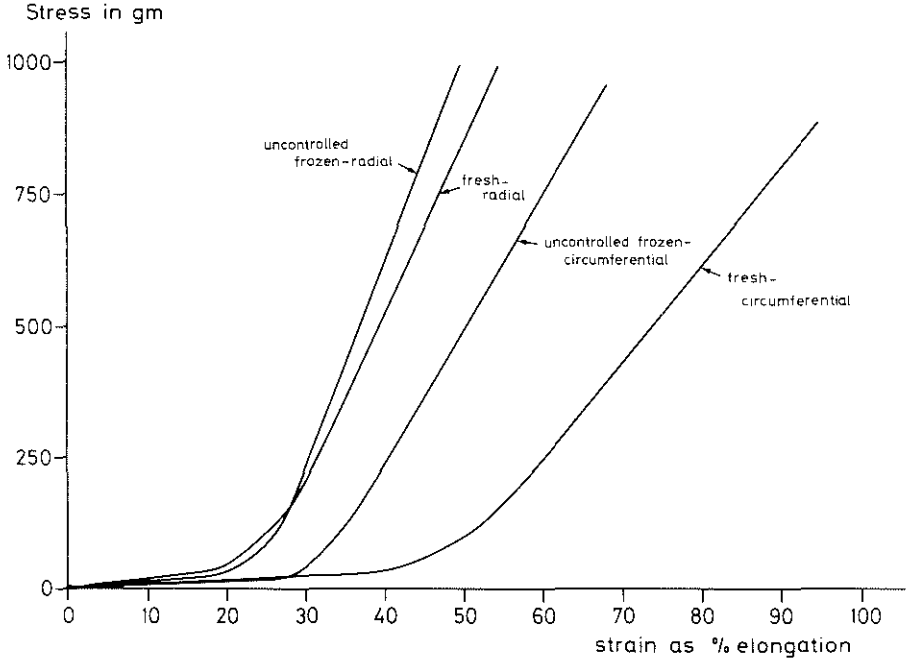


Fig. 2-10. Plot of average values of stress and strain for fresh and uncontrolled frozen human aortic wall strips in the radial and circumferential direction.

more compliant in the circumferential direction than in the radial direction. This is shown both by a lower initial modulus and a longer elongation at the point of transition. Uncontrolled freezing alters the initial elasticity modulus and the point of transition for aortic-wall strips taken in the circumferential direction and most importantly, the aorta looses the ability to elongate under low stress. As when compared with results obtained by Clark (1973) the highest compliance in the aortic-wall is found in the circumferential direction whereas in the aortic valve the highest compliance is observed in the radial direction. The decrease in compliancy after uncontrolled freezing in valve leaflets described by Clark is somewhat larger than measured in our experiments on aortic-wall.

These data show that the effect of sterilization and preservation procedures on the stress-strain behaviour can be reliably tested on aortic-wall provided that one takes into account that the direction of highest compliancy is circumferential and that the effect on aortic-wall is likely to be somewhat less than would be the case for aortic-valve leaflets.

The influence of the conditioning method of Carpentier on the stress-strain behaviour of aortic-wall strips taken in the circumferential direction has been tested. From the data illustrated in Table 2 - 3 and plotted in fig. 2-11 it is clear that this method of conditioning significantly reduces the compliance of the aortic-wall. This is shown by a shorter initial expansion period and a smaller elongation at transition, under the same stress as used for fresh aortic-wall strips. Rupture of the conditioned strips occurred at a lesser elongation than for fresh aorta strips. The most important difference between fresh and conditioned aortic-strips is the loss in ability to elongate under low stress. (Fig. 2-7.)

Parameter	Fresh		Conditioned according to Carpentier	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.
Initial expansion (per cent)	25	$\pm$ 7	6.7	$\pm$ 4
Elongation at transition (per cent)	44	$\pm$ 11	23	$\pm$ 5
Stress at transition (g)	258	$\pm$ 64	231	$\pm$ 70
Elongation at rupture (per cent)	60	$\pm$ 11	42	$\pm$ 8
Stress at rupture (g)	673	$\pm$158	627	$\pm$148

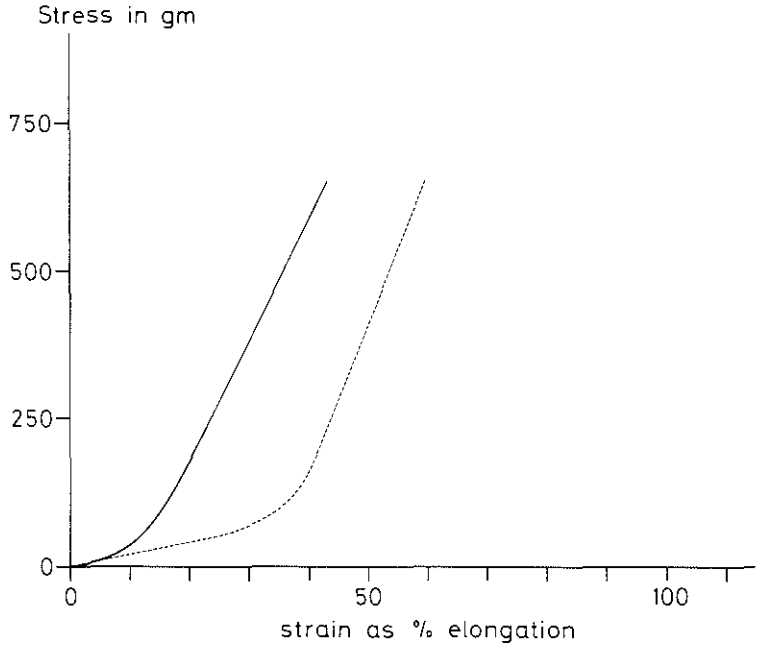


Fig. 2-11. Plot of average values of stress and strain for fresh (....) and conditioned (-) human aortic wall strips in the circumferential direction.

Conclusions

1. Sterilization by 2.5 megarads γ-irradiation, leads to cellular death and changes in the periodicity length of the collagenic microfibrils.
2. Combined sterilization and preservation by the method of Carpentier, et al. (1969) results in cell death and in functional impairment of the valve as indicated by a decreased compliancy.
3. Preservation in Hank's solution at $4^{\circ}C$ or by lyophillization with subsequent storage at $-40^{\circ}C$ or storage at $-40^{\circ}C$ after slow freezing all lead to a-cellular valves with structural alterations of the intercellular matrix of the valve.
4. Based on these effects on the (ultra) structural and on a functional test like the stress-strain behaviour it is unlikely that aortic valves sterilized and preserved with the methods described will exhibit a normal long-term function after transplantation.

Chapter III

FIBROBLAST FUNCTION IN THE MAINTENANCE OF THE AORTIC-VALVE MATRIX

INTRODUCTION

The incidence of valve related complications in aortic valve allografts is still troublesome. (Barratt-Boyes, et al., 1969; Bigelow, et al., 1967a; Barnes, et al., 1970; Wallace, et al., 1974; Lonescu, et al., 1972; Angell, et al., 1973; Carpentier, et al., 1974). Slight malpositioning of the valves produces turbulence and may increase the incidence of degenerative changes. Consequently, perfect insertion of the allograft valve assures more prolonged function. Degenerative changes, such as calcification and cusp rupture, are most likely related to methods of sterilization and preservation (Gonzalez-Lavin, et al., 1972). The disastrous influence of several sterilization and preservation techniques on cells and intercellular components has been illustrated in Chapter II.

Results obtained after transplantation of fresh aortic-valve allografts (Murray, 1956; Barratt-Boyes, 1964; Angel , et al., 1972; Angell, et al., 1973 and Gonzalez-Lavin, et al., 1972) indicate the importance of viable fibroblasts in the transplanted valve tissue. Long-term survival among the patients of Murray and Barratt-Boyes, superiority in long-term performance of viable grafts over non-viable allograft and prostheses as found by Angell (1973), and excellent short-term results of viable grafts reported by Gonzalez-Lavin (1972) all suggest a positive effect of the presence of viable fibroblasts for a good long-term function.

The importance of the fibroblast might be based on its role in the synthesis of

intercellular components. These are essential for the maintenance of an optimal function of the valve. To our knowledge no studies have been described on the function of the fibroblast in aortic valve tissue.

In this chapter experiments will be described by which we have investigated the function of the valve-fibroblast in matrix maintenance. Electronmicroscopy of the valve fibroblast showed the presence of rough endoplasmatic reticulum in these cells (Chapter II), which is indicative for active protein synthesis. This was tested by amino-acid incorporation studies using ^{3}H-proline as a marker and autoradiography as the localization technique. The production and "turn over" of extra cellular component was investigated by long-term incorporation experiments using ^{3}H-proline and ^{3}H-methionine as markers; the latter was used to discriminate between collagenous and non-collagenous products. Purification of calf valve collagen and subsequent amino-acid analysis had demonstrated that methionine is present in minute quantities. In the turn-over studies, autoradiography was used for the localization and liquid scintillation counting was used to obtain quantitative information of the incorporated amino acids. For further definition of the extra cellular products synthesized by the valve fibroblasts, separation of the collagenous from the non-collagenous components was carried out on material derived from rats which had been allowed to incorporate ^{3}H-proline during six weeks.

MATERIALS AND METHODS

Collagen extraction and amino acid analysis.

The aortic valves of five fresh calf hearts were used for collagen extraction according to the method of Harkness, et al., 1954. The valves were cut into small pieces with a scalpel, washed in a.d. for 24 hours and extraction was done by stirring. All steps were carried out at 4°C to prevent bacterial growth,

except stated otherwise. The main steps of the fractionation procedure are shown in Fig. 3-1.

The pieces of tissue were dried and extracted with 0.5 M Na Cl (2.9%) in 0.1M M phosphate, pH 7.4 for 24 hours. The mixture was centrifuged at 5000 rpm (2000 g) for 45 min, the supernate preserved and the extraction repeated with the residue. This method rendered the rough Na Cl - soluble extract (I) . The residue was washed in a.d. and extracted with 0.075 M Sodiumcitrate pH 3.7 for 24 hours after centrifugation at 5000 rpm (2000 g) for 45 min, the extraction was repeated. The supernates constituted the rough citrate-soluble fraction (II). After washing the residue was solubilized as gelatin by adding 20 volumes of water and autoclaving at 124°C for three hours. The remaining insoluble residue was removed from the gelated solution by filtering through a Buchner funnel; the gelatin constituted the insoluble collagen (III). For the purification of the rough Na Cl - soluble extract (I) it was treated with a 3.5 M Na Cl (20%) solution for 24 hours to precipitate the collagen, followed by centrifugation at 20,000 rpm (30,000 g) for 45 min. The procedure was repeated on the supernate the residues resolved in 0.01 M acetic acid and dialyzed against 0.01 M acetic acid to remove the salt. Lyophillization of the dialysate rendered the Na Cl - soluble collagen in pure form (I). For the purification of the citrate-soluble fraction (II), the supernates were dialyzed against 0.02 M trisodium hydrogen phophate, pH 8-9. After precipitation the content of the dialyze-bag was centrifuged at 20,000 rpm (30,000 g) for 45 min, after resolution of the residue in 0.10 M acetic acid the procedure was repeated. Lyophillization of the residue rendered the pure citrate-soluble collagen (II). The protein concentration in the Na Cl -soluble, citrate soluble and insoluble collagen extracts was determined with the Kjeldahl method. A volume of the protein solutions equivalent to about 2.0 u M protein was hydrolyzed by adding 5 ml 6 N HCL and heating in vacuo at 105°C for 22 hours. The HCL in the hydrolysate was removed by evaporation. On the hydrolysates amino acid analysis was done using equipment basically similar to the Beckman automatic analyzer; after amino acid separation in a column filled with ion exchange resins the amino

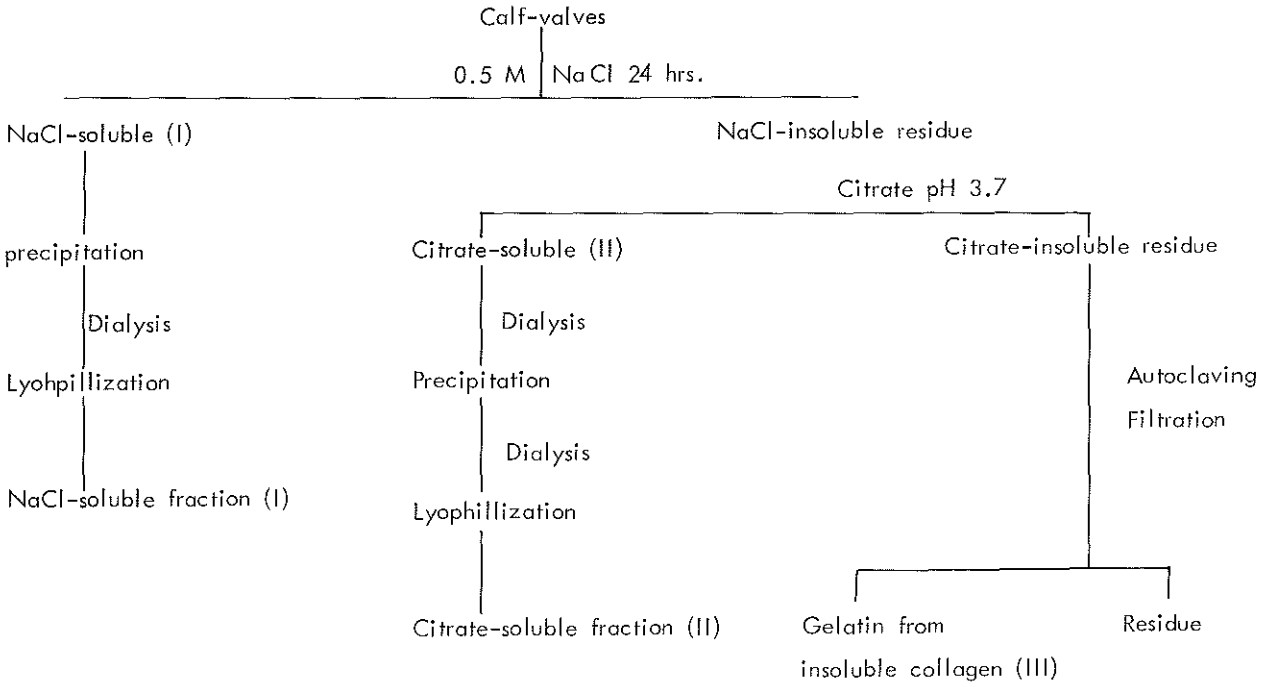


Fig 3-1. Scheme of fractionation of calf aortic-valves, All steps were carried out at $4^{o}C$.

acids were stained with ninhydrin, and the optical density of each reaction product at 570 um was measured by flow colorimetry. The values were plotted against time by a recorder. The absorption at 440 um of the peaks due to proline and OH-proline were plotted by an independent photometer.

INTERRELATION BETWEEN VALVE FIBROBLAST AND INTERCELLULAR MATRIX

A. Localization studies

The interrelation between valve fibroblast and matrix was investigated qualitatively in two main experiments. In the first experiment the incorporation of ^{3}H - proline (spec. act. 10 m Ci/ m mol, Amersham) in aortic valves and costal cartilage was studied. Costal cartilage was used as a reference because of the fact that proline incorporation in this tissue had been studied extensively by others (Malawista and Schubert, 1958; Eastoe, 1961). A group of glaxo-wistar rats (12 wks old, 250 gm) were injected intraperitoneally with 4 uCi ^{3}H-proline/gm body weight. Hearts and pieces of costal cartilage were dissected from the animals at $\frac{1}{2}$ - 1 - 24 - 72 and 168 hours after labelling.

Next to this short-term incorporation experiment two groups of glaxo-wistar rats (12 wks. old, 250 gm) were injected intraperitoneally with an activity of 1 uCi/ gm body weight of respectively ^{3}H-proline (spec. act. 6.6 m Ci/m mol and ^{3}H-methionine (spec. act. 250 uCi/m mol). Hearts were dissected from both groups at 30 min - 60 min - 1 day - 3 days and 1 - 2 - 4 - 8 weeks after labelling. Methionine incorporation was performed to investigate the behaviour of the non-collagenous protein pool.

In both short-term and long-term experiments the material (hearts and costal cartilage) was fixed in 4% formaline; after fixation paraffin sections (5um) of the hearts including the aortic valves and sections of costal cartilage were processed for dipping autoradiography using 1 L ford K-2 emulsion. After

hematoxylin and eosin staining the microscopic localization of the label in aortic valve and costal cartilage was determined. Two rats for each period of incorporation were used.

B. Quantitative experiments

Quantitative assessment of the interrelation between the valve-fibroblast and matrix was performed by a long-term incorporation experiment using ^{3}H-proline and ^{3}H-methionine as markers. Again ^{3}H-methionine was used to test the synthesis of non-collagenous proteins. In this experiment two groups of male glaxo-wistar rats, (12 wks old, 250 gm), were injected intraperitoneally with an activity of 1 u Ci/gm body weight of ^{3}H-proline (spec. act. 6.6m Ci/m mol) or ^{3}H-methionine (spec. act. 250 u Ci/m mol). The hearts were dissected from both groups at 10 - 60 - 180 - 360 min and 1 - 3 days and 1 - 2 - 4 - 8 weeks after labelling. For each period three to four rats sacrificed. The hearts were quickly frozen (- 70°C) and cryostat sections (30 um) were freeze-dried in vacuo. From the lyophillized sections aortic valves were dissected under the microscope and the segments were weighed on a quartzfibre balance according to procedures described by Galjaard, et al. (1970). Subsequently 50 - 150 ugm of isolated aortic valve material was introduced into a scintillation vial, dissolved in soluene and finally 15 ml of scintillation mixture (5 gm PPO plus 0.5 gm POPOP per litre of toluene) was added. One sample per rat was counted in a liquid scintillation counter (Packard), and the results were expressed as number of desintegrations per minute per unit dry weight of aortic-valve. All results were corrected for non-incorporated labelled amino-acids by trichloric-acid precipitation.

For a further characterization of the products still containing ^{3}H-proline six weeks after incorporation, the following experiment was carried out. Eight male glaxo-Wistar rats(12 wks old, 250 gm weight) were injected intraperitoneally with an activity of 2 u Ci/gm body weight of ^{3}H-proline (spec. act. 6.6 m Ci/ m mol). The hearts were dissected from all rats at six weeks after labelling and

aortic valve material was isolated under the microscope fromfrozendried sections according to the method previously described. From pooled aortic-valve material the collagen was solubilized as gelatin by adding 20 vol. of distilled water and autoclaving at 124°C for several hours. The remaining insoluble residue was removed from the gelatin solution by filtering through a Buchner funnel and both fractions were freeze dried. The number of desintegrations per minute per unit dry weight of aortic-valve was counted.

RESULTS

1. Determination of the amino acid composition of calf aortic valve collagen.

The amino acid composition of NaCl-soluble, citrate-soluble and insoluble calf aortic valve collagen is given in Table 3-1 together with the amino acid composition of collagen from human dura mater as a comparison.

As can be concluded from Table 3-1 the amino acid composition of the NaCl-soluble, citrate-soluble and insoluble collagen fractions of calf aortic-valves are basicly the same. There is a high glycine, alanine and (hydroxy) proline content. A 1 : 1 ratio of OH-proline and proline, a roughly 1 : 1 ratio of OH-proline plus proline and glycine and a relatively high number of hydroxylated lysine residues implying a considerable sugar content. There is a complete lack of methionine residues in all three fractions. The difference between calf aortic valve collagen and human dura mater collagen mainly concerns methionine and OH-lysine residues. It should be noted that the number of methionine residues is extremely low in dura mater, while no methionine could be demonstrated in calf aortic valve. In incorporation studies on the synthesis and turn-over of collagen, methionine, is therefore been used as a marker for non-collagenous protein (Carneiro, 1965). On the other hand (hydroxy) proline is known to be mainly incorporated in collagenous protein.

Table 3-1

AMINO ACID COMPOSITION OF CALF AORTIC VALVE COLLAGEN FRACTIONS AND HUMAN DURA MATER COLLAGEN

Amino acid	NaCl-soluble Collagen	Citrate-soluble Collagen	insoluble Collagen	Dura Mater Collagen Harding (1963)
OH-proline	115.8	114.3	112.2	102.0
Asparatic acid	33.9	32.6	33.5	48.9
Threonine	12.9	12.8	13.2	18.3
Serine	28.1	29.7	28.2	31.1
Glutaminic acid	85.3	83.6	84.5	71.6
Proline	115.1	115.6	112.2	125.0
Glycine	296.6	302.4	294.5	327.0
Alanine	131.1	129.0	130.1	106.0
Valine	22.1	21.0	21.3	26.2
Methionine	0.0	0.0	0.0	3.7
Isoleucine	12.9	13.6	13.8	10.7
Leucine	25.4	24.9	25.8	27.1
Norleucine	0.0	0.0	0.0	n.m.*
Tyrosine	1.1	0.9	1.2	4.0
Phenylalanine	13.4	12.8	13.5	14.7
Lysine	24.1	23.2	23.8	25.0
Histidine	3.9	4.2	4.2	5.1
Arginine	50.0	47.3	49.1	47.5
n OH-lysine	28.6	26.9	29.8	4.3
Allo-DH-lysine	8.5	7.8	8.9	n.m.*

*n.m.: not measured

2. Relation between fibroblast and intercellular matrix in aortic valve.

A Qualitative experiments

The relation between valve fibroblast and intercellular matrix was studied by two autoradiographic experiments. The first involved incorporation with ^{3}H-proline which was localized at different periods up to 7 days in aortic heartvalves and costal cartilage which was used as a control. In both tissues after an $\frac{1}{2}$ hour after incorporation labelling was found over the cells; this localization was more strict in the aortic valve than in costal cartilage where part of the incorporated proline had already entered the intercellular matrix (Fig. 3-2, top left and right). After one hour the label was localized over the cellular as well as over the intercellular compartment in both aortic valve and costal cartilage. (Fig. 3-2, middle left and right). At 24 hours after incorporation almost all radioactivity was found in the extracellular compartment in both aortic valve and costal cartilage (Fig. 3-2, bottom left and right). In costal cartilage the decrease of radioactivity in the cells and the concomitant increase of radioactivity in the intercellular compartment starts earlier than in the aortic valve (compare Fig. 3-2, top left and right). The total amount of radioactivity incorporated by the aortic valve seems to be less than in costal cartilage as suggested by the difference in grain intensity. (compare Fig. 3-2, left and right). Grain counts made of several parts of the rat aortic valve showed the highest values at the base of the leaflets i,e. at junction of the collagen layer of the aortic valve with the skeleton of the heart; the photomicrographs shown in Fig. 3-2 were taken from this site of the valve.

These data show the synthesis by the heart valve fibroblast of a proline containing product, which is deposited in the extracellular matrix. The pattern of labelling at various time intervals after incorporation in aortic-valve and costal cartilage suggest that this product is a collagenous protein.

The second experiment was designed to obtain further information on the

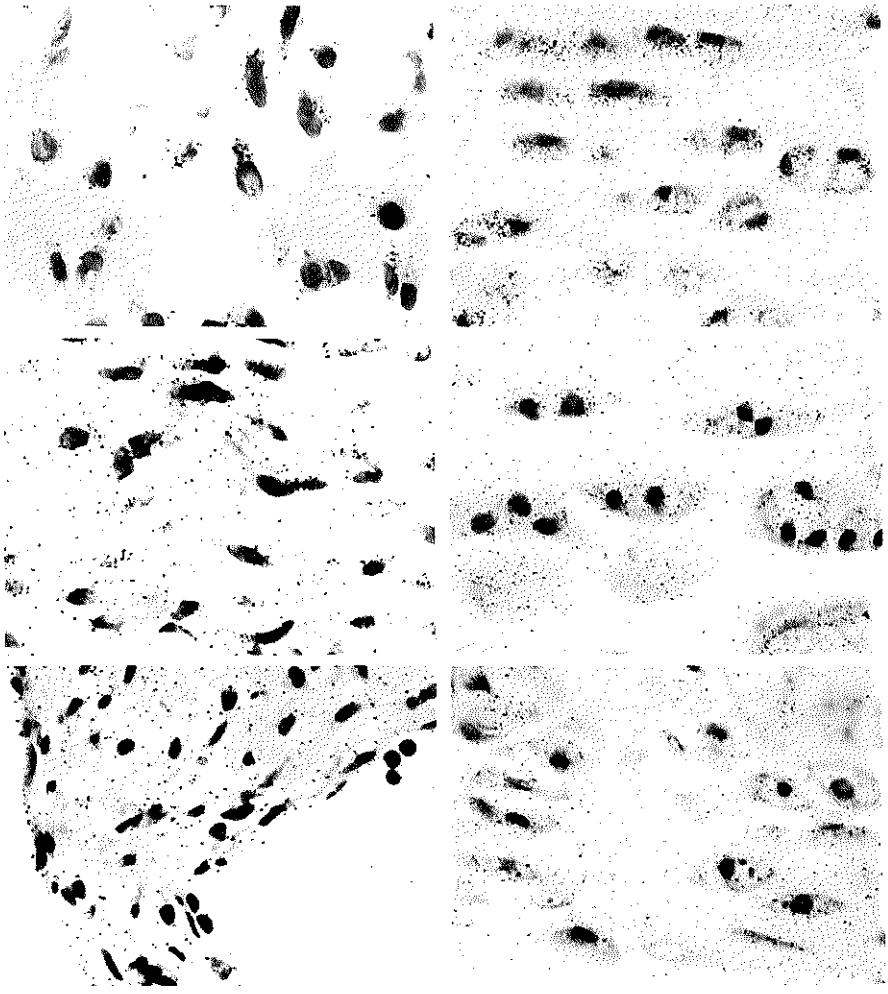


Fig. 3-2. Light microscopic autoradiographs of rat aortic valve (left) and costal cartilage right) after ^{3}H-proline incorporation. Top: At $\frac{1}{2}$ hour after labelling most of the label is localized over the cells. Middle: At 1 hour after labelling the isotope is localized over the cellular as well as over the extra cellular compartment. Bottom: Autoradiographs 24 hours after ^{3}H-proline incorporation show that labelling is merily localized over the extra-cellular compartment.

nature of the product synthesized by the fibroblast in the heart valve. Both ^{3}H-methionine and ^{3}H-proline were used and their incorporation was studied up to eight weeks to gain information about products with a long turn-over time. The behaviour of methionine was used as a marker for non-collagenous protein (see Table 3-1) and ^{3}H-proline incorporation is mainly representative for the synthesis of collagenous protein.

The data represented in Fig. 3-3 show that at two weeks after incorporation hardly any grains can be found over the valves of rats which had been injected with ^{3}H- methionine (Fig. 3-3, bottom right). In constrast in the valves of rats injected with ^{3}H-proline (Fig. 3-3, bottom left) labelling was found over the extracellular compartment at all time intervals even at eight weeks after incorporation (Fig. 3-4).

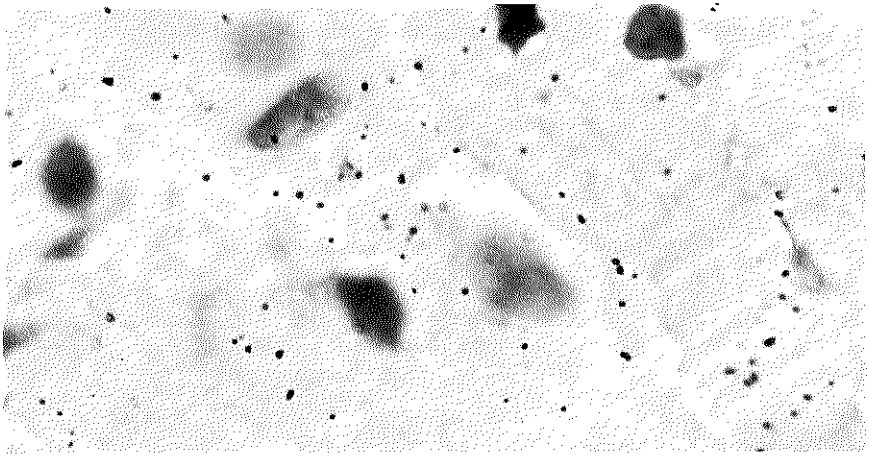


Fig. 3-4. Light microscopic autoradiograph of rat aortic valve 8 weeks after ^{3}H-proline incorporation.

Another difference between the two groups is the confinement of label to the cells in the case of ^{3}H-methionine (Fig. 3-3, d,e,), while most of the ^{3}H-proline label shifted within 24 hours from the cellular to the extracellular compartment of the valve.

B Quantitative analysis

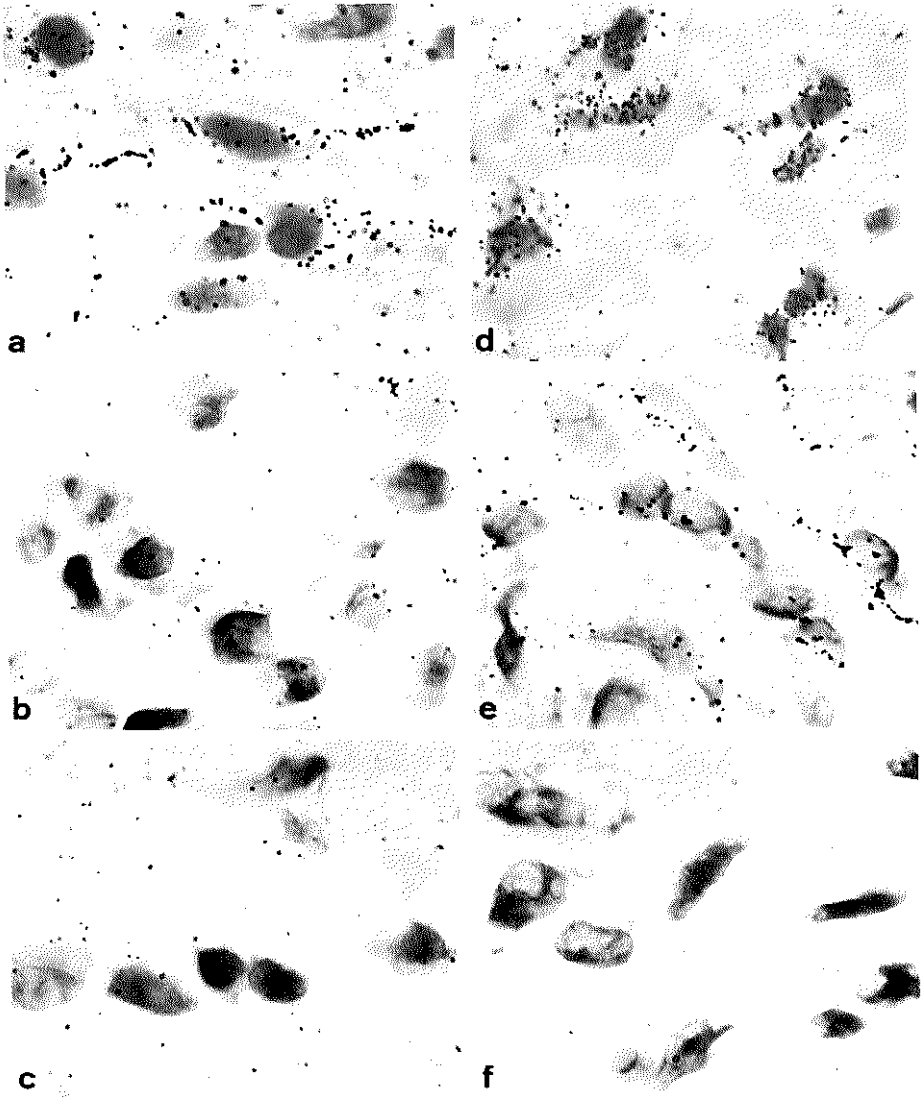


Fig. 3-3. Light microscopic autoradiographs of rat aortic valve after ^{3}H-proline (left) and ^{3}H-methionine (right) incorporation. Autoradiographs at one hour (a), one week (b) and two weeks (c) after injection with ^{3}H-proline. Autoradiographs at one hour (d), one week (e) and two weeks (f) after injection with ^{3}H-methionine.

The amount of incorporated radioactivity after ^{3}H-methionine and ^{3}H-proline injection was also measured using liquid scintillation counting. At times from 1 hour to 8 weeks intervals after labelling cryostat sections from the hearts were freeze-dried, aortic valves were dissected under the microscope and the dry weight of the pooled fragments was determined with a quartz fiber balance. The results of scintillation counting for two groups of animals are represented in Table 3-2.

Table 3-2

COMPARISON OF RADIOACTIVITY IN D.P.M. IN RAT AORTIC VALVE AFTER ^{3}H-METHIONINE AND ^{3}H-PROLINE INCORPORATION AT VARIOUS TIME INTERVALS AFTER ADMINISTRATION

Time interval	^{3}H-methionine	^{3}H-proline
10 min	6020 ± 590 a	4240 ± 380
60 min	5850 ± 720	6540 ± 470
180 min	4540 ± 450	13650 ± 1160
360 min	3370 ± 460	10070 ± 1550
1 day	2290 ± 260	7880 ± 870
3 days	970 ± 160	4830 ± 630
1 week	320 ± 100	3650 ± 210
2 weeks	b	3410 ± 490
4 weeks		3240 ± 300
8 weeks		2990 ± 450

[a]Deviations are represented as standard deviations derived from three analyses

[b]Activities were at background level

In Fig. 3-5 the incorporated radioactivity for ^{3}H-proline and ^{3}H-methionine is plotted against the different time intervals after injection.

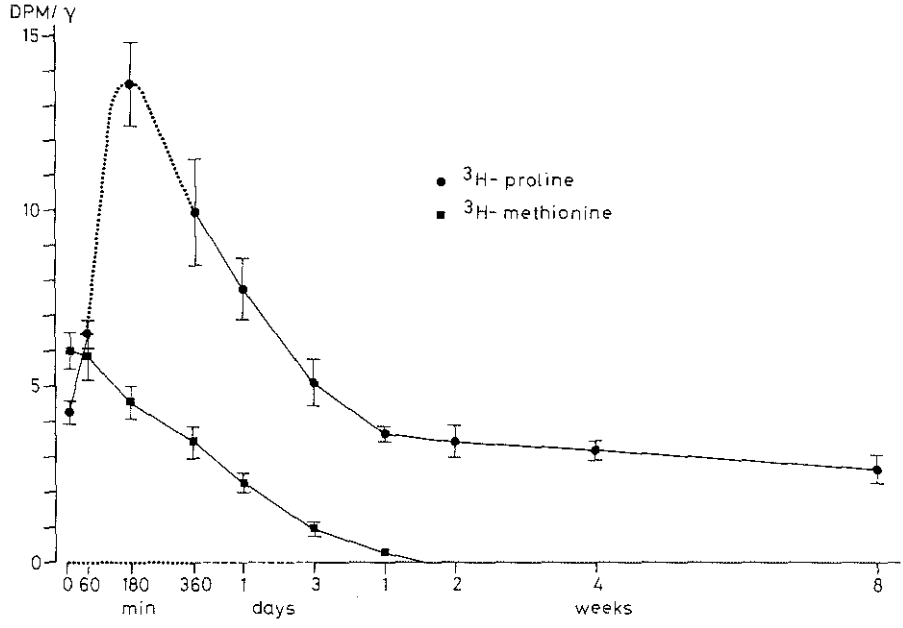


Fig. 3-5. Radioactivity (at different time intervals) after injection of ^{3}H-methionine and ^{3}H-proline. Values for sixty animals (three animals per point) are represented. Deviations are represented as standard deviations for three analysis.

The data show that after a time interval of one week the ^{3}H-methionine activity falls rapidly to background levels. The ^{3}H-proline activity is maintained at a fairly constant level up to eight weeks after an initial decline in activity during the first two weeks. These data combined with results of the autoradiography indicate that ^{3}H-methionine and part of the ^{3}H-proline are incorporated in non-collagenous intracellular products with a turn-over time of about two weeks. Part of the ^{3}H-proline is incorporated in a product that is secreted into the extracellular matrix and which has a turn-over time of at least eight weeks.

The nature of this latter constituent of the valve matrix, was further investigated by the following experiment. Aortic valve material from eight rats injected with ^{3}H-proline was sampled six weeks after labelling. After separation of the collagenous and non-collagenous material the radioactivity was determined in both fractions by scintillation counting (Table 3-3).

TABLE 3-3

RADIOACTIVITY IN FRACTIONS OF ISOLATED AORTIC HEART-VALVES SIX WEEKS AFTER ^{3}H-PROLINE LABELLING

Collagenous proteins	Non collagenous material plus rest activity of non-separated collagen
6530 $\pm$ 610	1070 $\pm$ 140

Radioactivity is expressed as mean value of D.P.M. per mg dry weight of isolated aortic heartvalves and standard deviation are indicated for 3 analyses.

About 90% of the radioactivity is found to be localized in the gelatinized collagenous fraction proving the synthesis of this extracellular protein in normal rat aortic valves. The 13% activity found in the other fraction may be derived from non-collagenous extracellular products or from non-separated collagen.

Conclusions

1. The amino acid composition of calf aortic valve collagen shows a relatively high content of glycine, (hydroxy) proline residues and there is a lack of methionine. This makes methionine acceptable as a marker for synthesis of non-collagenous protein, whereas proline is known to be a good marker in collagen.

2. autoradiography of rat aortic valves shows that the incorporation pattern of ^{3}H-proline is quite similar to that in costal cartilage. After initial incorporation into valve fibroblasts labelled products are secreted into the intercellular matrix. Radioactivity in the latter compartment can be shown (at least 8 weeks) after incorporation.

3. Autoradiography and scintillation counting after ^{3}H-proline and ^{3}H-methionine administration shows that the latter amino acid is incorporated intracellulary only, and its turn-over time is fairly rapid (radioactivity disappears within two weeks after labelling).

4. Separation studies six weeks after labelling show that merely 90% of the ^{3}H-proline incorporation occurs in the collagenous protein fraction of the heart valve.

5. These experiments demonstrate that the fibroblasts in the aortic valve are actively engaged in collagen synthesis and its secretion into the intercellular matrix. The collagen of the matrix is replaced slowly with a turn-over time which is longer than 8 weeks.

6. As collagen is one of the major constituent which is necessary for an optimal valve function, its production and hence the presence of viable fibroblasts seems mandatory for maintaining a good valve function after transplantation.

Chapter IV

DEVELOPMENT OF A PRESERVATION METHOD ALLOWING MAINTENANCE OF CELL-VIABILITY

INTRODUCTION

The viability of the valve fibroblasts is essential for maintaining the valve-matrix and hence for the valve function since proper functioning is dependent on the anatomical and functional integrity of the valve. Thus, in the preserved aortic-valve graft, the survival of the fibroblast is paramount.

The first allovital valve transplantations were performed with sterile grafts, which were temporary stored in a salt solution with or without antibiotics (Murray, 1956; Barratt-Boyes, 1964; Duran, et al., 1965; Barratt-Boyes, et al., 1969). Replacement of the mitral valve with aortic-allografts, fixed on a supportive strut, sterilized and stored in nutrient medium 199-IX containing antibiotics, was performed both experimentally and clinically by Angell, et al. (1967 and 1969a) and Bigelow et al. (1967b). The same procedure was used for multiple valve replacement in the mitral and aortic area (Angell, et al., 1968). A number of the first human allovital valve transplantations by Murray and Barratt-Boyes were successful. In the more extended series of Angell (1972) promising results were obtained with mitral valve replacement and especially with aortic-valve replacement
However such transplantations require at the moment of surgery the availability of allograft-valves with orifice diameters comparable to the diameter of aortic-valve ostium of the acceptor. This work was carried out with fresh material without a qualitative or qualitative assessment of its viability. Al-Janabi, et al. (1972) used ^{3}H-thymidine incorporation as a viability test for valves stored in a balanced salt solution with antibiotics.

The use of the nutrient medium 199-IX instead of the salt solution resulted in prolonged viability (Al-Janabi, and Ross, 1973). This method, however, can only be used in case of relative short preservation periods (up till 8 weeks) and is not suitable for long-term preservation which will be needed by many centres. Controlled freezing, to low temperature with protective agents has been used successfully as a preservation method retaining cell-viability. The method was introduced by Polge, et al. (1949) and used for the preservation of sperm (Bunge, and Sherman, 1953; Friberg, and Gemzell, 1973), erythrocytes (Lovelock, 1953), bone marrow (Schaefer, et al., 1972; O'Grady and Lewis, 1972), mammalian cell-lines (Mazur, 1965), skin (Billingham and Medawar, 1952; Bondoc and Burke, 1971) and other tissues. This controlled freezing technique renders the possibility for long-term preservation of aortic-heart valves with maintenance of cell-viability.

In experiments described in the next paragraphs we have investigated whether controlled freezing could also be used as a long-term preservation method for heart valves. The viability of the valve fibroblasts, their localization and their capacity of protein synthesis after ^{3}H-proline incorporation has been studied in relation to various freezing procedures. Also, the structural and functional characteristics of the valve-matrix were investigated, using lightmicroscopy, electronmicroscopy of isolated collagenic fibrils and measurements of the stress-strain characteristics. The sterilization method described by Lockey (1972) was used throughout the experiments after testing its validity with respect to sterilizing capacity and its effect on cell viability. The experimental part of this thesis will be concluded with the description of a method which enables any period of preservation of heart valves with maintenance of cell viability, throughout storage and which leaves the structural and functional integrity of the valve matrix intact.

MATERIALS AND METHODS

Aortic heartvalves wereobtained from dogs that were sacrificed for sampling of donorblood. Also autopsy material from people who had no diseases which primarily or secundarily involved the aortic-valves were used throughout the experiments. All valve systems were divided in six equal parts according to Fig. 2-1, chapter 2 and one piece was always used as a control.

Test for cell viability and protein synthesis

Control valves and valves preserved by different ways of controlled freezing were treated in the following way. Aortic valve tissue attached to a piece of aortic wall was incubated during 2,4,6 or 8 hours in Ham's F 10 nutrient medium containing 0.15 ml calf serum and 15 u Ci ^{3}H-proline (spect. act. 10 m Ci/ m mol) per ml. After incubation at 37^{o}C under air containing 5% CO_2 the tissue was washed three times 15 minutes with saline containing cold proline to remove all non-incorporated ^{3}H-proline. After fixation in 4% formalin paraffin sections (5 um) of the valves were processed for dipping autoradiography using IL ford K-2 emulsion and exposure at 4^{o}C for six weeks. After hematoxylin and eosin staining the microscopic localization of labelled fibroblasts was determined in three widely separated parts for each valve. The total number of labelled and unlabelled cells was scored in five non-selected fields of view (microscopic magnification 400 X). The percentage of cells which had incorporated ^{3}H-proline was considered as a good parameter for cell viability and ability for protein synthesis. The results for preserved valves were compared with those of the control valve. From this comparison percentages for cell-survival can be derived for valves which have been preserved accordingly to different methods. Assessment of cell-survival in the aortic wall was performed in those samples that had retained optimal survival of the fibroblasts in the aortic valve. To determine whether

the presence of ^{3}H-proline in the valves was dependent on cell viability only or was possibly related to insufficient removal of non-incorporated label, ^{3}H-proline incorporation was performed in valve pieces which were rapidly frozen and thawned thus destroying the cells. To ensure that the ^{3}H-proline incorporation found was an indication for protein synthesis a number of valves were treated with cycloheximide (20 ug/ml), 30 minutes before and during proline incubation; the results for valves where protein synthesis had been blocked were compared with those in non-treated control valves.

Aortic valve sterilization*

Aortic valves were obtained from autopsy within 24 hours after death and performed without any attempt at sterility. The dissected valve and several pieces of aortic wall were immersed in a solution, containing several antibiotics as described by Lockey, et al. (1972) (See Table 4-1). After 24 hrs at 4°C the pieces of aortic wall and 5 ml nutrient medium were removed under sterile precautions and these were tested for growth of aerobic and anaerobic bacteria as well as fungi and yeast at 4°C, 20°C and 37°C for 3 weeks.

The valve tissue was tested both before and after revoval of the antibiotics after 24 hours incubation in NHH solution (Table 4-1). Sterility was also tested after storage of heart valves under optimal conditions of preservation by controlled freezing.

To determine the influence of the sterilization procedure on the survival of valve fibroblasts, tests for cell viability as described above were performed on non-sterilized and sterilized aortic valves.

*Tests for growth of micro organisms were performed by the Department of Clinical Microbiology of the Erasmus University and University Hospital at Rotterdam (Prof. Dr. M.F. Michel).

TABLE 4-1

COMPOSITION OF THE NHH* SOLUTION ACCORDING TO LOCKEY, ET. AL., 1972 FOR STERILIZATION OF HEART VALVES

Gentamycine	4 mg/ml	
		In Ham's F 10 nutrient
Methicillin	10 mg/ml	
		medium + 10% calf serum
Erythromycin Lactobionate	6 mg/ml	The pH was adjusted to neutrality with sodium-bicarbonate
Nystatin	2,500 Units/ml	

*NHH: National Heart Hospital

Preservation procedures

As a method for preservation, controlled freezing was chosen especially for its advantage in long-term storage. The technique is based on the fact that freezing at a controlled rate in combination with cryoprotective agents prevents cell damage by intracellular ice-crystal formation and osmotically induced alterations.

Aortic valves were immersed in an open vial containing 5 ml Ham's F 10 nutrient medium supplemented with 15% calf-serum. After addition of the cryoprotective agent (dimethylsulfoxide, glycerol or ethylene glycol) during various time intervals and at different temperatures freezing was carried out at a rate of $1^{\circ}C$/min, found to be optimal for human skin by Billingham and Medawar (1952). The freezing rate was controlled by a cryoson biological freezer type BV-4 connected with a cryoson pressure tank type 200 VLR. When the temperature of the valves reached $-90^{\circ}C$ they were rapidly cooled down with liquid nitrogen till $-196^{\circ}C$. After storage at $-196^{\circ}C$ the vials were thawned rapidly in a $40^{\circ}C$ waterbath immediately thereafter replacement of the immersion medium by Ham's F 10 nutrient

medium without cryoprotective agent was started. The time period used for this procedure ranged from 15 to 60 minutes to determine its influence on cell-survival as reported by Schaefer et al., 1972. Subsequently the valves were tested for cell-viability and cell-function by ^{3}H-proline incubation studies. The following conditions for controlled freezing were investigated:

1. type and concentration of cryoprotective agent
2. incubation conditions of the valve with the cryoprotective agent prior to freezing
3. the removal of the cryoprotective agent after storage and prior to the use of the valve.

Effects of preservation on the valve matrix

The preservation of the structure and function of the valve-matrix throughout sterilization, freezing, storage and thawing was assessed by the following methods. Light microscopy after hematoxylin-azophloxin-saffran staining to study the cellular and intercellular components. Electron-microscopy of isolated collagenic microfibrils after negative staining was carried out for an analysis of the banding patterns according to the method described in Chapter II. Finally the effect of the various preservation methods on valve function was studied by analysing the stress-strain characteristics of strips of aortic-wall according to the method described in Chapter II.

RESULTS

1. Sterility of aortic heartvalves

fourteen human aortic valve systems and several pieces of aortic wall were sterilized in nutrient medium containing NHH antibiotic solution at 4°C for

24 hours according to Lockey, et al., 1972. The nutrient medium and pieces of aortic wall were tested for growth of aerobic and anaerobic bacteria, yeast and fungi. Cultures at 4°, 20° and $37^{\circ}C$ were prolonged to 3 weeks before sterility was concluded. Half of the samples were tested after removal of the antibiotic solution by repeated washing in saline. All samples except one were found to be sterile; the contaminated sample containing proteus mirables belonged to the valve group from which antibiotics had been removed. Five samples of aortic valve tested for contamination after storage in liquid nitrogen and thawing were all found to be sterile.

2. Incorporation of ^{3}H-proline in fibroblasts of "fresh" canine aortic valve

The percentage of fibroblasts labelled by autoradiography with ^{3}H-proline was estimated after incubation periods of 2 - 4 - 6 and 8 hours (see Fig. 4-1).

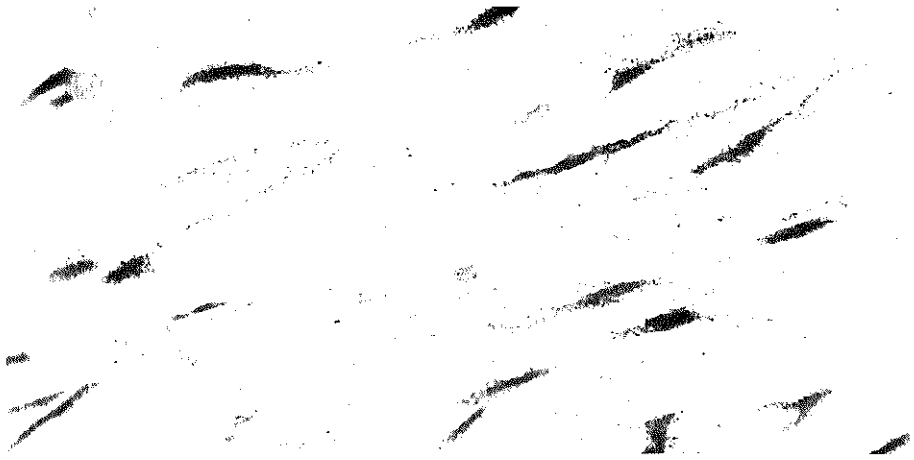


Fig. 4-1. Light-microscopic autoradiograph (magnification 400X) of dog aortic valve after incubation with ^{3}H-proline for six hours. A high number of labelled fibroblasts with thin cytoplasmic extensions is present.

For every time interval six valve pieces derived from different individuals were used. Fig. 4-2 shows that prolongation of the incubation period results

in an increasing percentage of labelled cells untill six hours.

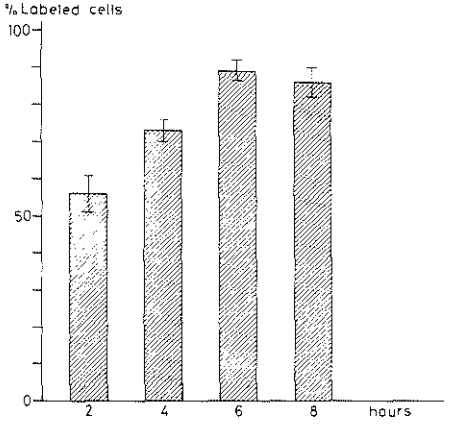


Fig. 4-2. Percentage labelled cells in canine aortic valves after incubation with ^{3}H-proline for different periods. Six valve pieces were used for every period tested. Standard error of the mean is indicated.

The percentage of labelled cells was 56 $\pm$ 5 after two hours incubation, 73 $\pm$ 3 after four hours, 89 $\pm$ 3 after six hours and 86 $\pm$ 4 after 8 hours. After repeated freeze-thawing and subsequent incubation with ^{3}H-proline valves did not contain significant amount of label after a six hour incubation period. The same result was found in valves treated with cycloheximide before proline incubation. This indicates that the labelling of fibroblasts after ^{3}H-proline incubation is a good maker for cell viability and for their ability of collagen synthesis (see also Chapter II).

3. Influence of sterilization on cell-viability

Of ten canine aortic valve systems one valve piece was incubated directly with ^{3}H-proline without previous sterilization whereas another piece was incubated with ^{3}H-proline after sterilization in NHH antibiotic solution. In all cases the number of labelled and unlabelled cells was determined; for each valve pair the percentage of labelled cells in the non sterilized valves was used as the hundred percent reference value. The results of this experiment represented in Table 4-2, show an average loss of viable cells of about 15 percent in valves kept in NHH antibiotic solution for 24 hours. If valves were kept in NHH antibiotic solution for longer periods (48 to 120

hours) a considerable greater cell-loss was found. If the period of sterilization was prolonged till 120 hours loss of viable cells was virtually 100 percent.

Table 4-2

INFLUENCE OF 24 HOURS STERILIZATION IN NHH ANTIBIOTIC SOLUTION AT 4°C ON CELL-SURVIVAL IN CANINE AORTIC VALVES

% labelled cells[a] before sterilization	% labelled cells after sterilization	% cell-loss in sterilized valves
94	88	7
86	68	21
96	72	25
82	67	18
75	66	12
87	77	11
88	70	20
91	86	6
90	76	16
85	73	14
$\bar{x} = 87$	$\bar{x} = 74$	$\bar{x} = 15$

[a]Valves were incubated with ^{3}H-proline for six hours

Since the sterilization capacity of 24 hours incubation in NHH antibiotic solution was found to be sufficient and as the loss of viable cell remains within acceptable limits we have selected a 24 hour sterilization period for the routine procedure.

4. The influence of different cryoprotective agents on cell-survival

The influence of the most commonly used cryoprotective agents, ethylene glycol, glycerol and dimethylsulfoxide on cell-survival was investigated by incorporation of ^{3}H-proline in canine aortic valve fibroblasts (see Fig. 4-3)

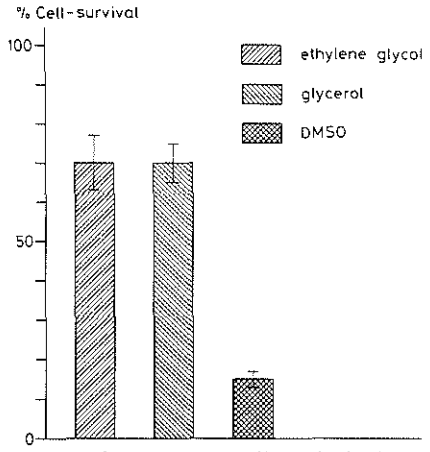


Fig. 4-3. Influence of cryoprotective agent on cell-survival. For each method tested five valve pieces were derived from different valve systems in such a way that factors like donor age, valve condition etc. were eliminated.

The concentration of the cryprotective agent, was 10 percent v/v with an incubation time of 30 minutes at 20°C and a period of removal of the cryoprotective agent after freezing and thawing of 60 minutes. The results in Fig. 4-3 show that 70% cell survival was found for glycerol and ethylene glycol and only 15% for dimethylsulfoxide (DMSO).

Following this initial test a number of experiments were performed with ethylene glycol, glycerol and DMSO as cryoproctective agents. The concentration of agent, the conditions of incubation, and the procedure of thawing and removal of the cryoprotective agent after freezing and thawing were varied according to table 4-3.

The influence of different incubation temperatures on cell-survival in canine aortic valves after controlled freezing with 10% ethylene glycol.

This influence was tested by ^{3}H-proline incorporation in valves that were frozenafter incubation during 30 minutes at 4°C, 20°C and 37°C with 10% ethylene glycol. Again the period of removal by dilution of the cryoprotective agent was 60 minutes. The results of this experiment are illustrated in Fig. 4-4. The results illustrate that the highest percentage of cell-survival (70%) is scored when incubation with ethylene glycol is

Table 4-3: Variation of the indicated parameters during the controlled freezing experiments

Cryoproctective agent	Concentration (percent v/v)	Incubation time (minutes)	Incubation temperature (°C)	Removal period after freezing, storage and thawing (minutes)
Ethylene glycol	5-10-15-20	1-15-30	4-20-37	15-30-60
Glycerol	5-10-15-20	1-15-30	4-20-37	15-30-60
Dimethylsulfoxide	5-10-15-20	1-15-30	4-20-37	15-30-60

performed at 20°C.

The influence of different incubation times with the cryoprotective agent on cell-survival was tested in the following way. Five pieces of different canine aortic valve systems were incubated with 10% ethylene glycol during 1,15 and 30 minutes at 20°C. The removal of the cryoprotective agent was carried out as described above. The results shown in Fig. 4-5 indicate that the highest percentage of cell-survival (79%) was obtained with a 15 minute incubation period.

The influence of different ethylene glycol concentrations on cell-survival was tested by comparing ^{3}H-proline incorporation after freezing with concentrations of 5, 10, 15 and 20% v/v. The temperature was kept at 20°C and the period of incubation was 15 minutes. Removal of the cryoprotective agent was done as described earlier. The result of this experiment is illustrated in Fig. 4-6. A concentration of 10% (v/v) yields the best results in terms of cell-survival (79%).

Finally the influence of different times of removal by dilution of the cryoprotective agent on cell-survival was tested in canine aortic valves. After freezing with 10% ethylene glycol incubated during 15 minutes at 20°C, the cryoprotective agent was removed by dilution during 15,30 and 60 minutes. The percentage of cell-survival as determined by ^{3}H-proline incorporation is illustrated in Fig. 4-7. It shows that a period of dilution of 60 minutes is required to obtain the best results (79%) concerning cell-survival.

The conclusion of all these experiments is that cell-survival is optimal (79%) for ethylene glycol if this is used in a 10% concentration, incubated during 15 minutes at 20°C and if it is removed after freezing by dilution during one hour.

In the following experiments different conditions of controlled freezing with glycerol as cryoprotective agent were tested. Conditions were varied according to table 4-2.

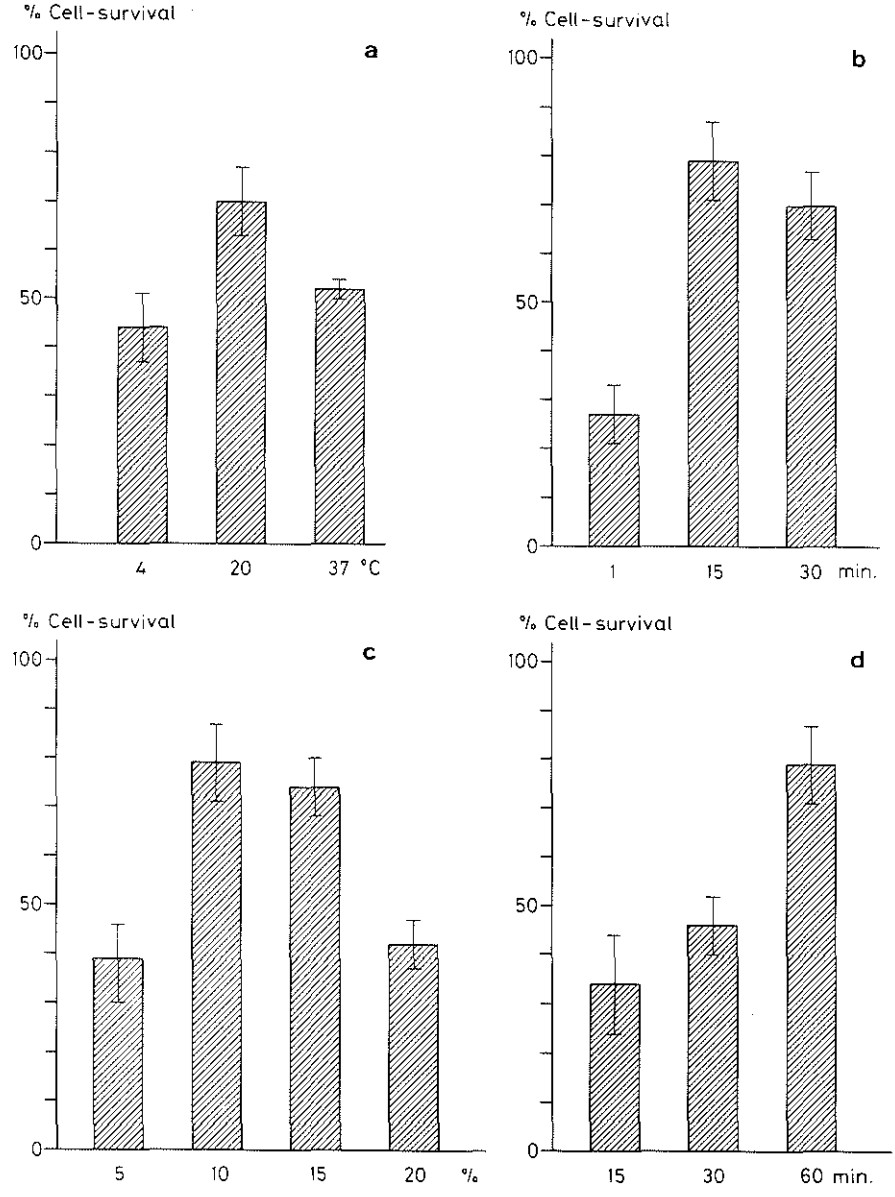


Fig. 4-4, -5, -6, -7. a. Effect of incubation temp. with ethylene glycol on cell-survival. b. Effect of incubation time with ethylene glycol on cell-survival. c. Effect of percentage ethylene glycol on cell survival. d. Effect of removal period of ethylene glycol on cell-survival.

Mean values of five valve pieces of different valve systems for every condition tested with standard error of the mean are represented.

The influence of different incubation temperatures on cell-survival in canine aortic valves after controlled freezing was tested by ^{3}H-proline incorporation after freezing with 10% glycerol. Incubation was carried out at 4°C, 20°C and 37°C during 30 minutes. ^{3}H-proline incorporation in valve fibroblasts was determined after removal of the cryoprotective agent during a period of 60 minutes. The results illustrated in Fig. 4-8 show that the highest percentage of cell-survival (70%) is scored when incubation with glycerol is performed at 20°C.

The influence of incubation time with 10% glycerol on cell-survival in canine aortic valves was tested at 37°C for periods of 1, 15 and 30 minutes. ^{3}H-proline incorporation in valve fibroblasts was measured after removal by dilution of the glycerol during an one hour period. The results of this experiment (Fig. 4-9) show that the highest percentage of cell-survival (78%) was obtained with a 30 minutes incubation period.

The influence of concentrations of 5, 10, 15 and 20 percent v/v of glycerol on cell-survival was tested for an incubation period of 30 minutes at 37°C. ^{3}H-proline incorporation was determined after removal of the cryoprotective agent as described above. The effects on cell-survival (Fig. 4-10) show that the best results are obtained when a 10% (v/v) glycerol solution is used. The difference with a 15% and 20% solution are, however, relatively small.

The influence of various removal periods was measured after controlled freezing with 10% glycerol and 30 minutes incubation at 37°C. The results in Fig. 4-11 show that the best results regarding cell-survival are obtained if the glycerol is removed during a dilution period of one hour. This is similar to the results obtained with ethylene glycol.

In conclusion optimal cell-survival (78%) with glycerol as a protective agent can be realized with a 10% solution, incubation during 30 minutes at 37°C and removal during one hour dilution.

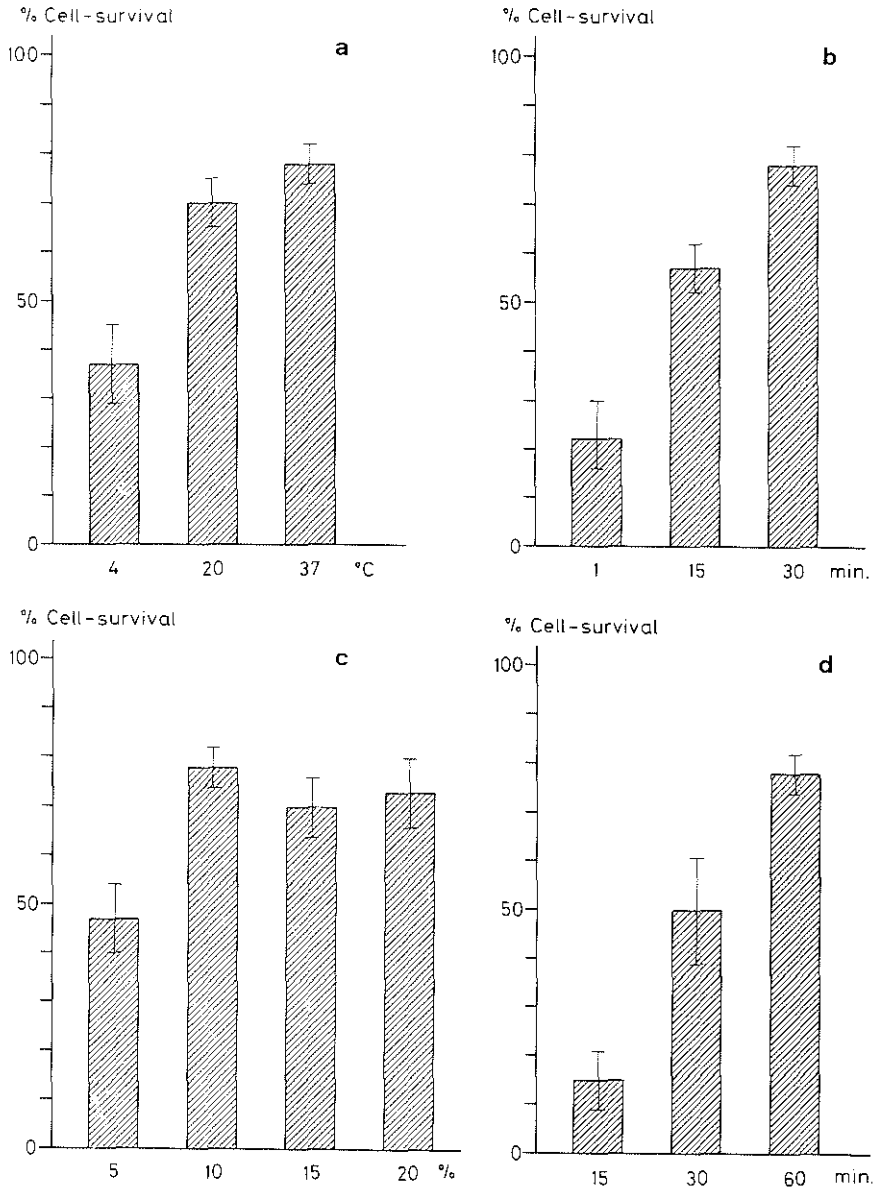


Fig. 4-8, -9, -10, -11. a. Effect of incubation temp. with glycerol on cell-survival. b. Effect of incubation time with glycerol on cell-survival. c. Effect of percentage glycerol on cell-survival. d. Effect of removal period of glycerol on cell-survival.

Mean values of five valve pieces of different valve systems for every condition tested with standard errors of the mean are represented.

Finally the effects of incubation time, incubation temperature and the concentration of the third cryoprotectiveagent, dimethylsulfoxide (DMSO) were tested. The conditions were varied according to table 4-2.

The results for cell-survival in canine aortic valves after controlled freezing with DMSO are presented in Fig. 4-12; 4-13; 4-14 and 4-15 respectively.

Contrary to the findings with other cryoprotective agents, with DMSO controlled freezing yielded the best results if incubation was performed at $4^{o}C$ (Fig. 4-12). Short incubation periods (1 min. - 15 min.) at $4^{o}C$ gave the best results in case of a 10% DMSO solution (Fig. 4-13). The DMSO concentration was optimal at ten to fifteen percent with an incubation period of 1 minute at $4^{o}C$. These conditions resulted in a cell-survival of 64% and 58% respectively as illustrated in Fig. 4-14. The influence of dilution time on cell-survival in canine aortic valves is shown in Fig. 4-15. The best results (81% cell-survival) are obtained when the cryoprotective agent is removed during a short dilution period of 15 minutes. These experiments were carried out after controlled freezing with a 10% DMSO solution and incubation during one minute at $4^{o}C$.

When comparing all different conditions with the three cryoprotective agents tested the highest percentage of cell-survival (81%) was obtained with a 10% DMSO solution, and incubation during one minute at $4^{o}C$. After freezing, storage and thawing removal of the DMSO has to be carried out by dilution during 15 minutes.

Throughout these experiments the total number of tritium labelled and non-labelled fibroblasts in frozen valve pieces was comparable to the total number of fibroblasts in control aortic valve pieces.

The optimal conditions for controlled freezing of canine aortic valves with ethylene glycol, glycerol and dimethylsulfoxide were tested for cell-suvival in human material by the following experiments.

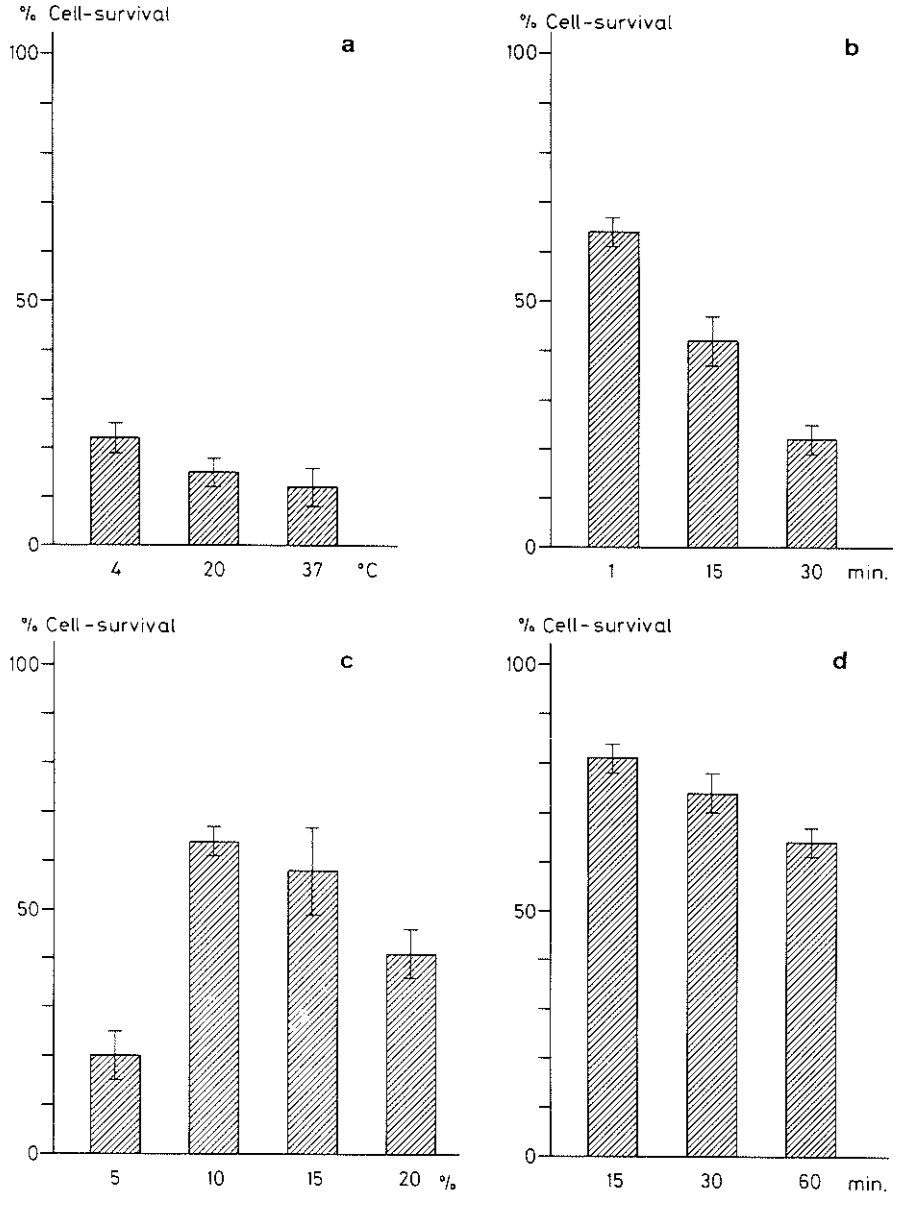


Fig. 4-12, -13, -14, -15. a. Effect of incubation temp. with DMSO on cell-survival. b. Effect of incubation time with DMSO on cell-survival. c. Effect of percentage DMSO on cell-survival. d. Effect of removal period of DMSO on cell-survival.

Mean values of five valve pieces of different valve systems for every condition tested with standard errors of the mean are represented.

The effect of dimethylsulfoxide (DMSO) on cell-survival in human aortic valve and aortic wall after controlled freezing

Twenty pieces from different human valve systems were incubated in Ham's F 10 nutrient medium containing 15% calf serum and 10% DMSO. Controlled freezing down to -90°C was carried out at a cooling rate of 1°C per minute. After snap freezing to -196°C the valves were thawned rapidly and the cryprotective agent was removed by dilution during a period of 15 minutes. Cell survival as calculated from ^{3}H-proline incorporation was compared for one minute and 15 minutes incubation periods with DMSO at 4°C. All experiments were carried out with human material placed in the glass vial illustrated in Fig. 4-16.

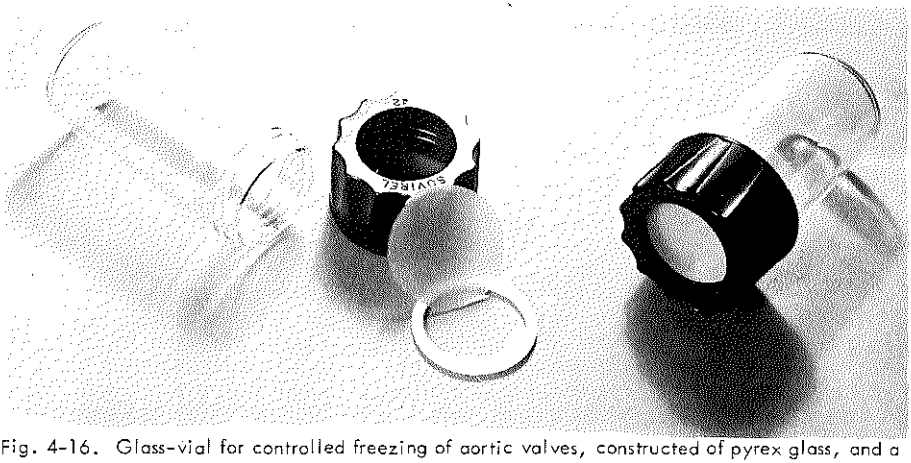


Fig. 4-16. Glass-vial for controlled freezing of aortic valves, constructed of pyrex glass, and a screw-cap of bakelite, with a silicone centre part to form a pressure-valve, necessary whenever liquid nitrogen has entered the vial. A teflon ring is used to assure good closure.

This vial was constructed to withstand both high temperatures necessary for sterilization of the vial and very low temperatures (-190°C) used for preservation. The results in cell survival in human aortic valve and aortic wall presented in Fig. 4-17 show that the highest values are obtained with a short incubation period. In aortic valve there is a mean cell-survival of 88% $\pm$ 1.5% after an incubation during 1 minute. In aortic wall cell-survival is much lower but again the best results are obtained with short incubation periods.

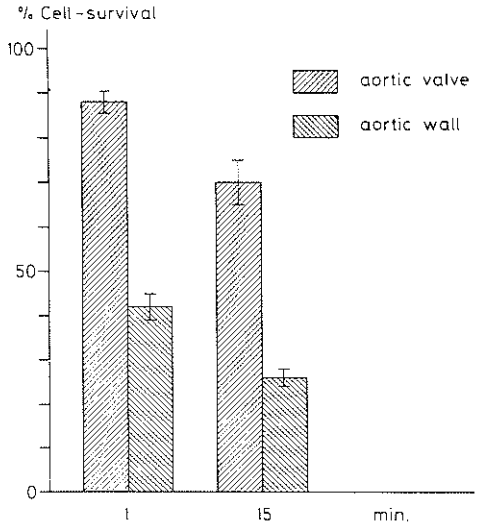


Fig. 4-17. Comparison of cell-survival in human aortic valve and aortic wall after various incubation times with 10% DMSO, at an incubation temp. of 4°C. Twenty valve pieces of different valve systems were used for every incubation period tested. Standard errors of the mean are represented.

The influence of ethylene glycol and glycerol on cell-survival in human aortic valves

The influence of ethylene glycol and glycerol on cell-survival in human aortic valves was tested under conditions that were found to be optimal in experiments on canine aortic valves. Ethylene glycol was used in a concentration of 10% v/v with an incubation time of 15 minutes at 20°C. Glycerol was used in a concentration of 10% v/v with an incubation time of 30 minutes after 37°C. The dilution time for removal of the cryoprotective agent was limited to 15 minutes in spite of better results obtained in canine aortic valve with longer periods (see Fig. 4-7 and 4-11). Limitation of the dilution time to short periods is mandatory in the practice of aortic valve transplantation because longer periods of valve preparation would imply a non desirable prolongation of surgery. The results illustrated in Fig. 4-18 show that the percentages of cell-survival are 16% and 23% for ethylene glycol and for glycerol respectively. These values correspond

with the low values obtained under similar conditions for canine aortic valve (Fig. 4-7; 4-11).

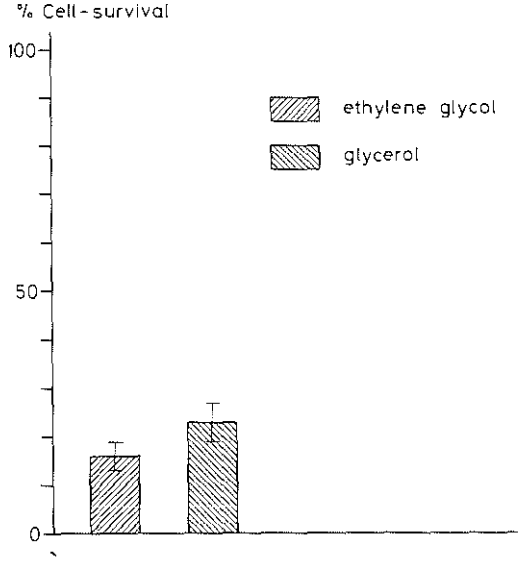


Fig. 4-18. Influence of ethylene glycol and glycerol on cell-survival in human aortic valve, after a removal period of 15 min. Ten valve pieces of different valve systems were used for every cryoprotective agent tested. Standard errors of the mean are indicated.

The experiments with controlled freezing of human aortic valves indicate that a high percentage of cell-survival (88% $\pm$ 1.5) can be obtained with DMSO as a cryoprotective agent. Other agents like ethylene glycol or glycerol, although yielding a good cell-survival under experimental conditions, cannot be used in practice in human valve transplantation surgery because of the long periods that are required to remove the cryoprotective agent before the tissue can be used.

5. Preservation of the valve matrix after controlled freezing

Light microscopic studies of human aortic-valves preserved by controlled freezing revealed no changes in the distribution or structure of the collagen and elastic fibers. Vacuolization of the matrix as present in lyophillized

valves was not found after controlled freezing. Even with careful handling of the valves it showed not to be feasible to retain a lining of endothelial cells. Only a very small number of endothelial cells remain viable throughout the controlled freezing procedure and the non-viable endothelial cells tend to disrupt from the basal membrane. As was shown in the preceeding paragraph most fibroblasts remain viable after preservation by controlled freezing. The periodicity length of collagenic fibrils in fresh aortic valves and in valves preserved by controlled freezing was determined by analyzing electronmicrographs (see also Fig. 2-4). For preservation by controlled freezing human aortic valve pieces were placed in a closed glass-vial (Fig. 4-16) containing F 10 nutrient medium supplemented with 15% calf serum and 10% v/v dimethylsulfoxide. Incubation was carried out for one minute at 4°C before freezing at a rate of 1°C/min. Directly after controlled freezing down to -90°C followed by snap freezing to -196°C (liquid nitrogen), the material was rapidly (3-4 min) thawned the cryoprotective agent was removed by a stepwise dilution within 15 minutes. For both groups six valve pieces were investigated providing six thousand measurements per group. The average value of periodicity length of 641 $\mathring{A}$ $\pm$ 8 $\mathring{A}$ found for fresh valves does not significantly differ from the average value found for the valves after controlled freezing (639 $\mathring{A}$ $\pm$ 11 $\mathring{A}$). Hence the ultrastructure of the collagenic fibrils does not seem to be affected under optimal conditions of this type of preservation.

Finally as a functional parameter stress-strain characteristics of strips of aortic-wall of fresh human thoraric aortas and strips sterilized in NHH solution and preserved by controlled freezing were determined. Strips were obtained according to the method described in chapter II (page 29-30) in the circumferential and radial direction and they were tested directly after procurement or after controlled freezing under optimal conditions with DMSO described above. Data of initial expansion at low stress, stress and elongation at transition and rupture are summarized in Table 4-4. From these these data and those plotted in Fig. 4-19, it can be concluded that

TABLE 4-4: PLOT OF AVERAGE VALUES OF STRESS AND STRAIN OF HUMAN AORTIC WALL BEFORE AND AFTER CONTROLLED FREEZING WITH DMSO

	fresh				controlled frozen			
	Circumferential		Radial		Circumferential		Radial	
Parameter	$\bar{X}$	S.D.	$\bar{X}$	S.D.	$\bar{X}$	S.D.	$\bar{X}$	S.D.
Initial expansion (per cent)	29	± 8	17	± 5	27	± 8	18	± 5
Elongation at transition (percent)	49	± 1	31	± 8	42	± 12	34	± 9,0
Stress at transition (gm)	200	± 73	289	± 69	262	± 57	303	± 77
Elongation at rupture (percent)	74	± 18	39	± 10	71	± 17	39	± 8
Stress at rupture (gm)	708	± 183	690	± 151	775	± 171	711	± 180

Transition point = point where the modulus of elasticity changes abruptly with additional stress. (see chapter 2 fig. 2)

controlled freezing under optimal conditions does not significantly alter human aortic wall material and thereby aortic-valve material (see chapter II) in its initial expansion or posttransition stress strain characteristics.

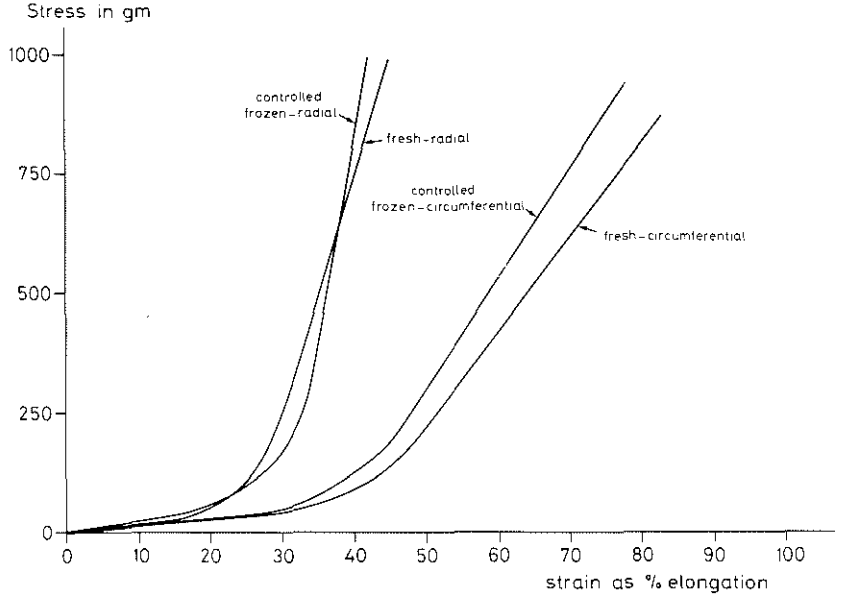


Fig. 4-19. Plot of average values of stress and strain for strips of fresh human aortic wall and after preservation by controlled freezing.

Conclusions

1. By immersion during 24 hours at 4°C in Ham's F 10 medium containing 15% calf serum and NHH antibiotic solution human aortic valves obtained at autopsy can be reliably sterilized.

2. The sterilization procedure hardly affects cell viability, 86% of the fibroblasts in sterilized valves were shown to incorporate ^{3}H-proline. Longer sterilization periods than 24 hours result in increasing loss of viable cells.

3. Long-term preservation of human aortic valves can be achieved by controlled freezing. Under optimal conditions of one minute incubation at 4°C with 10% dimethylsulfoxide (DMSO) as a cryoprotective agent survival

of 88% of the valve fibroblasts present was achieved. After storage the DMSO has to be removed by gradual dilution with Ham's F 10 medium during 15 minutes.

4. Cell-survival of the aortic wall after using the same procedure of controlled freezing is less (42%) than in the aortic valve; this is mainly due to the presence of different cell types.

5. Light microscopic studies after controlled freezing revealed no major alterations of the cells and of the intercellular matrix.

6. Electron microscopy of collagenic fibrils showed no effect of controlled freezing on the ultrastructure of these fibrils.

7. The stress-strain characteristics of aortic-wall strips, are not affected by controlled freezing.

8. The method developed seems to offer a reliable means of long-term preservation of humàn valve material with maintenance of cell viability, cell function and structural and functional integrity of the intercellular valve matrix.

Chapter V

GENERAL DISCUSSION

In order to obtain proper valve-function after transplantation, preservation of the structural integrity of the valve is of paramount importance. Optimal preservation of valve-integrity is likely to be achieved in case of allo-transplantation of fresh heartvalves derived directly after death of a fairly young individual, who has not suffered from diseases affecting the heartvalves. This procedure, although used in some instances (Murray 1956, Bigelow, et al., 1964), meets with several problems. Apart from the difficulty of availability of fresh valves at time of surgery, the diameter of the valve orifice in donor and acceptor valves has to be comparable. Although the acceptor orifice of the acceptor valve can be somewhat adapted to that of the donor's valve, by attaching the valve to a strut, the possibility of adaptation is limited. Furthermore the diameter of the acceptor aorta can only be estimated during the actual operation. To enable an optimal match between donor and acceptor valves, it is necessary that a number of valves with ranging orifice diameters are available at the time of surgery. This implies the necessity of a valve-bank and consequently the availability of procedures for sterilization and preservation of human cadaver aortic heartvalves.

A. Effect of sterilization and preservation procedures on aortic valve structure and function.

Several methods for preservation have been developed and their influence on the structure and function of the valves were tested. (Barratt-Boyes, 1964;

1965a; Harris, et al., 1968; Carpentier, et al., 1969). The results are represented in Chapter II. Preservation by freeze-drying with subsequent storage at -40°C in vacuo or dry storage at low temperatures -80°C and -40°C, resulted in valves with degenerated fibroblasts, embedded in an intercellular matrix consisting of disrupted collagen fibers and vacuolized by ice-crystal formation (Fig. 2-4). These results are in agreement with observations made by King, et al., 1967; Barnes, et al., 1970 and Donnelly et al., 1973, who described severe structural deformations of the valve fibroblasts at the light microscope and electronmicroscope level as well as disruption of collagenic and elastic fibers. Loss of tensile strength to an extent of about 30% in these valves has been observed by King, et al., 1967, Harris, et al., 1968 and Firor and Sharma, 1970. The valve failures after transplantation which have been frequently reported in follow-up studies (Heimbecker, et al., 1968; Barnes, et al., 1970; Smith, 1967) are most probably due to the severe cellular and structural alterations caused by the sterilization and preservation procedure.

Preservation in Hank's solution at 4°C for six months, resulted in completely a-cellular valves and a loss of the fibrillar aspect of the intercellular matrix, indicating severe changes of the collagenic and elastic fibrils. No studies of the effects of long-term storage in Hank's solution have been reported by other investigators, but Gavin et al., 1973 using the electron-microscope found degeneration of valve fibroblasts already after 7 days in Hank's solution. Several groups have described valve failure, caused by rupture or calcification of the leaflets (Barratt-Boyes, 1969; Ross et al., 1968; Gonzalez-Lavin, 1972), histological examination of these valves revealed degeneration of the valve matrix.

Sterilization of heart-valves by high doses of γ-irradiation has been frequently used (Harris, et al., 1968; Bowman, et al., 1969; Malm, et al., 1969). We have tested the effect of this procedure by electron microscopic investigations of human valves. A lengthening of the normal periodicity of

collagen fibrils was observed. Davidson and Cooper (1968) and Cooper and Russell (1968) exposed purified collagen to γ-irradiation with a dose of 10,000 rads and higher. They found a scission of peptide bounds and intermolecular covalent bond resulting in a decreased stability of the helical structure of the collagen fibrils; also a disruption of the ordered polymeric forms of tropocollagen was observed. This is another argument that high doses of γ-irradiation result in changes of the molecular structure of collagen. In the long-term such changes might affect the function of the heart-valve after transplantation. The clinical results of transplantation of heart valves sterilized by γ-irradiation show valve failure after a few years, (Malm, et al., 1969; Bowman, et al., 1969), possible due to dysfunction of the valve matrix.

A method for testing the integrity and function of the valve is the measurement of the stress-strain characteristics. A number of investigators did not find a significant decrease in the tensile strength of aortic-wall after γ-irradiation (King, et al., 1967; Malm, et al., 1969; Bowman, et al., 1969). However no recording of stress-strain characteristics after initial expansion were made while this would have rendered more relevant information about the integrity and function of the valve than merely measurement of the tensile strength as was shown by Clark (1973).

Carpentier, (1969) using xenografts as a valve transplant designed a method reducing the antigenecity of the valve by rendering the valve acellular and stabilizing the collagen fibers by tanning. We have observed that the tanning process causes substantial loss of elasticity at low stress (Fig. 2-11), which might explain the high failure rate (28%) of xenografts transplanted in the mitral position (Carpentier, et al., 1974). Besides structural changes in the extracellular compartment, cell death could be a causal factor in graft failure, since the valve fibroblasts are essential for the maintainance of valve integrity and hence valve function.

It can be concluded that all sterilization and preservation methods result in

acellular aortic-valves with (ultra)structural alterations of the matrix. It is likely that these changes are partly responsible for the valve-failures after long-term transplantations as reported by Barratt-Boyes, et al., 1969; Gonzalez-Lavin, et al., 1972; Barnes, et al., 1970; Carpentier, et al., 1974 and Wallace, et al., 1974.

B. The role of the fibroblasts in the maintenance of the aortic valve matrix.

The use of fresh human valve material has yielded good results in long-term (Angell, et al., 1972) and short-term follow-up studies, Gonzalez-Lavin et al., 1970. In these transplantation series non-preserved aortic allografts (Angell) and short-term preserved aortic allografts were implanted. In these methods cell degeneration is minimal and alteration of the valve matrix is avoided which might well be responsible for the good clinical results.

We have performed several experiments to elucidate the role of the fibroblasts in the aortic valve. ^{3}H-thymidine labelling of rat aortic valves indicated the occurence of fibroblast proliferation although the rate of proliferation was found to be low.

The light microscopic structure of many valve fibroblasts and their staining characteristics (basophilic) are indicative for an active metabolism of these cells. This is in contrast with some other connective tissues, like fascia lata and tendon tissue where nearly all fibroblasts show morphological characteristics of inactive cells. With the electronmicroscope we have observed quite an extensive rough endoplasmatic reticulum in the valve fibroblasts (chapter II, Fig. 2-5) which also indicates involvement in active protein synthesis. The role of the fibroblast in the production of extracellular components, has been established for various kinds of connective tissues. (see for reviews Ross and Benditt, 1962; Bornstein, 1974; Uitto, 1971; Gross, 1974a; Ten Cate and Deporter, 1975) As no such studies had

been performed for the aortic heart valve, we have investigated the role of the valve fibroblast in the production of one of the main components of the valve matrix; collagen which was found to comprise 56% of the total dry weight of the valve. The synthesis, secretion and turnover of collagenous proteins may be studied by the use of various radioactively labelled amino acids such as glycine, lysine and proline. None of these amino acids, however, is entirely "specific" for collagen. In cartilage 93% of the proline is incorporated in collagen which is then secreted into the intercellular matrix. (Malawista and Schubert, 1958; Eastoe, 1961) For this tissue it is reasonable to assume that the metabolic pathway of proline is representative for collagen formation. We have therefore studied the proline incorporation in rat costal cartilage as a control for our investigations on rat heart valves. In rat aortic valve the incorporation pattern of ^{3}H-proline was very similar to that observed in costal cartilage (chapter III, Fig. 3-2). Shortly after injection of ^{3}H-proline nearly all of the label is localized over the valve fibroblasts. Compared with the chondroblasts the label remains intercellular for a longer period. Also the amount of intracellular labelling is lower in the valve fibroblast than in the chondroblast. (Fig. 3-2) Those are indications for a lower rate of synthesis in the valve fibroblasts. The higher incorporation of proline in cells localized at the base of the leaflet compared with the rest of the valve may be correlated to the functional demands made upon this part of the leaflet. It cannot be excluded, however, that other factors may be responsible for these local differences in labelling intensity. Autoradiography at larger time intervals after labelling with ^{3}H-proline show that from a few hours on most of the label is localized in the extracellular matrix of the valve (Fig. 3-3). To identify the nature of the labelled product in the heart valve double labelling experiments with both ^{3}H-methionine and ^{3}H-proline were carried out. The results of autoradiography and scintillation counting showed that ^{3}H-methionine remained confined to the valve fibroblasts. Its activity was decreased to background values within a period of two weeks. In contrast

the activity of ^{3}H-proline started to shift to the extracellular compartment within an hour after labelling. The stabilization in the amount of radioactivity in the period for 1-8 weeks after labelling indicates a long turnover time of the labelled extracellular product. The high peak in radioacitivity observed the first hours after proline administration is due to incorporation of proline both in non-collagenous products and collagenous proteins itself. The correspondance of the subsequent decline in activity of proline with that of methionine suggest a fairly rapid turnover time of the non-collagenous products. The decrease in activity during the first two weeks after labelling is also due to the degradation of proline containing products in the extracellular matrix as has been observed for other connective tissues (Klein and Weiss, 1966; Nimni, et al., 1967).

Further characterization of the ^{3}H-proline labelled extracellular product was obtained by separation of collagen and non-collagenous proteins (Harkness, et al., 1954; Jackson, 1958) and subsequent determination of the radioactivity in both fractions. The results of this experiment (Table 3-3) showed that the vast majority of extracellular proline was incorporated in collagenous protein.

The main conslusion of these incorporation experiments is that the heart valve fibroblast is actively involved in the synthesis and secretion of collagen, which is the main component of the extracellular matrix. In normal connective tissue extracellular collagen is continously degraded by enzymatic action of collagenase, which is also produced by the fibroblast (Gross 1974b). Normally the loss of collagen is balanced by newly synthezised collagen. In an acellular valve no collagenase will be present and hence this enzymatic degradation will not occur. However, damage to collagen fibrils by preservation methods cannot be repared in acellular valves because collagen synthesis is absent. Even if the valve matrix is intact at transplantation mechanical trauma of collagen fibrils will occur during long-term valve functioning. Again, in acellular valves such

damages will not be compensated by de novo synthesis of collagen and can be causative for valve failure (Gonzalez-Lavin, et al., 1970; Wallace, et al., 1971; Carpentier, et al., 1974).

Repopulation of a-cellular donor valves by fibroblasts derived from the aortic wall of the acceptor would in principle enable long-term valve matrix maintenance. Conflicting reports on the occurrence of repopulation by ingrowth of cells have been published (Mohri, et al., 1968; Reichenbach, et al., 1969). It is however clear that whenever cell repopulation takes place this is only after a fairly long period after transplantation. This means a period of a-cellularity either or not followed by repopulation which never reaches a cell-density comparable to that of a normal aortic valve. A low cell-density will result in insufficient production of intercellular matrix components.

The main conclusion of our experimental work is that there is active collagen synthesis in the heart valve, and the fibroblast plays an essential role in the maintenance of the equilibrium between collagen production and degradation. To obtain optimal long-term results after valve transplantation it thus seems necessary to maintain cell-viability throughout the sterilization and preservation procedures.

C. Development of a preservation method maintaining cell viability and matrix integrity

After short term preservation during several days in Hank's solution with sterile procurement of the graft a number of investigators have observed the presence of viable cells in the valve. Al-Janabi, et al., 1972, used 48 hours ^{3}H-thymidine incorporation as a parameter and found 70% of the cells labelled. This would imply that the majority of valve fibroblasts are involved in cell proliferation which has never been observed for any connective tissue. Investigators from the same group also observed cell viability after preservation in nutrient medium uptill 6 to 8 weeks after sterilization

in an antibiotic solution (Lockey, et al., 1972; Al-Janabi and Ross, 1973). This result is surprising if one takes into account the short survival times in vitro which have been observed for various tissues. Our own results on this respect are more in agreement with the expectations that only a very low percentage of the valve fibroblasts proliferate .

To maintain long-term maintenance of cell viability it seems more appropiate to use preservation methods which have been successfully applied for the storage of various human cell types, i.e. controlled freezing. Mermet, et al., 1971 and our own group (van der Kamp, et al., 1972) were the first to use controlled freezing for the preservation of heartvalves. An advantage of controlled freezing is the possibility of storage at $-196^{o}C$ for infinite periods which enables the collection of suitable autopsy material with respect to the valve diameter and matching of tissue specific antigens. Sterilization of the autopsy material by the NHH antibiotic solution (Lockey et al., 1972) was found to be effective (see chapter IV) and a 86% cell-survival was found after 24 hours immersion of the graft in the antibiotic solution. Sterilization performed during shorter periods was found to be less effective according to Lockey, et al., 1972.

In spite of what is known about the action of cryoprotective agents on the prevention of intracellular icecrystal formation and about the influence of the freezing and thawing rate on cell-survival, the optimal conditions of controlled freezing have to be determined for each tissue. (LoveLock, 1953; Mazur, 1970; Leibo and Mazur, 1971; Meryman, 1971). Methods developed for cryopreservation of whole skin (Billingham and Medawar, 1952), cornea (Capella, et al., 1966), developing teeth (Bartlett and Reade, 1972) and bone marrow stem cells (Schaefer, et al., 1972) give guide lines for the type of cryoprotective agent, the rate of freezing and thawing and for the stepwise removal of the cryoprotective agent after thawing. Cryoprotective agents which act on the extracellular compartment and which have been successfully used for cell suspensions (Meryman, 1971)

do not provide sufficient protection for tissues and organs. This is probably related to the fact that in tissues solute influx in the cells is insufficient and hence dehydration and cell destruction occur (Merryman, 1971). The use of several cryoprotective agents that act intracellularily was found to be effective concerning the maintenance of cell viability in heart valves provided optimal conditions were applied. The differences in cell-survival between different agents as ethylene glycol, glycerol and dimethylsulfoxide are partly based on differences in their penetration rate into the cells. This is relatively slow for glycerol and high for dimethylsulfoxide (Meryman, 1971). Another explanation for the different behaviour is the different toxicity of the various agents which is relatively low for glycerol and high for dimethylsulfoxide (Meryman. 1971; Malinin, 1973). Differences in cell-survival as observed for heart valves preserved with different cryoprotective agents (chapter IV) have also been shown for cultured hamster cells (Leibo and Mazur, 1971), bone marrow stem cells (O'Grady and Lewis, 1972) and for whole skin (Lehr, et al., 1964; Athreya, et al., 1969). Within most organs or tissues different cell types occur and these may have a different susceptibility to injury after the same procedure of controlled freezing. In our experiments we have observed that cryopreservation of human aortic heartvalves under optimal conditions for leaflet fibroblasts (88%) resulted in 44% cell-survival for aortic wall cells and no survival of endothelial cells. This differential susceptibility is a well known phenomenon which is likely to be due to tissue and cell penetration of the cryoprotective agent, the cell-volume and the cell membrane permeability (Mazur, 1970; Pribor, 1974).

In our present study cell viability has been assessed by ^{3}H-proline incorporation. This offers several advantages over viability assessment by cell-cultivation (Mermet, et al., 1971). ^{3}H-proline incorporation studies enable exact counting of the numbers of surviving cells, determination of their intravalvular localization and at the same time it can be tested whether the fibroblasts are still capable of protein (collagen) synthesis.

Controlled freezing of human heart valves under the optimal conditions described in chapter IV permits long-term storage with 88% cell-survival and the majority of these fibroblasts were shown to be actively engaged in collagen synthesis after thawing. The preservation of fibroblast function during sterilization and storage guarantees optimal maintenance of the structural and functional integrity of the valve-matrix. The procedure developed had no damaging effect on the ultrastructural pattern of the collagenic fibrils. Finally the stress-strain characteristics and hence the aortic wall elasticity remained unaffected by the cryopreservation procedure. Follow-up studies after valve transplantation using controlled freezing as a preservation method have not yet been described.

D. Graft rejection

With the use of vaible allografts in heartvalve transplantation graft rejection may obviously be a problem. The long-term results obtained with allovital valve transplants by Barratt-Boyes (1969) and Angell, et al. (1972), suggest that aortic heartvalves do not incite a severe rejection process. In experiments with allovital canine valves a cell-loss was observed after an initial increase in cell number (Angell et al., 1967) with the same experimental approach Beal, et al. (1961), Lower, et al. (1966) and Duran and Whitehead (1966) described the presence of mild infiltrates of lymphocytes or plasma cells into the periphery of the valve leaflets during the first post-operatives months; later on no such cell infiltrations were identified. In the same experiments early hyalinization and cell-loss has been reported. These observations on valves located in their normal functional environment are suggestive for tissue alterations which may be related to a mild graft rejection process. Experimental evidence for valve-antigenicity has been found by several investigators but these concerned allovital aortic valves transplanted in subcutaneously or intramuscularly positions (Mohri, et al., 1967 and Baue, et al., 1968). Heslop, et al. (1973) implanted rat interstrain Ag B/Rt-H_1 incompatible aortic valve allografts into the

abdominal aorta and she found the production of lymphocytotoxic and hemagglutinating antibodies. Also, microscopic investigation showed mononuclear cell infiltration which was not present in intrastrain syngeneic grafts. Experiments performed with isolated leaflets, aortic wall and cardiac muscle revealed that antigenicity resited predominantly in the rim of cardiac muscle; valve leaflets were not found to be immunogenic. Allovital aortic valve transplants grafted distal to the pulmonic valves did not show any sign of rejection when no connection with the vasa vasorum of the pulmonic artery was present; however rejection could be initiated by partial stripping of the artery wall thus creating connection between the transplanted valve and the pulmonic wall circulation. (Donawick and Baue, 1970).

These experiments show that allovital heart valves have a low antigenicity. This is likely to be due to the non vascular character of the valve and the protection of its cells by the surrounding intercellular matrix. A similar situation exists in cartilage and cornea. For both of these tissues transplantations are successfully being carried out even without tissue typing. The protective role of the intercellular matrix in transplantation of cartilage has been shown by Moskalewski et al., 1966 and Heyner, 1973 who could induce a graft rejection by removing the intercellular matrix.

Preservation of heart valves by controlled freezing according to the procedure described in chapter IV results in an almost complete loss of the endothelial lining of the valve. Even if endothelial cells would be preserved they will be lost shortly after transplantation (Kosek, et al., 1969). The absence of endothelial lining may result in the formation of microthrombi and in insufficient nutrition of the valve. It seems therefore essential that after transplantation the endothelial lining of the valve is being restored. In principle this can be accomplished by overgrowth of endothelium from the graft host junction (Duran and Whitehead, 1966; Mohri, et al., 1968; Kosek, et al., 1969; Angell, et al., 1973; Gavin, et al., 1973a). Such overgrowth will probably be inhibited even by a mild

immunological reaction. The selection of grafts from a donor whose tissue antigens are comparabel to those of the acceptor, could possibly solve this problem. The formation of microthrombi as a result of insufficient endothelial lining after transplantation may also be an impediment for the overgrowth of host endothelial cells. This might however be prevented by administration of anticoagulents during the initial period after transplantation surgery.

The use of allovital valve transplants thus seem to necessitate matching of both the orifice diameter and tissue specific antigens. This requires the availability of a preservation method enabling long-term storage of autopsy material. The method developed and tested in the present thesis seems to fulfil this requirement. In addition it seems to be the only long-term preservation method where cell-viability and the structural and functional integrity of the valve matrix are maintained.

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