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TESTING OF RPMI-1640 AS A NUTRIENT MEDIUM FOR FRESH SFMILUNAR V--ETC(U)
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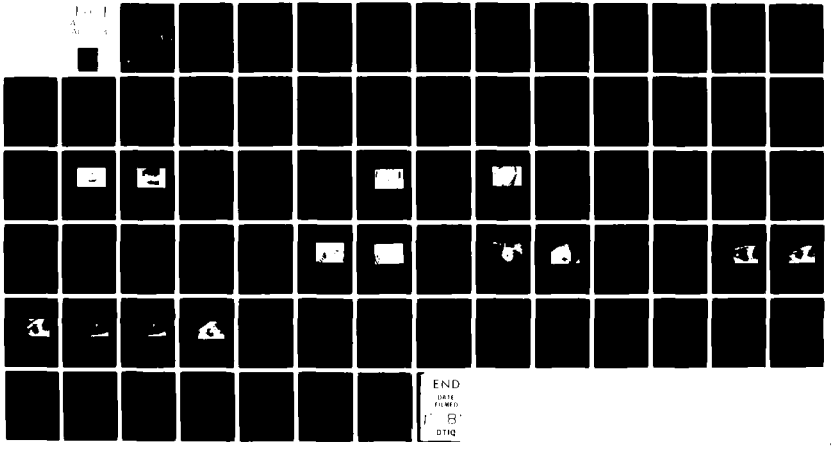

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

| REPORT DOCUMENTATION PAGE | READ INSTRUCTIONS BEFORE COMPLETING FORM |
|---|---|
| 1. REPORT NUMBER: 79-302T-S (6) | 2. GOVT ACCESSION NO.: AD-A106 824 |
| 4. TITLE (and Subtitle): Testing of RPMI-1640 as a Nutrient Medium for Fresh Semilunar Valve Storage. (10) (1) | 5. TYPE OF REPORT & PERIOD COVERED: THESIS/DISSERTATION |
| | 6. PERFORMING ORG. REPORT NUMBER |
| 7. AUTHOR(s): James Beverly Nichols (7) | 8. CONTRACT OR GRANT NUMBER(s) |
| 9. PERFORMING ORGANIZATION NAME AND ADDRESS: AFIT STUDENT AT: Michigan State Univ | 10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS |
| 11. CONTROLLING OFFICE NAME AND ADDRESS: AFIT/NR WPAFB OH 45433 | 12. REPORT DATE: (11) 1979 |
| | 13. NUMBER OF PAGES: 60 |
| 14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office): (12) AFIT/NR | 15. SECURITY CLASS. (of this report): UNCLASS |
| | 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE |

LEVEL II

16. DISTRIBUTION STATEMENT (of this Report)

APPROVED FOR PUBLIC RELEASE; DISTRIBUTION UNLIMITED

DTIC ELECTE NOV 9 1981 S

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

27 OCT 1981

Fredric C. Lynch

FREDRIC C. LYNCH, Major, USAF
Director of Public Affairs
Air Force Institute of Technology (ATC)
Wright-Patterson AFB, OH 45433

18. SUPPLEMENTARY NOTES

APPROVED FOR PUBLIC RELEASE: IAW AFR 190-17

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

ATTACHED

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The antibiotics added to the medium, sterilized all the valves and along with the RPMI 1640 did not cause appreciable tissue death. All valves remained viable. The histopathological estimates of heart valve viability ranged from normal to moderate degeneration. Ninety-one percent (10 out of 11 valves used) were normal or had only mild degeneration.

Results indicate that with RPMI 1640 medium and the selected antibiotics, valves could be maintained in a sterile, viable state for several weeks.

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By

James Beverly Nichols

Captain, United States Air Force / Veterinary Corps

61 Pages

MASTERS OF SCIENCE

Michigan State University

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# TESTING OF RPMI-1640 AS A NUTRIENT

# MEDIUM FOR FRESH SEMILUNAR VALVE STORAGE

BY

James Beverly Nichols

A Thesis

Submitted to

Michigan State University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

Department of Veterinary Surgery and Medicine

1979

*ACKNOWLEDGEMENTS*

The author wishes to thank Dr. George E. Eyster who guided the research as well as the development and preparation of this thesis. Without his help, this research and thesis would never have been accomplished.

In addition, I wish to thank Bonnie DeYoung for her expertise and help as well as her warm friendship extended to me and also, to thank the students on the cardiovascular team. A special thanks goes to Dr. Robert Bull for his help in preparation of the medium and to Dr. Harold Tvedten for his assistance in histopathology.

I would like to show my appreciation to my residency committee. Dr. Waldo Keller, committee chairman and advisor, suggested the project and has guided me through my years at Michigan State University. Drs. Gretchen Flo, Robert Langham and, again, George Eyster were not only members of my committee but, also, provided invaluable didactic and clinical instruction.

To Cathy Powers, who spent much time retyping the many drafts of this thesis, I extend my sincere thanks. And lastly, to Patricia Powell, who patiently spent many hours in the reconstruction of a scientific paper into this thesis, I will be forever grateful.

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# INTRODUCTION

A controversy exists over whether an alive tissue human allograft or a dead tissue porcine xenograft heart valves function better as a heart valve replacement in man. Stated simply, do live tissue transplants function better than fixed, dead tissue transplants? Much of the varying results dealing with live heart valves by different investigators may be due to varying degrees of necrosis from time of collection until time of transplantation. To circumvent this problem, various methods of preserving heart valves have been tried. To date, no data indicates preserved heart valves are viable after two weeks in the storage medium.

This study was undertaken to select a nutrient medium to maintain heart valves in an alive, fresh state for two or more weeks.

## LITERATURE REVIEW

A literature review was conducted with three goals to be accomplished. The first goal was to obtain a general overview of what had been done in the past with the use of replacement heart valves. The second goal was to study the different methods of sterilization and preservation of stored valves. And lastly, in order to decide on what nutrient medium and antibiotics to use, a search was made through the literature for ingredients readily available for the storage of heart valves.

### A. The use of valve grafts.

The use of replacement heart valves has proved valuable because it has allowed surgical treatment for previously noncorrectable heart defects. Some of the defects presently being corrected are left ventricular outflow hypoplasia, transposition of the great arteries with ventricular inversion and associated pulmonary stenosis. Other problems requiring valve replacement which have been treated are aortic valve defects, severe Tetralogy of Fallot, pulmonary atresia, ventricular septal defect, truncus arteriosis, transposition of the great arteries with intact ventricular septum and pulmonary stenosis, tricuspid atresia, and common ventricle.[1,2,3]

The transfer of tissue from one part of the body to another or from one animal to another has been tried from earliest times, and has gained

a increasing popularity since the turn of the century. By the 1950's reports of successful transplantation reached the literature, and by the 1960's many of the present day methods were developed.[4] An allograft heart valve was first used by Ross in 1962 in a clinical cardiac surgery.[5,6] In 1964, Kirklin used a valveless conduit constructed of pericardium as an out flow tract.[7] In 1966, Ross and McGoon started experimentation with the use of aortic valve allografts.[2,3,8] Later, Stewart used a combination of a tissue valve in a conduit with great success because it reduced the trauma that is required for the replacement of a valve in a heart.[3] This procedure also created a method of overcoming severe developmental abnormalities such as ventricular atresia, truncus arteriosis, and Tetrology of Fallot.[3]

Experimental work has been done utilizing many different animal models. Due to the inherited differences of each specie, the experimental results have varied greatly. The dog which is the animal model most used responds differently than man to conditions of hypovolemia, increased venous pressure, and tissue reaction.[9,10] These variations along with different techniques cause a wide variety of results when applied to the human patient.[9,10]

From early pioneering efforts, many approaches to valve replacement materialized. Mechanical valves were developed and tried in over a thousand human cases.[4,11] Although the mechanical valves solved many of the problems associated with tissue transplantation such as storage, obtainability, and sizes, there were still many disadvantages. The main disadvantages were mechanical failure and thromboembolism with the requirement of long term (up to lifetime) anticoagulation therapy.[2,11,12,13,14,15,16,17,18,19,20,21] So, the search continued for other methods of heart valve replacement.

The efforts of most investigators centered around the use of animal valves as replacements. The primary source of tissue valves were humans, pigs, calves, sheep, and goats.[4,22,23] There were many problems with each valve source. Sterilization, storage and deterioration were problems encountered with all valves. Procurement was an additional problem associated with human valves.

Human valves, however, have been and are used today. The London Heart Institute utilizes between six and twelve heart valves a week.[24] Due to different results obtained by a number of investigators, the human heart valve has lost favor with many cardiovascular surgeons who have converted to the use of the glutaraldehyde preserved porcine xenographs.[15,25]

Tissue valves, in general, have a number of prevailing difficulties associated with transplantation. One problem is thromboemboli. This phenomenon has been reported with both the mechanical and to a lesser frequency animal valve usage. Normally, long term anticoagulants (dicumerol) are used to reduce the occurrence of the thromboembolism. The anticoagulants are generally given for two to three months with mitral valve replacement. With aortic, pulmonary, and tricuspid valve replacement, they are usually not needed.[15]

A second problem is tissue rejection which can cause shrinkage leading to nonfunctional valves. Rejection begins as an inflammatory response typified by histiocytes, giant cells and macrophages. There is a degeneration of collagen and elastin with some calcium deposition. Calcification appears in the aortic wall of the graft and is visible radiographically after about six months. These deposits, however, do not

appear to impair heart valve function nor involve the valve cusps. The primary method of overcoming rejection is the administration of prednisolone and antilymphocyte serum. Alone, neither is effective, but in combination, they are quite successful.[2,22,25,26,27]

A controversy exists concerning the use of human allografts of live tissue[3,10,26,27,28,29,30,31,32,33,34] versus porcine xenografts of dead tissue.[3,15,16,22,23,31,35,36,37,38,39] The debate centers around whether live tissue or dead tissue function in a more normal manner with greatest durability.[15,28] The issue has not been solved due to conflicting reports, but basically it is a question as to whether live fibroblasts enhance the life and function of the heart valve. Some believe that when a viable human heart valve is placed in another human, over a period of time, the recipient's fibroblasts will replace the donor's fibrocytes, and eventually the valve continues to live throughout the recipient's life.[4,6,29]

Some researchers have found more thromboemboli with allografts, while others state that xenografts cause the most thromboemboli.[15,16] There is, also, a dispute over which causes the most tissue reaction/rejection.

Allografts require sterilization and preservation of the tissues as do xenografts. Using the theory that viable grafts are superior, the selection of sterilization methods and storage medium becomes very important. The viability of a graft can be increased or decreased depending on the method of sterilization chosen and the nutrient medium used.[29] An additional advantage to the use of live tissue is that their valvular function is hemodynamically superior to dead tissue. Although the leaflets close equally well on both types of valves, the live tissue

valves open more fully. The wider opening decreases the pressure gradient across the valves when used in the vertical position.[30]

A disadvantage in the use of human heart valves is obtainability. Authorization and legal justification require time consuming paperwork for each heart valve. Approval has to be obtained from the deceased's next-of-kin and once obtained, the heart valve has to be removed from the body which is generally stored in a morgue which is unsterile. Presently, once the heart valves have been removed an adequate storage method does not exist.

Xenografts of porcine origin have gained great popularity since their first use in 1965.[15] The one consistent finding with xenografts is that they are not replaced by the recipient but slowly and continually degenerate.[15,16] A major advantage of xenografts is that when they do fail, it is generally not abrupt but a slow progressive process that allows elective reoperation under optimal conditions.[16,25] Some investigators felt that xenografts function better because they are not involved in tissue turnover, thus making them stronger and longer lasting.[4,16,25]

Another favorable factor involved with the use of porcine xenograft heart valves is the absence of intravascular hemolysis that is seen with mechanical prosthetic valvular replacements.[39,40,41,42,43] A tremendous advantage of porcine xenografts is their ability to be stored and maintained in glutaraldehyde. Storage is a very simple, easy, effective procedure and will be discussed.

B. Sterilization and Storage of Heart Valves.

The single most important factor effecting durability of the heart valve is the method of preservation. Xenografts are generally stored in glutaraldehyde. Glutaraldehyde was first used effectively in 1968.[25] Glutaraldehyde has two advantages. It is a tanning agent which increases tissue stability by forming irreversible cross linkages between collagen molecules.[25] In addition, it markedly reduces the antigenicity of the graft tissue. Two disadvantages in using glutaraldehyde are that the preserved tissue has to be thoroughly washed before use and there have been reported outbreaks of Mycobacteria chelonei associated with the valves.[25,37]

The ideal method of sterilization and storage of allografts is still being sought. If viable tissue is better than dead tissue, the objective is to find a method of storage that causes the least amount of deterioration.

Early methods of sterilization were ethylene oxide, beta-propriolactone, formalin, and gamma irradiation.[6] These led to cusps being ruptured and torn because of tissue deterioration. Present methods utilize a nutrient medium with antibiotic-antifungal additives. Valves are placed in the medium at 37°C for 24 hours. Then they are kept at 4°C until they are used or frozen.[26,28,29]

Hanks solution was the first medium tried but later was replaced by a nutrient medium which decreased cell loss. One of the more popular media used to replaced Hanks solution was Medium 199 or Tissue culture 199. To this medium, calf serum and 4.4% sodium bicarbonate was added. Later 24 mM Hepes buffer and glutamine were added to the TC 199 and calf serum. At the end of two weeks in the medium, severe damage to the valves occurred

and after three weeks no viability was present.[6] As a result, valves kept in this nutrient medium must be used within one week. To overcome this time limitation, valves are generally frozen until used. In order to freeze the tissue, dimethyl sulfoxide was added to the nutrient medium and the temperature reduced to that of liquid nitrogen (-196°C).[6,10] This procedure also causes tissue damage due to cellular rupture during the freezing and thawing processes.

Realizing that most human heart valves were collected under less than sterile condition. A microbiological study was conducted. Escherichia coli and Streptococcus faecales were found to be the most common organisms associated with the heart valve tissue.[6,10] Also found were Klebsiella, Proteus, Pseudomonas and occasionally Staphylococcous.[6] About 85% of the valves collected were contaminated.[6,10] These data indicated the necessity for an adequate method of sterilization.

Early methods of sterilization caused deterioration of tissue until antibiotics were selected for use.[6] Several different studies were conducted to see which antibiotics were the most effective and caused the least tissue damage. Gentamicin, Methicillin, Erythromycin, and Nystatin worked excellently for several investigators. Colistimethate, Gentamicin, Kanamycin and Lincomycin are another combination used.[33] Polymyxin is used by some because of its rapid bacteriocidal effect even against inactive bacteria.[6,10,24,26,28,30,32,33,35]

C. Selection of a Nutrient Medium and Antibiotics.

Many different media formulas for heart valve storage are currently on the market. Since Medium 199 was the medium most widely used at the time of this study, it was chosen as a comparison for the selection of a replacement medium. RPMI 1640 is a recently developed medium being used for white cell culture. The formula is quite similar to Medium 199 with slight variations (see appendices A & B). It was felt that if white cells grew well in RPMI 1640, then perhaps it would serve as a replacement medium. Calf serum was added to RPMI 1640 as it had been to Medium 199.[44]

The antibiotics that were found to be most effective against organisms previously identified in heart valve tissues were then further compared according to their effects on heart valve viability.[45]

## METHODS AND MATERIALS

A. Collection of four semilunar valves for culture and sensitivity to determine antibiotics needed for normal flora of thorax and heart.

Four mix breed dogs of 12-15 kilograms were euthanized. All dogs were positioned in right lateral recumbancy. Three dogs were clipped and surgically prepared using iodine and alcohol alternating three times. Contamination from organisms around the incision could occur so the remaining dog was not surgically prepared. An incision was made with a scalpel knife at the level of the fourth intercostal space through all layers of the body wall, directly into the chest. Using aseptic technique, the surgeon grasped the heart in one hand while cutting the aorta, posterior and anterior vena cavae, pulmonary artery and veins. The heart was then removed from the dog and placed on a sterile towel. Using another sterile pair of scissors and a pair of Adson forceps, the surgeon excised the aortic semilunar valve from the heart. A longitudinal incision was made in the aorta, extending through the valve. A circular incision was then made around the valve. Excess muscle and aorta were trimmed from each valve. The valve was then placed in a sterile test tube and submitted to microbiology for culture and sensitivity. The microbiological results led to appropriate antibiotic additives to be used with the medium.

B. Preparation of nutrient medium.

The preparation of the nutrient medium was accomplished in several steps. First, all materials were assembled (Table 1). Second, the materials were mixed using sterile techniques followed by the third step of filtering the solution.

A five liter Erlenmeyer flask was sterilized. Four liters of sterile RPMI-1640 with 25 mM Hepes buffer were mixed with one liter of sterile fetal calf serum in the flask. Then, 125 milligrams of Colistimethate, 125 milligrams of Gentamicin, 375 milligrams of Kanamycin, 1.25 grams of Lincomycin and 12.5 milligrams of Amphotericin B were added to the medium.

This solution was placed into a sterile container on the millipore filter assembly. The medium was then pumped through a 10 micron millipore filter under pressure, sealed in sterile jars, and placed at 4°C until needed.

In order to prepare the required seven and one-half liters for the entire procedure, a second mixture of nutrient medium was prepared in a similar manner at half volume.

TABLE 1

NUTRIENT MEDIUM FORMULA

4 liters - RPMI 1640 with 25 mM Hepes Buffer - Grand Island Biological Company

1 liter - Fetal calf serum - Grand Island Biological Company

125 mg - Colistimethate Sodium (Coly-Mycin M) - Warner/Chilcott

125 mg - Gentamycin Sulfate (Garamycin)-Schering

375 mg - Kanamycin Sulfate (Kantrex) - Bristol

1.25 g - Lincomycin Hydrochloride Monohydrate (Lincocin) - Upjohn

12.5 mg - Amphotericin B. (Fungizone) - Squibb

C. Surgical procedure for harvesting donor valves.

Sixteen dogs were selected as donors. Nine were euthanized with T-61 Euthanasia Solution (National - .14ml/kg). The remaining dogs were anesthetized with Halothane and exsanguinated to ensure that the euthanasia solution did not affect the heart valves.

Each animal was placed in right lateral recumbancy. The left chest area was clipped and surgically prepared with povidone-iodine. After capping, masking, gowning, and gloving the surgeon four-cornered draped the animal.

A lateral thoracotomy was performed by incising just caudal to the scapula over the fourth intercostal space. The latissimus dorsi and scalenus muscles were transected, the serratus ventralis muscle was divided, and the intercostal muscles incised. Hemostasis was accomplished with electrocoagulation. The pleura was transected and the thoracic cavity entered.

Aseptically, all vessels leading to and away from the heart were cut 3-5 cm from the heart and the heart was placed on a sterile towel. The pulmonic and aortic valves were dissected from the heart and trimmed of excess muscle and fat.

D. Procedure for processing valves from time of harvesting until implantation.

After the semilunar valves were harvested utilizing aseptic technique, they were placed in a sterile cup containing 100 milliliters of nutrient medium. One cusp of the pulmonary semilunar valve was placed directly into 10% formalin and sent to pathology as a control. A 0.25 by 1.0 cm piece of myocardium that had been trimmed from the valves was sent to microbiology for culture and sensitivity. Two other portions of the same size myocardium were placed in the nutrient medium with the valves to be used for later culture.

The containers were stored for 24 hours at 37°C at which time the valves and portions of myocardium were transferred to another sterile container. One hundred (100) milliliters of fresh nutrient medium were added to the container. The valves were stored for an additional 24 hours at 37°C. Utilizing the same procedure, the valves were again transferred to fresh medium. One of the two segments of myocardium was sent to microbiology for culture and sensitivity. The container and tissues were then stored at 4°C from 13-133 days (Table 2).

Two days prior to implantation, the valves were mounted in a dacron conduit. Using strict aseptic technique a table for trimming valves was prepared. A waterproof sterile drape was placed on the table. A sterile cloth drape was placed on top of the waterproof drape, followed by four sterile towels. The remaining valves and myocardium were removed from the container with sterile Adson forceps. The myocardium was placed in a sterile test tube and sent to microbiology for culture and sensitivity. The remaining pulmonary semilunar valve cusps were placed

TABLE 2

THE NUMBER OF DAYS ALL HEART VALVES WERE IN THE MEDIUM

| | DONOR VALVES | DAYS IN MEDIUM |
|---|---|---|
| 1. | A | 29 |
| 2. | B | - |
| 3. | C | 36 |
| 4. | D | - |
| 5. | E | - |
| 6. | F | - |
| 7. | G | 23 |
| 8. | H | 72 |
| 9. | I | 135 |
| 10. | J | 64 |
| 11. | K | 77 |
| 12. | L | - |
| 13. | M | 16 |
| 14. | N | 15 |
| 15. | O | 22 |
| 16. | P | 30 |

Note: Valves B, D, E, F and L were utilized for microbiolgy culture and sensitivity testing only. These valves were kept for a total of 48 hours and then sent for microorganism testing.

in 10% formalin and submitted for histopathology to determine the viability of the tissue. Using tenotomy scissors, the remaining excessive connective tissue, fat, and myocardium were trimmed from the aortic valve. The aortic annulus was trimmed to 4 mm above the top of the commissure of the valve and 3 mm below the base of the cusps following the curved outline of the leaflets. The coronary ostii were included in the excision.

To insure no damage would be incurred to the tissue during the trimming process the leaflets and vessel lumen were not handled with instruments. The valve was dipped in the nutrient media every three minutes to prevent drying. Excessive tissue was removed in order to limit the rejection response in the recipient. Once the valve had been trimmed, it was ready to be mounted in the conduit.

An 18 mm diameter conduit was used to match the outer dimensions of all the valves selected, since all of the donors were 12-15 kilogram dogs. Ten cm was the length of conduit required to connect the right ventricle to the pulmonary artery.

The conduit was everted for two-thirds of its length. The ventricular side (inflow side) of the valve was placed on the conduit. Two stay sutures of 4-0 double-armed Ticron were placed through the conduit and vessel wall 180° apart. A simple continuous suture pattern was then placed from one stay suture around to the other and tied in a

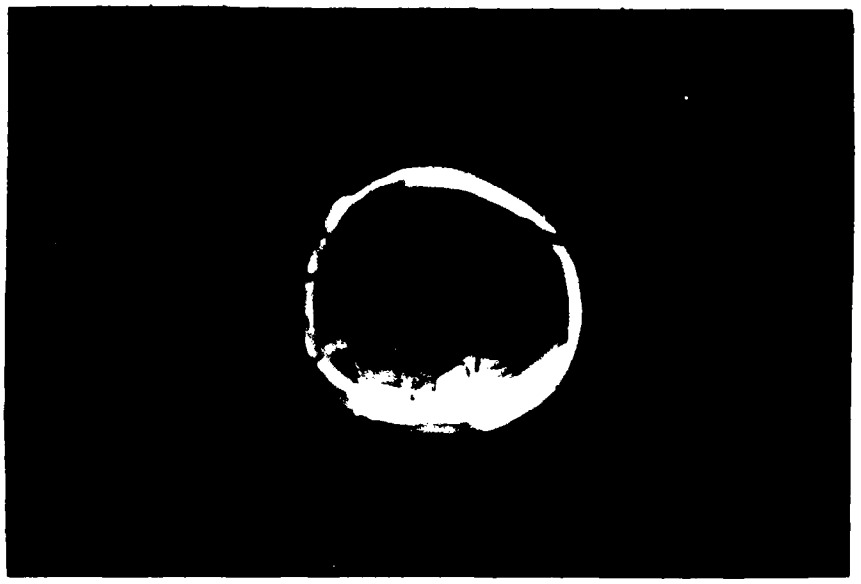
square knot. Utilizing the second stay suture, the same pattern was continued completing 360°. The sutures were spaced 2 mm apart.

The conduit was then reversed to the level of the outflow side of the valve. Three stay sutures of 4-0 Ticron were placed through the conduit into each of the apices formed by the leaflets. These stay sutures were not equidistant from each other due to the varying shapes of each leaflet. In a similar manner as before, the valve was sutured to the conduit incorporating all three stay sutures for a complete 360°. The conduit was returned to its normal shape, thus placing the valve on the inside of the dacron tube. (Figure 1 and 2)

Nutrient medium was poured into the conduit from the outflow side to evaluate valve function. The competent aortic valves did not allow medium to regurgitate. The mounted valve was placed in fresh nutrient medium and maintained at 4°C until time of implantation.

FIGURE 1

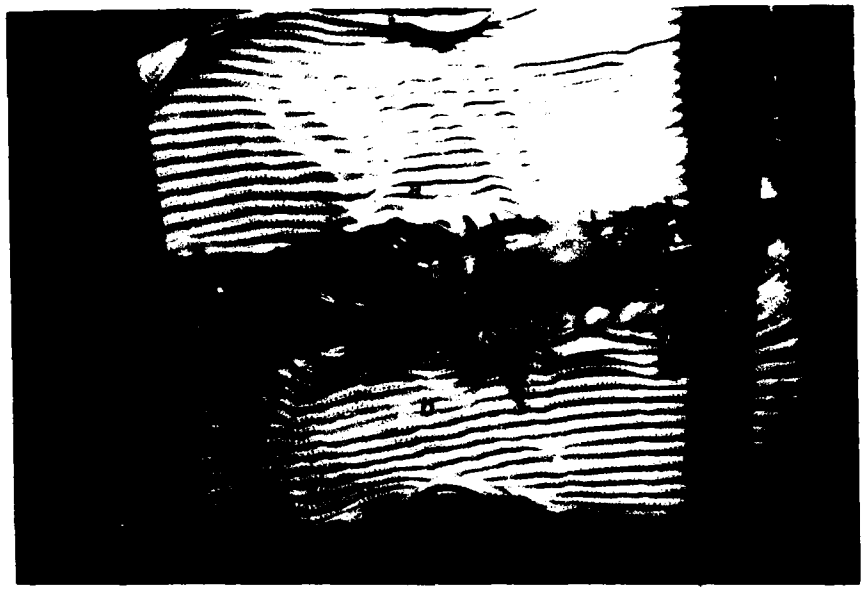
MOUNTED VALVE IN CONDUIT - END ON VIEW



Outflow side of aortic valve mounted in a dacron conduit. Note the three stay sutures (a) of 4-0 Ticron unequally placed. The leaflets (b) have been retracted open for photographic effect.

FIGURE 2

MOUNTED VALVE IN CONDUIT - INCISED LONGITUDINAL VIEW



The dacron conduit has been opened longitudinally. The inflow side of the valve (a) is a straight line. The outflow side (b) has three apices with curved lines rather than straight in between each apex. Due to the pull of the conduit being stretched for photographic illustration these curved lines are not readily obvious. Note the three apices (c).

E. Surgical procedure for implantation of donor valves.

Ten 3-7 months old conditioned mongrel dogs weighing from 11 to 30 kilograms, were selected as recipients (Table 3). The conditioning consisted of familiarization with cage life, canine distemper and hepatitis vaccinations, worming for internal parasites, and obtain one normal complete blood count.

Atropine sulfate (1 mg/30 kilograms body weight) was administered subcutaneously to the animal fifteen minutes before anesthesia. The dog was anesthestized with a 4% solution of sodium thiamylal (16 mg/kg) via the cephalic vein to a depth of anesthesia that would allow endotracheal intubation. A maintanence anesthesia mixture of 1% Halothane and oxygen was used. An intravenous catheter was placed in the cephalic vein and a drip (1 cc/5 min) of Lactated Ringers solution was administered.

An electrocardiogram (ECG) was monitored on the Electronics for Medicine DR8 recorder. The dog was placed in right lateral recumbency with the rear legs apart exposing both femoral groove areas. The femoral groove areas and the left side of the chest were clipped and surgically prepared with povidine-iodine. A catheter was inserted in the left femoral artery and connected to a Statham P 23Db pressure transducer, which provided electrical input for continuous arterial pressure monitoring to the Electronics for Medicine DR8 recorder.

A lateral thoracotomy was made just posterior to the scapula over the fourth intercostal space. The latissimus dorsi and the scalenus muscles were incised, the serratus muscle divided, and the intercostal muscles were transected. The pleura was incised and the thorax opened. The left

TABLE 3

LENGTH OF TIME VALVE PRESERVED IN MEDIUM AND LENGTH OF TIME VALVE TRANSPLANTED IN THE DOG

| RECIPIENT DOG # | DAYS IN MEDIA | CLINIC NUMBER | DONOR VALVE # | DAYS IN DOG |
|---|---|---|---|---|
| 5. | 15 | 169882 | N | 82 |
| 3. | 16 | 169643 | M | 82 |
| 6. | 29 | 167153 | A | 82 |
| 7. | 30 | 171377 | P | 84 |
| 1. | 36 | 166386 | C | 81 |
| 2. | 64 | 170674 | J | 89 |
| 4. | 77 | 169788 | K | 89 |
| *8. | 23 | 167155 | G | 81 |
| **9. | 22 | 169990 | O | 70+ |
| ***10. | 135 | 170521 | I | 21 |

*Patent pulmonary artery

**Disappeared one week before catheterization

***Died 21 days postsurgically due to infection

anterior lung lobe was reflected posteriorly to facilitate optimal visualization of the heart. Hemostasis was maintained with electrocoagulation.

The pericardium was incised between the vagus and the phrenic nerves. Four 00 silk sutures were placed through the pericardium near the incision, being careful not to damage the nerves thus forming a pericardial basket. (Figure 3) The porous conduit containing the donor valve was pre-clotted with 5 ml of recipient blood withdrawn directly from the right ventricle with a 22 gauge needle and 5 ml syringe.

A right angled Lehey bile duct forcep was used to dissect around the pulmonary artery proximal to its division into the left and right pulmonary arteries. Umbilical tape (3 mm wide) was placed around the artery. A Satinsky forcep was placed on the pulmonary artery to occlude half the diameter. A stab incision was made with a number eleven scalpel blade. The incision was lengthened with Potts scissors to equal the diameter of the conduit. The conduit was placed over the pulmonary artery and attached to the pulmonary artery by two stay sutures of 5-0 Dermalene placed 180° apart at the incision commissures. Beginning at one suture a simple continuous suture pattern was used 180° around and tied to the opposite stay suture. The second stay suture was then continued on in a similar manner to complete a 360° circle.

A second Satinsky forcep was placed 2 mm from the anastomosis site on the conduit. The clamp on the pulmonary artery was slowly released and evaluated for leaks. If leaks were present hemostasis was accomplished with simple interrupted 5-0 Dermalene sutures. Ten ml of Lidocaine were placed into the Lactated Ringers Solution and 5 ml were placed directly

FIGURE 3

LATERAL VIEW OF THORACIC CAVITY AND PERICARDIAL BASKET

Left lateral thoracic cavity at 4th intercostal space. Pericardium with 4 00 silk sutures (a) placed to form a basket used to elevate the heart (b). Note phrenic (c) and vagus (d) nerves, anterior lobes of lungs folded posteriorly (e) and left auricle (f).

on the right ventricle to decrease irritability. At the same time, the blood pressure and electrocardiogram were monitored. The trauma of putting a clamp on the ventricle caused ectopic foci leading to premature beats. These premature beats were controlled with Lidocaine, which was continually placed on the ventricle until the premature beats were no longer present. An incision similar to the one in the pulmonary artery was made into the ventricle. Following the stab incision, the opening was lengthened with Potts scissors. Two stay sutures of 5-0 Dermalene placed 180° apart on the ventricle anastomosed the conduit to the ventricle. One stay suture was used in a continuous pattern around one side of the conduit and tied to the other stay suture. The second stay suture continued the pattern to complete the 360°. A Satinsky forcep was placed across the conduit 2 mm from the anastomosis site. The Satinsky forcep on the ventricle was released to verify hemostasis. If bleeding occurred, it was controlled by simple interrupted sutures. Once hemostasis was established, air was withdrawn from the conduit through a 22 gauge needle into a 10 ml syringe. The Satinsky forcep on the pulmonary artery end of the conduit was released. Any remaining air was then withdrawn from the conduit. The Satinsky forcep on the right ventricle end of the conduit was released and blood pressure and electrocardiogram recorded.

When the blood pressure and the electrocardiogram returned to preoperative normal, the umbilical tape around the pulmonary artery was tightened causing hypotension. When the blood pressure and electrocardiogram returned to the preoperative normal, the umbilical tape was tied in a square knot completely closing the portion of the pulmonary artery between the two anastomoses. The pericardium was left open. (Figure 4)

FIGURE 4

CONDUIT CONNECTING THE RIGHT VENTRICLE TO THE PULMONARY ARTERY

Left lateral thoracic cavity at 4th intercostal space. The conduit (a) is anastomosed to the right ventricle (b) and the pulmonary artery which is underneath the left auricle (c). Note the phrenic nerve (d) and pericardium (e).

At this time, a chest tube is inserted to evacuate air from the thoracic cavity. A stab skin incision was made in an intercostal space posterior to the thoracotomy site. A Kelly forcep was placed through the incision and tunneled anteriorly, for two intercostal spaces, through subcutaneous tissue. At that point, the Kelly forcep was used to bluntly perforate the intercostal muscles and pleura. A #16 French urethral catheter was then passed retrograde from the thoracic cavity to the skin via the subcutaneous tunnel. A purse string suture of 3-0 Ethilon was placed in the skin around the catheter to make the incision air tight.

Continuous suction of 10 mm mercury pressure was then applied to the chest catheter. Number one medium chromic catgut was used to approximate the ribs. The serratus ventralis and scalenus were closed with a continuous pattern of 00 medium chromic catgut. The latissimus dorsi muscle and subcutaneous layer were each closed in a similar manner. The skin was sutured with 4-0 Ethilon in a continuous pattern. The chest catheter was left in the chest until less than 25 ml of fluid were removed per hour. The catheter was then removed and the purse string suture secured. Post-operatively, procaine penicillin (10,000 u/lb) was given intramuscularly once a day for 5 days.

F. Cardiac Catheterization Procedure.

Atropine sulfate (1 mg per 30 kilograms body weight) was administered subcutaneously fifteen minutes prior to anesthesia. An intravenous solution of sodium pentobarbital (one mg/kg) was administered. The animal was placed in left lateral recumbancy. A small area was clipped over the right jugular vein and the skin was incised with a #10 scalpel blade. The right jugular vein was bluntly dissected with hemostatic forceps. Two 00 silk sutures were placed around the vein. The cranial suture was tied and one throw was placed in the posterior suture. By lifting on the posterior suture, blood flow ceased completely. A stab incision into the vein was made with a #11 scalpel blade. A ventriculography catheter was introduced into the vein and advanced caudally to the location of the heart. The posterior suture was tied to prevent the leakage of blood around the catheter.

The catheter was connected to two 3-way stopcocks in series, which were in turn connected to a Statham P23Db transducer. The transducer was wired to an Electonics for Medicine DR-8 recorder. A General Electric image intensifier fluoroscope was used to determine catheter position.

The catheter was manually guided from the jugular vein into the anterior vena cava and on into the right atrium. From the right atrium it was passed to the right ventricle and into the conduit. When the catheter passed through the conduit, pressures on both sides of the conduit valve were recorded. As the catheter was withdrawn, pressures were recorded from the right ventricle and right atrium. The catheter was advanced again to the right ventricle where radiopaque dye was injected and a ventriculogram was recorded.

The catheter was then completely withdrawn and the vein tied. The animal was euthanized with an overdose (20 ml) of sodium pentobarital.

G. Necropsy

Immediately following euthanasia, the animal was transported to pathology for a necropsy. The animal was placed on its right side. An incision was made through the hair, skin, subcutaneous tissue, and muscles overlying the sternum. A stab incision was made on the ventral midline caudal to the ribs. A rib cutter was inserted into the incision to split the sternum. Another incision was made along the dorsal portion of the ribs and the ribs were cut, thus completely exposing the left thoracic cavity. The chest wall was removed after noting any lung adhesions. The thoracic cavity was observed and all gross pathology noted. The trachea, esophagus, anterior vena cava, and the anterior vessels from the aorta were transected cranial to the heart. The esophagus, posterior vena cava, and descending aorta were transected caudal to the heart. The heart, lungs, and the short segment of esophagus were removed. The lungs and esophagus were separated from the heart. The conduit and the areas around its anastomotic sites were excised. The remaining portion of the heart was examined for pathology. The conduit, myocardium, and valves were grossly examined and tested for function by back flushing. After this procedure they were placed in 10% formalin for histopathological examination.

## RESULTS

### A. MICROBIOLOGY.

The microbiological results from the four dogs used in the preliminary microbiological study indicates that microorganisms are present that could cause contamination (Table 4). These microorganisms are the same ones that were stated in the literature review. One of the antibiotics that was used in the nutrient medium and that the microorganism was sensitive to is listed in Table 4.

The donor valves produced no growth in culture after 48 hours in the nutrient-antibiotic medium. The seminlunar valves were maintained in the nutrient-antibiotic medium for an additional 13 to 133 days. When cultured just prior to implantation, all semilunar valves were still sterile (Table 5).

TABLE 4

PRELIMINARY CULTURE RESULTS OF HEART VALVES

| TEST ANIMAL | ORGANISMS | SENSITIVE TO |
|---|---|---|
| 1. | Escherichia coli | Gentamicin |
| | Enterobactera sp. | Gentamicin |
| | Stapylococcus aureus | Lincomycin |
| | Non-hem Streptococcus | Gentamicin |
| | Alpha hem. Streptococcus | |
| | Corynebacterium sp. | Kanamycin |
| | Micrococcus | Kanamycin |
| 2. | None | ---- |
| 3. | Micrococcus | Gentamicin |
| 4. | Bordatella bronchiseptica | Gentamicin |

TABLE 5

MICROORGANISMS CULTURED FROM DONOR HEART VALVES

| | DONOR VALVES | AT HARVEST | 48 HOURS AFTER HARVEST | AT TIME OF IMPLANTATION |
|---|---|---|---|---|
| 1. | A | nonhem. Micrococcus | None | None |
| 2. | B | nonhem. Micrococcus | None | --- |
| 3. | C | nonhem. Micrococcus | None | None |
| 4. | D | Alkaligenes | None | --- |
| 5. | E | None | None | --- |
| 6. | F | Alkaligenes | None | --- |
| 7. | G | None | None | None |
| 8. | H | None | None | None |
| 9. | I | None | None | None |
| 10. | J | None | None | None |
| 11. | K | None | None | None |
| 12. | L | Moraxella | None | --- |
| 13. | M | None | None | None |
| 14. | N | Bordetella bronch. | None | None |
| 15. | O | Bordetella bronch. | None | None |
| 16. | P | Moraxella | None | None |

--- No culture taken

B. PATHOLOGY OF VALVES.

Semilunar valve samples sent to pathology for assessment of tissue viability were processed and stained with Hematoxylin and Eosin. Although 16 valves were collected only 11 were tested histologically. The others were used only for microbiological study.

Viability of the heart valve tissue was determined by the staining quality and the shape of fibrocyte nuclei. In addition, the staining quality and nuclei characteristics of the myocardial tissue associated with each heart valve was evaluated. These findings were compared to similar data acquired from a control valve which had been taken from a donor dog and immediately placed in formalin.

The results of the histopathological study indicate that all tissues submitted were viable. Some tissue appeared normal up to 72 days. One valve (N) had a very small amount of necrosis at 15 days. Only the tissue maintained in the nutrient medium for 135 days had a mild to moderate amount of necrosis.

The necrosis of the tissue was evidenced by pyknotic nuclei or nuclei undergoing karyolysis. A few nuclei were associated with clear vacuoles, giving a signet-ring appearance. Other nuclei were of a somewhat granular, dirty appearance. One valve (I) that had been in the nutrient medium for 135 days, had an associated myocardium that stained more basophilic and the cross-striations were not as prominent as in other samples.

To act as controls, two heart valves with adjacent myocardium were placed in saline for 40 days. Both controls had extensive necrosis with no viability as evidenced by pale staining quality and coagulation necrosis of the tissues.

C. PATHOLOGY OF VALVE TRANSPLANTS.

Ten dogs were selected as recipients for the heart valve transplants. Results generated from three of these dogs could not be used for this study for the following reasons. One (#10) died 21 days postsurgically due to pneumonia. On necropsy, the valve transplant appeared functional and had no bearing on the death. Whether the pneumonia was a result of the surgery or a disease contracted after the surgery could not be established.

A second dog was used mistakingly for another experiment (#9). He was euthanized and no necropsy was performed. Prior to that time, the animal appeared normal with no audible murmur during the 70 days the valve transplant had been in place.

The third dog (#8) that could not be used in this study had a patent pulmonary artery. Standard surgical procedures were used, but the umbilical tape around the pulmonary artery had not been tied securely. As a result, the donor valve was nonfunctional because it adhered to the sides of the conduit due to inadequate blood flow through the conduit.

At the time of euthansia, all dogs used in the study appeared to be healthy, growing, and gaining weight. At gross examination, there was a very mild to moderate foreign body reaction with each valve transplant. In every study dog, the left lung lobes were adhered to the chest wall pleura and the dacron conduit. The lungs appeared functional except for adhesions. (Figure 5 and 6)

FIGURE 5

ADHESIONS OF LUNGS TO PLEURA

Left lateral thoracic cavity demonstrating typical adhesions of the lungs (a) to the pleura (b) as seen in most thoracic surgery. Note posterior lobe of left lung (c), heart (d) and diaphragm (e).

FIGURE 6

SEVERE ADHESIONS AND TISSUE REACTION TO THORACIC SURGERY AND VALVED CONDUIT IMPLANT

Left lateral view of thoracic cavity demonstrating typical fibrous connective tissue adhesions (a) of the lungs (b) and the conduit (c). Note left auricle (d) and posterior lobe of left lung (e).

In each case the conduit was covered with 1 - 4 mm of fibrous connective tissue and the pericardial sac was adhered to the heart. All other thoracic organs appeared normal.

When the conduits were cut open lengthwise, a thin layer of connective tissue lined each conduit. In all cases, the valves appeared functional. As noted in Table 6 some of the valves had a few calcium placques, which is a typical finding of foreign body reaction. (Figure 7 and 8)

Histological sections confirmed the gross impression. The anastomotic sites appeared to be healthy and intact. There were live fibrocytes present indicating the valves were still viable. A very small amount of degeneration was present as evidenced by the pale staining quality of the tissue. The primary histopathological changes seen involved a chronic reaction to a foreign body. This change involved a fibrous connective tissue reaction with some mineralization and granulomatous inflammation. Dense fibrous connective tissue surrounded the conduit and some was dispersed in the surrounding myocardium. Fibroblastic activity was present on the valves and along the inside surface of the conduit. The inflammatory response consisted of lymphocytes, plasma cells, neutrophils and macrophages.

FIGURE 7

DISSECTED HEART AFTER CONDUIT IMPLANTATION

Dissected heart after removal from the dog at necropsy. Note the conduit (a), anatomosis of conduit to right ventricle (b), and right ventricle (c).

FIGURE 8

VALVED CONDUIT DISSECTED AT NECROPSY

The conduit has been incised longitudinally to illustrate the fibrous connective tissue reaction on the outside (a) and inside (b) of the conduit. A few calcium plaques (c) can be seen. The outflow side of the valve (d) can be seen and the leaflets were functional. The anastomotic site of the conduit-pulmonary artery (e) is smooth with no necrosis present.

TABLE 6

PATHOLOGY OF HEART VALVES TRANSPLANTED IN DOGS

| DONOR VALVE | DAYS IN MEDIUM | DAYS IN DOG | PATH. AFTER MEDIUM | PATH. AFTER TRANSPLANT |
|---|---|---|---|---|
| A | 29 | 82 | Mild myocard. degen. | Mild to moderate foreign body reaction. |
| C | 36 | 81 | Mild myocard. degen. | Mild to moderate foreign body reaction. |
| G | 23 | 81 | Normal | Not used. Patent Pulmonary artery. |
| H | 72 | | Normal | |
| I | 135 | 21 | Mild to mod. degen. | Died 21 days Post-op. |
| J | 64 | 89 | Near normal | Mild foreign body reaction. |
| K | 77 | 89 | Mild degen. | Moderate foreign body reaction. |
| M | 16 | 82 | Normal | Mild foreign body reaction. |
| N | 15 | 82 | Very mild degen. | Relatively normal with minimal foreign body reaction. |
| O | 22 | | Normal | Missing. |
| P | 30 | 84 | Normal | Very minimal foreign body reaction. |

D. CARDIAC CATHETERIZATION RESULTS.

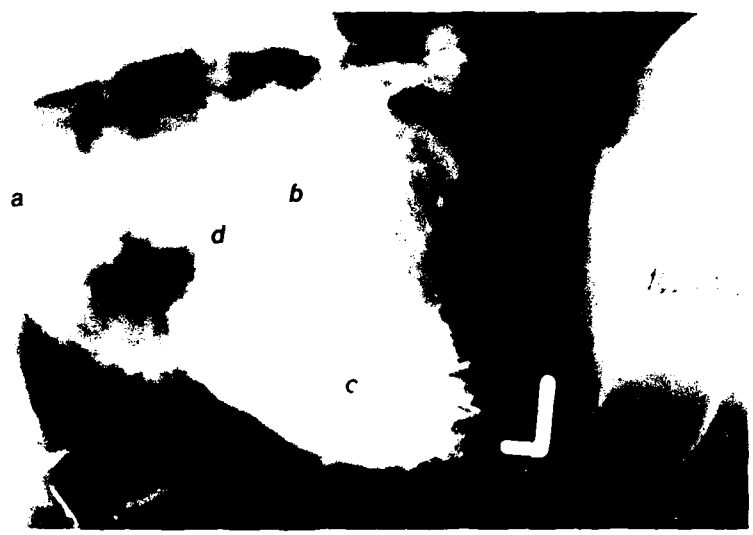
The cardiac catheterization data is listed in Table 7. In all cases where data could be recorded, a gradient was present. This gradient was primarily due to the size of the conduit and not because of tissue reaction or valve function. In the one case (#1) where data could be taken from both sides of the valve in the conduit, there was an approximate 20 mm Hg gradient. The valves appeared functional and in good condition. Figure 9 shows a ventriculogram demonstrating the valved conduit to be functional.

One dog (#7) died during the catheterization procedure. The death occured at the time of dye injection.

FIGURE 9

VENTRICULOGRAMS OF VALVED CONDUIT IMPLANTS IN THE HEART

a.




The ventriculography catheter is seen entering the heart through the anterior vena cava (a) into the right atrium (b) and into the right ventricle (c). The area of the occluded pulmonary artery can be seen (d).

FIGURE 9 (CONTINUED)

VENTRICULOGRAMS OF VALVED CONDUIT IMPLANTS IN THE HEART

b.


The ventriculography catheter is seen entering the heart through the anterior vena cava (a) into the right atrium (b) and into the right ventricle (c). The area of the occluded pulmonary artery can be seen (d). The catheter tip is in the conduit (e).

FIGURE 9 (CONTINUED)

VENTRICULOGRAMS OF VALVED CONDUIT IMPLANTS IN THE HEART

c.


The ventriculography catheter is seen entering the heart through the anterior vena cava (a) into the right atrium (b) and into the right ventricle (c). The area of the occluded pulmonary artery can be seen (d). The catheter tip is in the conduit (e).

FIGURE 9 (CONTINUED)

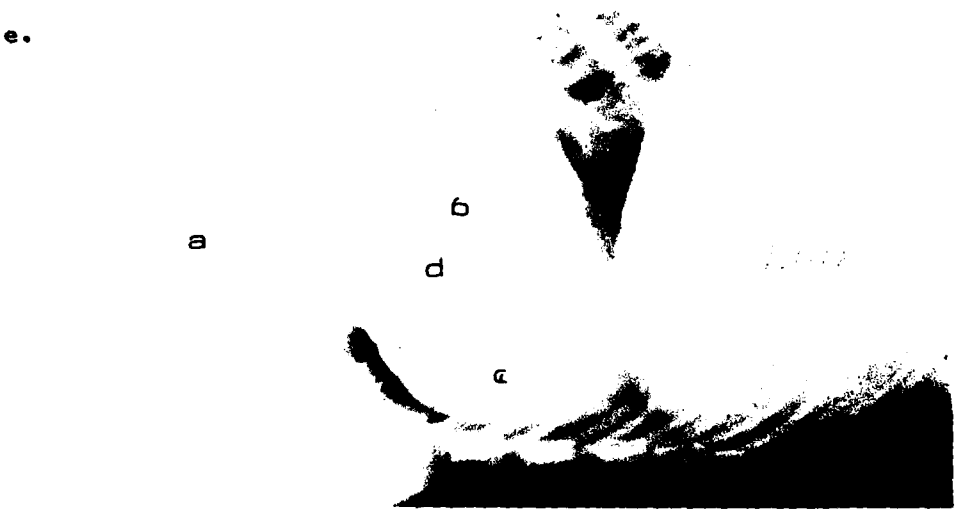
VENTRICULOGRAMS OF VALVED CONDUIT IMPLANTS IN THE HEART

d.


The ventriculography catheter is seen entering the heart through the anterior vena cava (a) into the right atrium (b) and into the right ventricle (c). The area of the occluded pulmonary artery can be seen (d).

FIGURE 9 (CONTINUED)

VENTRICULOGRAMS OF VALVED CONDUIT IMPLANTS IN THE HEART




The ventriculography catheter is seen entering the heart through the anterior vena cava (a) into the right atrium (b) and into the right ventricle (c). The area of the occluded pulmonary artery can be seen (d). The catheter tip is at the valve (e).

FIGURE 9 (CONTINUED)

VENTRICULOGRAMS OF VALVED CONDUIT IMPLANTS IN THE HEART

f.


The ventriculography catheter is seen entering the heart through the anterior vena cava (a) into the right atrium (b) and into the right ventricle (c). The area of the occluded pulmonary artery can be seen (d). The catheter tip is at the valve (e).

TABLE 7

CARDIAC CATHETERIZATION RESULTS OF DOGS

WITH IMPLATED ALLOGRAFT HEART VALVES

| RECIPIENT DOG # | RIGHT ATRIUM | RIGHT VENTRICLE | PRE-VALVE PRESSURE | POST-VALVE PRESSURE |
|---|---|---|---|---|
| 1. | 10 | 60 | 45 | 28 |
| 2. | 15 | 130 | -- | 35 |
| 3. | 5 | 75 | -- | -- |
| 4. | -- | 35 | Died | -- |
| 5. | -- | 45 | -- | 15 |
| 6. | 8 | 60 | -- | 55 |
| 7. | Died at time of dye injection | | | |

-- No data obtained

## DISCUSSION

From the time of collection to the time of implantation, many factors can act on a valve to decrease its viability. First, contamination and sterilization can affect a valve. In addition, viability is affected by storing the valves for several weeks until a recipient is found.

The major efforts of this study were directed at the viability problems associated with contamination, sterilization, and storage of heart valves. As a follow up, the valves were implanted in a recipient merely to test the viability of the valves 75 days after implantation. Tissue rejection was not one of the prime considerations of the study, so its effect on tissue viability was not tested and the amount of deterioration caused by it was not determined.

The first objective of sterilization was accomplished. No bacterial or fungal growth was present on any of the valves after 48 hours or at the time of implantation. Further, from the valves tested and found to be essentially normal when compared to a fresh untreated valve, it can be stated that there was no damage to the fibrocytes by the antibiotics. In dealing with microorganism contamination and antibiotic sterilization, one has to keep in mind that each contribute somewhat to tissue deterioration. Good, aseptic surgical technique should always prevail in the collection of donor heart valves - the less contamination and surgical trauma, the less tissue deterioration from the start.

In the actual situation where human heart valves are generally collected at a city morgue, contamination often occurs. All efforts should be made to keep contamination to a minimum. In this study, most semilunar valves were harvested in an aseptic environment. No attempt was made to include a test for specific microorganisms, but primarily to test the amount of deterioration caused by the antibiotics used and the nutrient medium. The organisms found on the valves were controlled by antibiotics present in the medium, as well as acting as a prophylactic agent against microorganism growth during the storage phase. In a control valve that was placed in sterile saline, both bacterial and fungal growth occurred. Table 8 lists the antibiotics used for sterilization and the microorganisms they have been found to be effective against as determined by the manufacturer.

The second point of this study dealt with the viability of the valvular tissue after different storage periods in the nutrient medium. Several methods of testing for viability have been used by different investigators. Due to time, cost involved, and sophisticated equipment required to do enzyme studies, autoradiography, and tissue cultures[8,21], it was decided that only the histological appearance would be used in this study for determination of viability. To help determine the viability, controls were used for comparison. Fresh valve tissue was placed directly into formalin and then processed. Other fresh valves first were placed in a saline solution for forty days, before being exposed to formalin and processing.

The results of this study indicate that five of the 11 semilunar valves tested were normal. The rest were either near normal or had mild

TABLE 8

SENSITIVITY OF ANTIBIOTICS USED IN THE MEDIUM

| ANTIBIOTICS GENERIC | ORGANISMS SENSITIVE |
|---|---|
| Colistimethate | Enterobacter aerogenes, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa. |
| Gentamicin | Escherichia coli, Proteus sp., Pseudomonas aeruginosa, Klebsiella sp., Enterobacter sp., Serratia group, Citrobacter sp., Staphylococcus sp., Salmonella sp., Shigella sp. |
| Kanamycin | Staphyloccus aureus, Staphylococcus epidermidis, Neisseria gonorrhoeae, Hemophilus influenzae, Eicherichia coli, Enterobacter aerogenes, Shigella sp., Klebsiella pneumoniae, seratia Marcescens, Mima-Herellea, Proteus sp. |
| Lincomycin | Staphylococcus aureus, Staphylococcus allius, Beta hemolytic Streptococcus, Streptcoccus viridans, Diplococcus pneumoniae, Clostridium tetani, Clostridum perfringens, Corynebacterium diphtheriae. |
| Amphotericin | Histoplasma capsulatum, Coccidiodes immitis, Candida sp., Blastomyces dermatitides, Rhodotorula, Cryptococcus neoformans, Sporotrichum schenckii, Mucor mucido, Asperigillus fumigatus. |

degenerative changes. They were all viable. This would indicate that the antibiotics which were selected had little or no effect on the viability of the semilunar valves.

The reason these antibiotics were selected was because of their efficacy and minimal effect on tissues as proven in previous studies.[6,10,24,26,28,30,32,33,37] Also, antibiotics had been shown in previous studies to cause less tissue deterioration than other methods of sterilization such as formalin, beta-propiolactone, and gamma irradiation.[6]

The nutrient medium (RPMI-1640 plus fetal calf serum) was selected due to its effectiveness in preserving white blood cell cultures. The use of this medium to preserve heart valves had not been previously reported. It was felt that if it worked well on white blood cells then it might be effective with heart tissue.

The results of this study indicate this medium was effective in preserving the viability of semilunar valves as determined by the degree of necrosis present in the myocardial tissue. The semilunar valve leaflets were also checked for fibrocytic activity and in all cases, the valves were viable in appearance.

The last phase of the study involved the actual implantation of the semilunar valves for 75 days or more in a recipient dog. Due to the amount of tissue reaction associated with the implantation, it was difficult to evaluate the results as they pertain to the heart valve viability. Histologically, the semilunar valves were viable with only mild structural changes. These changes primarily were associated with

tissue rejection. A further study is recommended in which the animals are treated for tissue rejection in order to completely evaluate the changes made only by the antibiotic-nutrient medium on the semilunar valve. Viability was, however, demonstrated in all cases by the amount of normal fibrocytic activity indicating that the goal of this study was accomplished - that of improving the storage quality of fresh valves.

From this study, it was shown that the selected antibiotics effectively sterilized the tissue with little detrimental effect on the viability of the semilunar valve. These antibiotics proved to be an excellent combination and should be used in future studies. This study indicates that this nutrient medium is a better formula than previously published ones for maintaining viability of fresh valves. Further studies are needed to more effectively evaluate the medium. With different test results obtained in dogs versus humans, the medium should be tested using human valves. If these results are similar to this canine study, a more realistic evaluation can be made as to whether the best tissue for heart valve replacement is from live tissue of human allografts or dead tissue of procine xenografts.

## SUMMARY

The medium selected to store canine heart valves utilized RPMI-1640 (a white cell tissue culture medium) with fetal calf serum and antibiotics added. The valves were stored at 4C for periods from 15 to 135 days. When removed from the medium the pulmonic semilunar valves were tested for viability and sterility. Viability was determined by histopathological changes and sterility by microbiological cultures. The aortic valves were mounted in dacron conduits. Through a left lateral thoracotomy they were surgically implanted between the pulmonary artery and the right ventricle. The section of the pulmonary artery between the anastomoses was occluded. After 81 to 89 days the recipient dogs were catheterized and euthanasia followed. Results indicate that all valves were sterile and viable with 91% being normal or having only mild degeneration.

# APPENDICES

# RPMI Media 1640

| COMPONENT | mg/L |
|---|---|
| **INORGANIC SALTS** | |
| $CaCl_2$ | 100.00 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | 400.00 |
| KCl | |
| $MgSO_4$ | 100.00 |
| $MgSO_4 \cdot 7H_2O$ | 6000.00 |
| NaCl | 2000.00 |
| $NaHCO_3$ | |
| $NaH_2PO_4 \cdot H_2O$ | |
| $Na_2HPO_4$ (anhyd.) | 1512.00 |
| $Na_2HPO_4 \cdot 7H_2O$ | |
| **OTHER COMPONENTS** | |
| Bacto-peptone | 2000.00 |
| Glucose | 1.00 |
| Glutathione (reduced) | 5.00 |
| Phenol red | |
| **AMINO ACIDS** | |
| L-Alanine | 200.00 |
| L-Arginine (free base) | 50.00 |
| L-Asparagine | 20.00 |
| L-Aspartic acid | |
| L-Cysteine HCl[b] | 50.00 |
| L-Cystine | 20.00 |
| L-Glutamic acid | 300.00 |
| L-Glutamine | 10.00 |
| Glycine | 15.00 |
| L-Histidine (free base) | 20.00 |
| L-Hydroxyproline | 50.00 |
| L-Isoleucine (Allo free) | 50.00 |
| L-Leucine (Methionine free) | 40.00 |
| L-Lysine HCl | 15.00 |
| L-Methionine | 15.00 |
| L-Phenylalanine | 20.00 |
| L-Proline (Hydroxy L-Proline free) | 30.00 |
| L-Serine | 20.00 |
| L-Threonine (Allo free) | 5.00 |
| L-Tryptophan | 20.00 |
| L-Tyrosine | 20.00 |
| L-Valine | |
| **VITAMINS** | |
| Ascorbic acid | 0.20 |
| Biotin | 0.25 |
| D-Ca pantothenate | 3.00 |
| Choline Cl | |

| COMPONENT | mg/L |
|---|---|
| **VITAMINS Cont'd.** | |
| Folic acid | 1.000 |
| Folinic acid | |
| i-Inositol | 35.000 |
| Nicotinamide | 1.000 |
| Nicotinic acid | |
| Para-aminobenzoic acid | 1.000 |
| Pyridoxal HCl | |
| Pyridoxine HCl | 1.000 |
| Riboflavin | 0.200 |
| Thiamine HCl | 1.000 |
| Vitamin $B_{12}$ | 0.005 |

## MEDIUM 199

Ref.: Proc. Soc. Exp. Biol. Med., 73:1 (1950).

| COMPONENT | HANKS' SALTS #115H (1X) #118H (10X) | EARLE'S MODIFIED SALTS #115E (1X) #118E (10X) | EARLE'S #115EE (1X) #118EE (10X) |
|---|---|---|---|
| | mg/L | mg/L | mg/L |
| NaCl | 8000.0 | 6800.0 | 6800.0 |
| KCl | 400.0 | 400.0 | 400.0 |
| $MgSO_4 \cdot 7H_2O$ | 200.0 | 200.0 | 200.0 |
| $Na_2HPO_4 \cdot 2H_2O$ | 60.0 | — | — |
| $NaH_2PO_4 \cdot H_2O$ | — | 125.0 | 140.0 |
| $KH_2PO_4$ | 60.0 | — | — |
| Glucose | 1000.0 | 1000.0 | 1000.0 |
| Phenol red | 20.0 | 20.0 | 20.0 |
| $CaCl_2$ (anhyd.) | 140.0 | 200.0 | 200.0 |
| $NaHCO_3$* | 350.0 | 1250.0 | 2200.0 |

| Component | mg/L | Component | mg/L |
|---|---|---|---|
| L-Arginine HCl | 70.000 | DL-Isoleucine | 40.000 |
| L-Histidine HCl | 20.000 | DL-Valine | 50.000 |
| L-Lysine monohydrochloride | 70.000 | DL-Glutamic acid monohydrate | 150.000 |
| DL-Tryptophan | 20.000 | DL-Aspartic acid | 60.000 |
| DL-Phenylalanine | 50.000 | DL-Alpha-Alanine | 50.000 |
| DL-Methionine | 30.000 | L-Proline | 40.000 |
| DL-Serine | 50.000 | L-Hydroxyproline | 10.000 |
| DL-Threonine | 60.000 | Glycine | 50.000 |
| DL-Leucine | 120.000 | L-Glutamine | 100.000 |

* *Omitted from 10X preparations.*

### Medium 199 (con't.)

| Component | mg/L | Component | mg/L |
|---|---|---|---|
| Sodium acetate | 50.000 | i-Inositol | 0.050 |
| L-Cystine | 20.000 | Ascorbic acid | 0.050 |
| L-Tyrosine | 40.000 | Folic acid | 0.010 |
| L-Cysteine HCl | 0.100 | Para-Aminobenzoic acid | 0.050 |
| Adenine Sulfate | 10.000 | Ferric nitrate $Fe(NO_3)_3$ | 0.100 |
| Guanine HCl | 0.300 | d-Biotin | 0.010 |
| Xanthine | 0.300 | Menadione | 0.010 |
| Hypoxanthine | 0.300 | Glutathione | 0.050 |
| Uracil | 0.300 | Vitamin A | 0.100 |
| Thymine | 0.300 | Calciferol | 0.100 |
| Disodium alpha tocopherol phosphate | 0.010 | Cholesterol | 0.200 |
| Thiamine HCl | 0.010 | Tween 80** | 20.000 |
| Pyridoxine HCl | 0.025 | Adenosinetriphosphate (Di-Sodium salt) | 1.000 |
| Riboflavin | 0.010 | Adenylic acid | 0.200 |
| Pyridoxal HCl | 0.025 | Desoxyribose | 0.500 |
| Niacin | 0.025 | Ribose | 0.500 |
| Niacinamide | 0.025 | Choline Cl | 0.500 |
| Ca pantothenate | 0.010 | | |

** *Trademark of Atlas Powder Co.*

## APPENDIX B

### List of Materials and Pharmaceuticals Used in the Study

Povidone-Iodine - (Betadine Surgical Scrub) Purdue Frederick Co., Yonkers, N.Y.

RPMI 1640 with 25 mM Hepes Buffer, Grand Island Biological Co., Grand Island, N.Y.

Fetal Calf Serum, Grand Island Biological Co., Grand Island, N.Y.

Colistimethate Sodium (Coly-Mycin M) Warner/Chilcott, Morris Plains, N.J.

Gentamycin sulfate (Garamycin) Schering Corp. Kenelworth, N.J.

Kanamycin sulfate (Kantrex) Bristol Laboratories, Syracuse, N.Y.

Lincomycin hydrochloride monohydrate (Lincocin) Upjohn, Kalamazoo, MI

Amphotericin B (Fungizone) Squibb and Sons, Princeton, N.J.

T-61 Euthanasia Solution - National Laboratories Corp., Summerville, N.J.

Dacron conduit - United States Catheter Instrument, Inc., Billerica, MA

4-0 Ticron - Davis-Geck, Pearl River, N.Y.

Atropine sulfate - Bel-mar Laboratories Inc., Inwood, N.Y.

Sodium Thiamylal (Surital) Parke Davis and Co., Detroit, MI

Halothane (fluothane) Ayerst Laboratories, Inc., New York, N.Y.

Statham P23Db Pressure Transducer, Statham Laboratories, Inc., Hato Rey, Puerto Rico.

5-0 Dermalene Davis Geck, Pearl River, N.Y.

Lidocaine - Veratex Corp., Detroit, MI

Urethral catheter (chest tube) Sherwood Medical Industries, Inc., St. Louis, MO

Medium chronic catgut - Ethicon, Somerville, N.J.

4-0 Ethilon - Ethicon, Somerville, N.J.

Procaine Pencillin - Squibb and Sons, Princeton, N.J.

Lehman Ventriculography catheter - United States Catheter Instrument Corp., Billerica, MA.

Diatrizoate Meglumine and Diatrizoate Sodium (Hypoque-M75%) Winthrop Laboratories, New York, N.Y.

I.V. Catheter - Becton, Dickinson and Co., Rutherford, N.J.

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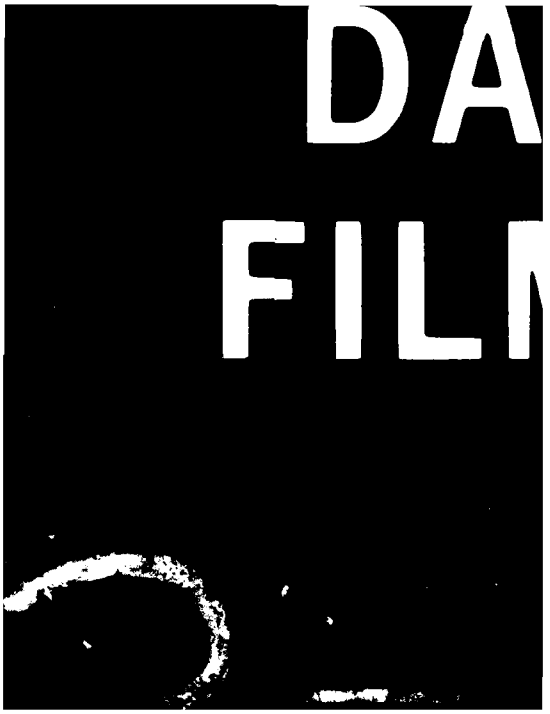
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