INVESTIGATION OF PROSTHETIC VASCULAR GRAFT INFECTIONS IN AN OVINE MODEL

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ABSTRACT

This thesis examines the *in-vitro* and *in-vivo* effect of rifampicin (a known anti-staphylococcal agent) following prosthetic graft impregnation, to prevent and treat *Staphylococcus epidermidis* (MRSE) and Staphylococcus aureus (MRSA) prosthetic vascular graft infections. In addition the effects of rifampicin on intimal hyperplasia were analysed.

Using a disc diffusion technique, one square centimetre segments of Gelsoft, Gore-Tex, Fluoropassiv or Thoratec were impregnated with rifampicin at concentrations of 1.2mg/ml, 10 mg/ml or 30 mg/ml and placed on a bacterial lawn of MRSA or MRSE. With increasing rifampicin concentration, all grafts displayed increased initial zones of inhibition and length of time of antibacterial activity. Fluoropassiv and Gelsoft were the superior grafts at all studied rifampicin concentrations.

Using an established ovine model in which grafts were interposed in the carotid artery of sheep, it was shown that all grafts studied *in-vitro* were easily infected with an overwhelming dose of MRSA or MRSE, and once infected would harbour the infective micro-organism.

From our preliminary *in-vitro* and *in-vivo* results Gelsoft was chosen as the graft of choice for the subsequent *in-vivo* (ovine model) experimentation.

Interposition Gelsoft grafts at concentrations of 1.2mg/ml or 10 mg/ml rifampicin was inoculated with 10^8 colony forming units (CFU) of MRSE or MRSA and compared to infected non-impregnated Gelsoft grafts. Grafts were harvested at three weeks.

Our findings showed that rifampicin at both 1.2 mg/ml and 10 mg/ml reduced the incidence of abscess formation, anastomotic disruption and graft thrombosis for both

MRSE and MRSA. In addition overall positive cultures were significantly reduced with increasing rifampicin concentration for MRSE and MRSA infected grafts.

MRSE or MRSA infected Gelsoft grafts were replaced at three weeks with 1.2 mg/ml or 10 mg/ml rifampicin impregnated Gelsoft grafts. The replacement grafts were subsequently removed following a further three weeks. No significant improvements were noted for the recorded macroscopic or bacteriological parameters with increasing rifampicin concentration with the MRSA infected grafts. However, with regards to *S. epidermidis*, a concentration of 10 mg/ml effectively reduced the total number of infected grafts compared to both the control group and the 1.2 mg/ml rifampicin group.

Varying the concentration of rifampicin between nil, 1.2mg/ml and 10 mg/ml had no significant impact on the formation of intimal hyperplasia or platelet aggregation.

These results demonstrated that 10 mg/ml rifampicin impregnation of Gelsoft grafts is an effective means of preventing MRSA and MRSE prosthetic graft infection and treating established MRSE graft infection without contributing to intimal hyperplasia.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

List of tables

List of figures

ABBREVIATIONS

CFU	colony forming units
CNS	coagulase negative staphylococci
C.T.	computerised tomography
ECs	endothelial cells
E. coli	Escherichia coli
ePTFE	expanded polytetrafluoroethylene
IgG	immunoglobulin G
IH	intimal hyperplasia
MRSA	Methicillin resistant staphylococcal staph aureus
MRSE	Methicillin resistant staphylococcal epidermidis
P. aeruginosa	Pseudomonas aeruginosa
PRP	platelet rich plasma
PET	polyethylene terephthate
PTFE	polytetrafluoroethylene
S. aureus	Staphylococcal aureus
S. epidermidis	Staphylococcal epidermidis
SMC	smooth muscle cells

PUBLICATIONS AND PRESENTATIONS TO LEARNED SOCIETIES

Publications

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Vicaretti, Hawthorne, W. J., Ao, P.Y., Fletcher, J.P. (1997). Does replacement of an *S. epidermidis* infected vascular graft with a rifampicin soaked gelatin sealed Dacron graft reduce the incidence of subsequent infection? *Surgical Research Society of Australasia, Freemantle*

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DECLARATION

I declare that the research presented in this thesis is original and my own work with such assistance as is acknowledged elsewhere. None of the work has been submitted to this or any other university for a higher degree.

[Redaction]

Mauro Vicaretti

Chapter 1

PROSTHETIC VASCULAR GRAFT INFECTIONS, AETIOLOGY, TREATMENT AND COMPLICATIONS.

1.1 HISTORICAL REVIEW

1.1.1 Arterial Vascular Surgery

The credit for the beginning of vascular arterial surgery lies with two comparatively unknown men, Rufus of Ephesus (ca. 100 AD) and Antyllus (ca 140 AD) who both propounded the principles of haemostasis, including compression, cautery and ligature (Osler 1915). The Christian saints of surgery, Cosmas and Damian, who reached martyrdom in 303 AD, have been credited with the first arterial and venous anastomoses when they replaced their patient's cancerous, gangrenous leg with the leg of a just dead Moor (Callow 1978). In 1552, Ambroise Paré (1510-90), regarded as the leading figure of Renaissance surgery rediscovered the ligature (Tracy 1988) and introduced the true ancestor of the artery forceps, the "bec de corbin". Despite Hallowell's initial success in 1759 to control arterial haemorrhage and preserve the lumen of a damaged brachial artery (Dale, 1974), Assmann in 1773 (Assmann, 1773, cited in Callow 1982) and LeConte the following year (LeConte, 1774 cited in Callow 1982) found that the repair of arteries with the intention of lumen patency invariably led to thrombosis and permanent lumen obliteration. It is in light of these observations that ligation of damaged arteries became the standard of care for the next century. Jassinowksy in 1891 (Jassinowksy, 1891, cited in Callow 1978) demonstrated that arterial wounds could be repaired with lumen preservation. Queirolo and Masini (1895), Nitze (1897), Murphy (1897), Jaboulay (1898), Dörfler (1899), Abbe (1892), and Payr (1901) all made major contributions to the advancement of arterial anastomosis (Callow 1978). Alexis Carrel in the early 1900's working with Jaboulay in France and later with Guthrie in the United States of America was a major contributor to the beginnings of successful vascular surgery (Callow 1978). Carrel devised the technique of triangulation for the suturing of arterial ends; described a patch technique to anastomose a smaller artery to the side of a larger artery (Carrel patch) and is considered the pioneer of organ transplantation (Barker 1993). In 1912, Carrel received the Nobel Prize in recognition of his contribution to vascular anastomosis and

organ transplantation (Friedmann 1993). In 1906, José Goyanes reported the first use of a patient's vein as a conduit in the repair of the arterial defect following excision of a popliteal artery aneurysm (Goyanes, 1906, cited in Barker 1993).

The World Wars contributed little to the progress in vascular surgery and the period up to the 1940's was recognised more for the development of anaesthesia, blood transfusion, surgical sympathectomy and anticoagulation (Dale 1974). However, the period following World War II saw rapid developments in vascular surgery particularly with the use of arterial homografts. Dubost, Allary and Oeconomos in 1952 described the first successful resection of an abdominal aortic aneurysm and replacement with a homograft. By the early 1960's the future of arterial homografts was poor because of the low patency rates, degenerative changes occurring in the preserved arterial homografts, their limited supply and problems of harvesting, sterilisation and preservation (Wesolowski & Dennis 1963).

Voorhess, Jaretski, and Blakemore (1952) heralded the modern day era of reconstructive synthetic arterial surgery. They fashioned and inserted Vinyon "N" cloth tubes of varying length into the abdominal aorta of dogs that were killed 19-153 days later. On removal of the synthetic tubes they found the inner lining to be similar to the inner lining of the abdominal aorta but without elastic and smooth muscle elements. This paved the way to the development of newer arterial conduits especially for large artery replacement.

The 1960's saw the introduction of the balloon embolectomy catheter by Thomas Fogarty for the extraction of clot in the management of embolisation (Fogarty, Cranley, Krause, Strasser & Hafner 1963) and percutaneous angioplasty (Dotter & Jenkins 1964).

The 1970's was revolutionised by Stanley Crawford who pioneered the management of thoracoabdominal aneurysms (Crawford 1974).

The eighties and beyond have been revolutionised by endovascular technologies allowing for an increasing emphasis on the management of arterial disease by percutaneous means.

In addition, the growth of non-invasive investigative modalities and a better understanding of arterial disease pathology have allowed the vascular surgeon to accurately diagnose and better manage arterial disease.

1.1.2 Arterial Prostheses

The characteristics of the ideal vascular prosthesis are that it is available in varying sizes and lengths, easily handled and sutured, durable, non-thrombogenic, of low infectivity, has a high patency rate and has viscoelastic properties similar to the host artery. To date no autogenous or prosthetic conduit is available that meets all these characteristics (Zelt & Abbott 1994). The development of a prosthesis with these characteristics has been an exciting period for vascular surgery because of the novel approaches by numerous researchers to develop the perfect graft. Following Voorhess, Jaretski, and Blakemore's (1952) development of the Vinyon "N" cloth tubes prostheses, the interest in synthetic conduits accelerated. Such was the enthusiasm in experimenting with different textiles that Wesolowski (1978) commented that "There followed a rather unscientific interest in arterial prostheses during the subsequent 5 years when vascular surgeons raided yard-goods stores and empirically screened all sorts of materials for use as artificial arteries" (p.27). In addition Barker (1993) stated that "the transcripts of vascular surgery meetings of the late 1950's might be mistaken for a textile journal, as various weaves, deniers, calendarizing, and the advantages of braid versus knit versus taffeta weaves were discussed" (p.9). Edwards and Tapp (1955) introduced braided and crimped nylon tubes, but it was soon recognised that nylon was unsuitable because of its loss of tensile strength. With all the differing materials under investigation, a committee appointed by the Society of Vascular Surgery found that many of the materials lost significant tensile strength following implantation except for two materials namely Dacron (polyethylene terephthate) and Teflon (polytetrafluoroethylene) whose tensile strength remained essentially unchanged following implantation (Creech, Deterling, Edwards, Julian, Linton & Shumacker 1957). Since, the majority of fabric vascular prostheses in current use are

constructed from either polyethylene terephthalate (PET, e.g. Dacron®) or polytetrafluoroethylene (PTFE) (Callow, 1982).

DeBakey, Cooley, Crawford, and Morris (1957) introduced the knitted Dacron graft, which is the basis for the current grafts in use today. Modifications to the first knitted Dacron graft have included the addition of velour, providing a trellis for fibrin and fibroblasts to adhere to (Sauvage, Berger, Mansfield, Wood, Smith & Overton 1974), and impregnating the Dacron with albumin (Guidoin, et al. 1984), collagen (Quiñones-Baldrich, Moore, Ziomek, & Chvapil 1986) or gelatin (soluble collagen) (Jonas, Ziemer, Schoen, Britton, & Friefeld 1988) to reduce graft porosity. Teflon was largely replaced with Dacron as the prosthesis of choice in reconstructive vascular surgery but Teflon's nontextile extruded form, namely ePTFE, was introduced by Soyer, Lempinen, Cooper, Norton, and Eiseman (1972). With subsequent modifications ePTFE become the preferred/ suitable alternate for the reconstruction of diseased arteries (Kempczinski 1995).

1.1.2.1 PET grafts

PET grafts are manufactured in three basic constructions, woven, knitted and braided. In woven grafts fabric threads are interlaced in an over and under fashion in both lengthwise and circumferential directions (Brewster 1995). The woven prosthesis is tightly constructed and as a result is relatively strong and stiff with little or no stretch in any direction. As a consequence, the advantages of the woven fabric are reduced bleeding and graft dilatation. The disadvantages are that the graft is less compliant with a tendency to fray at the edges and is associated with a reduced perigraft reaction (Horrocks, Beard & Mohammed 1998).

The knitted prosthesis is produced by looping yarns around a needle to form a continuous interlocking chain of loops. The prosthesis may be orientated in either a longitudinal (warp

knitting) or circumferential (weft knitting) direction. Pore dimensions are related to the size of the needles used and the radius of the curvature taken by the yarn as it bends around the needles. This allows for a greater range of possible porosities and in general account for the increased porosity with knitted grafts when compared to woven grafts. Warp knitted grafts account for the majority of commercially available knitted grafts. The knitted prostheses are generally more conformable, are easier to handle and suture, are less susceptible to fraying and are therefore better at retaining sutures than woven prostheses. The disadvantage of knitted grafts are that increased porosity leads to increased bleeding unless the graft is preclotted or modified and that the grafts are less strong (Brewster 1995).

Braided grafts are no longer used because they require heavier yarn, fray easily, are bulky and relatively nonporous.

1.1.2.2 PTFE grafts

Robert W. Gore in 1969 discovered a method of extruding Teflon polymers and as a consequence developed PTFE. Soyer et al (1972) first introduced PTFE by replacing the inferior vena cava with PTFE in experimental animals. The first clinical use of PTFE was reported in 1976 by Campbell, Brooks, Webster and Bahnson.

The molecule of PTFE is a polymerised chain of repeating unit of a carbon atom-carbon atom (C-C) bonding with four attached fluorine atoms (Boyce 1982). Its thermal degradation begins at 350°C, enabling repeated sterilisation in high temperature (Campbell et al, 1976). The material is produced by a heating and mechanical stretching followed by extrusion (ePTFE) through a dye producing a porous material of characteristic structure that has solid nodes interconnected by fine fibrils. Fibril length determines pore size. This material is chemically inert, highly electronegative and hydrophobic. The advantages of the material are its resistance to aneurysmal dilatation, the no need to preclot the graft and having a smooth luminal surface (Brewster 1995). The new stretch ePTFE are thin walled

allowing for ease of handling, better conformability and improved compliance (Horrocks et al 1998).

1.1.2.3 Polyurethanes

These are thin walled grafts with a very smooth non-thrombogenic inner surface that are prone to aneurysm formation (Horrocks et al 1998). Its chemical composition also allows self sealing ("Thoratec Graftways" 1994) which is advantageous when the graft is used as a conduit for vascular access (Allen, Yuill, Nankivell, & Francis 1996).

1.1.2.4 Biological Prosthetic Grafts

A number of biologically derived conduits have been studied including bovine heterograft and human umbilical cord vein allograft. Rosenberg introduced bovine heterografts in 1966, but this particular type of graft was abandoned because of its tendency to thrombosis and aneurysmal dilatation (Kempczinski 1995). Interest in human umbilical cord vein grafts has been present for over 30 years. Despite the ongoing interest, it lacks patency superiority when compared to other commercially available prostheses, is technically difficult to implant and potentially can biodegrade resulting in aneurysmal dilatation (Brewster 1995). The use of the previously abandoned arterial homograft has been advocated by some in the management of infected aortic prosthesis (Kieffer, et al. 1993) but experience to date is in its infancy and it is uncertain if progress will limited by similar problems encountered in the early 1960's.

1.2 ARTERIAL GRAFT INFECTIONS

1.2.1 Incidence

It was not long following the introduction of the Vinyon "N" cloth tubes prostheses by Voorhess et al., (1952) that the first complication of a prosthetic graft was recorded. Claytor, Birch, Cardwell, and Zimmerman (1956) reported in 1956 on the suture rupture of a nylon aortic bifurcation graft into the small bowel. A recognised cause being prosthetic graft infection. Since this initial report, and reviewing the literature on medline, several thousand cases of infected vascular grafts have been reported individually or as collective reviews.

The true incidence of vascular prosthetic graft infections is difficult to quantify as it is relatively uncommon and may not manifest itself until many years after implantation (Back & Klein 1994). Numerous retrospective reports in the literature quote the incidence of prosthetic vascular graft infections in the range of 1.3 to 6 per cent (Table 1) with the incidence generally higher for vascular prostheses placed in the groin (Back & Klein 1994).

Although prosthetic vascular graft infections are relatively uncommon they are a dreaded occurrence confronting the vascular surgeon (Liekweg & Greenfield 1977; Bunt 1983a; Back & Klein 1994).

The mortality rate, despite aggressive antibiotic therapy and surgical management, remains high, ranging between 20 to 75 percent (Hoffert, et al. 1965; Fry & Lindenauer 1967; Szilagyi, et al. 1972; Rubin, Yao, Thompson & Bergan 1985b; Back & Klein 1994; Bandyk & Bergamini 1995; Moore & Deaton 1993). Mortality occurs in approximately one third of all graft infections (Back & Klein 1994) with mortality highest when an aortic prosthesis is involved (Bandyk & Bergamini 1995; Moore & Deaton 1993). As many as 75 percent of survivors of infected aortic prosthesis require amputation of a limb (Moore & Deaton 1993), with the incidence of amputation highest when the infection involves more distal prosthetic grafts (Buckels & Wilson 1994).

Table 1.1 Incidence of prosthetic graft infection according to location

Author and Year	Location of Graft	Incidence (%)
Hoffert, Gensler and Haimovici (1965)	Aortoiliac/ femoral	2.2
	Femoropopliteal	6
Fry and Lindenauer (1967)	Aortoiliac/ aortofemoral	1.34
Szilagyi, Smith, Elliot and Vrandecic (1972)	Aortoiliac	2.2
	Aortofemoral	1.6
	Femoropopliteal	3.0
Criado (1982)	Carotid- subclavian	1.5
Lorentzen, et al. (1985)	Aortofemoral	3.0
	Femoropopliteal	3.5
Rubin, Yao, Thompson and Bergan (1985b)	Femoropopliteal	2.4

1.2.2 Natural history of prosthetic vascular graft infections

Early prosthetic vascular graft infections typically occurring in the first four months following placement (Back & Klein 1994), once accounted for the majority of prosthetic vascular graft infections (Szilagyi, et al. 1972; Bouhoutsos, Chavatzas, Martin & Morris, 1974; Goldstone & Moore, 1974). Today, these are relatively uncommon with an

incidence of less than 1% (Bandyk & Esses 1994). In addition, they generally involve grafts connecting the femoral artery (Szilagyi, et al. 1972; Bouhoutsos, et al. 1974; Bandyk 1985; Lorentzen, et al. 1985), usually present as wound infections (Seabrook 1990) and are caused by the more virulent micro-organisms, such as *S. aureus, E. Coli, Pseudomonas, Klebsiella, Proteus and enterobacter* (Back & Klein 1994).

Late prosthetic vascular graft infections are the result of two possible mechanisms. Firstly, a normally incorporated and uninfected graft may become infected following haematogenous seeding from a septic focus elsewhere (Moore & Cole 1991) or become infected with enteric contents following a graft- enteric erosion (Seabrook 1990). The haematogenous mechanism of graft infection is supported by animal experimental work performed by Malone, Moore, Campagna and Bean (1975) who inserted prosthetic grafts in the abdominal aorta of dogs then infused 1 X 10^7 organisms of *S. aureus*. At one month 100 percent of the grafts were infected. When the infusion was performed one year following graft insertion the incidence of graft infection fell but the incidence remained high at 30 percent. A similar experiment performed by Leport, et al. (1988) who infused 6 X 10^8 CFU of *S. aureus* found graft segments heavily contaminated with *S aureus* in those grafts removed up to two months following insertion. In those grafts removed at 6 months, less than 50 CFU/cm^2 of *S. aureus* were detected on the grafts. On viewing the graft electron microscopically they noticed that the degree of staphylococcal entrapment was related to the amount of fibrin deposits that were in abundance early in the process of healing. They concluded that graft colonisation was especially to be feared in the first weeks after graft implantation. In both the haematogenous and graft-enteric erosion situations the usual causative organisms are those with high virulence and clinical manifestations are signs and symptoms of sepsis. The second mode of presentation usually is insidious and is caused by the less virulent coagulase negative staphylococci such as *S. epidermidis* with contamination likely to occur at the time of implantation (Back & Klein 1994). Clinically the mode of presentation of these late appearing infections are as sinus tracts, perigraft fluid collection and anastomotic pseudoaneurysm formation (Seabrook 1990).

1.2.3 Mechanisms of graft contamination at operation

The potential sources of graft contamination at the time of graft insertion are numerous and include the patient's natural skin flora, division of lymph nodes and or lymphatic channels, intestinal bag contents, aneurysmal wall contents, break in aseptic technique especially in emergency procedures and concurrent nonvascular surgery (Buckels & Wilson 1994).

There is general agreement that the most common mechanism by which grafts become infected is by bacterial contamination at the time of implantation. The source of the bacterium may be the result of inadequate sterilisation of the graft and the surgical instruments or more commonly the bacteria originating from the surgical team or the patient undergoing the insertion of the graft (Moore and Deaton 1993). Levy, et al., (1990) in a sequential analysis of staphylococcal colonisation of body surface cultures on patients undergoing vascular surgery found that the majority of patients undergoing arterial revascularisation were colonised with coagulase negative staphylococci. Colonisation of patients with nosocomial bacteria is enhanced when the preoperative hospitalisation is lengthy (Perry 1991).

The evidence substantiating that the division of lymph nodes and or lymphatic channels, especially in the inguinal region, potentiates graft infection is conflicting. Bunt (1983b) reported in a review of patients with distal infection, positive infected inguinal nodes in 22% of which two were associated with graft infection. Bouhoutsos, et al. (1974) in a similar group of patients found that when the inguinal nodes were cultured there was no bacterial growth. Support for this mechanism of graft contamination is evident in animal experimentation by Rubin, Malone, and Goldstone (1985a) who carefully transected or preserved the lymphatics in the groin of dogs and found that seven from eight dogs from each group had experimental graft infection as opposed to one from five graft infection in those dogs where the lymphatics were ligated and lymph nodes excised.

Cultures of the transudated fluid from the contents of bowel bags during aortic surgery have been positive in 2.5 to 14 percent of cases although no recorded graft infection has occurred in these cases (Russell, Barnes & Baker 1975; Ernst, Campbell, Daugherty, Sachatello and Griffin 1977; Scobie, McPhail, Barber and Elder 1979).

Ernst, et al. (1977) postulated that the aneurysm wall and laminated thrombus were potential sources of infection. In a study of 80 patients, they found that the incidence of positive cultures was significantly greater in patients with ruptured as compared to nonruptured aneurysms. In addition the incidence of late graft sepsis (greater than 6 months) developed in 10 percent of positive culture specimens as opposed to 2 percent in those with negative arterial wall cultures. A similar finding was noted by Buckles, Fielding, Black, Ashton, and Slaney (1985) but in addition they demonstrated that if antibiotics were given prophylatically in the preoperative period the incidence of positive intraoperative cultures was zero. The organism most commonly isolated from the aortic wall is *S. epidermidis* (Macbeth, Rubin, McIntyre, Goldstone & Malone 1984; Lakla, et al. 1989). The arguments against the importance of positive aortic wall cultures are demonstrated by Brandimarte, et al. (1989) who in a study of 90 patients found no significant increase for subsequent graft infection in those patients (twenty-eight) who had positive intraoperative cultures. The suggestion that if intra-aortic wall infection were the main source of infection one would expect similar infection rates following aortoiliac and aortofemoral bypass, which is clearly not the case (O'Brien and Collin 1992) (refer to Table 1.1). Similarly, Stonebridge, Mutirangura, Clason, Ruckley and Jenkins (1990) obtained positive cultures in the intraluminal thrombus of 11.3 percent patients with infrarenal aneurysms. There was no statistical correlation between the culture result and the incidence of graft infection.

Jamieson, DeWeese, and Rob (1975) reported the incidence of graft infections to be 7.5 percent following emergency aneurysmorrhaphy for ruptured abdominal aortic aneurysms as compared to a risk approximating 1 percent for elective intra- abdominal grafting.

Concurrent nonvascular surgery, particularly intraabdominal is not recommended and should be avoided as the risk of graft contamination by enteric organisms is increased (Buckels & Wilson 1994).

1.3 PATHOGENESIS OF GRAFT INFECTIONS

The introduction of prostheses for arterial reconstruction has unfortunately not been without its problems. One of the more serious complications which dramatically alters patient outcome is graft infection. Prosthetic vascular grafts are foreign bodies and their interstices are a nidus for organisms (O'Brien & Collin 1992). The exact aetiology of vascular graft infections is not completely understood and is likely to be multifactorial as indicated in figure 1.

According to Bandyk and Esses (1994) the risk of foreign body infections as demonstrated by animal models can be predicted by the formula:

$$\text{Risk of biomaterial infection} = \frac{\text{Dose of bacterial contamination X virulence}}{\text{Host resistance}}$$

Essentially the steps involved in biomaterial associated infection include the following: a/ bacterial adherence, b/ microcolony formation within a biofilm, c/ activation of host defences and d/ the inflammatory response involving perigraft tissues and the graft artery anastomoses (Bandyk & Bergamini 1995).

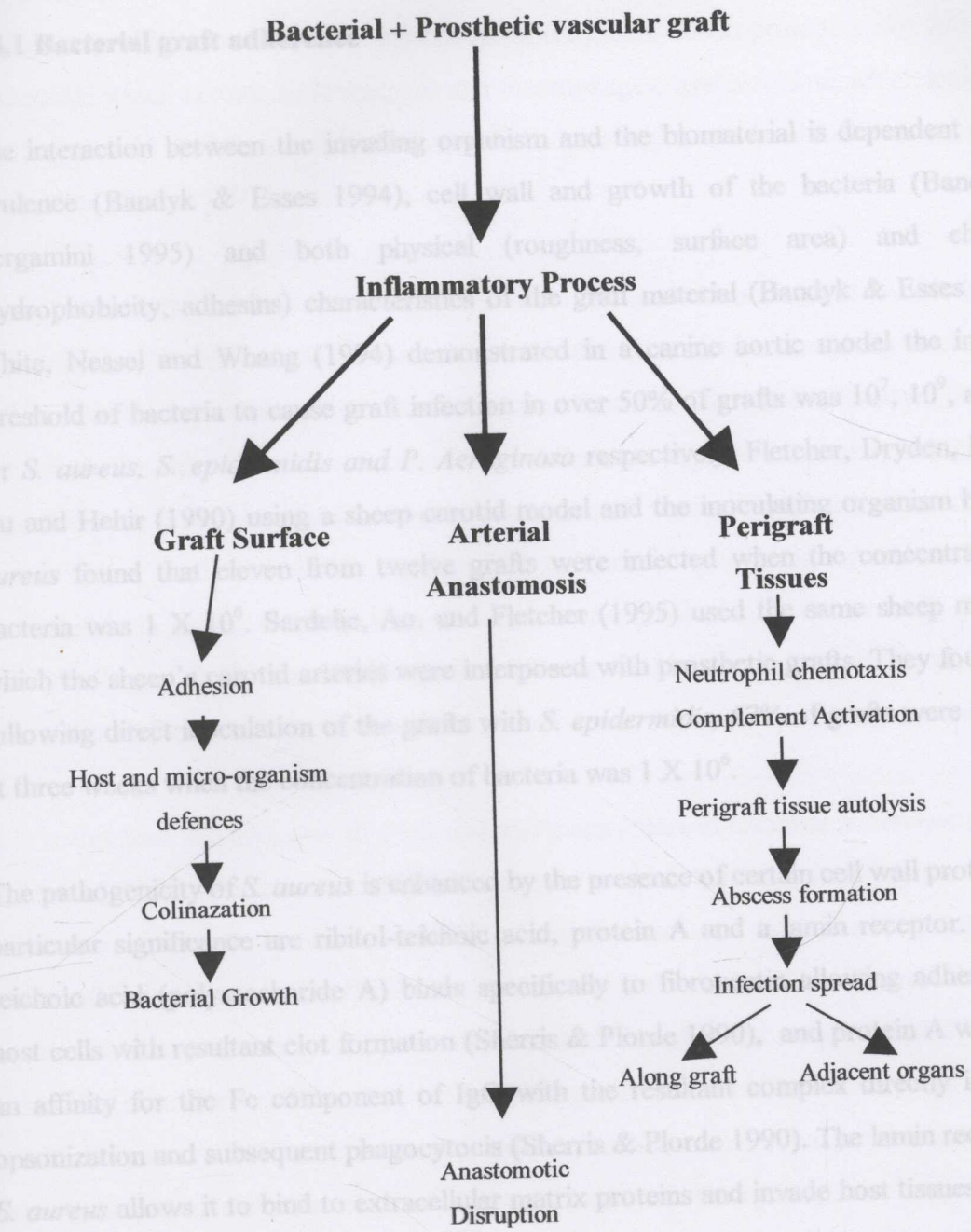

Figure 1.1

Pathogenesis of vascular biomaterial- associated infections (modified from Bandyk & Bergamini 1995).

1.3.1 Bacterial graft adherence

The interaction between the invading organism and the biomaterial is dependent on the virulence (Bandyk & Esses 1994), cell wall and growth of the bacteria (Bandyk & Bergamini 1995) and both physical (roughness, surface area) and chemical (hydrophobicity, adhesins) characteristics of the graft material (Bandyk & Esses 1994). White, Nessel and Whang (1994) demonstrated in a canine aortic model the infective threshold of bacteria to cause graft infection in over 50% of grafts was 10^7, 10^9, and 10^2 for *S. aureus, S. epidermidis and P. Aeruginosa* respectively. Fletcher, Dryden, Munro, Xu and Hehir (1990) using a sheep carotid model and the inoculating organism being *S. aureus* found that eleven from twelve grafts were infected when the concentration of bacteria was 1 X 10^6. Sardelic, Ao, and Fletcher (1995) used the same sheep model in which the sheep's carotid arteries were interposed with prosthetic grafts. They found that following direct inoculation of the grafts with *S. epidermidis,* 67% of grafts were infected at three weeks when the concentration of bacteria was 1 X 10^6.

The pathogenicity of *S. aureus* is enhanced by the presence of certain cell wall proteins. Of particular significance are ribitol-teichoic acid, protein A and a lamin receptor. Ribitol-teichoic acid (polysaccharide A) binds specifically to fibronectin allowing adherence to host cells with resultant clot formation (Sherris & Plorde 1990), and protein A which has an affinity for the Fc component of IgG with the resultant complex directly inhibiting opsonization and subsequent phagocytocis (Sherris & Plorde 1990). The lamin receptor of *S. aureus* allows it to bind to extracellular matrix proteins and invade host tissues (Lopes, dos Reis & Brentani 1985).

The virulence of the micro-organism is often directly associated with the production of secreted toxins and enzymes. *P. aeruginosa* produce proteases that digest elastin, collagen, fibronectin and fibrin with a resultant decline in structural integrity of the artery wall (Bandyk & Esses 1994). *S .aureus* utilises a number of toxins and enzymes to control

the perigraft environment and cause graft infection (White, et al. 1994). These include alpha toxin which causes lysis of erythrocytes, coagulase which promotes clot formation, leukocidin which is toxic to leukocytes and macrophages, hyaluronidase which hydrolyses hyaluronic acid in tissues and lipases and fibrinolysin which are involved in dissolution of fibrin clots (Cohen 1991).

Gram positive organisms, such as staphylococci are known to produce an extracellular glycocalyx or "mucin" which promotes bacterial adherence to biomaterials in much greater (10 to 1000 times) numbers than gram negative organisms (Bergamini, Bandyk, Govostis, Vetsch, & Towne 1989). *S. epidermidis* is a coagulase negative staphylococci, generally regarded as a low virulence organism associated with vascular graft infections (Bandyk, Berni, Thiele, & Towne 1984) but its slime or "mucin" producing subtype adheres to biomaterials in higher numbers than the non- mucin producing *S. epidermidis* (Schmitt, Bandyk, Pequet & Towne 1986b; Muller, Takeda, Goldmann, & Pier 1991; Malangoni, Livingston & Peyton 1993).

It is recognised that the type of graft material plays a part in bacterial adherence (Bandyk & Esses 1994). Schmitt, et al. (1986b) using an in- vitro model comparing the bacterial adherence of four strains of bacteria (*S. aureus*, "mucin" and "non-mucin" producing *S. epidermidis* and *E. coli*) to ePTFE, woven Dacron and velour knitted Dacron found that bacterial adherence was greatest to velour knitted Dacron and least with ePTFE. They postulated a number of differences between the grafts that could account for the observed results. Expanded PTFE represented a smaller surface area for bacterial attachment, is more hydrophobic and thus less likely to form bonds with the hydrophobic bacterial walls, and is relatively non-porous. A further observed result was that adherence of the mucin producing *S. epidermidis* to ePTFE and the velour knitted Dacron grafts was increased by 10-100 times when compared to *S aureus* and the non- mucin producing *S. epidermidis*. Shmitt et al (1986b) found that "mucin" producing *S. epidermidis* adhered to Dacron 10 to 100 fold compared to PTFE.

The differential adherence of staphylococci is believed to be related to capsular adhesins as antibody inhibition of these adhesins has resulted in inhibition of biomaterial adherence (Bandyk & Bergamini 1995).

1.3.2 Microcolony formation within a biofilm

Many bacterial strains, including *S. epidermidis, S. aureus and P. aeruginosa* (Bandyk 1985) are known to produce extracellular polymer substances, (predominately carbohydrates) forming a capsule if the bacteria are suspended in fluid or a "slime" layer (glue like matrix) incorporating the bacteria if they are adherent to a surface. The resultant surface-bacterial-matrix complex is referred to as a biofilm (Richards & Gagnon 1993). This biofilm protects the micro-organism against host defences and antibiotic therapy (Richards & Gagnon 1993).

The bacterial biofilm particularly with low virulence organisms such as the coagulase negative staphylococci, creates a symbiotic infection with no early clinically recognisable signs of infection (Bergamini 1990), with clinical infection occurring months to years following implantation (Bergamini 1990; Bandyk, Bergamini, Kinney, Seabrook, & Towne 1991). In effect the biofilm creates a reservoir of micro-organisms that can produce chronic or late appearing infections (Schmitt, Bandyk, Pequet, Malangoni, & Towne 1986).

1.3.3 Activation of host defences

It is the interaction between the graft and the host that produces inflammatory stimuli activating the host's humoral and cellular immune system with the release of cytokines and polymorphonuclear granulocytes (Bandyk & Bergamini 1995). In addition, the acidic, ischaemic, microenvironment produced by the immune foreign body reaction to the synthetic vascular grafts promotes bacterial biofilm formation and proliferation (Bandyk & Bergamini 1995).

1.3.4 The inflammatory response involving perigraft tissues and the graft artery anastomoses

The interaction between the graft and the host is a dynamic one, whereby the host isolates the synthetic graft instead of incorporating it into the surrounding tissue (White, et al. 1994). Following implantation of the prosthetic graft a pseudointima develops on the luminal surface which comprises an abnormal proliferation of vascular smooth muscle cells and an extracellular connective tissue matrix (Davies & Hagen 1994). Graft luminal blood flow provides little metabolic or immunologic support for the pseudointima especially the deeper layers. A thin layer of lymphatic fluid surrounds the external surface of the graft and the injury of implantation activates fibroblasts and macrophages with the development of a graft capsule composed of fibrous tissue. (White, et al. 1994).

The isolation of the graft and the microenvironment produced, which prevents antibiotics and immune complexes exerting their maximal effect all contribute to the ultimate manifestation of graft infection that being tissue autolysis, arterial vessel wall or anastomotic disruption and haemorrhage (Bandyk & Bergamini 1995).

1.4. BACTERIOLOGY OF VASCULAR GRAFT INFECTIONS

1.4.1 Overview

Gram positive, gram negative, anaerobic and fungal micro-organisms all have the potential to infect a vascular prostheses but in general the majority of infections are the result of a small number of micro-organism. Staphylococci are the most prevalent organism associated with prosthetic graft infection (Goldstone & Moore 1974; Liekweg & Greenfield 1977; Bunt 1983a; Bandyk 1985; Lorentzen, et al. 1985; Golan 1989; Fletcher, Dryden & Sorrell 1991; Bandyk & Bergamini 1995). Of the staphylococci, *S. aureus* is generally regarded as the most common causative bacteria (Bunt 1983a; Bandyk 1985;

Golan 1989; Bandyk & Bergamini 1995), particularly MRSA (Fletcher, et al. 1991). In some series *S. epidermidis* is now being recognised as the leading cause of vascular graft infection, particularly chronic and late onset infections (Bandyk, et al. 1984; Bandyk 1985; Bandyk, et al. 1991; Calligaro, Westcott, Buckley, Savarese & DeLaurentis 1992a; O'Brien & Collin 1992).

Staphylococci are common skin commensals (Lorentzen, et al. 1985; Sleigh & Timbury 1986; Liekweg & Greenfield 1977), so common that Levy, et al. (1990) demonstrated that the majority of patients undergoing arterial revascularisation were colonised with mucin producing strains of coagulase negative staphylococci.

The gram negative organisms, *E. Coli, Pseudomonas, Klebsiella, Enterobacter* and *Proteus,* although relatively uncommon causative organisms for graft infections are of particular interest and concern because of their high virulence and their tendency to destroy the vessel wall (Geary, et al. 1990; Bandyk & Esses 1994; Calligaro, Veith, Schwartz, Savarese, & DeLaurentis 1992).

Candidia, Mycobacteria, and Aspergillus infections are uncommon but pose a significant risk to patients who are immunocomprimised (Bandyk & Bergamini 1995). Although uncommon they are all expected to increase infrequency because of their increasing resistance to standard prophylatic antibiotics (Treiman 2000).

There is an association between the type of infecting organism, the type of vascular complication and the arteries that are involved in the anastomosis to the prosthetic graft. Bandyk and Bergamini (1995) in a collective survey of 1258 patients who had a vascular graft infection found that the majority of aortoenteric fistulas were the result of either *Streptococci or E. Coli* and if the anastomosis involved the femoral artery, the thoracic aorta, the subclavian, carotid or innominate arteries *S. epidermidis or S aureus* was the likely causative organism. *E. Coli, Enterococci and Enterobacter* were the more likely organisms to be involved in aortoiliac anastomoses.

1.4.2 *Staphylococci*

The genus *Staphylococcus* belongs to the broad *Bacillus- Lactobacillus- Streptococcus* cluster (Kloos & Lambe Jr 1991). The *Staphylococci* are gram positive, non- sporing, non- motile cocci, 0.5μm- 1.5μm in diameter, characteristically arranged in clusters. They are generally catalase positive, aerobic or facultatively anaerobic, with limited capsular formation (Sleigh & Timbury 1986; Cohen 1991; Kloos & Lambe Jr 1991; Locksley 1991). They appear as circular, opaque, smooth colonies that in general will grow on most laboratory media that support gram- positive organisms (Finegold & Baron 1986).

The genus is currently divided into 27 species (Kloos & Lambe Jr 1991) but traditionally they have been divided into two groups according to their ability to clot blood plasma, the "coagulase" test.

S. aureus is a coagulase positive *staphylococcus* and the remaining staphylococci are predominately coagulase negative of which *S. epidermidis* is the most important member, particularly in regards to vascular graft infections (Cohen 1991).

Staphylococci are widespread but are mainly found on skin, skin glands, and mucous membranes of mammals and birds (Kloos & Lambe Jr 1991). They are usually transmitted by person to person transfer via contaminated hands (Locksley 1991). They produce many extracellular toxins and enzymes which are often associated with virulence (refer to section 1.3). Although they are usually associated with self- limiting skin infections such as furuncles, carbuncles, cellulitis, impetigo, and post-operative wound infections, they have the potential to cause a number of more serious infections including staphylococcal bactereremia, septic shock, endocarditis, meningitis, arthritis, osteomyelitis, pneumonia, abscesses in any organ, scalded skin syndrome and infection of prosthetic devices and catheters (Kloos & Lambe Jr 1991; Locksley 1991). They are responsible for more than 80% of suppurative diseases confronting the clinician in medical practice (Willett 1992).

Since the introduction of penicillin in the 1940's resistance amongst the genus to antibiotic therapy rapidly proliferated. Originally sensitive to penicillin, by the 1960's the vast majority of hospital strain staphylococci were penicillin resistant (Mehtar 1994). The mechanism of the resistance was the production of a β-lactamase (penicillinase) enzyme that inactivates penicillin (Sleigh & Timbury 1986; Brumfitt & Hamilton-Miller 1989). This penicillin resistance can be transferred between staphylococci by plasmid coded transduction via bacteriophages (Sleigh & Timbury 1986; Cohen 1991). Methicillin, introduced in 1961 was effective chemotherapy for penicillin resistant staphylococci but resistance to methicillin and other antibiotic classes such as macrolides, aminoglycosides and β lactams has emerged as a serious clinical and epidemiological problem (Kloos & Lambe Jr 1991). Resistance to the β lactams antibiotics is not fully understood but is believed to occur via a chromosomally mediated alteration to the penicillin binding protein (Brumfitt & Hamilton-Miller 1989; Locksley 1991). Resistance to other commonly used antibiotics is brought about either by chromosomal changes or by plasmid induction (Brumfitt & Hamilton-Miller 1989). Fortunately, at present, no resistance to the glycopeptides group of antibiotics, such as vancomycin and teicoplanin has emerged so that serious MRSA infections can still be treated (Duckworth 1993). This resistance is not only associated with *S. aureus* but is a growing problem with coagulase negative staphylococci in particular *S. epidermidis* (Sleigh & Timbury 1986; Brumfitt & Hamilton-Miller 1989; Kloos & Lambe Jr 1991; Locksley 1991; Cohen 1991; Willett 1992; Duckworth 1993; Mehtar 1994; Eykyn 1996). Up to 80% of infected cardiac prosthetic valves have been due to methicillin resistant *S. epidermidis* (Locksley 1991). With the growing reliance on prosthesis for medical therapy and the predilection for *S. epidermidis* to colonise biomaterials, the emergence of resistance amongst coagulase negative staphylococci could potentially be disastrous from a medical and an economic point of view.

1.4.2.1 *S. aureus*

S. aureus is a transient coloniser of the anterior nares in up to 90% of humans (Locksley 1991), although colonisation of other skin areas (perineal, axillary and vaginal regions) and mucous membranes (throat, gut) occurs less frequently (Sleigh & Timbury 1986; Locksley 1991). Sleigh and Timbury (1986) refer to *S. aureus* as being "ubiquitous and always present in the hospital environment" (p. 57). This may explain the higher carriage rates encountered amongst hospital employees and hospitalised patients (Locksley 1991). Other groups of people with higher carriage rates include persons with atopic dermatitis, and people who have repeated skin punctures (insulin dependent diabetes mellitus, dialysis dependency, and drug abusers) (Locksley 1991). In addition certain body sites are particularly resistant to MRSA eradication, including tracheostomes, wounds and leg ulcers (McDonald 1997).

Colonies of *S. aureus* are typically golden but their pigmentation may vary from white to orange (Sleigh & Timbury 1986). The distinguishing feature of *S. aureus* is that it is coagulase positive and it is this characteristic that often is sufficient to identify the organism as being *S. aureus* (Finegold & Baron 1986). Other differentiating characteristics of *S. aureus* which are atypical of coagulase negative staphylococci are that S. *aurues* is phosphatase positive, can hydrolyse DNA and when cultured on mannitol salt agar (a media that contains mannitol, high salt concentrations and a phenol red pH indicator) and converts mannitol to acid (Finegold & Baron 1986; Sleigh & Timbury 1986).

S. aureus has characteristic group-specific and type-specific antigens that can aid in identification. Protein A, and polysaccharide A are group specific cell wall antigens of *S. aureus* that are implicated in vascular graft infections (refer to section 1.3.1) are usually identified by serum-gel diffusion (Cohen 1991) whilst the type-specific antigens used for serotyping *S. aureus* are identified by phage typing.

1.4.2.2 *Coagulase negative staphylococci*

Coagulase negative staphylococci (CNS) represent a major part of the normal skin flora of humans (Kloos & Lambe Jr 1991) although they are also residents of mucous membranes and lower bowel (Locksley 1991). Thirteen species of CNS are recognised amongst humans and although considered of low pathogenicity as a group, a number of CNS species are recognised as opportunistic human pathogens (Kloos & Lambe Jr 1991). *S. epidermidis* has the greatest pathogenic potential and diversity (Kloos & Lambe Jr 1991) and with *S. aureus* have become the most frequent cause of nosocomial infections in United States hospitals (Locksley 1991).

Colonies of CNS are in general similar to *S. aureus* in morphological appearance (Finegold & Baron 1986). Colonies of *S. epidermidis* are relatively small compared to *S. aureus* with an average size of 2.5 to 6mm in diameter as opposed to an average size of 6 to 8mm in diameter (Kloos & Lambe Jr 1991) and usually do not produce pigment (Sleigh & Timbury 1986; Kloos & Lambe Jr 1991). Some of the slime producing strains of *S. epidermidis* are sticky and adherent to the agar surface (Kloos & Lambe Jr 1991) a characteristic that favours biomaterial adherence.

Coagulase negative staphylococci do not produce the number of extracellular toxins produced by *S. aureus* although some species produce haemolysin, possess an antiphagocytic capsule (Finegold & Baron 1986; Eykyn 1996) and contain in their cell wall an α-linked glucosyl glycerol teichoic acid named polysaccharide B (Cohen 1991). It is the production of slime or mucin by these organisms that makes them important in the pathogenesis of vascular graft infections (refer to section 1.3.1).

1.5 INVESTIGATIONS FOR DETECTION OF PROSTHETIC GRAFT INFECTIONS

1.5.1 History and physical examination

The presentation of vascular graft infections is often subtle (Bandyk & Bergamini 1995), and improvements in prognosis are only possible with the early diagnosis and treatment of

graft infections (Perdue, Smith, Ansley & Constantino 1980) especially in equivocal cases, where diagnosis is directed towards proving that no infection is present (Bandyk & Bergamini 1995).

The diagnosis of infected superficially placed grafts (groin, thigh) in general is clinically easier and the patient may present with evidence of infection (Murray & Goldstone 1994). The clinical diagnostic clues include an inflammatory perigraft mass, overlying cellulitus, presence of exposed prosthetic graft, a sinus tract with persistent purulent drainage and or bleeding and or a palpable anastomotic pseudoaneurysm (Goldstone & Moore 1974; Moore & Deaton 1993; Buckels & Wilson 1994; Murray & Goldstone 1994; Bandyk & Bergamini 1995). Other less common presentations include graft thrombosis and distal septic embolisation (Buckels & Wilson 1994). The difficulty in these superficially placed grafts lies not so much in the diagnosis of infection but determining the proximal extent of the infection (Becker & Blundell 1976).

The diagnosis of an infected intraabdominal prosthetic graft is usually much more difficult as the presentation is of an inflammatory process with non-specific signs and symptoms and or upper or lower gastrointestinal haemorrhage (Murray & Goldstone 1994). Other important features in the history and examination of the patient include fever of unknown origin, septicaemia, abdominal pain and or the development of a pulsatile mass (Moore & Deaton 1993). Goldstone and Cunningham (1990) in a review of patients with aorto-enteric fistulas found that 38 percent of patients with aortic graft infection had graft-enteric fistulas with two thirds of these patients presenting with gastrointestinal bleeding. Bleeding from a graft-enteric fistula is usually acute and associated with massive blood loss, although, one third of patients may present with chronic gastrointestinal bleeding (O'Brien & Collin 1992).

Occasionally indirect evidence (Murray & Goldstone 1994), such as the presence of hydronephrosis (Schubert, et al. 1985) or osteomyelitis of the spine (McHenry, Rehm,

Krajewski, Duchesneau, Levin, & Steinmuller 1991) may be indicative of perigraft inflammation.

Ongoing infection involving a prosthetic graft that is not controlled eventually leads to graft disruption and haemorrhage (Moore & Deaton 1993).

1.5.2 Laboratory investigations

Routine laboratory studies such as white cell count with differential, erythrocyte sedimentation rate, blood cultures, urinalysis, stool guaic test for blood are obtained (Bandyk & Bergamini 1995; Moore & Deaton 1993) but the results may be non-specific and even normal for perigraft infections secondary to *S. epidermidis* (Bandyk & Bergamini 1995).

Microbiological identification is important to confirm the diagnosis and to commence appropriate antimicrobial therapy (Bandyk & Bergamini 1995). Where possible aspirates of pus or perigraft exudate, blood cultures, wound cultures, and tissue specimens (fibrous, adipose, arterial wall and graft material) should be sent for microbiological culture (Gröschel & Strain 1994). All solid samples need to be mechanically disrupted either by tissue grinding or by ultrasonic disruption as this is recognised as the most sensitive way of detecting S. *epidermidis* graft infections (Tollefson, Bandyk, Kaebnick, Seabrook, Towne 1987; Bergamini, Bandyk, Govostis, Kaebnick, & and Towne 1988; Bergamini et al. 1989; Wengrovitz, Spangler, & Martin 1991). Bergamini, et al. (1989) when culturing the mechanically disrupted graft in broth media were able to recover bacteria in 83% of cases when gram staining and culture of the perigraft tissue and fluid were negative. In addition when the biofilm concentrations were less than 100 CFU per cm^2 only culture in broth media reliably recovered micro-organisms (Bergamini, et al. 1989). Sardelic, et al. (1995), in establishing a *S. epidermidis* infection model in the sheep carotid artery similarly were only able to retrieve 80% of *S. epidermidis* when grafts were inoculated with 10^8 CFU of *S. epidermidis*. Padberg, Smith and Eng (1992) in an assessment of the

optimal method of culturing vascular prosthetic grafts compared ultrasonic bath treatment, direct ultrasonic disruption and agitation on a vortex mixer as methods of enhancing bacterial recovery. They concluded that the ultrasonic bath treatment released bacteria consistently greater by one log than the other methods.

1.5.3 Diagnostic Imaging

Various diagnostic modalities, some of which are discussed briefly below, are used to help the vascular surgeon determine the extent of vascular graft infection and plan definitive treatment but in less obvious cases surgery may be the only means of diagnosis.

1.5.3.1 Ultrasonography

Ultrasonography is portable, non-invasive, relatively cheap, and free of ionising radiation that makes it a very suitable diagnostic modality in the initial assessment of the patient with a prosthetic graft infection (Murray & Goldstone 1994). Ultrasonography, particularly duplex ultrasonography is of benefit in the identification of perigraft fluid, pseudoaneurysm formation and absence of graft incorporation especially with the femoral anastomosis (Gooding, Effney & Goldstone 1981; Polak, Donaldson, Whittemore, Mannick, & O'Leary 1989; Bandyk, & Esses 1994). Despite these advantages it is limited by the fact that it is operator dependent, and evaluation can be difficult especially if there is obesity and overlying bowel gas (Maloney, Pairolero, Smith, Hattery, Brakke, & Spitell 1977).

1.5.3.2 Computerised Tomography

Contrast enhanced CT is the preferred initial investigative modality when the aorta is suspected to be involved in the infective process (Bandyk & Bergamini 1995). The CT features suggestive of prosthetic graft infection include perigraft fluid or abscess collection, perigraft gas, focal bowel wall thickening, loss of normal retroperitoneal tissue

planes, pseudoaneurysm formation, hydronephrosis and or vertebral osteomyelitis (O'Hara, Borkowski, Hertzer, O' Donavan, Brigham, & Bevan 1984; Bandyk & Bergamini 1995). Periprosthetic gas is a normal feature up to six weeks following implantation and beyond this period should alert the clinician to the possibility of a periprosthetic graft infection or a graft-enteric fistula (Qvarfordt, Reilly, Mark, Goldstone, Wall, Ehrenfeld & Stonney 1985).

1.5.3.3 Magnetic Resonance Imaging

Magnetic resonance imaging is a relatively new means of diagnosing prosthetic vascular graft infections whose major advantage is the ability to differentiate between high attenuation fluid and soft tissue inflammation (Olofsson, Auffermann, Higgins, Rabahie, Tavares, & Stoney 1988). Olofsson, et al. (1988) compared magnetic resonance imaging with CT in patients in whom graft infections were confirmed or excluded by surgery. Magnetic resonance imaging was performed in eighteen patients and was able to identify perigraft infection in fourteen of sixteen patients and correctly exclude infection in two patients. Computerised tomography was performed in only twelve patients with a correct diagnosis in five.

1.5.3.4 Leucocyte or immunoglobulin labelled scanning

These radionuclide studies are useful in the diagnosis of vascular graft infection and rely on accumulation of labelled leucocytes or polyclonal IgG at the site of graft infection.

Gallium-67 citrate has been reported to be more specific than CT and is recommended as a complementary test to CT to increase specificity (Johnson, Russ, Bair, & Friefeld 1990) although it is limited by gastrointestinal, surgical incision uptake and two to three day delay in acquisition of scan information (Lawrence, Dries, Alazraki, & Aldo 1985). Sedwitz, Davies, Pretorius, and Vasquez (1987) in a study evaluating the clinical value of indium- 111 labelled white blood cell scans reported that abnormal indium-111 scans are

common with prostheses involving a groin anastomosis but unusual when infection is confined to the abdominal aorta and in the absence of clinical suspicion the scan is not reliable in predicting graft infection because of its low specificity of 50 percent. However, LaMuraglia, Fischman, Strauss, Keech, Wilkinson, and Callahan (1989) investigated the utility of indium 111-labelled human IgG in the detection of focal vascular graft infection in 25 patients with suspected vascular infections of which 22 involved prosthetic grafts. All scans were correct when correlated with surgical findings, other imaging modalities and or clinical follow-up except one patient with an aortoduodenal fistula whose scan was a false negative result. They concluded that this method of diagnosis was a non-invasive means of localising vascular infections.

1.5.3.5 Others

Contrast sinography especially when the infection involves the femoral region can demonstrate the extent of the perigraft cavity but is generally not recommended because of the risk of introducing further graft contamination and the perigraft cavity may not be reflective of the full extent of the infection (Bandyk & Bergamini 1995).

Oesophagogastroduodenoscopy and or colonoscopy is of value to exclude other gastrointestinal causes of bleeding in patients with aortic prosthesis and gastointestinal bleeding. Although, the presence or absence of a gastrointestinal lesion does not rule out prosthetic graft infection (Murray & Goldstone 1994).

Angiography is of use only in the identification of complications of infection namely graft disruption, graft thrombosis and or pseudoaneurysm formation (Bandyk & Bergamini 1995).

1.6 MANAGEMENT OF PROSTHETIC GRAFT INFECTIONS

1.6.1 Prevention

Although the incidence of prosthetic graft infection is relatively low (refer to section 1.2.1) simple measures prior to graft implantation may reduce the incidence of infection and avoid the need for management of infected grafts.

The routine use of skin preparations containing povidine-iodine or chlorhexidine (Cruse & Foord 1973), the use of a depilatory agent for hair removal (Seropian & Reynolds 1971), limiting the length of preoperative hospitalisation (Perry 1991), operating time and intensive care stay all contribute to the reduction in wound infection and more importantly the chance of developing resistant multiple nosocomial infections (Cruse & Foord 1973).

The use of antimicrobial prophylaxis is justified in vascular surgery as it prevents bacteria from extrinsic sources attaching to the prosthesis (Ehrenkranz 1993) and has been shown to reduce wound infections in vascular surgery (Bandyk 1991). Since the majority of graft infections are secondary to a limited number of bacteria, narrow spectrum antibiotics against these micro-organisms are recommended to prevent the emergence of antibiotic resistance (Law & Gelabert 1993). Parenteral antimicrobial prophylaxis should be completed as close to the time of incision as possible and repeated in the event of haemorrhage, and operations lasting more than four hours and for every four hours thereafter (Ehrenkranz 1993). The duration of prophylatic antibiotics postoperatively is controversial. Some groups continue prophylaxis until all lines, tubes and catheters are removed (Herbst, Kamme, Norgren, Qvarfordt, Ribbe, Thörne 1989) although the data supporting greater than 24 hours of postoperative antibiotics is not strongly supported (Bandyk & Bergamini 1995).

Attention to sterile technique, and haemostasis is essential to avoid bacterial contamination of the graft and encourage tissue incorporation of the graft (Beard & Wilmshurst 1991).

Experimentation with various antibiotics, protein carriers and graft types have been undertaken by a number of groups in an attempt to find the combination that provides

graft protection from bacterial contamination. Clark and Margraf (1974) developed polyester grafts treated with a silver allontoin-heparin preparation and noted that in an experimental model, infection was prevented for twenty-four hours. These silver compounds act on bacteria by slowly releasing ionic silver, which is bacteriocidal in neutral to mildly alkaline aqueous environments (Clark & Margraf 1974). Shah, et al. (1987) when comparing norfloxacin and silver norfloxacin coated PTFE grafts with triododecylmethylammonium chloride as a cationic surfactant and *S. aureus* or *E. Coli* as the bacterial contaminant found that the silver norfloxacin coated grafts had significant *in-vitro* antibacterial activity to 15 days compared to 24 hours for the norfloxacin only coated grafts. Similarly, Benvenisty, et al. (1988) prepared PTFE grafts by combining silver with oxacillin or amikacin using an organic solvent. When these grafts were implanted in the abdominal aorta of dogs and removed for analysis at one week, they found that the silver amikacin and silver oxacillin prepared PTFE grafts had significantly less growth of *S. aureus* when compared to the non treated PTFE prostheses. Kinney, Bandyk, Seabrook, Kelly and Towne (1991) found that bonding of silver-ciprofloxacin on PTFE grafts by either a surfactant mediated or direct bonding method provided concentrations of ciprofloxacin in excess of the minimum inhibitory concentration of *S. epidermidis* at both 7 and 14 days. They concluded that this method of graft treatment would be of value for bypass grafting in contaminated wounds or for the *in-situ* replacement of coagulase negative staphylococcal infected prosthetic grafts. Harvey and Greco (1981) used benzalkonium chloride or tridodecylmethylammonium salts to bind penicillin, oxacillin or cephazolin to PTFE with success but a later study by Prahlad, Harvey, and Greco (1981) found that because of *in-vivo* elution minimal antibacterial activity was present at 24 hours. Webb, et al. (1986) undertook an analysis of PTFE grafts following pre-treatment with 1 mg/ml oxacillin, and varying combinations of 95% ethanol pre-treatment and benzalkonium chloride followed by exposure to high concentrations of *S. aureus*. Despite the presence of bacteriostatic concentrations of the attached oxacillin, adhesive bacteria could still be seen on all the PTFE grafts regardless of the type of pre-treatment. Grafts treated with benzalkonium chloride at 90^0 Celsius demonstrated a twofold reduction in bacteria numbers, which was significant when compared to the other treatments. Sobinsky

and Flanigan (1986) using a glucosaminoglycan-keratin luminal coated PTFE graft bound with cefoxitin in a dog model found significant protection against a concentration of 1 X 10^8 *S. aureus* when compared to non treated PTFE grafts. In addition, antibiotic elution occurred exponentially with no detectable antibiotic activity after 10 days. Ney, et al. (1990) soaked PTFE grafts implanted in the abdominal aorta of dogs with a fibrin glue antibiotic suspension containing cryoprecipitate, bovine thrombin, aminocaproic acid and tobramycin and then contaminated the grafts with 3 X 10^8 organisms of both *S. aureus* and *E. Coli.* They reported reductions in both vascular graft infection and pseudoaneurysm formation after exposure to the bacterial inoculum. Shenk, Ney, Tsukayama, Olson and Bubrik (1989) used N-butyl –2 cyanoacrylate as a vehicle to deliver tobramycin to contaminated (*S. aureus* and *E. coli*) PTFE grafts that had replaced the infrarenal aorta in a canine model. They found the antibiotic/ N-butyl –2 cyanoacrylate combination to be effective in the prevention and treatment of PTFE graft infections. Recently, Okahara, et al. (1995) were able to bind ofloxacin to a sheet of PTFE by impregnation and then cut the impregnated sheet of PTFE into fine threads. These fine PTFE threads were then coiled around a ridged outer wall of PTFE. The grafts were then interposed in the inferior vena cava of rabbits and compared to non treated PTFE grafts. The entire graft was covered with a concentration of 3 X 10^7 *E. Coli.* They demonstrated significant *in-vitro* antibacterial activity for more than two weeks and concluded from their *in-vivo* model results that the ofloxacin bonded PTFE grafts exhibited marked infection-resistant properties.

Active methods for binding antimicrobials have also used Dacron prostheses. Shue, et al. (1988) by a surfactant mediated bonding technique bonded oxacillin to Dacron. In a dog model, they exposed interposed Dacron grafts in the infrarenal aorta to a concentration of 1 X 10^8 *S. aureus*. The incidence of infected grafts was significantly reduced in the treated Dacron prostheses compared to both the control grafts with or without parenteral oxacillin. Haverich, et al. (1992) inserted Dacron grafts into the aorta of pigs. The Dacron grafts were pre-treated with gentamicin and fibrin or gentamicin and fibrin alone. Following graft insertion the grafts were directly inoculated with four drops of 10^8 *S.*

aureus. They found that the combination of gentamicin and fibrin allowed for bacterial bioactivity for at least one week and suggested it would ".....serve as a prophylactic tool against perioperative vascular graft infection." (p.187). Phaneuf et al (1993) using a thermofixation procedure were able to bind the ciprofloxacin (a member of the quinolone class of antibiotics) to Dacron and demonstrated in an *in-vitro* model anti-staphylococcal activity for greater than 336 hours. Using the same technique Ozaki et al. (1993) inserted the ciprofloxacin/Dacron grafts into the subcutaneous tissues of New Zealand white rabbits and compared them with plain Dacron grafts. Following insertion into the subcutaneous tissues 0.1 ml of 10^6 *S. aureus* was carefully dispersed over the Dacron. After one week the grafts were removed. The treated Dacron grafts demonstrated significantly reduced wound infections and number of adherent bacteria to the inserted Dacron pieces as compared to the non-treated Dacron pieces.

During the early 1980's interest in passive means of antibiotic incorporation into prosthetic grafts were emerging. Moore, Chvapil, Seiffert and Keown (1981) experimented with uncrimped, filamentous velour Dacron that had been prepared by bonding amikacin-enriched collagen. The grafts were then inserted into the abdominal aorta of thirteen mongrel dogs and compared with a control group of dogs receiving a control graft. All dogs received an intravenous infusion of 10^8 organisms of *S. aureus*. The incidence of infection in the treated group was significantly less (8%) than the control group (100%) when the grafts were removed at three weeks. Only one of the treated grafts displayed bacterial inhibitory activity at three weeks. They concluded this was a promising means of preventing graft infection. Kempczinski (1981) who added cephalothin to the blood used to preclot Dacron grafts highlighted the problem with passive means of graft pre-treatment. In his series of *in-vitro* and *in-vivo* experiments it was noted that the concentration of cephalothin in the preclotted grafts were comparable to the concentrations reached clinically but once the grafts were soaked in buffered saline solution the measurable amount of cephalothin in the grafts were negligible after only 45 seconds. The *in-vivo* aspect of the experiments demonstrated negligible antibiotic activity and no antibiotic activity at two and four minutes respectively.

Rifampicin is a hydrophobic semi synthetic substance derived from *Streptomyces mediterranei,* which inhibits DNA dependent RNA polymerase activity in bacterial cells without effecting mammalian cells (Farr & Mandell 1982). It has a high affinity for gelatin used to coat vascular grafts (Ashton, Cunningham, Paton & Maini 1990) and is usually active against the methicillin resistant strains of staphylococci (Turnbridge & Grayson 1993). Passive incorporation of rifampicin into the Gelseal graft has been shown to be resistant to experimental bacterial contamination (Powell, Burnham & Johnson 1983; MacDougal, Burnham & Johnson 1986; Avramovic & Fletcher 1991; Chervu, Moore, Gelabert, Colburn, and Chvapil 1991b) with *in-vitro* activity of approximately 22 days (Chervu, Moore, Chvapil & Henderson 1991a), although the *in-vivo* graft bioactivity is significantly less, lasting only up to 4 days (Goeau-Brissonniere, Leport, Bacourt, Lebrault, Comte & Pechre 1991; Lachapelle, Graham & Symes 1994; Gahtan, Esses, Bandyk, Nelson, Dupont & Mills 1995;). It is these qualities plus its excellent tissue and intracellular penetration (Turnbridge & Grayson 1993) that make rifampicin an ideal antibiotic to be bonded to prosthetic grafts in order to prevent subsequent graft infection.

1.6.2 Established Infection

1.6.2.1 Antibiotic therapy

Once the diagnosis or suspicion of prosthetic vascular graft infection is made then broad spectrum antimicrobial therapy is initiated and subsequently converted to organism specific antibiotics (Moore & Deaton 1993). The length of antibiotic therapy following excision of the infected graft is unclear but Bergamini and Bandyk (1995) advocate parenteral antibiotics for two weeks and oral for six months.

1.6.2.2 Operative

The most important principles in the management of infected arterial prosthetic grafts are the excision of all infected graft and surrounding tissue, use of monofilament sutures in the repair of involved arteries, antibiotics and the restoration of adequate arterial flow (Gelabert & Moore 1994).

The "gold standard" in the management of the infected vascular prosthetic graft especially when it involves the aorta is total excision of the graft and surrounding infected tissue and extraanatomical bypass (Curl & Ricotta 1994). Although considered the gold standard, it is technically challenging, may be impossible to reconstruct by extra-anatomical bypass, is associated with an increased risk of graft thrombosis, fatal aortic stump rupture, infection of the new prosthesis and significant morbidity and mortality (Gelabert & Moore 1994). In light of these drawbacks, interest has generated in more conservative approaches to the management of the infected prosthetic graft. The most conservative of treatments is aggressive local wound care with graft preservation providing the graft and anastomoses are intact and the patient has no systemic features of sepsis (Calligaro, et al. 1992b). Calligaro, Veith, Schwartz, Savarese and DeLaurentis (1992c) in a report of a series of patients who had graft preservation concluded that with the exception of *Pseudomonas,* vascular graft infections could be managed with debridement, antibiotic therapy and wound closure. The skeletonized prosthetic graft can be covered using viable regional rotational flaps (Turnispeed & Dibbel 1994). The graft if clearly infected may be excised and directly replaced with cadaveric arterial allografts (Kieffer, et al. 1993), venous autografts (Clagett, et al. 1993), cryopreserved saphenous vein homografts (Fujitani, Bassinouny, Gewertz, Glagoz & Zarins 1992), autogenous arteries and or veins (Seeger, Wheeler, Gregory, Snyder & Gayle 1983) or prosthesis (Towne, Seabrook, Bandyk, Freischlag & Edminston 1994). The major drawback with in situ reconstruction is sepsis (Robinson & Johansen) but in certain instances will become the method of choice of managing graft infections (Gelabert & Moore 1994).

1.7 INTIMAL HYPERPLASIA

1.7.1 Overview

The normal arterial wall essentially consists of three layers. An inner single layer of endothelial cells (tunica intima) supported by a thin collagenous layer with elastic fibres, a middle layer of smooth muscle cells (tunica media) and an outer collagenous layer, the tunica adventitia. The amount of elastic fibres and smooth muscle are dependent on the artery's position and role in the circulatory system (Wheater, Burkitt & Daniels 1987).

IH was first described by Carrel and Guthrie (1906) who noticed a 'glistening substance' that developed in the operated sites of vascular bypass operation a few days following operation. They further studied the lesion microscopically and found that it covered the sutures at the anastomoses and its contents had a similar appearance to that of the normal endothelium.

Intimal hyperplasia is recognised as the causative factor in 50% of late failures involving endarterectomy and bypass procedures (Imparato, Bracco, Kim & Zeff 1972). Although its exact pathophysiology is not completely understood it is characterised by the activation and migration of quiescent smooth muscle cells of the media into the intima following intimal injury with subsequent intraluminal thickening and luminal narrowing (Colburn & Moore 1993). Considering that vascular graft infections are a relatively rare occurrence (Section 1.2.1) it would be imperative that the antibiotic which was to be impregnated onto the prosthesis would not contribute to the formation of intimal hyperplasia.

1.7.2 Histopathology

Macroscopically the chronic IH lesion appears firm, pale and homogeneous (Davies & Hagen 1994). It may be diffuse throughout the vessel or graft, focal at the anastomotic

site, or accumulate within the body of the vessel (Davies & Hagen 1994). Microscopically, IH locates in between the endothelium and the internal elastic lamina of an artery, or between the endoththelium and the medial smooth muscle cell layer of a vein graft, giving a smooth surface over the involved area (Chervu & Moore 1990). It demonstrates pathological features of abnormal migration and proliferation of vascular SMCs and fibroblasts with associated deposition of ECM (Davies & Hagen 1994).

1.7.3 Pathogenesis

It is generally agreed that damage to the endothelium of the vessel is the initial trigger for the consequent events in the development of IH, (Clowes & Reidy 1991). The development of IH may consist of three phases according to time course (Davies & Hagen 1994). The first phase occurs minutes to hours after intervention, in which ECs and SMCs are disrupted and denuded from the vessel wall with resultant release of basic fibroblast growth factor (bFGF). At the same time the platelet adherence and degranulation takes place with release of platelet-derived growth factor (PDGF). PDGF and bFGF are so called "growth promoters" which activate the SMCs from the quiescent phase to proliferate and migrate. The second phase occurs hours to weeks following injury to the artery. In this phase, the main features are medial SMC replication, migration and EC ingrowth. Re-endothelisation occurs in the third phase weeks to months later. SMCs replicate in the intima and synthesise extracellular matrix. The current hypothesis of the mechanism for the development of IH is illustrated in Fig. 1.2.

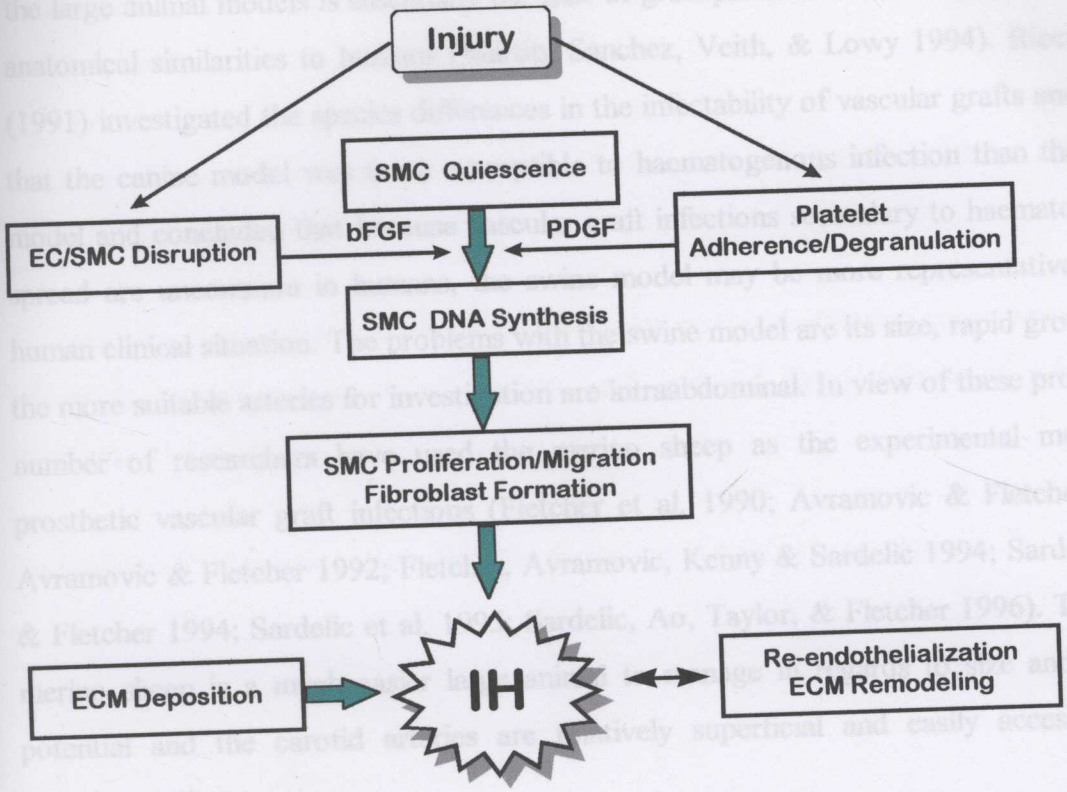

Fig. 1.2
Diagram illustrates the hypothetical mechanism of IH formation. Injury might cause endothelial cell (EC) disruption and release of mitogens such as basic fibroblast growth factor (bFGF) which in turn stimulates smooth muscle cell (SMC) proliferation and migration. Injury also causes platelet reactions and release platelet derived growth factor (PDGF) which regulates SMC growth. Deposition of extracellular matrix (ECM) also contributes IH formation. (Modified from Clowes 1995)

1.8 ANIMAL MODELS OF GRAFT INFECTIONS

A variety of small (rabbit, mice, rats and guinea pigs) and large animals (pigs, dogs and sheep) have been used to investigate vascular prosthetic graft infections. The advantage of

the large animal models is essentially the ease of graft placement and the physiologic and anatomical similarities to humans (Martin, Sanchez, Veith, & Lowy 1994). Ricci, et al. (1991) investigated the species differences in the infectability of vascular grafts and found that the canine model was more susceptible to haematogenous infection than the swine model and concluded that because vascular graft infections secondary to haematogenous spread are uncommon in humans, the swine model may be more representative of the human clinical situation. The problems with the swine model are its size, rapid growth and the more suitable arteries for investigation are intraabdominal. In view of these problems a number of researchers have used the merino sheep as the experimental model for prosthetic vascular graft infections (Fletcher et al. 1990; Avramovic & Fletcher 1991; Avramovic & Fletcher 1992; Fletcher, Avramovic, Kenny & Sardelic 1994; Sardelic, Ao, & Fletcher 1994; Sardelic et al. 1995; Sardelic, Ao, Taylor, & Fletcher 1996). The adult merino sheep is a much easier large animal to manage in regards to size and growth potential and the carotid arteries are relatively superficial and easily accessible for experimentation.

1.9 CONCLUSIONS FROM THE LITEATURE AND AIMS OF THIS PROJECT

Prosthetic arterial vascular graft infections are relatively uncommon but pose significant management problems with associated significant morbidity and mortality. The predominant organisms involved are the staphylococci, which are often methicillin resistant. In light of these problems and using an established sheep carotid model, the principle aim was to determine means of further reducing the incidence of graft infection so that the catastrophic complications can be prevented. To achieve these goals, a number of issues were addressed both at an *in-vitro* and *in-vivo* level with the specific aims of the thesis being:

To determine the optimal vascular graft material and dose of rifampicin soaking to prevent graft infection.

To compare a number of commercially available prosthetic grafts in the ovine model as to their infectivity.

To determine if an increased concentration of rifampicin reduced the incidence of MRSA and MRSE prosthetic vascular graft infections.

To determine if replacement of an infected vascular graft with differing concentrations of a rifampicin soaked gelatin sealed Dacron graft reduced the incidence of subsequent infection.

To establish if rifampicin has a role in the formation of intimal hyperplasia.

Chapter 2

GENERAL MATERIALS AND METHODS.

2.1 OVINE MODEL

Adult female Merino sheep weighing an average weight of 35 kilograms were used as the animal model for all *in-vivo* experimentation. From previous experience they are readily available, inexpensive and easier to manage compared to other medium to large sized animals such as dogs and pigs (Fletcher et al 1990; Avramovic & Fletcher 1991; Avramovic & Fletcher 1992; Sardelic, et al. 1995). In addition, the common carotid artery of the sheep is anatomically superficial in the neck of the sheep allowing easy operative access and its size of approximately 4-5 mm in diameter is comparable to the medium sized arteries of humans.

2.2 ANIMAL HUSBANDRY

The Animal Care and Ethics Committee of the Western Sydney Area Health Service approved all animal experimentation. All husbandry and experimental procedures were performed in accordance with the "Australian code of practice for the care and use of animals for scientific purposes" of the National Health and Medical Research Council of Australia (Australian Government Publishing Service 1994) and "principles of laboratory animal care" (NIH 1985). Following delivery to the Westmead Hospital research facilities, the sheep were quarantined for one week during which time they were drenched with ivomectin 0.8 grams/litre (2.5 mls/10 kg liveweight; Merck Sharpe & Dohme (Australia) Pty. Ltd.) and underwent a general health inspection by a veterinarian. Following quarantine the sheep were allowed to pasture in the research holding paddocks with their diets supplemented with daily lucerne and lamb and ewe nuts (Y. S. Feeds Pty. Ltd., Young, Australia).

2.3 GRAFT MATERIALS (Figure 2.1)

2.3.1 Gelatin Sealed Dacron (Gelsoft™)

Dacron in general is a multifilament yarn containing many small continuos filaments which may be fabricated by either knitting, weaving or braiding (Stanley, Lindenauer, Graham, Zelenock, Wakefield, & Cronenwett 1993).

The earlier forms of knitted Dacron were porous, mechanically compliant, with excellent handling characteristics but were disadvantaged by the fact they required preclotting prior to graft implantation (Brewster 1995). The addition of biological substances such as collagen, albumin or gelatin has rendered the Dacron grafts leak-proof (Horrocks, Beard & Mohammed 1998).

Gelsoft™ (Gelsoft, Vascutek Ltd, Scotland) is a modified form of knitted Dacron. It is a zero porosity polyester graft that achieves its zero porosity by a patented (U. S. Patent No. 4,747,848) impregnation of modified mammalian gelatin (gelatin being the heat-denatured form of collagen) which hydrolyses without inciting an inflammatory response. In vitro activity of the gelatin sealant has been shown to last between 7 and 9 days with in-vivo activity in an aortic canine model lasting 5 to 10 days (Drury, Ashton, Cunningham, Maini & Pollock 1987). It is its unique structure that allows it to act as a reservoir for antibiotics, particularly rifampicin (Strachan 1993).

2.3.2 Fluoropassivated Dacron (Fluoropassiv™)

Fluoropassiv™ is a further modification of the knitted gelatin sealed Dacron graft. It thus has all the advantages discussed for Gelsoft but in addition it has been modified by coating each polyester fibre with fluoropolymer molecules which confers inertness to the graft (Guidoin et al 1994). Fluoropassiv has been shown to cause less thrombus formation and tissue reaction in an animal model (Rhee, Gloviezki, Camria & Miller 1996).

2.3.3 Polytetrafluroethylene (PTFE)

The PTFE prosthesis utilised for both *in- vitro* and *in- vivo* experimentation was Gore-Tex® (W.L. Gore and Associates, Flagstaff, Arizona). Campbell, Brooks, Webster and

Bahnson (1976) reported the first clinical use of ePTFE. Following minor modifications it is regarded by some as the conduit of choice for lower limb revascularisation where vein is not available (Brewster 1995).

PTFE is a chemically inert polymer, inciting little tissue or blood reaction, has an electronegative surface which is regarded by some investigators to be "nonthrombogenic" and is impervious to blood (Hanel, McCabe, Abbott, Fallon & Megerman 1982). In addition, it is easy to handle and does not require preclotting although suture hole bleeding may be troublesome (Hanel et al 1982).

Initial experimentation with ePTFE was complicated by graft aneurysmal dilatation (Owens, Shinaberger, Wilson, & Wang 1978) which has been overcome by reinforcing the grafts with a thin external layer of PTFE (Tabbara & White 1994).

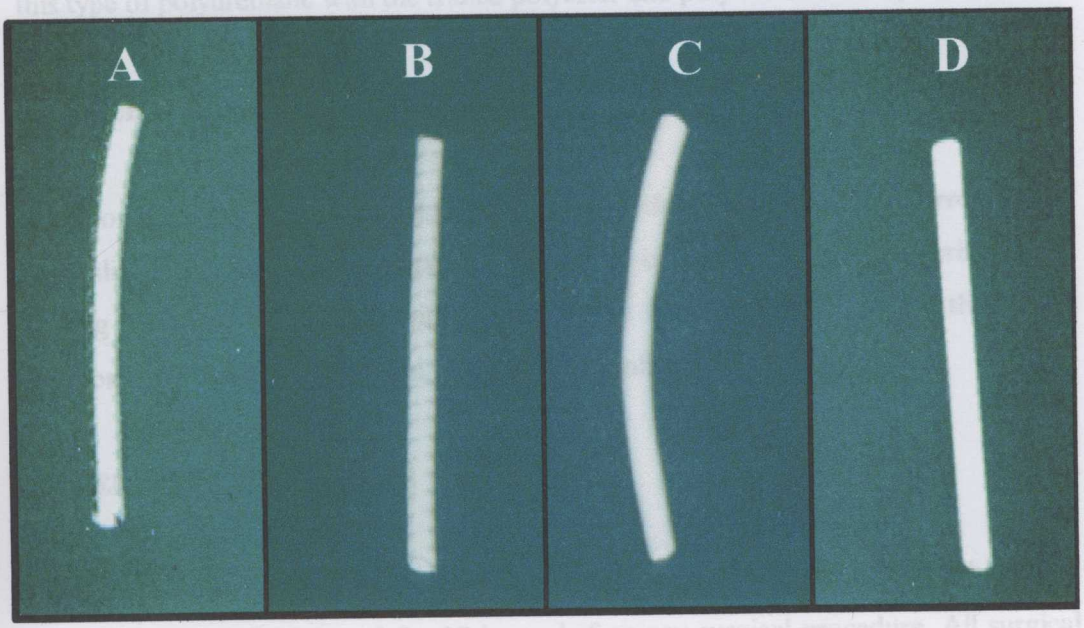

Fig. 2.1
Photograph of the four synthetic vascular prostheses. **A.** Gelsoft® **B.** Fluoropassiv™. **C.** PTFE. **D.** Thoratec®.

2.3.4 Polyurethane (Thoratec™)

Thoratec® VAG (Vascular Access Graft), (Thoratec Laboratories Corporation, Berkeley, USA) has been recently developed and subsequently introduced into Australia in 1992 for haemodialysis access (Allen, Yuill, Nankivell & Francis 1996). It is a three- layered polyurethane graft, with a middle layer composed of a solid sheath of BPS- 215M, an elastometric biomaterial, which confers elasticity, and compliance eliminating suture hole bleeding. Its chemical composition also allows self sealing ("Thoratec Graftways" 1994) which is advantageous when the graft is used as a conduit for vascular access (Allen et al 1996).

Although polyurethanes are infrequently used, they are commonly used at the institution in which the thesis was undertaken, namely Westmead Hospital. There use is at present confined to dialysis access. As a result, there is an opportunity to compare this type of polyurethane with the trialed polyester and polytetrafluoroethylene grafts.

2.4 PREPARATION OF RIFAMPICIN

The commercially available standard vial of Rifampicin (Rifadin, Merrell Dow, Australia) is 600mg, this is diluted to 60 mg/ml by adding 10 mls of sterile water. Mixing the 60 mg/ml solution with an appropriate volume of sterile normal then made solutions of 1.2 mg/ml, 10 mg/ml, and 30 mg/ml saline.

2.5 PERIOPERATIVE CARE

Sheep were allowed to graze normally up to the day prior to operation at which time they were housed and fasted for 18 hours before any surgical procedure. All surgical procedures were performed with the sheep under general anaesthesia. A premedication of thiazine 0.5 mg/kg (Xylazine, Nature Vet Pty Ltd, Sydney, Australia) and atropine sulphate (Atropine Sulphate, Parnell Labs Aust Pty Ltd, Australia) 0.03 mg/kg was given intramuscularly 30 minutes prior to induction of anaesthesia. Following premedication the sheep were induced with sodium thiopentone (15 mg/kg; Boehringer

Ingelheim Pty Ltd, Sydney, Australia) and were intubated endotracheally with size 9 endotracheal tubes. General anaesthesia was maintained with oxygen (2 litres/minute) and 1-2% Halothane® (ICI Pharmaceutical Division, Melbourne, Australia). Sheep respiration was monitored by an apAlert (MBM Enterprises, Australia) connected to the endotracheal tube. Whilst under general anaesthesia sheep were placed on their right sides, their left front legs restrained to the operating table and their left neck region shaved and prepared with chlorhexidine 0.5% in alcohol 70% and betadine (povidone iodine 10%). The prepared area was isolated with sterile drapes to ensure sterility throughout the procedure.

2.6 OPERATIVE PROCEDURE

2.6.1 General

A longitudinal neck incision was performed exposing and mobilising the left jugular vein. Dissection was continued posteromedially to the jugular vein identifying the common carotid artery and overlying vagus nerve (Figure 2.2).

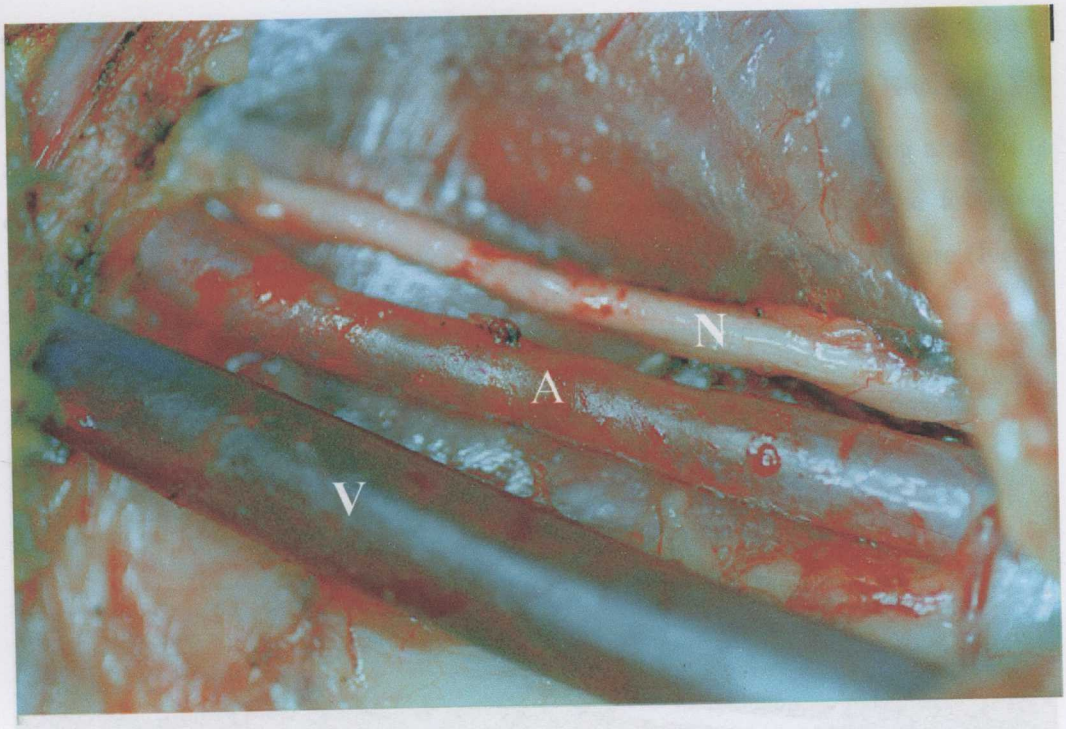

Fig. 2.2
Freely mobilised jugular vein (V), carotid artery (A) and vagus nerve (N).

The vagus nerve was dissected carefully from the common carotid artery to allow manipulation of the artery without inducing bradycardia in the sheep. In addition this manoeuvre allowed unimpeded arterial anastomosing. Intravenous heparin (5000 units; David Bull Laboratories Melbourne, Australia) was given directly via the jugular vein. Following a period of five minutes the common carotid artery was clamped with bulldog clamps proximally and distally to allow for graft anastomosis.

Following the specific arterial procedures to be outlined, the subcutaneous tissues were closed with 2/0 vicryl and the skin closed with 2/0 polypropylene suture. The wound was sprayed with antiseptic and insect repellent (Triclovet, Parnell Laboratories Pty. Ltd., Alexandria). The heparin was not reversed and no antibiotics were given during or following the surgical procedure. Following wound closure the Halothane® and oxygen were both ceased and the sheep allowed to recover. The endotracheal tube was removed when limb movements occurred usually coinciding with the sheep chewing on

the endotracheal tube. When the sheep were ambulatory they received a single dose of intramuscular buprenorphine analgesia (Temgesic®, Reckitt & Colman, Hull, U.K.) at a dose of 0.005 mg/kg. The sheep were kept indoors overnight only being allowed to drink water. All sheep and particularly their wounds were reviewed the following morning and if the assessment was satisfactory they were released to pasture (Figure 2.3).

Fig. 2.3
Sheep at pasture following the operative procedure.

2.6.2 Patch grafting

A vertical arteriotomy in the common carotid artery was performed between the bulldog clamps. The arterial lumen and vagus nerve were both irrigated with lignocaine 2% (Astra Pharmaceuticals Pty. Ltd, North Ryde, Australia) to remove blood from the lumen of the artery and to prevent arterial spasm. A 5 cm x 8 mm elliptically shaped Gelsoft patch graft was fashioned by cutting a 5 cm segment of graft in half longitudinally and then cutting this into an ellipse. This patch graft was sutured to the arteriotomy site using continuos 6/0 polypropylene suture (Figure 2.4). Prior to graft implantation the graft was soaked in a known concentration of rifampicin for 15 minutes at room temperature. The control group for this study was a control group performed as part of another study (Ao, Hawthorne, Vicaretti & Fletcher 2000) at our institution by the same investigators using identical methodology in which Gelsoft

patch grafts without any rifampicin soaking were anastomosed to the common carotid artery. On completion of the anastomosis the vascular clamps were slowly released re-establishing arterial flow in the artery. Gauze swabs were gently packed over the patch to control minor stitch hole bleeding and removed when haemostasis was obtained.

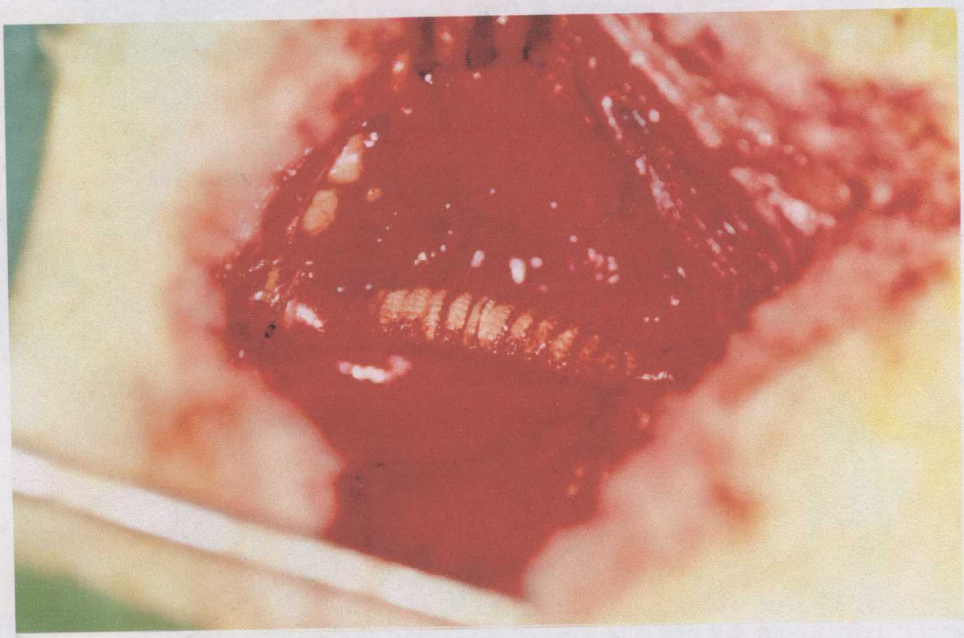

Fig. 2.4
Completed Gelsoft patch graft (control) to left common carotid artery.

2.6.3. Interposition grafting

A two centimetre segment of common carotid artery was excised between the bulldog clamps. The vagus nerve and both lumens of the carotid artery were irrigated with lignocaine 2%. The excised artery was replaced with a two centimetre by five millimetre Gelsoft™ graft interposed between the divided native artery and sutured to the native artery using interrupted sutures of 6/0 polypropylene. Prior to graft implantation grafts were soaked in rifampicin at various predetermined concentrations for 15 minutes at room temperature. On completion of the anastomosis, the graft

surface was directly inoculated with either 10^8 colony forming units of MRSA or 10^8 CFU of a known slime producing strain of methicillin resistant *S. epidermidis* (Figure 2.5).

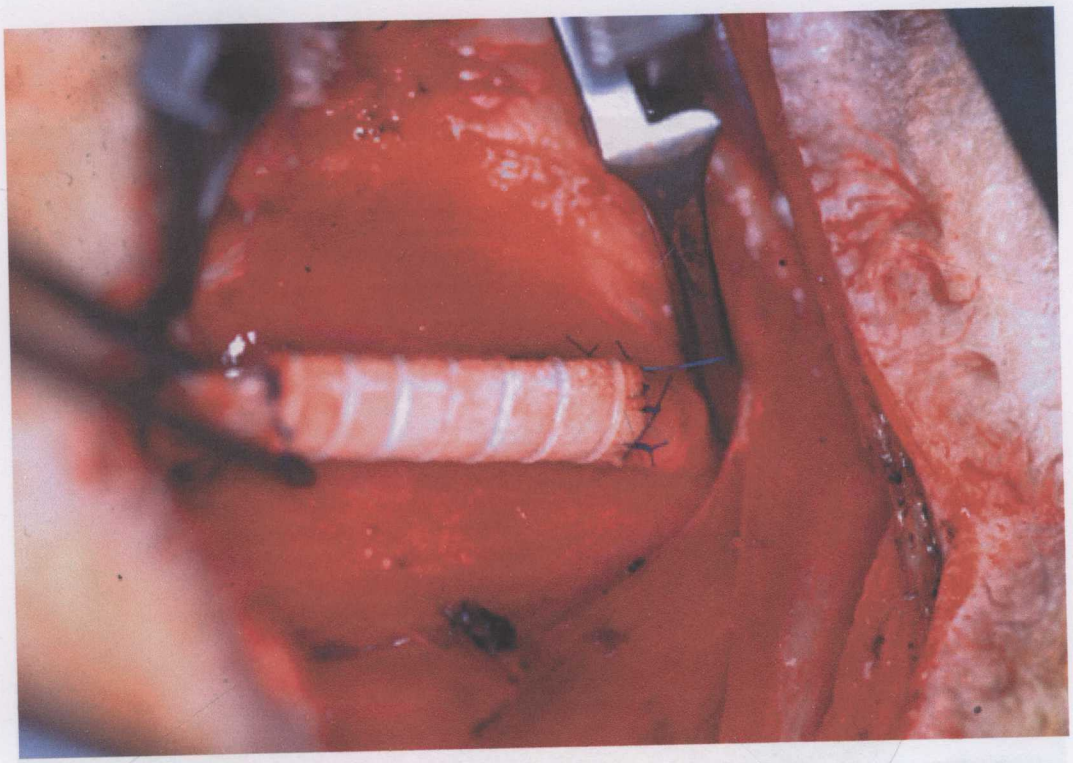

Fig. 2.5
Completed interposition graft without rifampicin impregnation.

2.6.4 Replacement of infected grafts with rifampicin impregnated Gelsoft grafts

The initial operative procedure in these experiments was carried out similarly to that outlined in 2.6.3. with the exception that the Gelsoft graft had not been soaked in rifampicin. Bacterial contamination of the graft with either 10^8 CFU of MRSA or 10^8 CFU of a known slime producing strain of methicillin resistant *S. epidermidis* was as carried out in 2.6.3.

Contaminated grafts (Figure 2.6) and adjoining stenosed or occluded native artery were removed three weeks following graft insertion and replaced with Gelsoft grafts that had been soaked in various predetermined concentrations of rifampicin for 15 minutes at room temperature (Figure 2.7).

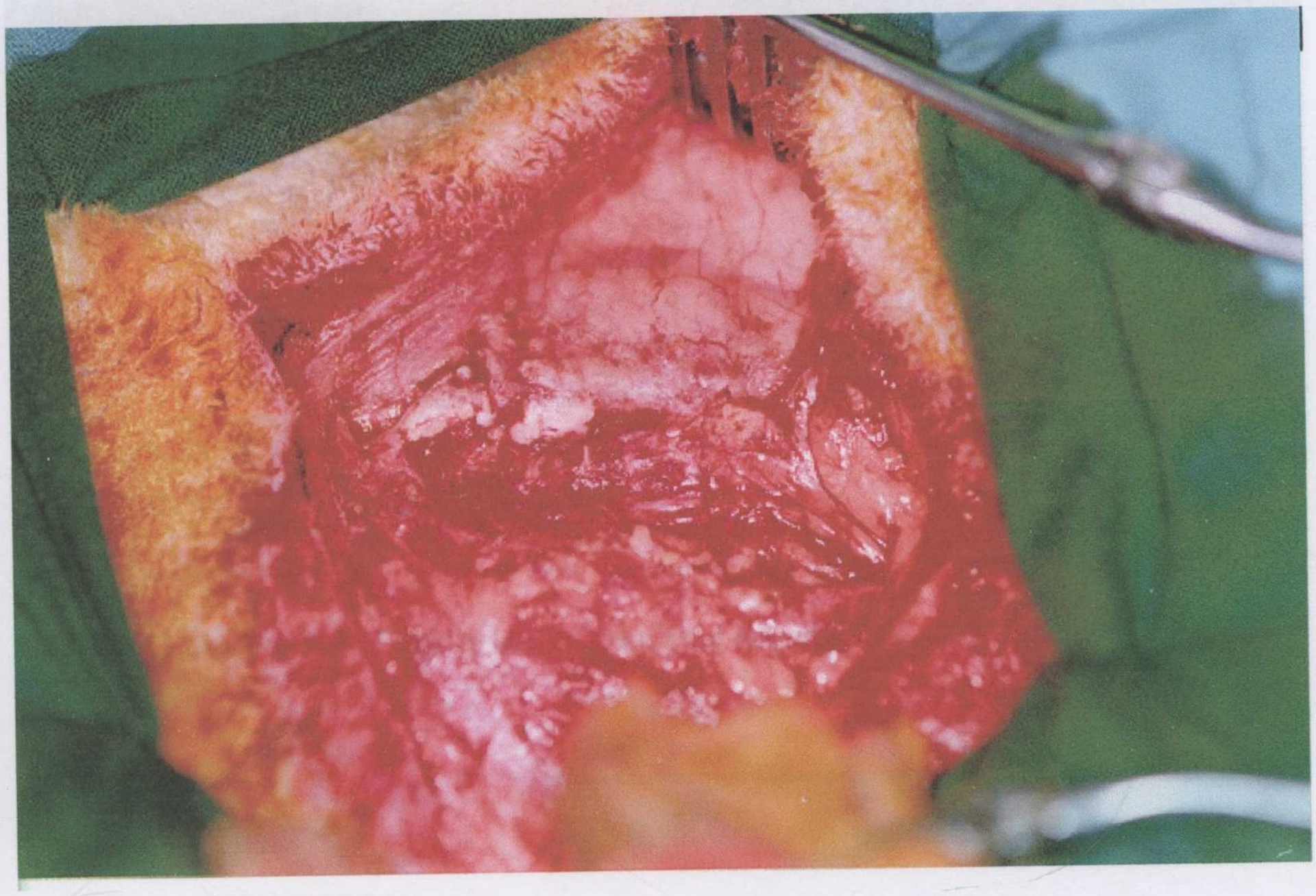

Fig. 2.6
Infected Gelsoft graft prior to mobilisation and removal.

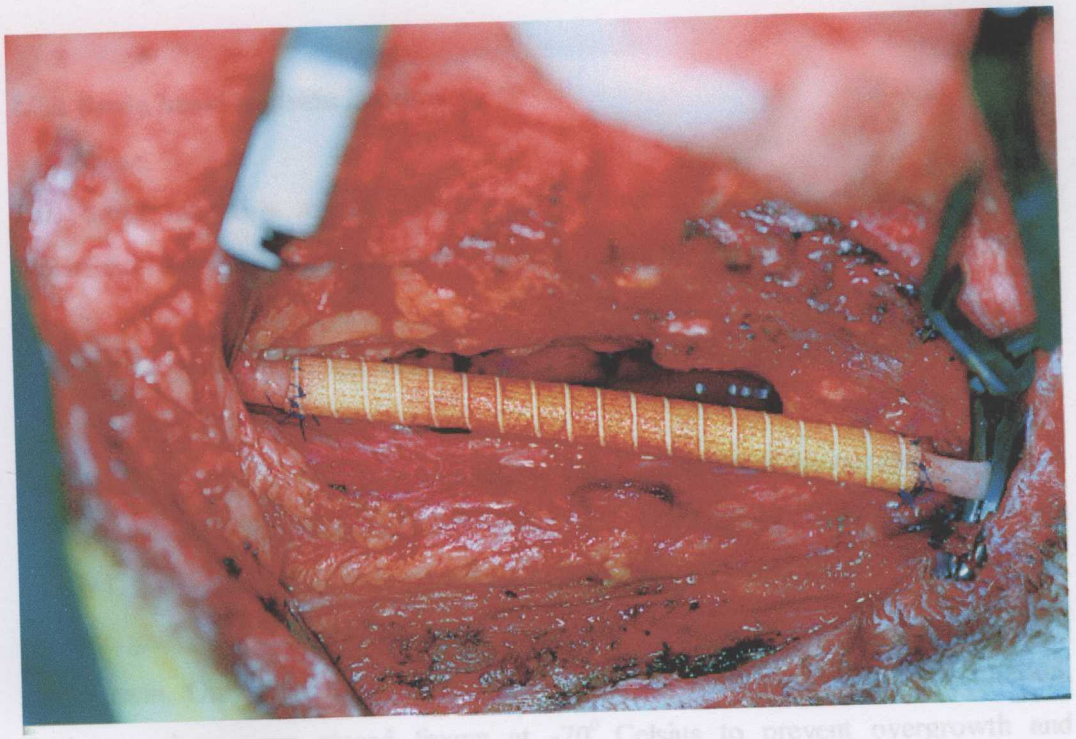

Fig. 2.7
Replaced infected Gelsoft graft with a 10mg/ml rifampicin soaked Gelsoft graft.

2.7 CULTURE MEDIA

2.7.1 Horse blood agar

Sheep blood agar plates are traditionally used as the artificial culture media for the bacterial inoculations as it is a supportive differential medium allowing the growth of most medically significant bacteria (Finegold & Baron 1986; Isenburg et al 1991). However, the blood agar plates used for bacterial culturing in all of the experimental studies utilised horse blood instead of sheep blood as it is the preferential media manufactured and used by the Clinical Microbiology Department at Westmead Hospital. There are essentially no differences between the two media in respect to growth of micro-organisms.

2.7.2 Brain heart infusion broth

This broth media is manufactured by the Clinical Microbiology Department at Westmead Hospital and is a highly nutritious medium for the support of most bacteria.

2.8 BACTERIAL PREPARATION

The MRSA was obtained from a positive blood culture in a haematological patient with an infected central line and the *S. epidermidis* was a known slime producing strain (strain D336) obtained from Department of Infectious Diseases, University of Sydney. Both organisms were resistant to methicillin but sensitive to rifampicin and vancomycin. Only these two organisms were used for all bacterial experimentation. Both organisms were stored frozen at -70^0 Celsius to prevent overgrowth and contamination.

The freeze stored bacteria were thawed 24 hours prior to the experiment. A one millilitre inoculum of bacteria was then inoculated onto a horse blood agar plate as described in section 2.9.2 and incubated at 37^0 C overnight. On the morning of the experiment variable numbers of cultured bacterial colonies were added to three millilitres of 0.9% normal saline solution to obtain a concentration of bacteria of 10^8 CFU per ml as determined by comparison with McFarland standards (Koneman, Allen, Dowell, & Sommers 1983). The concentration of bacteria was confirmed using an ATB 1550 photometer (bioMérieux, Lyon, France). The concentration of the inoculum was confirmed by tenfold serial dilutions of the inoculate. Ten microlitres of the fifth such dilution was cultured onto a horse blood agar plate and incubated at 37^0 Celsius overnight. Resultant manual colony counts were performed the following morning.

2.9 INOCULATION TECHNIQUES

2.9.1 Graft inoculation

Following graft anastomosis a one millilitre solution of 10^8 CFU of either MRSA or *S. epidermidis* was aspirated into a one millilitre syringe and gently irrigated onto the entire external luminal surface.

2.9.2 Culture plate inoculation for bacterial preparation and or identification

The culture plate inoculation is as described by Koneman, et al. (1983). A platinum wire fashioned into a loop was used for primary inoculation of bacteria. A primary inoculum was made in one quadrant of the agar plate and the inoculum successively streaked into the remaining quadrants by rotating the plate by 90 degrees. The loop was sterilised by flaming and air cooling between each successive quadrant streak. (Figure 2.8)

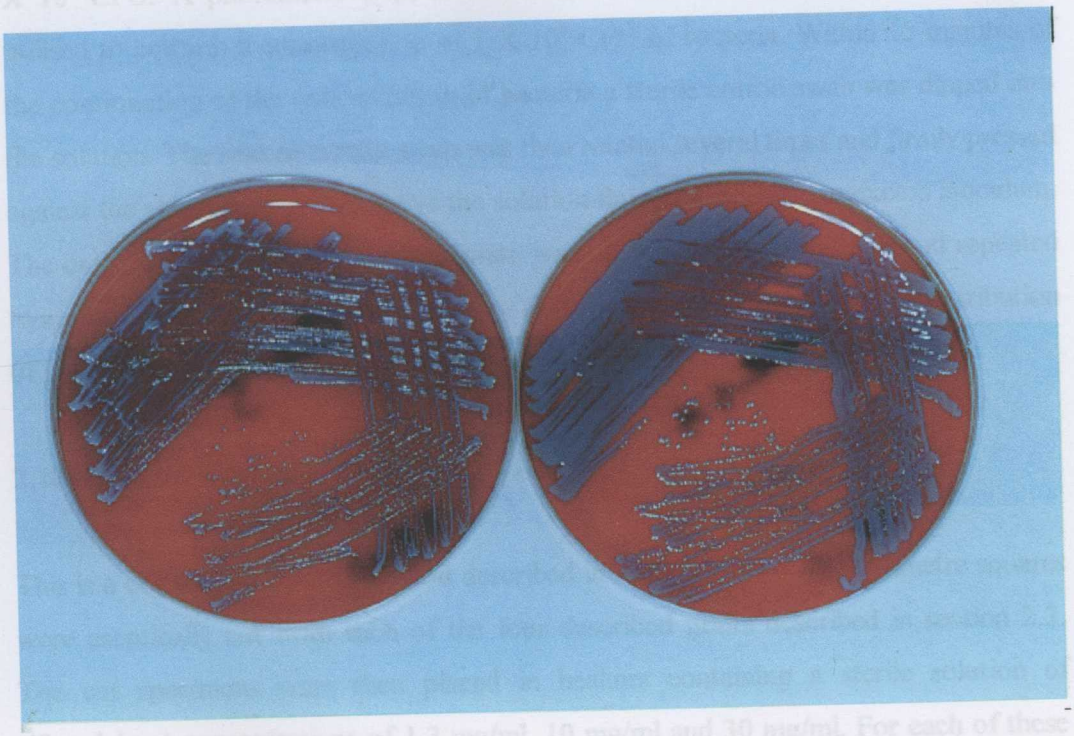

Fig 2.8
Cultures of *S. epidermidis* (left plate) and MRSA (right plate).

2.9.3 Culture plate inoculation for in-vitro analysis

The procedure for culturing agar plates for the in-vitro analysis to determine the optimal graft and dose of rifampicin was a modification of the procedure for performing disk diffusion testing by the direct colony suspension method as described by the National Committee for Clinical Laboratory Standards (NCCLS) (1997). The procedure used with its modifications is as follows. An inoculum of freeze stored MRSA and *S. epidermidis* was thawed and plated out onto an agar plate as described in section 2.9.2. Plates were incubated at 37^0 C for 24 hours. Isolated colonies of the same morphological type of organism were selected from each agar plate and placed into 10 millilitres of a sterile solution of normal saline. The suspension was then adjusted as determined by comparison with McFarland standards (Koneman, et al. 1983) to match a turbidity of 0.5. This corresponds to a bacterial concentration of 1-2 X 10^8 CFU. A photometer (Api ATB 1550, bioMérieux, Lyon, France) was then utilised to confirm a concentration of 1 X 10^8 CFU of bacteria. Within 15 minutes of the confirmation of the concentration of bacteria a sterile cotton swab was dipped into the solution. The soaked cotton swab was then rotated several times and firmly pressed against the solution container above the solution fluid level to remove excess inoculum. The entire surface of the blood agar plate was inoculated with the swab and repeated twice by rotating the plate approximately 60^0 each time to ensure an even distribution of inoculum. The rim of the agar plate was finally swabbed.

2.10 DISC DIFFUSION TESTING

This is a continuation of the method described in section 2.9.3. One centimetre squares were aseptically cut from each of the four described grafts described in section 2.3. The cut specimens were then placed in beakers containing a sterile solution of rifampicin at concentrations of 1.2 mg/ml, 10 mg/ml and 30 mg/ml. For each of these concentrations of rifampicin and each micro-organism six specimens of each graft material were cut. The cut specimens were left in the solution for 15 minutes at room temperature. The specimens were removed from the solution, blotted dry on a sterile linen drape and aseptically placed onto a previously prepared bacterial lawn of MRSA or S. *epidermidis* (refer to section 2.9.3). A maximum of three disks were placed on

each plate. The plates were placed in an incubator set to 37^0 C. At 24 hour intervals the plates were reviewed and the minimum distance from the soaked graft to the advancing or retreating front of micro-organisms was measured using a sterile ruler calibrated to the nearest millimetre (Figure 2.9). The discs were then systematically numbered, removed from the agar plate and placed onto a new agar inoculated with the same micro-organism as described in section 2.9.3. This procedure of measuring the minimum distance, removing the grafts and placing them onto a newly prepared bacterial lawn was repeated at 24 hour intervals until the front of bacteria had reached the graft. The time taken for bacteria to reach the graft was deemed the end-point.

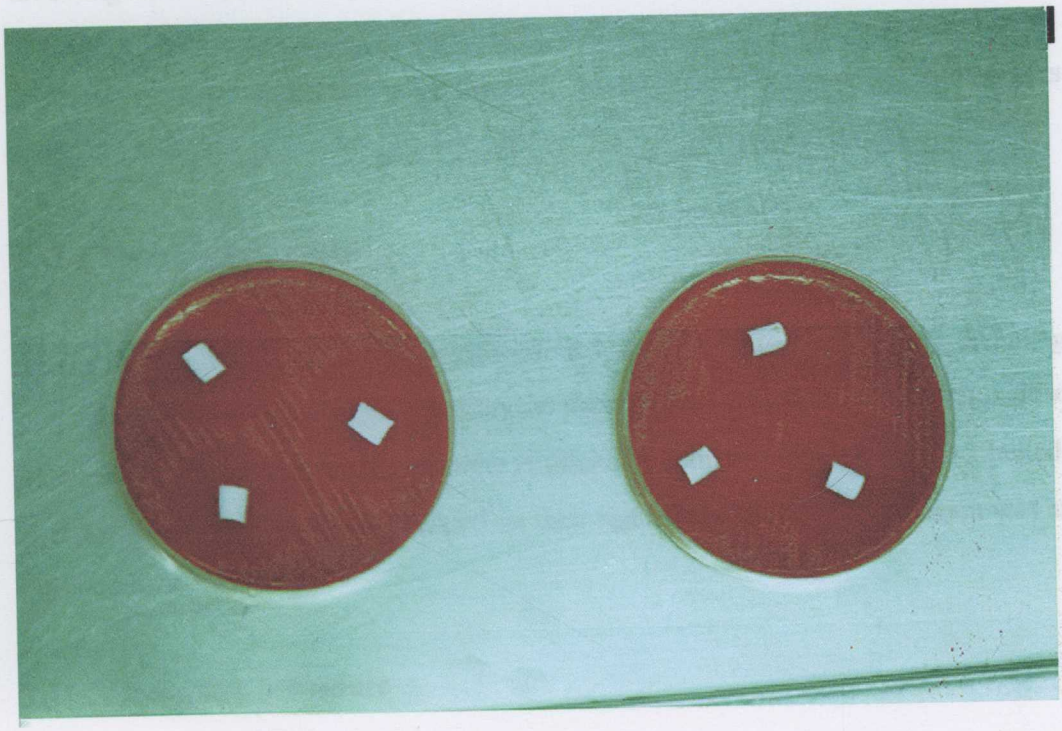

Fig. 2.9
PTFE graft segments on a lawn of *S. epidermidis* illustrating the disc diffusion technique.

2.11 OPERATIVE GRAFT HARVESTING

2.11.1 Timing

Grafts in the sheep undergoing patch grafting were harvested four weeks following graft insertion.

In the studies in which infected interposition grafts were replaced with rifampicin impregnated Gelsoft grafts the infected grafts were replaced with rifampicin soaked grafts at three weeks. These rifampicin soaked grafts were also removed three weeks following their implantation procedure.

In all other *in-vivo* infection studies the grafts in the sheep were removed three weeks following insertion.

2.11.2 Terminal procedure

Similar procedures as outlined in Section 2.5 were followed up to and including premedication. Following premedication the sheep were euthanased with a bolus dose of 60 mg/kg IV sodium pentobarbitone (Euthatal®, Pitman-Moore Australia Limited, Sydney, Australia). Sheep were placed on their right sides and neck preparation was as outlined in Section 2.5.

2.11.3 Harvesting procedure

The initial operative procedure for the identification of the carotid was identical to that outlined in Section 2.6.1. In difficult cases where the extent of the inflammatory reaction was extensive and no clear tissue planes were identifiable by sharp dissection the mobilisation of the carotid artery with the implanted graft was undertaken by blunt dissection. In these circumstances the prosthetic graft could be palpated through the inflammatory tissue and dissection carried out down towards the graft.

When the artery and/or prosthetic graft were identified dissection was continued along the line of the artery cranially and caudally so that the prosthesis with its proximal and distal anastomosis could be mobilised. The native artery was then clamped proximally and distally to the prosthesis and the prosthesis and adjacent native artery removed.

2.11.4 Macroscopic assessments of grafts

All features of the grafts were recorded with special attention being paid to the presence or absence of subcutaneous and perigraft abscess formation. When the artery and prosthesis were identified any evidence of anastomotic disruption was recorded. Finally on removal of the implanted graft, the lumen of the graft was inspected to determine if the lumen was occluded with thrombus.

2.12 HARVEST SPECIMENS COLLECTED FOR BACTERIAL ANALYSIS

The following section only applies to the *in-vivo* studies in which grafts were deliberately infected with MRSA or *S. epidermidis*.

2.12.1 Perigraft tissue sample

Swabs of the perigraft tissues and/or any encountered abscess were taken and smeared across the surface of a horse blood agar plate.

2.12.2 External graft sample

Once the graft with adjacent native artery was removed the external surface of the graft was directly smeared across the entire surface of a horse blood agar plate.

2.12.3 Internal graft sample

If the removed graft and arterial segment was patent, one millilitre of normal saline was irrigated into the lumen and the resultant solution inoculated directly onto a blood

horse agar plate. If the graft was non-patent, it was opened longitudinally and the internal surface of the graft with attached thrombus smeared onto the agar plate.

2.12.4 Graft segment

A 3-5 millimetre segment of the removed graft was excised and directly placed into a brain heart infusate broth. For the *S. epidermidis* infected grafts, to disrupt the biofilm and increase the yield of micro-organisms, the remainder of the graft was mechanically disrupted for 5 minutes with a mortar and pestle using 5 millilitres of normal saline to aid in grinding. The resultant ground graft and solution was then placed in brain heart infusate broth.

2.13 BACTERIAL GROWTH AND IDENTIFICATION OF MICRO-ORGANISMS

All specimens cultured in section 2.10 were incubated at 37^0 C. The specimens placed in the brain heart infusate were incubated for 48 hours. One millilitre of the incubated solution was then irrigated onto an agar plate. All plates were viewed for up to 96 hours.

2.13.1 Gram Staining

The gram-stain technique used is a modification of Lillie's method (cited in Cowan & Steel 1993). The modified procedure is as follows. Slides were flamed and allowed to cool. A thin smear of bacteria was then prepared on the flamed side of the slide by mixing a preselected colony from the horse blood agar plate with a drop of normal saline. The slide was fixed by again passing the slide through the flame. Ammonium oxalate-crystal violet stain was flooded onto the slide and left for 30 seconds. The slide was washed in running water and subsequently flooded with iodine. The iodine solution was left for 30 seconds, drained but not washed off the slide. Decolourisation was performed using acetone ensuring that it was removed within 2-3 seconds by washing thoroughly with running water. Counterstaining was performed using carbol fuchsin for 30 seconds. The slide was blotted dry using filter paper. The resultant slide

was examined under the light microscope at total magnifications of 100X and oil immersion (1000X). The resultant gram positive organisms stained blue or purple and gram negative organisms stained red.

2.13.2 Coagulase testing

The coagulase testing technique is a modification of that described by the Subcommitee on taxonomy of staphylococci and micrococci (1965) (cited in Cowan & Steel 1993). The procedure is as follows. A single colony from an blood agar plate is emulsified in 1 millilitre of undiluted fresh citrated human plasma supplied by the Haematology Department Westmead Hospital. The emulsant is then incubated at 37^0 Celsius in a water bath and results read at 1,4 and 24 hours. The coagulase test was interpreted as being positive if there was evidence of definite clot formation. Comparisons were also made between the positive controls of *S. aureus* and the negative controls of *S. epidermidis*.

2.14 HISTOLOGICAL PREPARATION

Histological specimens were only undertaken in the intimal hyperplasia study.

Graft specimens together with adjacent 5 mm artery harvested from the intimal hyperplasia study were initially irrigated with 10% buffered formalin to test for patency of the graft and then completely immersed in a solution of 10% buffered formalin. The following day each graft segment was divided equally into eight transverse sections. The specimens were dehydrated and prepared as paraffin blocks. Slides were then cut from the transected specimens and stained with haematoxylin and eosin.

2.15 INTIMAL HYPERPLASIA ANALYSIS

Each stained transverse section was viewed using a computerised image analysis system (Optimas, USA). The histopathology slide was first viewed under a light microscope and the area of interest analysed. The image analysis camera mounted on the microscope then obtained the image, which was stored by the computer and

viewed on the video screen. The area of intimal hyperplasia (IH) overlying the internal surface of the graft was carefully traced out and a recording made. The width of the graft similarly was traced and its measurement recorded (Figure 2.10). To account for the magnification of the section a graticule of known width and length was viewed using an identical set-up and the magnification value determined. True recordings of the area of intimal hyperplasia and graft width were then calculated accounting for magnification. Intimal hyperplasia was expressed as a ratio value by dividing the area (mm^2) of IH over the graft by the graft width (mm).

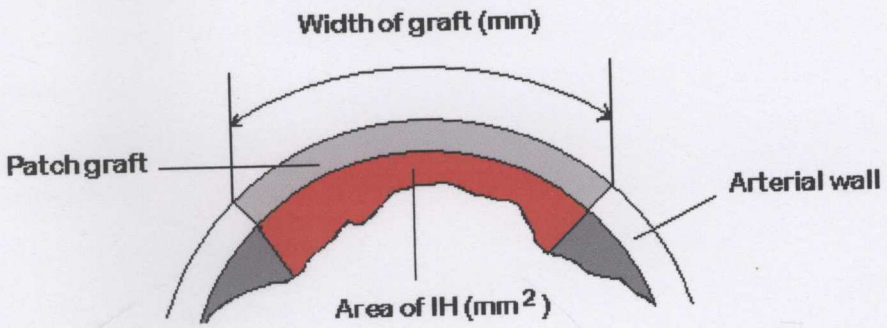

Fig. 2.10
Measurement of intimal hyperplasia (IH). In the cross section, the area of IH developed on the inner surface of the graft and the width of the graft were measured. IH index = area of IH (mm^2) divided by the width of the graft (mm).

2.16 STATISTICAL ANALYSIS

All data were entered into spreadsheet format utilising a software program (Microsoft Excel 5.0, Microsoft Corporation, USA). The data was converted to a file so that statistical analysis could be undertaken using Statistical Package for Interactive Data Analysis (SPIDA, Statistical Computing Laboratory, Eastwood, Australia).

Specific statistical tests are described in the methodology of relevant chapters.

Critical values of the χ^2 distribution, student-t-test and for Fisher's exact test were those outlined by Matthews and Farewell (1988).

Chapter 3

IN-VITRO STUDY DETERMINING THE OPTIMAL VASCULAR MATERIAL AND DOSE OF RIFAMPICIN IMPREGNATION TO PREVENT GRAFT INFECTION.

3.1 INTRODUCTION

There is general agreement that the most common mechanism of graft infection is by bacterial contamination at the time of graft implantation (Moore & Deaton 1993). To overcome the risk of infection at the time of surgery and in the early post-operative period it is postulated that the impregnation of the implanted graft material with a suitable antibiotic may be adequate treatment to prevent prosthetic graft infection by offering local bacterial protection. As yet no single prosthetic graft material with or without antibiotic impregnation has demonstrated protection from bacterial contamination. A number of groups have experimented with different antibiotics and protein carriers in both *in-vitro* and *in-vivo* models to offer graft protection from bacterial contamination (Clark & Margraf 1974; Shah, et al. 1987; Harvey & Greco 1981; MacDougal, et al. 1986; Benvenisty, et al. 1988; Chervu et al 1991a; Kinney, et al. 1991; Ozaki, et al. 1993; Okahara, et al. 1995;). Graft materials used for experimentation have included polyester (Clark & Magraf 1974), PTFE (Benvenisty, et al. 1988; Kinney, et al. 1991; Shah, et al. 1987; Sobinsky & Flanigan 1986) and Dacron (Kempczinski 1981; Powell, et al. 1983; MacDougal, et al 1986; Avramovic & Fletcher 1991; Sardelic, et al. 1994; Sardelic, et al. 1996). No *in-vitro* experimentation has been published on newer graft materials (namely Thoratec and Fluoropassiv) now available to the vascular surgeon with respect to the efficacy of antibiotic binding to the graft.

In this *in-vitro* study, we evaluated the effect of soaking four commercially available prosthetic grafts (PTFE™, Gelsoft™, Thoratec™ and Fluoropassiv™) in known concentrations of rifampicin against staphylococcal (MRSA and *S. epidermidis*) infection.

3.2 MATERIALS AND METHODS

The graft materials used in this study were four currently commercially available grafts used in vascular surgery, Gelsoft, Thoratec, PTFE and Fluoropasssiv. The descriptions of each of these graft materials are outlined in Section 2.3.

The methodology for this study is outlined in Sections 2.9.3 and 2.10. In general studied graft segments were soaked in concentrations of rifampicin of 1.2 mg/ml, 10 mg/ml or 30 mg/ml and placed on a bacterial lawn of either MRSA or *S. epidermidis*.

Daily replacement of the grafts onto a new lawn of micro-organisms was undertaken as it was felt that that any antibiotic that had eluted from the graft into the agar would reside in the agar affecting the rate of progression of the bacterial front and would not be a true reflection of the residual rifampicin in the graft.

3.3 STATISTICAL ANALYSIS

At twenty four hour intervals, measurements were taken from each graft to the advancing front of micro-organisms. For identical specimens the average minimum distance from the edge of the grafts to the bacterial front was recorded for each twenty four hour interval.

The average distances in millimetres for each twenty four hour interval were then compared to assess if there were any differences between graft material at a known concentration and to assess if increasing concentration had any effect on the anti-staphylococcal activity for each individual type of graft. The statistical tool utilised was two way analysis of variance with replication and a p value of less than 0.05 was taken as being significant.

3.4 RESULTS

Results are presented graphically and depict comparisons of graft material for set rifampicin concentration and comparison of individual graft material for differing rifampicin concentration (Figures 3.1 – 3.14).

At the concentration of 1.2 mg/ml rifampicin (Fig3.1& Fig 3.2) and adjusting for the staphylococcal species all the grafts were significantly different from each other. The rank order of grafts from best to worst as is evident from the graphs was Fluoropassiv, Gelsoft, Thoratec and PTFE.

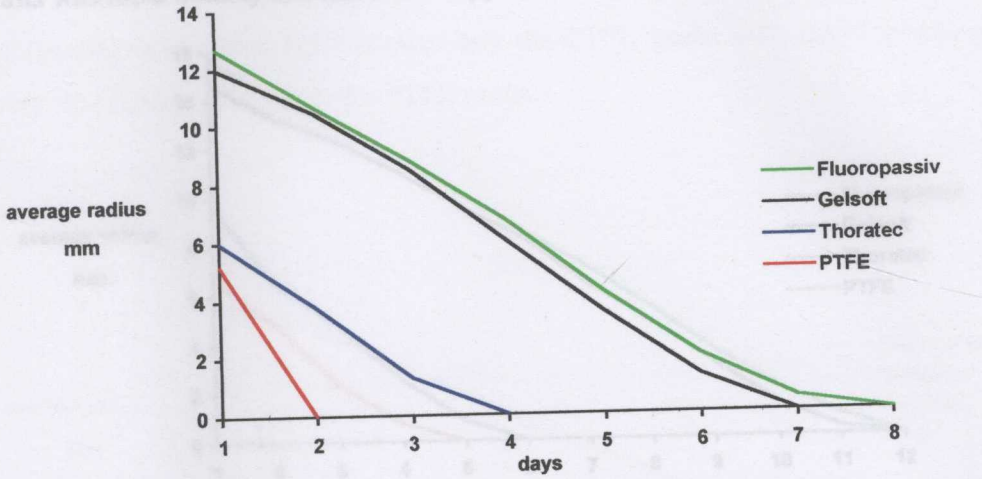

Figure 3.1

MRSA + 1.2 mg/ml rifampicin:

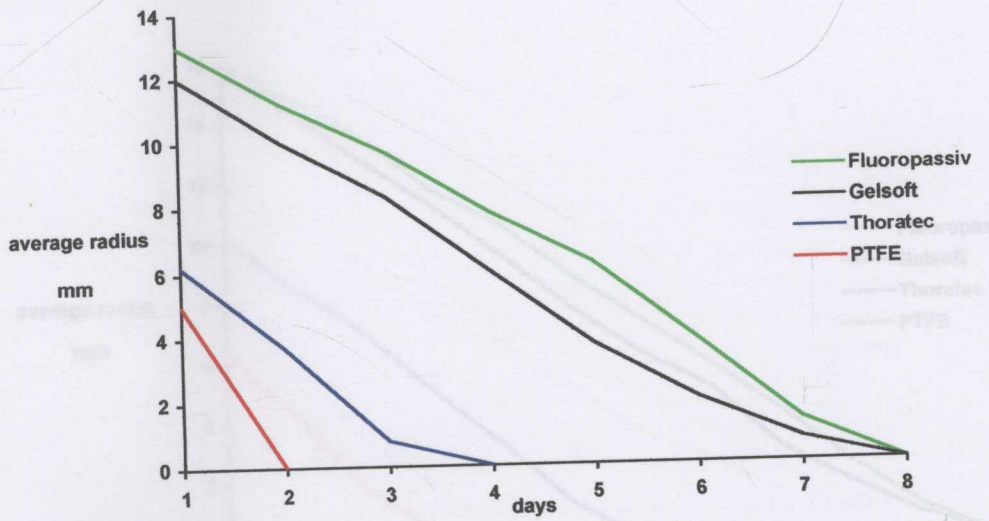

Figure 3.2

S. epidermidis + 1.2 mg/ml rifampicin

At the concentration of 10 mg/ml rifampicin (Fig 3.3 & Fig 3.4) and adjusting for the staphylococcal species, Fluoropassiv and Gelsoft were similar. Both were significantly better than the Thoratec and the PTFE with the Thoratec graft being significantly better than the PTFE grafts. The antibacterial activity of the Gelsoft and Fluoropassiv grafts was most notably increased from approximately eight days to twelve days.

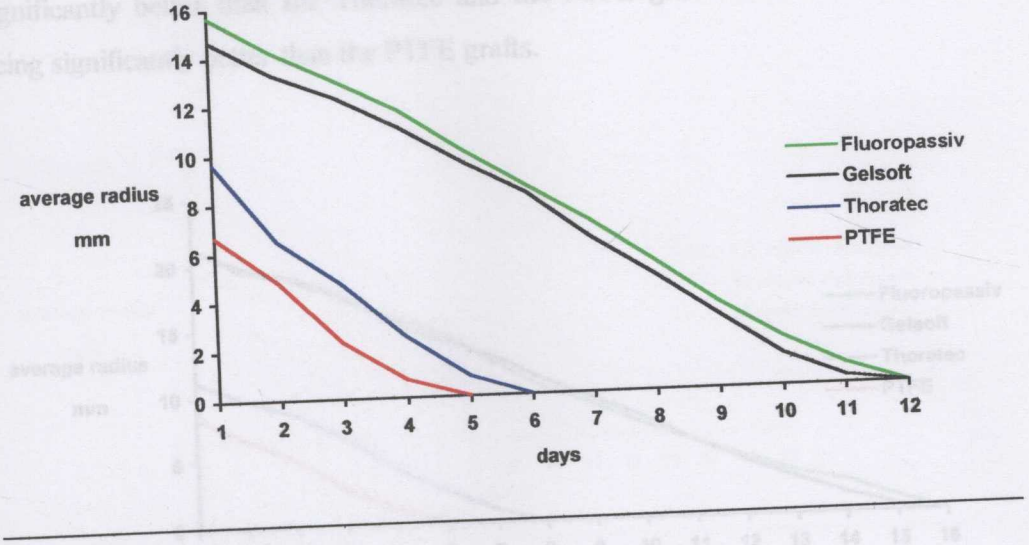

Figure 3.3

MRSA+ 10 mg/ml rifampicin

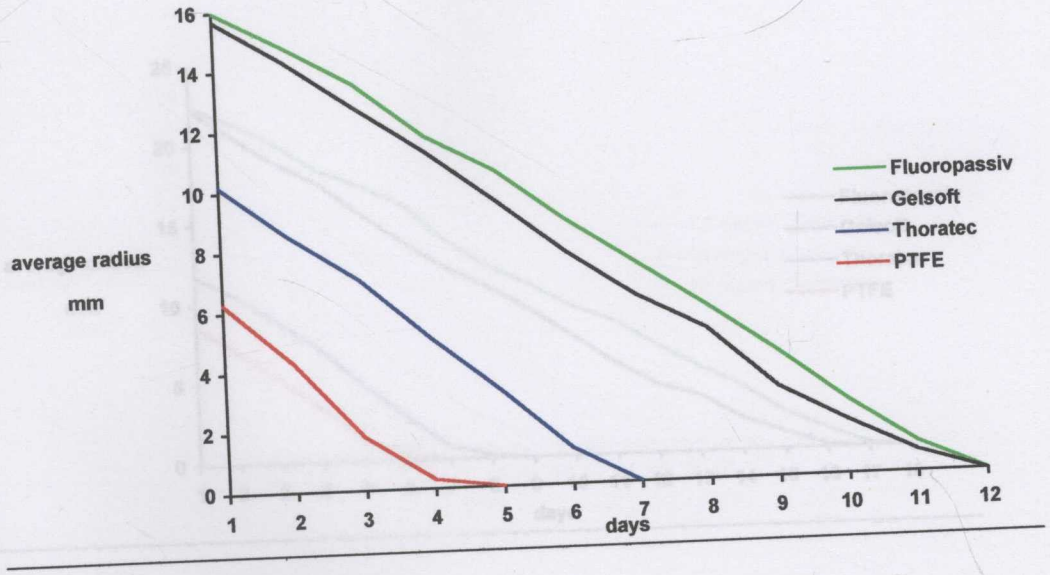

Figure 3.4

S. epidermidis + 10 mg/ml rifampicin:

With the highest concentration of 30 mg/ml rifampicin (Fig 3.5 and Fig 3.6) and adjusting for the staphylococcal species, no significant differences were evident between the Fluoropassiv and Gelsoft with similar average zones of inhibition at day one and similar length of antibacterial activity. Additionally, as was apparent with the lower rifampicin concentrations, the Gelsoft and Fluoropassiv grafts were both significantly better than the Thoratec and the PTFE grafts with the Thoratec graft being significantly better than the PTFE grafts.

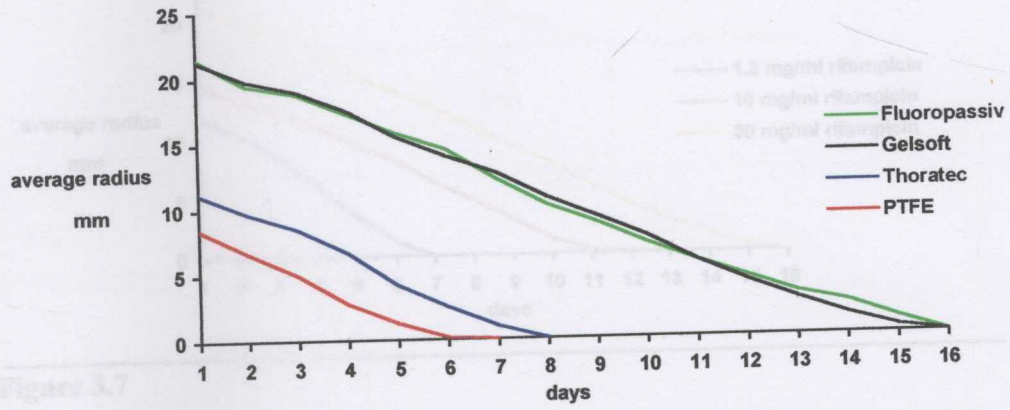

Figure 3.5

MRSA + 30 mg/ml rifampicin.

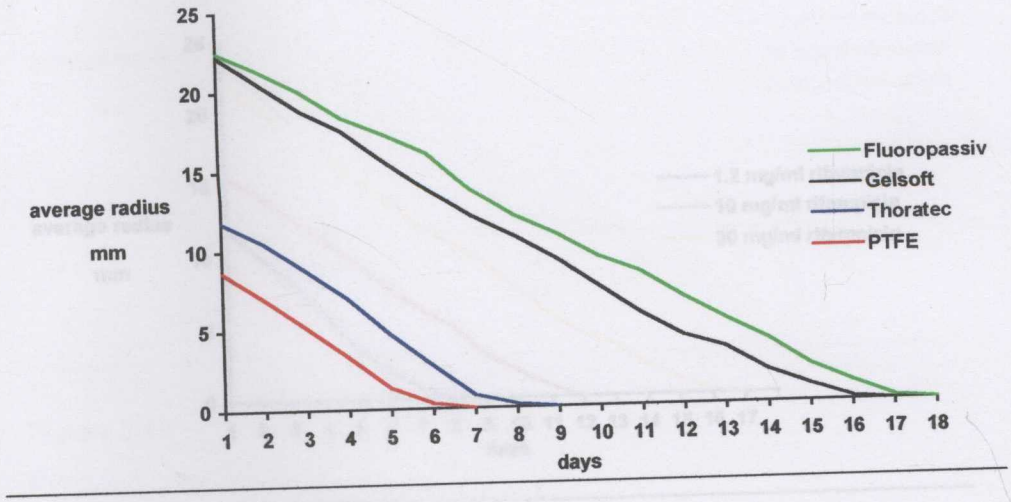

Figure 3.6

S. epidermidis + 30 mg/ml rifampicin.

In all the graft types with increasing rifampicin concentration there was a significant increase in the average zone of inhibition and the total antibacterial activity of the rifampicin soaked graft (Fig3.7-Fig 3.14).

No significant differences were apparent between the staphylococcal species when adjusting for graft type and rifampicin concentration.

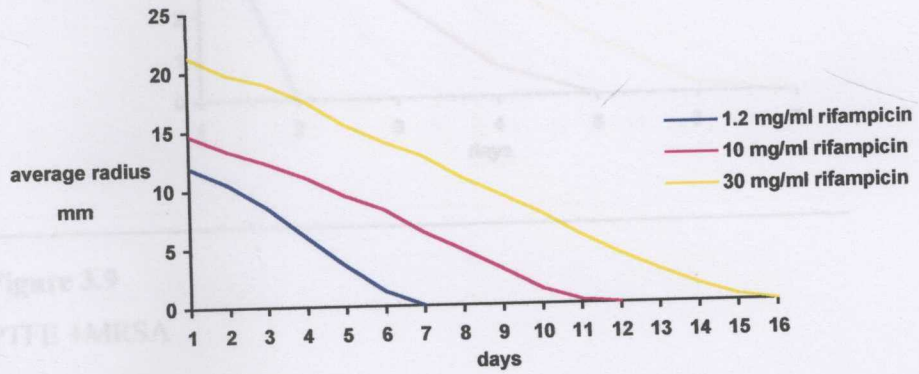

Figure 3.7

Gelsoft + MRSA

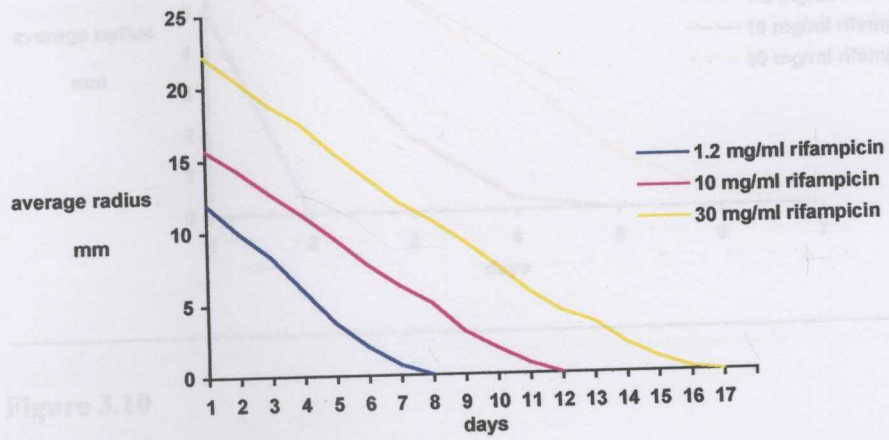

Figure 3.8

Gelsoft + S. epidermidis

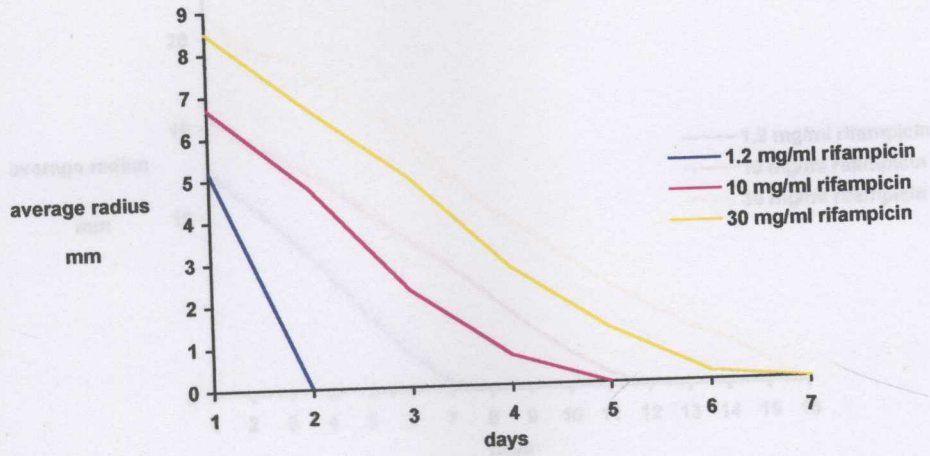

Figure 3.9
PTFE +MRSA

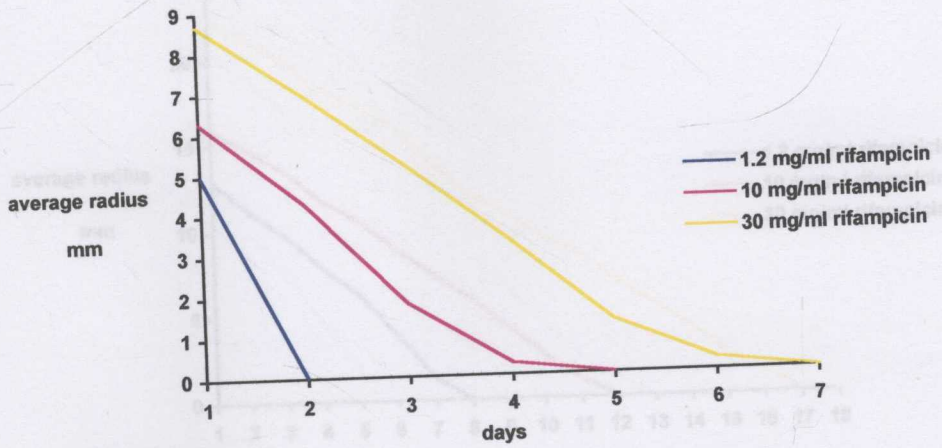

Figure 3.10
PTFE + *S. epidermidis*

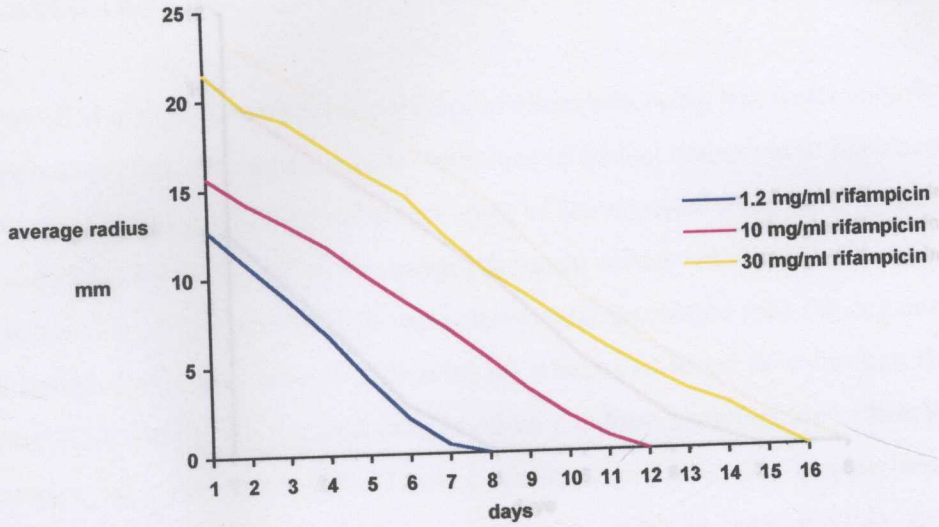

Figure 3.11

Fluoropassiv + MRSA

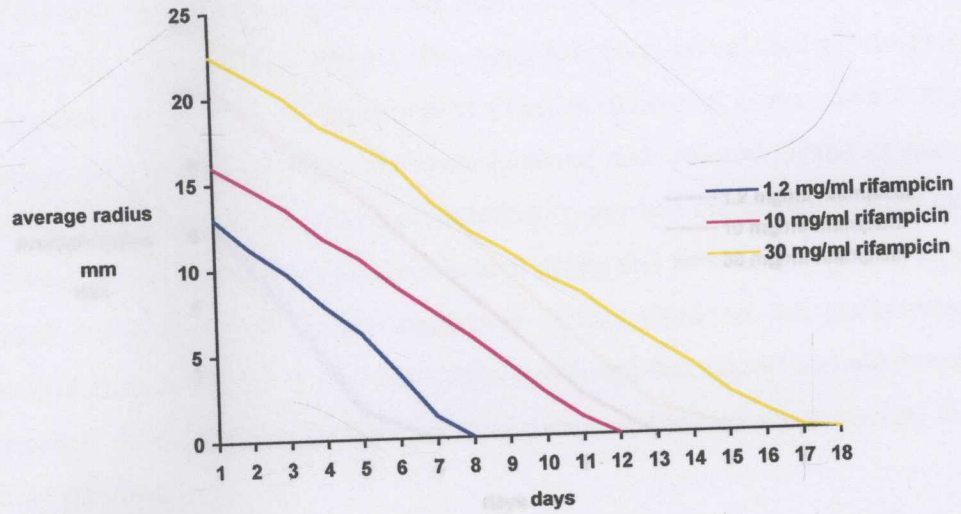

Figure 3.12

Fluoropassiv + *S. epidermidis*:

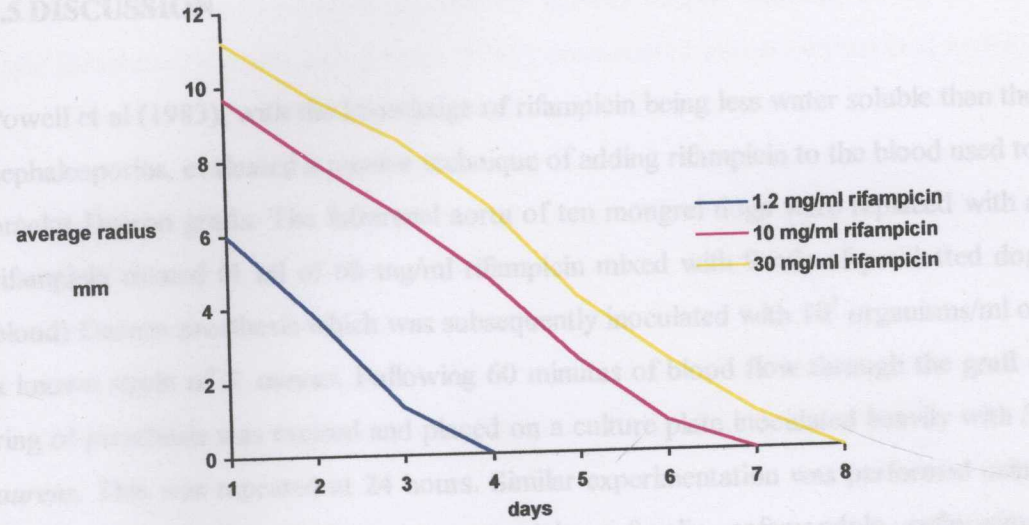

Figure 3.13

Thoratec + MRSA

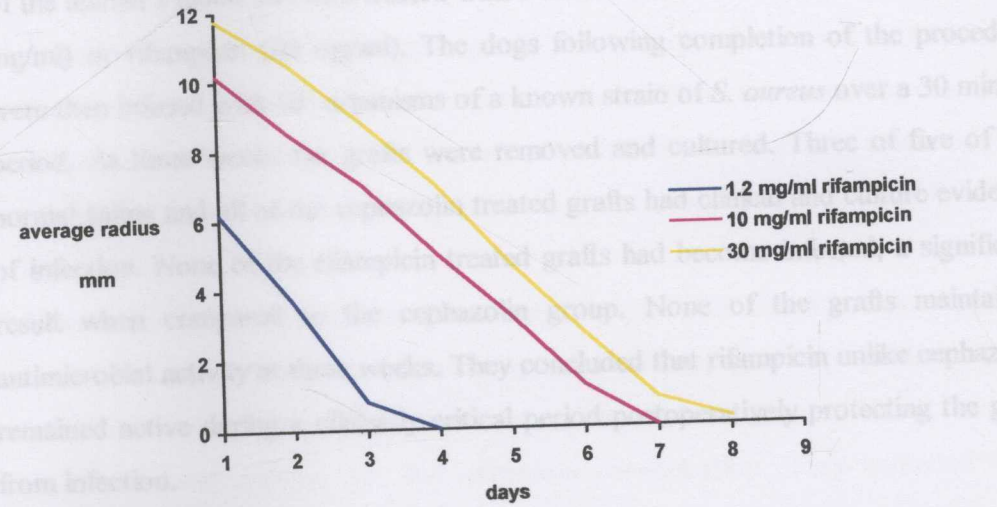

Figure 3.14

Thoratec + *S. epidermidis*

3.5 DISCUSSION

Powell et al (1983), with the knowledge of rifampicin being less water soluble than the cephalosporins, evaluated a passive technique of adding rifampicin to the blood used to preclot Dacron grafts. The infrarenal aorta of ten mongrel dogs were replaced with a rifampicin treated (1 ml of 60 mg/ml rifampicin mixed with 9 mls of preclotted dog blood) Dacron prosthesis which was subsequently inoculated with 10^7 organisms/ml of a known strain of *S. aureus*. Following 60 minutes of blood flow through the graft a ring of prosthesis was excised and placed on a culture plate inoculated heavily with *S. aureus*. This was repeated at 24 hours. Similar experimentation was performed using tobramycin, oxacillin, clindamycin, gentamicin, cefazolin, cefamondole, cefatoxime, and tetracylcline. At 24 hours all the grafts prepared with antibiotics other than rifampicin showed no activity. The mean inhibition at 60 minutes and 24 hours for the rifampicin treated grafts was 94% and 91% of that at time zero. MacDougal, et al. (1986), in a similar experiment to Powell, et al. (1983) replaced the infrarenal aorta of 15 mongrel dogs with a Dacron prosthesis . The prostheses were preclotted with 9 mls of the animal's blood and then treated with 1 ml of either normal saline, cefazolin (237 mg/ml) or rifampicin (60 mg/ml). The dogs following completion of the procedure were then infused with 10^7 organisms of a known strain of *S. aureus* over a 30 minute period. At three weeks the grafts were removed and cultured. Three of five of the normal saline and all of the cephazolin treated grafts had clinical and culture evidence of infection. None of the rifampicin treated grafts had become infected, a significant result when compared to the cephazolin group. None of the grafts maintained antimicrobial activity at three weeks. They concluded that rifampicin unlike cephazolin remained active during a clinically critical period postoperatively protecting the graft from infection.

With the development of protein sealants it became possible in the 1990's to use the sealant as a means of antibiotic incorporation into prosthetic grafts. Rifampicin by its nature of being relatively water insoluble, when combined with protein sealed (gelatin or collagen) Dacron grafts, has been shown to form ionic bonds with resultant drug accumulation and prolonged drug release (Gahtan, et al. 1995). Ashton, et al. (1990)

showed in *in-vitro* experiments that 1 mg/ml rifampicin soaking of a gelatin sealed Dacron graft (Gelsoft) retained antibacterial activity despite washing for up to four days. Strachan, Newsom and Ashton (1991) documented anti-staphylococcal activity in a rifampicin soaked (60 mg/ml) gelatin sealed Dacron graft in a post-mortem specimen of a patient three weeks following insertion. Others have demonstrated in animal models (Avramovic & Fletcher 1991; Goeau-Brissonniere, et al. 1991; Freyrie, et al. 1992; Sardelic, et al. 1994; Gahtan, et al. 1995; Sardelic, et al. 1996) and in humans (Torsello, Sandmann, Gehrt and Jungblut 1993; D'Addato, et al. 1994; French, Chard, Sholler and Cartmill 1994; Naylor, et al. 1995; Strachan 1995) the use of rifampicin impregnated grafts in the prevention of graft infection.

Our experimentation was solely concerned with the comparison of the *in-vitro* anti-staphylococcal activity of four commercially available prosthetic vascular grafts when soaked with known concentrations of rifampicin. Rifampicin was chosen as the antibiotic as it has activity against the micro-organisms commonly implicated with graft infection, namely staphylococci. The organisms were methicillin resistant *S. aureus* and *S .epidermidis.*

The Dacron type grafts, namely Fluoropassiv and Gelsoft, were superior to the other grafts at all studied rifampicin concentrations. In addition, by increasing rifampicin concentration to 30 mg/ml, the initial zones of inhibition were increased in size and the length of time of antibacterial activity for all the grafts was extended.

Gahtan, et al. (1995) evaluated the *in-vitro* antistaphylococcal activity of three types of Dacron prostheses (plain low porosity, gelatin, and collagen impregnated). Their conclusions were that the most important determinant of the *in-vitro* antistaphylococcal activity was the rifampicin concentration. They extended their experimentation to a rifampicin concentration of 60 mg/ml but were unable to use higher concentrations because of the relative insolubility of rifampicin. We encountered similar problems in the solubility of rifampicin even at the lower concentration of 30 mg/ml, with crystallisation of the rifampicin on the graft surface. Gahtan, et al. (1995) also concluded that, of the various Dacron prosthesis studied, the gelatin impregnated Dacron (Gelseal) demonstrated superior *in- vitro* bioactivity compared to the other

Dacron grafts in the study. It is interesting to note that although the 60 mg/ml solution of rifampicin was superior to all the other tested concentrations and the 30 mg/ml solution was superior to the 1 mg/ml solution, the 15 mg/ml soaked grafts contained rifampicin for approximately 24 hours longer than the 30 mg/ml solution. The *in-vivo* bioactivity was present for only up to 48 hrs after implantation. We did not set out to determine *in-vivo* bioactivity in this experiment but it is of note that others (Strachan, et al. 1991) have reported *in-vivo* bioactivity at three weeks.

Passive bonding of rifampicin with Thoratec or PTFE has never been documented and it would be expected that the *in-vitro* bioactivity of 8 days for the Thoratec and 7 days for the PTFE grafts when soaked with 30 mg/ml rifampicin would not be representative of the *in-vivo* situation as the rifampicin would be expected to elute with arterial flow through the prosthesis. It may be of importance to investigate the potential of vascular graft wash-out studies prior to further *in-vivo* work.

It is difficult to make direct comparisons with previous *in-vitro* and *in-vivo* experimental studies as they have compared different rifampicin concentrations (Gahtan et al 1995), used different species or multiple species of micro-organism (Ney, et al. 1990, Okahara, et al. 1995), used micro-organisms at varying concentrations (Chervu, et al. 1991, Gahtan, et al. 1995), used different graft materials for experimentation (Shah, et al. 1987), and have employed different methodology for their *in-vitro* analysis (Chervu, et al. 1991, Gahtan, et al. 1995). Nevertheless, there was increased *in-vitro* anti-staphylococcal activity of both the Fluoropassiv and Gelsoft grafts at all the studied concentrations when compared to the Thoratec and the PTFE grafts. This coupled with the fact that the technique of passive binding of the rifampicin to the Fluoropassiv or Gelsoft graft is easily performed, makes Fluoropassiv and Gelsoft the most suited grafts for rifampicin soaking in the prevention of staphylococcal prosthetic graft infection.

Chapter 4

AN *IN-VIVO* COMPARISON OF THE SUSCEPTIBILITY TO INFECTION OF FOUR COMMERCIALLY AVAILABLE PROSTHETIC GRAFTS.

4.1 INTRODUCTION

Prosthetic vascular grafts are foreign bodies whose interstices are a nidus for the settlement of micro-organisms with subsequent development of infection (O'Brien & Collin, 1992). Since the first use of synthetic material for vascular reconstruction in the 1950's numerous synthetic materials have been developed and applied clinically but to date no synthetic prosthesis currently available to the vascular surgeon is theoretically free from the risk of infection. According to Bandyk and Esses (1994) the risk of foreign body infections as demonstrated by animal models can be predicted by the formula:

$$\text{Risk of biomaterial infection} = \frac{\text{Dose of bacterial contamination X Virulence}}{\text{Host resistance}}$$

The equation, in a sense, attempts to simplify the risk of biomaterial infection, the pathogenesis of which is not fully understood. One important variable omitted from this equation is the influence the prosthesis has on resisting, harbouring and or potentiating micro-organism growth. In view of the *in-vitro* results (Chapter 3) and an *in-vivo* model, we set out to determine if any of these previously studied grafts were less likely to become infected when challenged with 10^8 CFU of either MRSA or methicillin resistant *S. epidermidis*.

4.2 MATERIALS AND METHODS

4.2.1 Animal Husbandary

Adult female merino sheep were used with average weights of 35 kg. Animal husbandary and ethics approval were performed as outlined in section 2.2.

4.2.2 Perioperative Care

The perioperative care of all the sheep was identical as that outlined in section 2.5

4.2.3 Operative Procedure

The operative procedure for this experimental work has been described in Sections 2.6.1 and 2.6.3. In addition to the insertion of Gelsoft interposition grafts, 2cm X 5mm grafts of Thoratec, PTFE and Fluoropassiv were individually interposed into the left common carotid artery of the sheep. No graft was soaked in rifampicin prior to insertion into the common carotid artery.

This experiment included no control group. The results for the Gelsoft interposition grafts were taken from previous studies (Sardelic et al 1994; Sardelic et al 1996) in which the efficacy of rifampicin impregnated and control Gelsoft grafts were compared for their effect in reducing either methicillin resistant Staphylococcus aureus or *S. epidermidis* vascular graft infection.

4.2.4 Bacterial Contamination

The preparation of both the MRSA and *S. epidermidis* micro-organisms is outlined in Section 2.8.

4.2.5 Harvest and graft analysis

Graft harvest is as outlined in Section 2.11. Macroscopic assessment of each graft, at three weeks was noted and the relevant bacterial samples as described in Section 2.12 taken. The growth and the identification of cultured micro-organisms has been outlined in Section 2.13.

4.3 STATISTICAL ANALYSIS

Chi-squared analysis was used to examine for any possible association between the number of positive samples at each site when the graft was harvested and the graft type.

4.4 RESULTS

One sheep from the PTFE group inoculated with *S. epidermidis* died one day following the procedure and was excluded from the study. No bacterial analysis was taken from this sheep.

For both the MRSA and *S. epidermidis* inoculated grafts there was no significant differences detected between the graft types for the macroscopic parameters of perigraft abscess, anastomotic disruption and or graft thrombosis (Tables 4.1 and 4.2). Similarly no significant differences were detected between grafts for the bacterial cultures obtained. This is outlined in Table 4.3 (MRSA) and Table 4.4 (*S. epidermidis*).

Table 4.1 Comparison of operative findings between grafts inoculated with MRSA.

	PTFE (n=5)	Gelsoft(n=10)	Fluoropassiv (n=5)	Thoratec (n=4)
Perigraft Abscess	4	8	4	3
Anastomotic Disruption	3	4	3	2
Graft Thrombosis	3	7	4	0

Table 4.2 Comparison of operative findings between grafts inoculated with *S. epidermidis*.

	PTFE (n=4)	Gelsoft(n=10)	Fluoropassiv (n=5)	Thoratec (n=4)
Perigraft Abscess	2	4	3	3
Anastomotic Disruption	0	2	4	2
Graft Thrombosis	2	7	3	0

Table 4.3 Comparison of microbiological findings between grafts inoculated with MRSA.

	PTFE (n=5)	Gelsoft (n=10)	Fluoropassiv (n=5)	Thoratec (n=4)
Perigraft	4	10	5	4
External	5	9	4	4
Internal	5	10	4	4
Graft	5	9	5	4
Total	19/20	38/40	18/20	16/16

Table 4.4 Comparison of microbiological findings between grafts inoculated with *S. epidermidis.*

	PTFE (n=4)	Gelsoft (n=10)	Fluoropassiv (n=5)	Thoratec (n=4)
Perigraft	3	5	3	3
External	3	5	3	3
Internal	2	5	2	4
Graft	4	7	3	4
Crush	3	8	4	4
Total	15/25	30/50	15/25	18/20

4.5 DISCUSSION

The pathogenseis of biomaterial associated infection is complicated and not fully understood. In general the mechanisms involved in the establishment of biomaterial infection involve a combination of the following; bacterial adherence, microcolony formation within a biofilm, activation of host defences and the inflammatory response involving perigraft tissues and the graft artery anastomoses (Bandyk & Bergamini 1995).

This *in-vivo* analysis set out to determine the infectivity of four commercially available prosthetic grafts to an overwhelming infective load of either a virulent micro-organism (MRSA) or non-virulent micro-organism (*S. epidermidis*). White, et al. (1994) demonstrated in a canine aortic model that the infective threshold of bacteria to cause graft infection in over 50% of grafts was 10^7, and 10^9 for *S. aureus* and *S. epidermidis* respectively. Sardelic, et al. (1995) using sheep as a model and interposing prosthetic grafts in the sheep's common carotid artery found that following direct inoculation of the grafts with *S. epidermidis,* and increasing the concentration of inoculum from 10^4 to 10^8 the rate of infection increased. They did however, find that they could not infect all grafts, their reasoning for the absence of universal graft infection was related to the production of a bacterial biofilm layer making bacterial retrieval more difficult. This is in contrast to the work performed by Fletcher et al (1990) that showed universal graft infection with 10^8 CFU of *S. aureus*. These findings by both Sardelic, et al. (1995) and Fletcher, et al. (1990) were evident in this experimental work which showed that at the bacterial concentration of 10^8 CFU near total infectivity occurred with MRSA whilst the total infectivity of the grafts inoculated with *S. epidermidis* neared 75% with the exception of the Thoratec grafts in which 90% were infected. Although exhibiting the greatest percentage of infected specimens for both MRSA and *S. epidermidis*, the Thoratec grafts were associated with zero graft thrombosis which is in contrast to the propensity of the sheep carotid to thrombose in the presence of infection as found by Avramovic and Fletcher (1991). These differences though were not statistically significant and no conclusions can be drawn with regards to the potential for infectivity of the four grafts studied.

The interaction between the microbacteria and the biomaterial is not only dependent on bacterial characteristics such as virulence but also on cell wall characteristics (Bandyk & Esses 1994; Bandyk & Bergamini 1995) and the physical and chemical characteristics of the biomaterial (Bandyk & Esses 1994). A fundamental step in the establishment of graft infection is the adherence of the bacteria to the graft surface with microcolony formation (Schmitt, et al. 1986b).

Gram positive organisms, such as staphylococci are known to produce an extracellular glycocalyx or "mucin" which promotes bacterial adherence to biomaterials in much greater (10 to 1000 times) numbers than gram negative organisms (Bergamini, et al. 1989). *S. epidermidis* is a coagulase negative staphylococci, generally regarded as a low virulence organism associated with vascular graft infections (Bandyk, et al. 1984) but its slime or "mucin" producing subtype adheres to biomaterials in higher numbers than the non- mucin producing *S. epidermidis* (Schmitt, et al. 1986b; Muller, et al. 1991; Malangoni, et al. 1993).

It is recognised that the type of graft material plays a part in bacterial adherence (Bandyk & Esses 1994). This was shown by Schmitt, et al. (1986b) in an *in- vitro* model comparing the bacterial adherence of four strains of bacteria (*S. aureus*, "mucin" and "non-mucin" producing *S. epidermidis* and *E. coli*) to ePTFE, woven Dacron and velour knitted Dacron. They found that bacterial adherence was greatest to velour knitted Dacron and least with ePTFE. They postulated a number of differences between the grafts that could account for the observed results. Expanded PTFE represented a smaller surface area for bacterial attachment, is more hydrophobic and thus less likely to form bonds with the hydrophobic bacterial outer membrane, and is relatively non-porous. A further observed result was that adherence of the mucin producing *S. epidermidis* to ePTFE and the velour knitted Dacron grafts was increased by 10-100 times when compared to *S aureus* and the non- mucin producing *S. epidermidis*. In addition Shmitt, et al. (1986a) found that "mucin" producing *S. epidermidis* adhered to Dacron in 10 to 100 fold greater numbers compared to PTFE. Bandyk and Bergamini (1995) have postulated that the differential adherence of staphylococci relates to capsular adhesins. This is based on the observation that antibody inhibition of these adhesins has resulted in inhibition of biomaterial adherence. Malagnoni, et al. (1993) from their results suggested that the production of slime even without a biofilm layer remained a determinant in bacterial adherence.

The end point of the experiment was the presence of bacterial growth on any retrieved specimen. Bacterial counts were not performed on any of the specimens as it was considered that if any bacterial growth occurred on any of the harvested specimens then the graft was considered infected. Like Sardelic, et al. (1995) who compared

PTFE to Dacron to *S. epidermidis* for susceptibility to infection no statistical differences were apparent. In this experiment no differences were apparent for any of the studied grafts for the type of inoculating organism.

In conclusion, this *in-vivo* experiment demonstrated no statistical significant differences between the infectivity of four commercially available prosthetic grafts in a sheep model. The results nevertheless show that all graft types can be easily infected and once infected will harbour the infective micro-organism. This may lead to perigraft abscess formation, anastomotic disruption and/or graft thrombosis.

Chapter 5

DOES INCREASED RIFAMPICIN CONCENTRATION REDUCE THE INCICDENCE OF METHICILLIN RESISTANT STAPHYLOCOCCAL AUREUS AND EPIDERMIDIS VASCULAR GRAFT INFECTIONS?

5.1 INTRODUCTION

As previously reported in section 1.2.1, despite the infrequency of prosthetic vascular graft infections, vascular graft infections are associated with significant morbidity and mortality. To avoid the potentially disastrous complications associated with graft infections and the recognition that the majority of infections are acquired at the time of surgery it is not surprising that various combinations of grafts and antibiotics have been studied using both *in-vitro*, and *in-vivo* models (See Section 3.1). The fact that so many different combinations have been trialed with varying degrees of success highlights the lack of consensus, as to the appropriate graft with or without antibiotic impregnation. With our knowledge that the predominant organisms implicated in the genesis of vascular graft infections are of the genus staphylococcus (see Section 1.4) and rifampicin is especially active against these organisms (Turnbridge & Grayson 1993) it would seem that a rifampicin bonded prosthesis would be a suitable option in the prevention of graft infection. From our *in-vitro* results the most suited graft for incorporation with rifampicin would be one of the Dacron type prostheses, namely Gelsoft or Fluoropassiv. From from our *in-vitro* studies, we demonstrated Fluoropassiv to be a better infection resistant prosthesis for 1.2 mg/ml rifampicin when compared to Gelsoft and no different at the other concentrations. Since Gelsoft is a product more widely used today in the clinical setting, it would be the best prosthesis to be studied.

Previous work at our institution has demonstrated that Gelsoft vascular grafts soaked at a concentration of 1.2 mg/ml of rifampicin and interposed in the sheep carotid artery reduced subsequent graft infection following challenges with high concentrations of *S. epidermidis* (Sardelic et al 1996) and *S. aureus* (Sardelic et al 1994). In view of the results of Sardelic et al (1994, 1996) and our *in-vitro* results it was our intention to see if increasing the concentration of rifampicin to 10 mg/ml would further reduce the incidence of graft infection for both methicillin resistant *S. aureus* and *S. epidermidis* The 30 mg/ml rifampicin concentration was not trialed in the *in-vivo* setting because of the *in-vitro* occurrence of rifampicin crystallisation.

5.2 MATERIALS AND METHODS

5.2.1 Animal husbandry

We used female adult merino sheep weighing 30-35 kg. All animal husbandry was carried out as detailed in Section 2.2.

5.2.2 Perioperative care

As outlined in Section 2.5.

5.2.3 Operative procedure

The operative procedure and the insertion of prosthetic grafts are described in Section 2.6.1 and more specifically Section 2.6.3.

5.2.4 Bacterial preparation

The preparation of both the MRSA and the *S. epidermidis* micro-organisms are outlined in Section 2.8.

5.2.5 Harvest and graft analysis

Graft harvest is as outlined in Section 2.11. Macroscopic assessment of each graft at three weeks was noted and the relevant bacterial samples as described in Section 2.12 taken. The growth and the identification of cultured micro-organisms have been outlined in Section 2.13.

5.3 STATISTICAL ANALYSIS

Results were compared to previous studies (Sardelic, et al. 1994; Sardelic, et al. 1996) with identical methodology investigating a control group with no rifampicin soaking and a lower concentration of rifampicin 1.2 mg/ml group in the prevention of

subsequent MRSA and *S. epidermidis* infection. The total number of infected grafts and number of infected specimens per culture type were analysed statistically using Chi square analysis and Fisher exact testing respectively between treatment groups for each inoculated organism.

Multiple logistic regression analysis was used to examine the effect of the type of staphylococcal species on the incidence of infection between graft harvest specimens adjusting for rifampicin concentration and to test for any differences in the response between concentrations after adjusting for the type of inoculating micro-organism.

5.4 RESULTS

Twenty sheep were successfully grafted all receiving Gelsoft grafts soaked in 10 mg/ml of rifampicin. Graft surfaces of ten sheep were directly inoculated with either MRSA or *S. epidermidis*. There was no morbidity or mortality arising from the procedures in either of the study groups. All grafts were harvested at three weeks as intended.

The rate of abscess formation, anastomotic disruption or thrombosis between control and treatment arms for *S. epidermidis* is outlined in Table 5.1. There was a reduction with increasing rifampicin concentration for the macroscopic parameters of graft thrombosis, perigraft abscess and anastomotic disruption for *S. epidermidis*. This, however, was not statistically significant.

Table 5.1 Comparison of operative findings between control and treatment arms for *S. epidermidis.*

Operative Finding	Control (no rifampicin) (n=10)	1.2mg/ml rifampicin (n=9)	10 mg/ml rifampicin (n=10)
Abscess	4	0	1
Anastomotic disruption	2	0	0
Graft thrombosis	7	5	4

n= number of sheep

For MRSA, graft thrombosis was significantly reduced in the higher concentration group compared to both lower concentration and control groups (p=0.002 and p=0.002, respectively). Abscess formation and anastomotic disruption were significantly reduced with the 10 mg/ml rifampicin group (p=0.0004 and p=0.04 respectively) when compared to the control group only (Table 5.2).

Table 5.2 Comparison of operative findings between control and treatment arms for *MRSA.*

Operative finding	Control (no rifampicin) (n=10)	1.2 mg/ml rifampicin (n=8)	10 mg/ml rifampicin (n=10)	10 mg/ml v's control *P*	10 mg/ml v's 1.2 mg/ml *P*
Abscess	8	0	0	0.0004*	NS
Anastomotic disruption	4	1	0	0.04*	NS
Graft thrombosis	7	6	0	0.002*	0.002*

n= number of sheep

*Fisher's exact test

NS= not significant

For the *S. epidermidis* arm (Table 5.3), in the 10 mg/ml rifampicin group, only four out of a possible fifty cultures were positive. These positive cultures were obtained from the one sheep. This finding was statistically significant when compared to the control group of 30 / 50 (Chi square 30.1, $p < 0.05$) and the 1.2 mg/ml group of 13 / 45 (Chi Square 8.0, $p < 0.05$).

Table 5.3 Comparison of microbiological findings between control and treatment arms for *S. epidermidis.*

Type of Culture	Control (no rifampicin) n=50	1.2 g/ml rifampicin n=50	10 mg /ml rifampicin n=50	10 mg/ml v's control *P*<	10 mg/ml v's 1.2 mg/ml *P*<
Perigraft swabs	5	1	0	0.01*	NS
External surface	5	2	1	NS	NS
Internal surface	5	2	1	NS	NS
Graft segment	7	4	1	0.01*	NS
Ground graft segment	8	4	1	0.01*	NS
Total positive cultures	30	13	4	0.05†	0.05†

* Fisher exact test

† χ^2 test

NS= not significant

n= number of possible samples

For the MRSA group (Table 5.4), in the 10 mg/ml treatment group, 6 from a possible 40 cultures were positive. The reduction in the total number of possible cultures with the higher concentration group was significant when compared to the control group of 38/40 (Chi square 48.5, $p < 0.05$) and the 1.2 mg/ml group of 19/32 (Chi Square 13.8, $p < 0.05$). Five grafts had no infection on any of the cultures. There was a significant reduction in all the culture types with the higher concentration compared to the control group. When compared to the 1.2 mg/ml rifampicin group there was a statistically significant reduction in graft segments with positive cultures, but not the "indirect" cultures, namely the perigraft tissues, external surface and internal washings.

Table 5.4 Number of positive cultures in the control and treatment arms for MRSA.

Type of Culture	Control (no rifampicin) n=40	1.2 g/ml rifampicin n=40	10 mg /ml rifampicin n=40	10 mg/ml v's control *P*<	10 mg/ml v's 1.2 mg/ml *P*<
Perigraft swabs	10	4	1	0.001*	NS
External surface	9	4	2	0.05*	NS
Internal surface	10	3	1	0.001*	NS
Graft segment	9	8	2	0.05*	0.001*
Total positive cultures	38	19	6	0.05†	0.05†

* Fisher exact test

† χ^2 test

NS= not significant

n= number of possible samples

Table 5.5 is a summary of the multiple logistic regression analysis for each macroscopic and bacteriological parameter following adjustments for the effect of the type of staphylococcal species on the incidence of infection between graft harvest specimens adjusting for rifampicin concentration and the response between concentrations after adjusting for the type of inoculating micro-organism.

This table demonstrates that with increasing rifampicin concentration and adjustments for the type of staphylococcal species there was a statistical significant reduction in both macroscopic and bacteriological parameters with the 10 mg/ml rifampicin group significantly better in all recorded parameters compared to the control group. When comparisons for the staphylococcal species were made with adjustments for concentration statistically significant reductions were only evident for *S. epidermidis* when compared to MRSA for only the bacteriologic and not the macroscopic parameters.

Table 5.5 Summary of Logistic Regression Analysis Results (p values):

	S. epi v's MRSA adjusted for rifampicin concentration	Conc 2 v's Conc 1 adjusted for staph species	Conc 3 v's Conc 1 adjusted for staph species	Conc 3 v's Conc 2 adjusted for staph species
Abscess	NS	0.001	0.003	NS
Anastomotic disruption	NS	NS	0.02	NS
Graft thrombosis	NS	NS	0.003	0.008
Perigraft swabs	0.005	0.01	0.001	0.05
External surface	0.03	0.04	0.001	NS
Internal surface	$p<0.05$	$p<0.007$	$p<0.001$	NS
Graft segment	$p<0.02$	NS	$p<0.001$	$p< 0.001$
Total positive cultures	$p< 0.001$	$p< 0.001$	$p< 0.001$	$p< 0.001$

S. epi *S. epidermidis*
Conc 1 controls
Conc 2 1.2 mg/ml rifampicin
Conc 3 10 mg/ml rifampicin
Staph Staphylococcus

5.5 DISCUSSION

The exact aetiology of vascular graft infections has not completely been established and is most likely that causation is multifactorial (See Section 1.2.1). The majority of graft infections are believed to occur at the time of graft insertion with the source of the bacterium being the surgical team or the patient (Moore & Deaton 1993). In an attempt to offer local protection and overcome the risk of infection at the time of surgery and in the early post-operative period many groups over several decades have developed methods of incorporating antibiotics and or protein carrier molecules into grafts (Section 3.1).

Rifampicin is a hydrophobic semi synthetic substance with a high affinity for gelatin coated grafts (Ashton et al 1990) and is usually active against the methicillin resistant strains of staphylococci (Turnbridge & Grayson 1993). It has demonstrated *in-vivo* bioactivity of up to four days (Gahtan, et al. 1995; Goeau-Brissonniere, et al. 1991; Lachapelle, et al. 1994) although bioactivity up to three weeks has been reported (Strachan, et al. 1991). These qualities make rifampicin the most suitable antibiotic for study in the prevention of vascular graft infections.

Our findings showed that rifampicin at both 1.2 mg/ml and 10 mg/ml resulted in a reduction in the incidence of abscess formation, anastomotic disruption and thrombosis when the inoculating organism was *S. epidermidis* (Table 5.1) although this was not statistically significant. With the MRSA group, both 10 mg/ml and 1.2 mg/ml rifampicin concentrations reduced the rate of abscess formation, anastomotic disruption and graft thrombosis when compared to the control group. Additionally, the rate of graft thrombosis in the 10 mg/ml group was reduced compared to the 1.2 mg/ml group. Though the usual presentation of *S. epidermidis* graft infection is with perigraft abscess and/or graft-cutaneous sinus formation (Bergamini 1990) this was not evident in our study in which low numbers of perigraft abscess were encountered. This may be in keeping with the pathogenicity of *S. epidermidis, which* is regarded as a low virulence pathogen. Alternatively, the bacterial load per square centimetre of graft may have been insufficient to cause an invasive graft infection. The single perigraft abscess that was encountered was associated with graft thrombosis. Sardelic, et al. (1995) in establishing the *S. epidermidis* vascular graft infection sheep model

similarly found an association with perigraft abscess formation and graft thrombosis although this association was not significant. In a previous study utilising an ovine model, Avramovic and Fletcher (1991) suggested that when the inoculating micro-organism was MRSA, vascular graft infections in sheep inevitably lead to graft thrombosis. This observation may explain the reduction in graft thrombosis in MRSA infected grafts with increasing rifampicin concentration and subsequent reduction in infected grafts. Marsan, Curl, Lakshmikumar, Gutierrez and Ricotta (1996) in a small clinical series speculated that thrombosed prosthetic grafts might be more susceptible to infection. Mohammad (1994) noted that there was a close association between infection and thrombosis although was uncertain if thrombosis supported infection or infection predisposed to thrombosis. In previous studies of *S. epidermidis* anastomotic pseudoaneurysm formation or disruption was usually associated with contaminants, namely *E. Coli, S. aurues and P. aeruginiosa* (Martin, et al. 1989; Geary, et al. 1990; Bergamini, et al 1988). These findings are in concordance with our findings that showed that the reduction of MRSA and not *S. epidermidis* graft infection was associated with a reduction in anastomotic disruption. It is interesting to note that when comparisons between *S. epidermidis* and MRSA were made with adjustments made for the rifampicin concentration no differences were apparent in regards to all the macroscopic parameters that were recorded.

The total number of infected cultures was significantly reduced both for *S. epidermidis* and MRSA when compared to both the control and 1.2 mg/ml rifampicin soaked groups. *S. epidermidis* causing graft infection is difficult to diagnose and consequently treat because of its ability to reside within an extracellular glycocalyx, more commonly known as a bacterial biofilm (Bandyk, et al. 1984; Bandyk, et al. 1991; Bergamini, et al. 1988). This difficulty in diagnosing *S. epidermidis* graft infection was recognised by Sardelic et al (1995) when the sheep carotid artery model was being established. In the model there was 80% retrieval of *S. epidermidis* when grafts were inoculated with 10^8 colony forming units of *S. epidermidis*. It is generally recognised that culturing a graft segment after graft disruption is the most sensitive way of detecting *S. epidermidis* (Bergamini et al 1988). Surprisingly, grinding the graft segment did not increase the yield of *S. epidermidis* graft infection in both the rifampicin treated groups and only marginally increased the yield in the control group. Some may consider the yield to be improved by using sonication as the means of

mechanical disruption (Wengrovitz, et al. 1991). In contrast, when the grafts were inoculated with 10^8 colony forming units of *S. aureus* there was universal graft infection (Fletcher 1990). Sardelic, et al. (1995), when investigating Gelsoft grafts soaked in 1.2 mg/ml rifampicin and inoculating them with *S. epidermidis,* found a reduction only in the total number of positive cultures and no statistically significant difference for culture type. It is thus encouraging to note the reduction in ground and non-ground segment positive cultures for *S. epidermidis* with the higher concentration of rifampicin (10 mg/ml) compared to controls. Just as encouraging was the fact that all the positive cultures in the *S. epidermidis* study group were from the same sheep. Similarly, in regards to the MRSA study, it was again of note that there was a reduction when compared to the control and 1.2 mg/ml group was in the graft segments and that only five of the ten sheep had any infected cultures.

One of the potential concerns with the increased rifampicin concentration is the development of rifampicin resistance. Sardelic et al (1995, 1994) using Gelsoft grafts soaked in 1.2 mg/ml of rifampicin and inoculating the grafts with either 10^8 colony forming units of *S. epidermidis* or MRSA found no development of rifampicin resistance. We did not set out to determine if rifampicin resistance occurred with the cultured isolates of staphylococci. Garrison et al (1997) using three different strains of *S. epidermidis* bound to knitted Dacron in low (10^4 / cm^2) or high (10^7 / cm^2) concentrations found that, *in-vitro*, near total killing of all the bacterial strains occurred with the low and not the high concentration group. More importantly, resistance was shown to develop in all three strains of the *S. epidermidis* with the high initial bacterial biofilm concentrations. Their *in-vivo* studies with a particular strain of *S. epidermidis* concluded that the optimal concentration of rifampicin for bacterial killing and the prevention of resistance was a rifampicin concentration of between 4 and 100X minimum inhibitory concentration.

In conclusion, an increased concentration of rifampicin significantly reduces the incidence of prosthetic vascular graft infection following a challenge of MRSA or slime producing *S. epidermidis*.

Chapter 6

DOES IN-SITU REPLACEMENT OF A STAPHYLOCOCCAL INFECTED VASCULAR GRAFT WITH A RIFAMPICIN IMPREGNATED GELATIN SEALED DACRON GRAFT REDUCE THE INCIDENCE OF SUBSEQUENT INFECTION?

6.1 INTRODUCTION

The infrequency of vascular graft infections allows the vascular surgeon limited experience in the management of such a difficult problem. This is evident by the numerous anecdotal reports in the literature of small numbers of patients undergoing various management techniques but no documentation of any truly randomised clinical trial. The optimal management of the infected vascular graft is thus in a sense controversial. Nevertheless, the most important principles in the management of infected arterial prosthetic grafts are the excision of all the infected graft and surrounding tissue, antibiotics and the restoration of adequate arterial flow (Gelabert & Moore 1994). The reputed "gold standard" in the management of the infected vascular prosthetic graft is total excision of the graft and surrounding infected tissue and extra-anatomical bypass (Curl & Ricotta 1994). Other surgical options, which are considered conservative, have included aggressive local wound care with graft preservation (Calligaro, et al. 1992b), replacement of the infected graft with cadaveric arterial allografts (Kieffer, et al. 1993), venous autografts (Clagett, et al. 1993), cryopreserved saphenous vein homografts (Fujitani, et al. 1992), autogenous arteries and or veins (Seeger 1983) and prosthesis (Towne, et al. 1994). In some situations, especially with suprarenal and/or abdominal visceral artery involvement extra-anatomical bypass may be impossible to undertake leaving the vascular surgeon with the only option of a conservative approach (Gelabert & Moore 1994). Despite its appeal, and in some cases technically less difficult, the major drawback with the in situ reconstruction is sepsis (Robinson & Johansen 1991).

In view of the more conservative approaches to the management of prosthetic vascular graft infections and the knowledge that in some series, chronic and late onset graft infections are being caused by the more insidious *S .epidermidis* (Bandyk, et al. 1984; Bandyk 1985; Bandyk, et al. 1991; Calligaro, et al. 1992a; O'Brien & Collin 1992) we set out to determine if the replacement of a staphylococcal infected vascular graft with a graft impregnated with rifampicin, a known anti-staphylococcal agent, would be considered appropriate surgical management in preventing early recurrent infection.

6.2 MATERIALS AND METHODS

6.2.1 Animal husbandry

Adult merino sheep weighing 30-35 kg were used. Animal husbandry and ethics approval was as detailed in Section 2.2.

6.2.2 Perioperative care

This has been previously outlined in Section 2.5.

6.2.3 Operative procedure

The operative procedure and the insertion of prosthetic interposition grafts are described in Section 2.6.1 and more specifically Section 2.6.4.

6.2.4 Bacterial preparation

The preparation of both the MRSA and the *S. epidermidis* micro-organisms was outlined in Section 2.8.

6.2.5 Harvest and graft analysis

Graft harvest was performed as outlined in Section 2.11. Macroscopic assessment of each graft was performed at three weeks and the relevant bacterial samples taken (Section 2.12). The growth and the identification of cultured micro-organisms have been outlined in Section 2.13. If the culture from all examined specimens was negative for any sheep, that sheep was excluded for analysis at six weeks.

Identical procedures were performed on all grafts regardless of whether they were removed at 3 or 6 weeks.

6.3 STATISTICAL ANALYSIS

Within each study group of either MRSA or *S. epidermidis*, Fisher exact testing was used to detect any differences for the macroscopic and microbiological parameters tested with increasing rifampicin concentration. Chi squared analysis was used to detect any significant difference within each staphylococcal infected group for the total number of infected specimens.

Multiple logistic regression analysis was used to examine the effect of the type of staphylococcal species on the incidence of infection between harvested specimens adjusting for rifampicin concentration. In addition, any statistical differences in the response between concentrations, after adjusting for the type of inoculating micro-organism was examined using this statistical method.

6.4 RESULTS

6.4.1 MRSA

Twenty- nine sheep received Gelsoft interposition grafts in the left common carotid artery that had been directly inoculated with 1 ml of 10^8 CFU of MRSA. All the 29 sheep survived the operative procedure, with all grafts successfully harvested at three weeks. At three weeks the previously inserted grafts were replaced with Gelsoft grafts soaked in rifampicin. Sheep were randomised to three groups, twelve sheep receiving grafts soaked in 1.2 mg/ml rifampicin, ten sheep receiving grafts soaked in 10 mg/ml and seven sheep served as controls receiving plain Gelsoft grafts. There were two immediate post-operative deaths as a result of aspiration pneumonia, one each from the rifampicin treated groups, both these sheep being excluded from the final results. All grafts were successfully harvested following a further three weeks in the remaining twenty- seven sheep. At the first harvest all grafts were infected with MRSA with 90/116 cultures positive. At the second harvest, the number of positive cultures were; control group 20/28, 1.2 mg/ml group 31/44 and 10 mg/ml group 23/36. Although there was a notable trend in the rifampicin treated grafts for both anastomotic

disruption and perigraft abscess formation (Figure 6.1) there was no statistical significant difference between the groups for any of the macroscopic parameters recorded. When total positive cultures were analysed and compared between the groups (Figure 6.2), statistically significant differences were not detected. In addition, in regards to the microbiological assessment (Figure 6.3), minor improvements were noted with the perigraft and the graft samples but again this was not statistically significant.

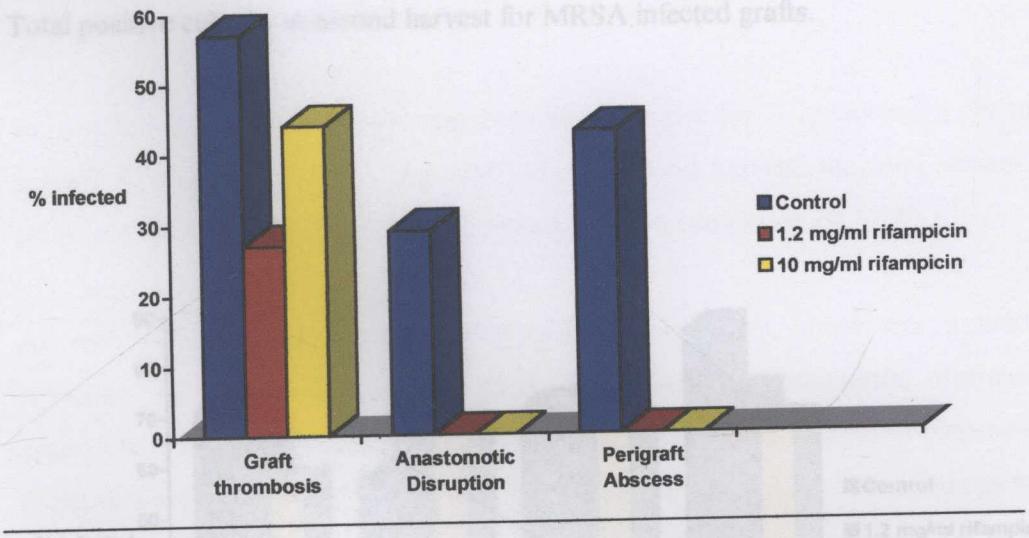

Figure 6.1

Macroscopic appearances of MRSA infected grafts at second harvest.

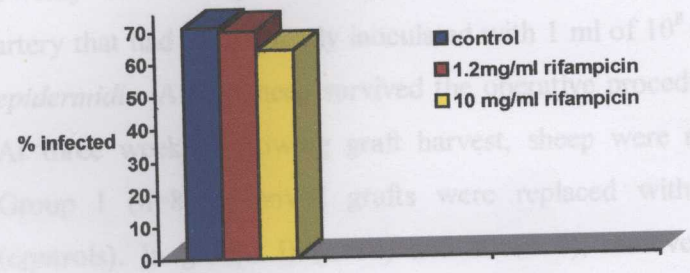

Figure 6.2

Total positive cultures at second harvest for MRSA infected grafts.

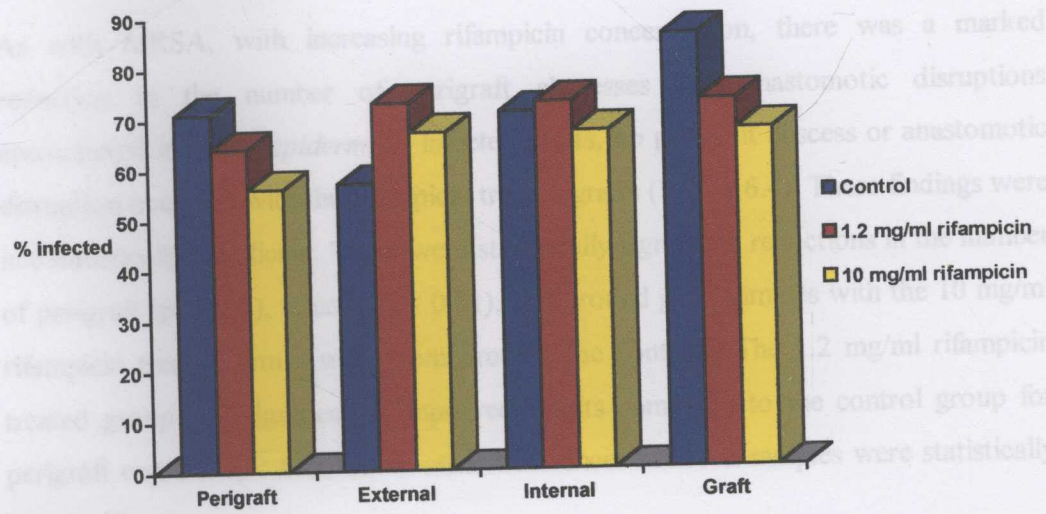

Figure 6.3

Culture positive results for MRSA infected grafts second harvest.

6.4.2 *S. epidermidis*

Twenty- seven sheep received Gelsoft interposition grafts in the left common carotid artery that had been directly inoculated with 1 ml of 10^8 CFU of a slime producing *S. epidermidis*. All 27 sheep survived the operative procedure with all grafts harvested. At three weeks, following graft harvest, sheep were randomised to three groups. Group I (n=8), removed grafts were replaced with non-treated Gelsoft grafts (controls). In groups II (n=10) and III (n=9), removed grafts were replaced with Gelsoft grafts soaked in rifampicin at concentrations of 1.2 mg/ml and 10 mg/ml respectively. One sheep from Group III died two days following the first harvest from aspiration pneumonia and was subsequently excluded from the study. All other grafts were successfully harvested following a further three weeks.

At first harvest, all ground graft segments were positive for *S. epidermidis*, the total number of positive cultures being 105/135. At second harvest, the total number of positive cultures were: Group I 27/40, Group II 30/50 and Group III 11/40.

As with MRSA, with increasing rifampicin concentration, there was a marked reduction in the number of perigraft abscesses and anastomotic disruptions encountered in the *S. epidermidis* infected grafts, no perigraft abscess or anastomotic disruption occurred with the rifampicin treated grafts (Figure 6.4). These findings were not statistically significant. There were statistically significant reductions in the number of perigraft ($p< 0.05$), internal ($p< 0.01$), and ground graft samples with the 10 mg/ml rifampicin treated group when compared to the controls. The 1.2 mg/ml rifampicin treated group had significantly improved results compared to the control group for perigraft cultures ($p< 0.001$). All other differences between samples were statistically not significant.

There was however, statistically significant reduction in the number of total infected specimens (Figure 6.6) in group III when compared to both Group I, ($p<0.001$) and Group II, ($p<0.005$).

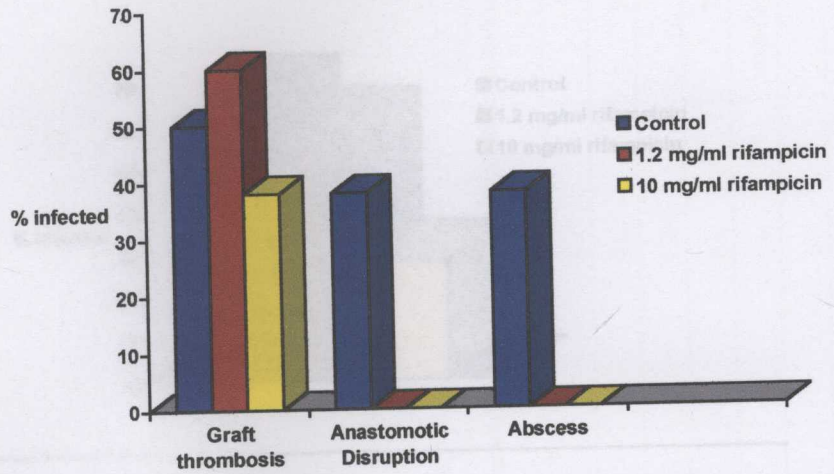

Figure 6.4

Macroscopic appearances of *S. epidermidis* infected graft at second harvest.

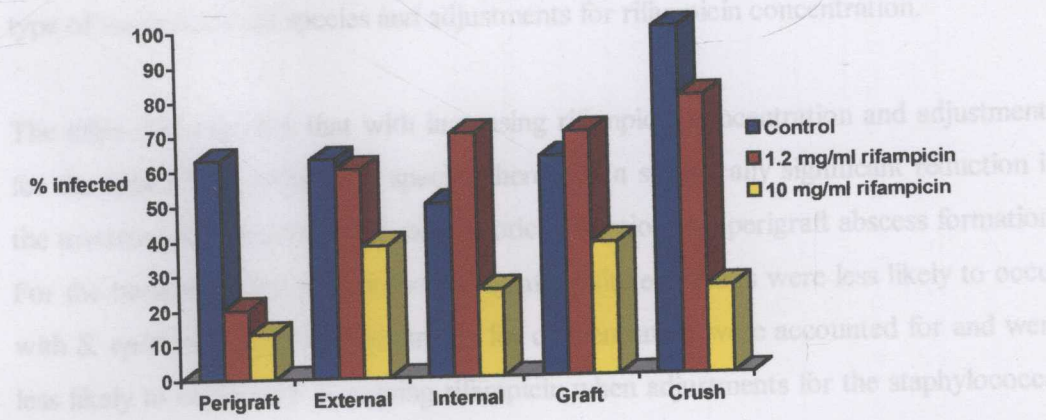

Figure 6.5

Culture positive results of *S. epidermidis* infected grafts at second harvest.

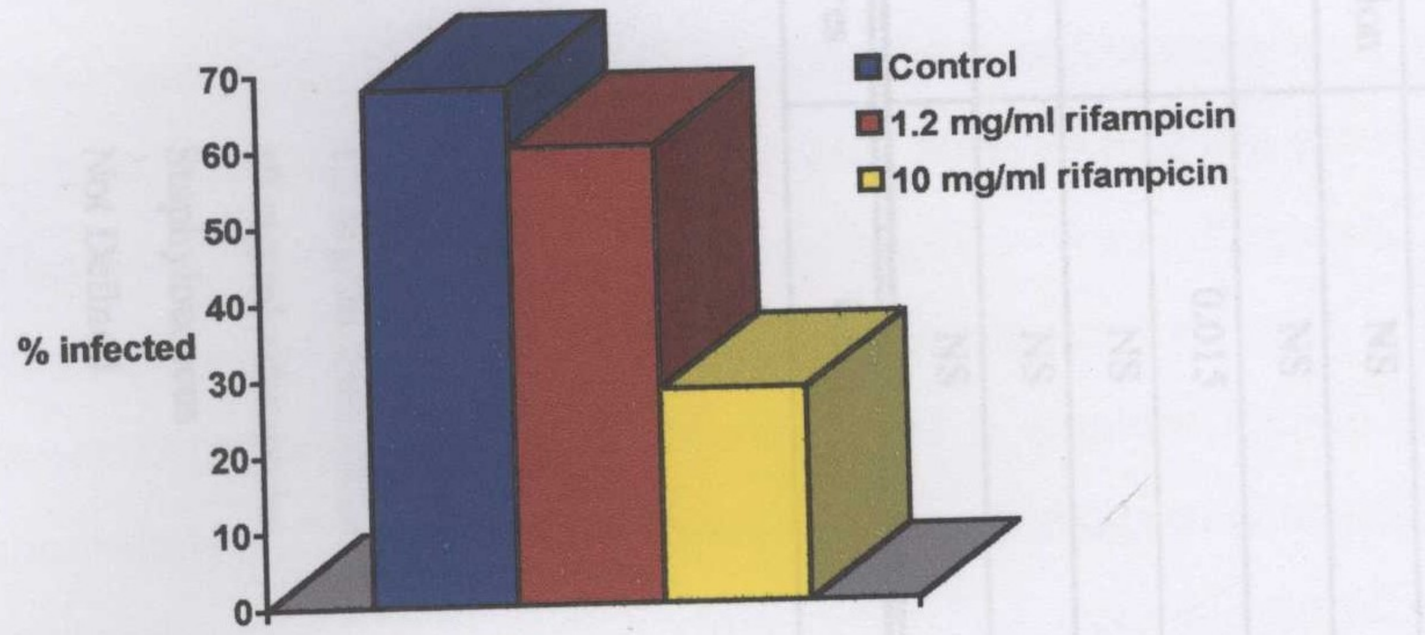

Figure 6.6

Total positive cultures for *S. epidermidis* infected grafts at second harvest.

Table 6.1 is a summary of the multiple logistic regression analysis for each macroscopic and bacteriological parameter following adjustments for the effect of the type of staphylococcal species and adjustments for rifampicin concentration.

The table demonstrates that with increasing rifampicin concentration and adjustments for the type of staphylococcal species there was a statistically significant reduction in the macroscopic parameters of anastomotic disruption and perigraft abscess formation. For the bacteriological parameters, perigraft positive cultures were less likely to occur with *S. epidermidis* when adjustments for concentration were accounted for and were less likely to occur with increasing rifampicin when adjustments for the staphylococcal species were made. As with the Chi squared analysis, when the micro-organism was adjusted for increasing concentration of rifampicin, the total number of specimens infected were statistically significantly reduced.

Table 6.1 Summary

	S. epi v's MRSA adjusted for rifampicin concentration	Conc 2 v's Conc 1 adjusted for staph species	Conc 3 v's Conc 1 adjusted for staph species	Conc 3 v's Conc 2 adjusted for staph species
Abscess	NS	0.002	0.006	ND
Anastomotic disruption	NS	0.007	0.02	ND
Graft thrombosis	NS	NS	NS	NS
Perigraft swabs	0.015	NS	0.052	NS
External surface	NS	NS	NS	NS
Internal surface	NS	NS	NS	NS
Graft segment	NS	NS	NS	NS
Total positive cultures	0.007	NS	0.002	0.008

S. epi	S. epidermidis
Conc 1	controls
Conc 2	1.2 mg/ml rifampicin
Conc 3	10 mg/ml rifampicin
Staph	Staphylococcus
ND	Not Defined

6.5 DISCUSSION

Vascular prosthetic graft infections are a relatively rare occurrence confronting the vascular surgeon. Not only are they relatively rare but the presentation may be subtle making the diagnosis difficult (Murray & Goldstone 1994). The spectrum of presentation is wide and varied, ranging from septicaemia with perigraft purulence to a chronic indolent inflammatory process surrounding the prosthesis (Bandyk 1994). The rarity in presentation, the difficulties with diagnosis and the modes of presentation all combine to make the management of such a problem difficult. In general, treatment is ideally individualised to account for the anatomical position of the prosthesis, the presenting signs, the microbiological characteristics of the infecting organism and/or organisms, involvement of adjacent structures such as bowel, skin or adjoining native artery and the condition of the patient with particular regards to their immunological status (Bandyk 1994).

It appears that the greatest likelihood of a favourable outcome for the patient and the vascular surgeon is when the method of management involves excision of the infected vascular prosthetic graft, extra-anatomical revascularisation and long-term antibiotic therapy (Buckels & Wilson 1994). Despite improvements, the overall morbidity and mortality of this form of treatment stills remains high (Reilly, Stoney, Goldstone, Ehrenfield, 1987; Ricotta, et al. 1991). As a consequence, a great deal of interest has been generated in selective graft preservation or in situ reconstruction (Bandyk & Bergamini 1995).

Calligaro, et al. (1992b) have advocated complete graft preservation with aggressive local wound care providing the graft and anastomoses are intact, and the patient has no systemic features of sepsis. Calligaro, et al. (1992c) in a report of a series of patients who had graft preservation, concluded, that with the exception of *Pseudomonas,* vascular graft infections could be managed with debridement, antibiotic therapy and wound closure. Further to these recommendations, Calligaro, et al. (1994) believed that two conditions mandated graft removal, these being infection involving the intracavitary portion of an aortofemoral graft and systemic sepsis. Morris, et al. (1994) treated ten patients with major aortic graft infection with graft preservation, surgical debridement, prolonged high dose local antibiotic irrigation and

systemic antibiotics with 67% four year survival and no limbs amputated. Their experience was limited to early graft infection where suture line integrity was maintained, but concluded that this method of management had a place in the treatment of early and late panprosthetic graft infections. Kwaan and Connolly (1981) successfully managed prosthetic graft infection in the groin with aggressive debridement and local wound care with povidone-iodine irrigation. Similarly, Matley, Beningfield, Lourens and Immelman (1991) have reported success in the management of an infected thoracoabdominal aortic graft by percutaneous catheter drainage, a situation where extra-anatomical bypass would not be feasible. A number of groups (Nielsen, et al. 1971; Bailey, Bundred, Pearson & Bell 1987; Reilly, Grigg, Cunningham, Thomas, & Mansfield 1989) have reported success with the placement of gentamicin beads around the graft in the treatment of localised graft infection.

In- situ revascularisation is appealing as an alternative to conventional management as it is reported to involve shorter operating times with less surgical stress, with fewer perioperative and postoperative complications (Gelabert & Moore 1994). The major risk of such management is recurrent infection (Robinson and Johansen 1991). Although, superficially appealing, its role is considered by some as limited (Bandyk 1994). The guidelines for the management of graft infection with *in-situ* revascularisation are that the bacterial pathogen is of low virulence, the infection is localised to the site treated and that all infected tissues are aggressively and completely debrided (Bunt 1994). The difficulty with these guidelines is that diagnosis of the low virulent micro-organism such as *S epidermidis* can be difficult unless the graft surface biofilm is adequately disrupted (Bergamini et al 1989). Additionally, the proximal extent of graft infection is at times undetermined with current methods of diagnosis (Becker & Blundell 1976).

In-situ revascularisation may be performed with the use of either autogenous or prosthetic material. Our experimentation was solely concerned with prosthetic in-situ revascularisation in the situation of overwhelming staphylococcal infection. The prosthesis used, Gelsoft, from our results in Chapter 3 and previously reported studies (Powell, et al. 1983; Ashton, et al. 1990; Avramovic & Fletcher 1991; Chervu, et al. 1991b) is shown to offer significant staphylococcal protection when impregnated with rifampicin. Others (Towne, et al. 1994) prefer the use of PTFE grafts as the conduit of

choice for revascularisation as they have shown in their laboratory that bacterial adherence of slime producing *S. epidermidis* was significantly less to PTFE than to knitted or woven Dacron. Bergamini, et al. (1988) in a canine model showed that, despite reduced bacterial adherence when PTFE was used as the graft for revascularisation, this was not reduced when compared to the dogs that received Dacron grafts for *in-situ* revascularisation.

Our findings, demonstrated no statistically significant improvement of either the macroscopic features or the bacteriological parameters with increasing rifampicin concentration with the MRSA infected grafts. These findings are consistent with the above guidelines with regards to MRSA, which is considered a high virulence bacteria (Back & Klein 1994).

However, with regards to *S. epidermidis*, a concentration of 10 mg/ml effectively reduced the total number of infected grafts compared to both the control group and the 1.2 mg/ml rifampicin group. Additionally, the incidence of perigraft infection was reduced compared to the MRSA group when rifampicin concentration was adjusted for. The reduction in perigraft infection for the 10 mg/ml rifampicin group when compared to the control group neared significance ($p=0.052$). At the higher concentration despite a statistically significant reduction in the total positive culture count, 3 from 8 eight grafts had growth *of S. epidermidis.* The concentration of *S. epidermidis* was deliberately high so as to create an overwhelming infection and may not be truly reflective of the clinical situation. *S. epidermidis* typically resides within a biofilm in low numbers of the order of 10^{3-4} CFU/cm^2 of graft surface (Bandyk 1994), a situation where treatment with a 10 mg/ml rifampicin impregnated Gelsoft prosthesis would be expected to further reduce the incidence of subsequent infection following revascularisation. Bergamini, et al. (1988) in a canine model of aortic *S. epidermidis* graft infection undergoing in-situ revascularisation and parenteral antibiotics for two weeks similarly reported 22% of 36 replaced grafts grew *S. epidermidis* when the biofilm was disrupted, signifying that recurrent infection was a real risk.

To date a number of groups (Torsello, et al. 1993; Naylor, et al. 1995) have successfully managed prosthetic graft infections with rifampicin impregnated grafts

with zero mortality, no requirement for limb amputation and to date no recurrence of infection.

Established *S. epidermidis* bacterial biofilm graft infections in a sheep model can be adequately treated by the in-situ replacement of the infected prosthesis with a 10 mg/ml rifampicin impregnated Gelsoft graft and would be recommended in the clinical setting. However, such management for MRSA established infections cannot be recommended from the results obtained in this particular animal model.

Chapter 7

THE ROLE OF RIFAMPICIN IN INTIMAL HYPERPLASIA.

7.1 INTRODUCTION

The characteristics of the ideal prosthetic graft are that it is durable, non-thrombogenic, of low infectivity, has a high patency rate and viscoelastic properties similar to the host artery (Zelt & Abbott 1994). In the early twentieth century, Carrel and Guthrie (1906) noted that the sutures they were using in vascular surgery were being covered by an endothelium like substance within days of the surgery. They are in a sense, credited with the first description of intimal hyperplasia, although, this observation is considered by others as more of a representation of the normal arterial response to injury (Colburn and Moore 1993). The true description of intimal hyperplasia is the abnormal migration and proliferation of vascular smooth muscle following vessel wall injury, which can lead to luminal stenosis and occlusion (Davies & Hagen 1994).

The predominant cellular response following vascular endothelial injury is the transformation of quiescent smooth muscle cells in the media adjacent to the injured endothelium (intima) into proliferating smooth muscle cells which migrate into the adjacent intima with subsequent deposition of extracellular matrix (Davies & Hagen 1994). It is this abnormal vascular response to injury that has been implicated in the development of arterial stenosis, eventual thrombosis and occlusion of small to medium-sized reconstructive arterial surgery (Chervu & Moore 1990). In prosthetic grafts, it has been recognised that this hyperplastic response develops at or near the distal arterial anastomosis (Echave, Koornick, Haimov & Jacobson 1979; LoGerfo, Quist, Nowak, Crawshaw, & Haudenschild 1983). This response in the prosthetic grafts is the result of cellular migration from the adjacent native vessels (Clowes 1994) and is the commonest cause of prosthetic graft failure (Taylor, McFarland, & Cox 1987; Clowes 1994). Despite our increased understanding of the mechanisms involved in intimal hyperplasia, the exact sequence of events between blood constituents and the injured vascular

endothelium that results in a hyperplastic response and not a normal vascular regenerative response still remains unclear.

From our results in Chapter 5 we have shown that by increasing the concentration of rifampicin soaking of a Gelsoft graft from 1.2 mg/ml to 10 mg/ml significantly reduced the incidence of subsequent staphylococcal graft infection. In addition, it was noted that with the higher concentration of rifampicin the incidence of graft occlusion at three weeks was significantly reduced for both MRSA and *S. epidermidis* inoculated grafts. With this background we set out to determine if rifampicin coating of a Gelsoft graft contributed to intimal hyperplasia.

7.2 MATERIALS AND METHODS

7.2.1 Animal husbandry

Adult merino sheep weighing 30-35 kg were used. Animal husbandry and ethics approval was as detailed in Section 2.2.

7.2.2 Perioperative care

As outlined in Section 2.5.

7.2.3 Operative procedure

The operative procedure and the insertion of prosthetic patch grafts are described in Section 2.6.1 and more specifically Section 2.6.2. The concentration of rifampicin used was 1.2 mg/ml and 10 mg/ml.

7.2.4 Harvest and graft analysis

Graft harvest is that outline in Section 2.11. The graft analysis is outlined in Section 2.14 and Section 2.15.

7.2.5 Platelet aggregation study

This was undertaken to assess if rifampicin at the previously studied concentrations of 1.2 mg/ml, 10 mg/ml and 30 mg/ml had any influence on platelet aggregation.

Twenty millilitres of fresh blood was obtained from five sheep and placed in citrated tubes. The blood was centrifuged to 900 rpm (200g) (no brake) at room temperature for 10 minutes to generate a Platelet Rich Plasma (PRP). The PRP was aspirated without disturbing the red cell pellet using plastic sterile pipettes. A platelet count was then obtained. The ideal platelet count for study is approximately 250 X 10^6 platelets per millilitre. A range of 200-300 X 10^6 platelets per millilitre is acceptable. The residual citrated blood was centrifuged (2500 rpm, 2000g) at room temperature to obtain a Platelet Poor Plasma in the event that there was an insufficient platelet count in the PRPs. The aggregometer was then turned on and allowed to heat up to 37^0 Celsius. The aggregometer was calibrated so that the recording chart speed was 2.5 cm/minute. Four hundred and fifty microlitres of PRP was combined with 50 µl of a known concentration of collagen (10 µg/ml) and placed in a cuvette containing a small magnetic stirrer bar moving at an approximate speed of 1,500 rpm. The collagen acted as the positive aggregator control. The procedure was repeated using 50 µl of rifampicin at a known concentration. The sheep platelets were analysed with relation to the effect of rifampicin on platelet aggregation.

7.3 STATISTICAL ANALYSIS

The intimal hyperplasia index was calculated as described in section 2.15. The mean and standard deviation of the intimal hyperplasia index for each graft was

calculated. The Student *t*-test was used to test for differences in the data between the groups.

A p value of less than 0.05 was accepted as being statistically significant.

7.4 RESULTS

7.4.1 Intimal hyperplasia (IH)

A total of twenty sheep were utilised in this study. Five sheep received Gelsoft patches soaked in 1.2 mg/ml rifampicin, five sheep received Gelsoft patches soaked in 10 mg/ml rifampicin and ten sheep served as a historical control having Gelsoft patch grafts inserted without rifampicin soaking.

There were no deaths amongst the sheep in any of the groups under investigation. Macroscopically, on harvesting the grafts, there was no graft disruption, graft thrombosis or perigraft abscess formation. The normal histopatholgy of the sheep artery (Fig 7.1) and the resultant formation of intimal hyperplasia (Fig 7.2 and Fig 7.3) following patch grafting are pictured.

The area of each of the eight transverse sections was calculated and the mean intimal hyperplasia index for each treated and control sheep as indicated in Table 7.1 was recorded.

A total of 10 control sheep had a mean IH index of 0.287 ± 0.077, this was used as a baseline comparison with the treated groups. The mean intimal hyperplasia for the rifampicin treated groups, namely 1.2 mg/ml (n=5) and 10 mg/ml (n=5) were 0.273 ± 0.057 and 0.248 ± 0.092 respectively. These results, when compared to each other using *t*-test analysis assuming equal variance, were not statistically significantly different.

Fig. 7.1
Cross section of a normal Merino sheep carotid arterial wall. The arrow indicates the single layer of endothelial cells (tunica intima). (Magnification × 63).

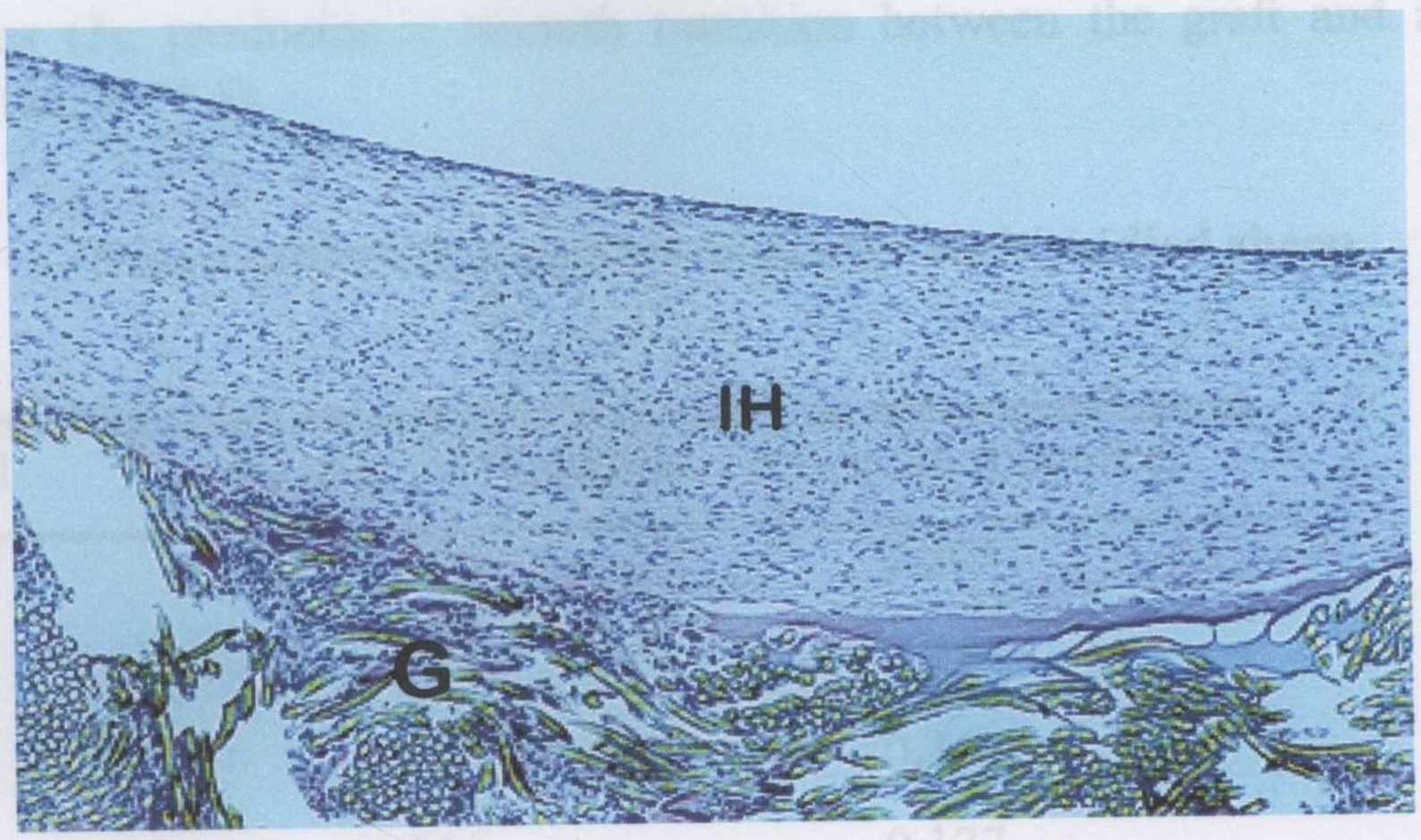

Fig. 7.2
Cross-section of an implanted Gelsoft graft (G), showing the development of intimal hyperplasia (IH) on the inner surface of the graft. (Magnification × 63).

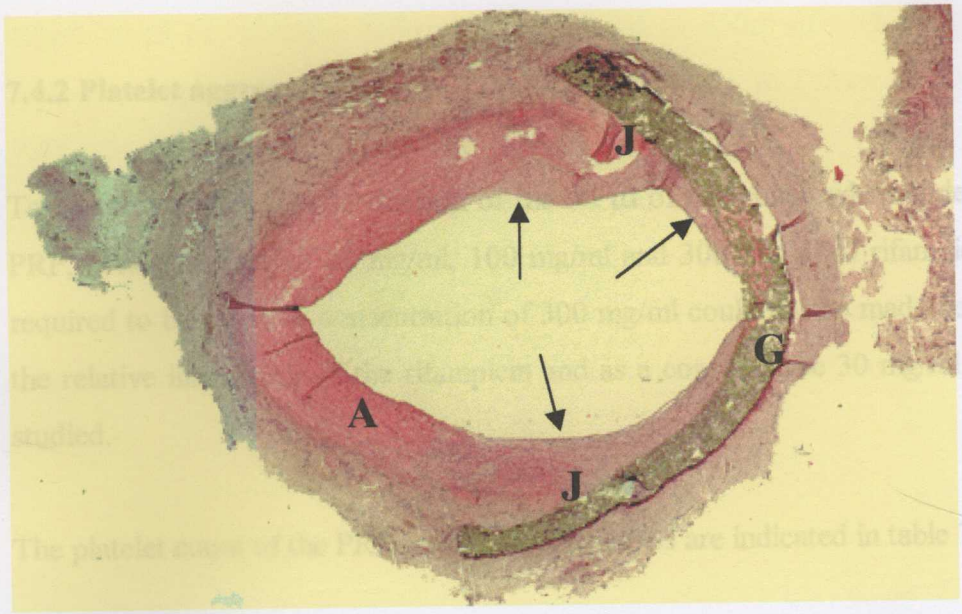

Fig. 7.3
Cross-section of the grafted arterial wall showing distribution of IH (arrows). IH is dominant on the internal surface of the Gelsoft (G) and most marked in the areas near the anastomoses (J) of the graft and artery (A). IH extends across the anastomoses (J), producing a smooth transition between the graft and artery. (Magnification × 12.5).

Table 7.1 Mean intimal hyperplasia index for each studied sheep

Control	1.2 mg/ ml rifampicin	10 mg/ml rifampicin
0.407	0.233	0.205
0.361	0.300	0.25
0.158	0.240	0.360
0.27	0.231	0.127
0.24	0.361	0.313
0.3		
0.268		
0.348		
0.194		
0.326		

7.4.2 Platelet aggregation study

To account for the tenfold dilution of the 50 µl of rifampicin when added to the PRP, concentrations of 12 mg/ml, 100 mg/ml and 300 mg/ml of rifampicin were required to be made. A concentration of 300 mg/ml could not be made because of the relative insolubility of the rifampicin and as a consequence 30 mg/ml was not studied.

The platelet count of the PRP for the sheep samples are indicated in table 7.2.

Table 7.2 PRP concentration for each analysed sheep.

Sheep Number	PRP (X 10^6 /ml)
1	250
2	260
3	270
4	260
5	250

As indicated in Table 7.2, the PRP from each sheep was adequate so that a platelet poor plasma was not required to supplement the PRP.

When 50 µl of collagen (10µg/ml) was added to 450µl of each sheep PRP a typical monophasic response occurred (Tillman, Carson, & Talken 1981), (Figure 7.4).

When 50 µl of 12mg/ml rifampicin was added to the 450 µl of each of the sheep PRP no effect was discernible even after 15 minutes of study. When the rifampicin concentration was increased to 100 mg /ml and added to each sheep PRP the result was identical to that observed with the lower concentration of rifampicin (Figure 7.5-7.7).

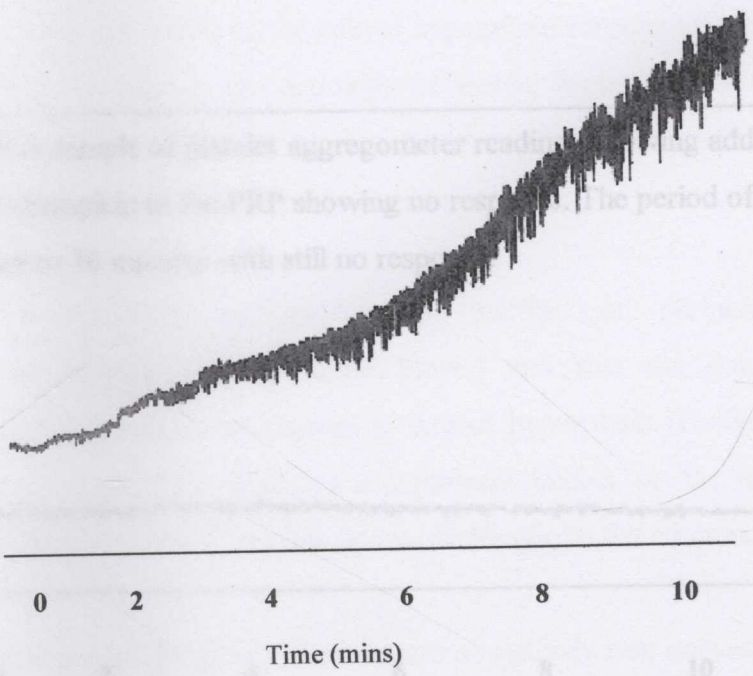

Figure 7.4

Representative sample of platelet aggregometer reading following addition of 50µl of 10µg/ml collagen to PRP showing the typical monophasic response.

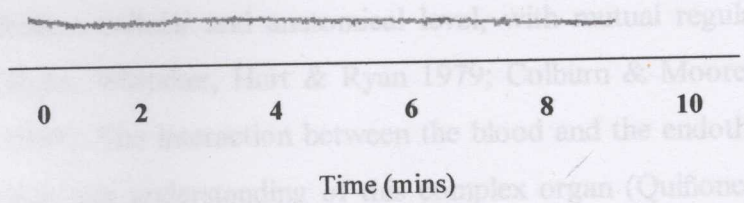

Figure 7.5

Representative sample of platelet aggregometer reading following addition of 50µl of 12mg/ml rifampicin to the PRP showing no response. The period of examination was extended to 30 minutes with still no response.

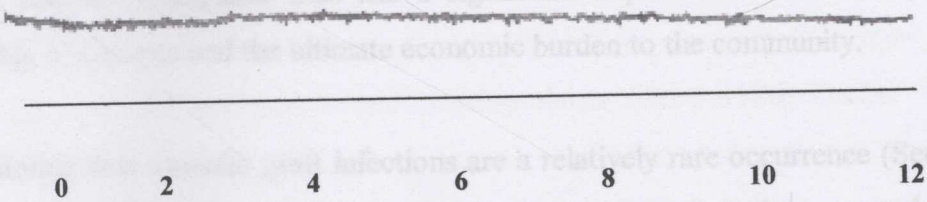

Figure 7.6

Representative sample of platelet aggregometer reading following addition of 50µl of 100mg/ml rifampicin to the PRP showing no response. As for the 12mg/ml rifampicin the period of examination was extended to 30 minutes with still no response.

7.5 DISCUSSION

Conventional understanding of the structure of the artery was that each layer was independent. With our increasing knowledge of the interactions between the individual arterial layers, it is apparent that the artery is a much more complex organ from a cellular and anatomical level, with mutual regulation between its layers (Ryan, Whitaker, Hart & Ryan 1979; Colburn & Moore 1993; Davies & Hagen 1994). The interaction between the blood and the endothelial layer further complicates our understanding of this complex organ (Quiñones-Baldrich 1993). To a large extent, the aetiology of intimal hyperplasia remains unproven, but in a sense the basic premise to the aetiology of intimal hyperplasia as described by Quiñones-Baldrich (1993) "...is the disordered communication and loss of local control systems" as a consequence of vessel injury.

Approximately 500,000 patients undergo cardiac or peripheral vascular reconstructive surgery yearly (United States) and that the common culprit adversely affecting long term success is intimal hyperplasia (Colburn & Moore 1993). Intimal hyperplasia thus has a significant impact on the morbidity and mortality of patients and the ultimate economic burden to the community.

Considering that vascular graft infections are a relatively rare occurrence (Section 1.2.1) it would be imperative that the antibiotic which was to be impregnated onto the prosthesis would not contribute to the formation of intimal hyperplasia.

From our results in Chapter 5, it was noted that, not only did the higher concentration (10 mg/ml) of rifampicin soaking of Gelsoft grafts reduce the incidence of both subsequent MRSA and *S. epidermidis* graft infection when compared to both a control group and a lower (1.2 mg/ml) rifampicin concentration group, but more interestingly, the higher concentration of rifampicin

(10 mg/ml) soaking resulted in a significantly reduced incidence of graft thrombosis for both MRSA and *S. epidermidis* graft infections.

Lundell, et al. (1992) using the carotid arteries of sheep and restricting the blood flow to 25 ml min^{-1} indicated that acute thrombogenicity was increased in rifampicin soaked Dacron grafts compared with control Dacron grafts soaked in saline. Chervu, et al. (1991b) and Lundell, et al. (1992) demonstrated no statistically significant difference in graft patency between Dacron grafts bonded with rifampicin/collagen and Gelseal grafts respectively in a restricted flow setting. Our model, on completion of the anastomosis, had no restrictions to blood flow, but the degree of restriction with evolving intimal hyperplasia during the following 4 weeks was not assessed. A number of studies, (Imparato, et al. 1974; Berguer, Higgins, Reddy 1980; Kohler, Kirkman, Kraiss, Zierler & Clowes 1991) have implicated flow velocity in the development of intimal hyperplasia. Others (Kamiya & Togawa 1980; Morinaga, et al. 1985; Zairns, Zatina, Giddens, Ku, & Glagov 1987; Zwolak, Adams & Clowes 1987) have suggested that the tangential or wall shear stress and not the flow rate may be the most likely haemodynamic predisposing factor to intimal hyperplasia.

Platelets play an important role in the response of the vessel wall to injury and subsequent intimal hyperplasia and or thrombosis (Ross 1986; Harker 1987; Chervu & Moore 1990). An adverse reaction of prolonged systemic usage of rifampicin given systemically is thrombocytopaenia (MIMS, 1996). A number of groups, (Moore, et al 1976; Friedman, et al. 1977) have deliberately induced thrombocytopaenia in sheep by the use of sheep antirabbit, antiplatelet serum, and have shown that the intimal hyperplastic response is reduced following arterial balloon catheter injury or the presence of an indwelling aortic catheter. No previous studies have addressed the effects of rifampicin in inducing thrombocytopaenia or its effects on intimal hyperplasia, as well as the local interactions of rifampicin impregnated grafts and platelets. Lundell, et al. (1992) in

their restricted sheep carotid artery flow model found the platelet activity to be significantly higher at the distal anastomosis in those grafts soaked in rifampicin compared to the control grafts. In addition, they found an increased rate of occlusion secondary to graft thrombosis amongst the rifampicin treated group and concluded that rifampicin soaked grafts had a higher incidence of thrombogenicty.

Though the concentration of rifampicin in the grafts was relatively low and its *in-vivo* activity would be restricted to a few days (Goeau-Brissonniere, et al. 1991), the actual effect on the local platelet activity at the graft site would be difficult to determine.

Although haematological functions in humans and sheep are similar (Tillman, Carson & Talken 1981) it was apparent that a major difference was present in regards to platelet aggregation. Tillman, et al. (1981) noted that platelet aggregation in response to epinephrine, collagen and ristocetin in sheep was less than in humans and characterised by a monophasic response with no secondary phase of aggregation typical of human platelet aggregation. In their experimentation, they used an aggregometer without a magnetic stirrer bar in the cuvette. Nevertheless, our platelet aggregation studies showed a similar monophasic response to collagen but no platelet response was apparent with the addition of rifampicin to the PRP for any of the sheep studied.

Profound differences have been reported in graft patency between species, especially sheep and dogs with sheep having a much higher graft occlusion rate (Örtenwall, Bylock, Kjellström, & Risberg 1988).

In conclusion, in our assumed unrestricted sheep carotid artery model, varying the concentration of rifampicin between nil, 1.2mg/ml and 10mg/ml had no clinically significant impact on the formation of intimal hyperplasia. This finding would

support the use of rifampicin soaked Gelsoft grafts in the prevention and treatment of prosthetic arterial graft infections.

Chapter 8

SUMMARY, GENERAL DISCUSSION AND FUTURE DIRECTIONS

SUMMARY AND GENERAL DISCUSSION

This thesis has attempted to address the problem of vascular graft infections, a problem that although infrequently encountered is associated with significant morbidity and mortality (Hoffert, Gensler and Haimovici 1965; Fry and Lindenauer 1967; Szilagyi, et al. 1972; Rubin, et al. 1985b; Moore & Deaton 1993; Back & Klein 1994; Bandyk & Bergamini 1995). This is compounded by the knowledge that the presentation of vascular graft infections is often subtle making diagnosis difficult (Bandyk & Bergamini 1995).

The management of vascular graft infections is often complex and in a sense is complicated by the lack of consensus amongst all vascular surgeons highlighted by the numerous publications outlining their management. The lack of consensus stems from the fact a randomised trial investigating all the possible treatment arms currently available would not be feasible requiring many thousands of patients to participate, a feat difficult to undertake because of the relative infrequency of the condition.

To gain some insight to the potential of these treatment arms both *in-vitro* and *in-vivo* models have been generated so those conclusions may be extrapolated to the human clinical setting.

Fletcher, et al. (1990) established the *in-vivo* model, namely an ovine carotid artery model. This model has been the basis of continuing research into the management of staphylococcal graft infections. The model has proven to be a technically easy model to work with, and the sheep requiring minimal essential post-operative management.

Rifampicin is a hydrophobic semi synthetic substance derived from *Streptomyces mediterranei,* which inhibits DNA dependent RNA polymerase activity in bacterial cells without effecting mammalian cells (Farr & Mandell 1982). It has a high affinity for gelatin used to coat vascular grafts (Ashton et al 1990) and is usually active against the methicillin

resistant strains of staphylococci (Turnbridge & Grayson 1993). It is these qualities plus its excellent tissue and intracellular penetration (Turnbridge & Grayson 1993) that make rifampicin an ideal antibiotic to be bonded to prosthetic grafts in order to prevent subsequent graft infection.

The basis of the thesis was the management of vascular graft infections with rifampicin impregnated prosthetic grafts.

Initially an *in-vitro* investigation was undertaken to evaluate the anti-staphylococcal (MRSA and *S. epidermidis*) activity of four commercially available prosthetic vascular grafts (PTFE, Gelsoft, Thoratec and Fluoropassiv) following soaking with known concentrations of rifampicin (1.2 mg/ml, 10 mg/ml, and 30 mg/ml). One centimetre squares were aseptically cut from the four commercially described and soaked in the predetermined rifampicin concentrations. These squares where paced onto a bacterial lawn of either MRSA or *S. epidermidis* which acted as discs as in a disc diffusion study (NCCLS 1997).

The Dacron type grafts namely Fluoropassiv and Gelsoft were superior to the other grafts trialed at all studied rifampicin concentrations with antibacterial activity to 16 days at a rifampicin concentration of 30 mg/ml. In addition with increasing rifampicin concentration to 30 mg/ml the initial zones of inhibition were increased as was the length of time of antibacterial activity for all the grafts. Gahtan, et al. (1995) who evaluated the in-vitro antistaphylococcal activity of three types of Dacron prostheses (plain low porosity, gelatin, and collagen impregnated), has documented similar findings. Their conclusions were that the most important determinant of the *in-vitro* antistaphylococcal activity was the rifampicin concentration and that the gelatin impregnated Dacron (Gelseal) demonstrated superior *in-vitro* bioactivity compared to the other Dacron grafts in the study.

All graft types investigated at the *in-vitro* level were assessed in regards to their infectivity in the established ovine model. These grafts were interposed in the carotid arteries of sheep and inoculated with an overwhelming infection (10^8 CFU) of either MRSA or *S. epidermidis*.

Although number of animals within the study groups were not equal because of constraints on the use of sheep, on discussion with our statistician, Dr Karen Byth, that because of the high rate of infectivity for the total samples for the individual grafts studied that the numbers used in the study were appropriate. In an ideal situation, experiments could have been repeated with increased sample numbers to add power to the statistics.

For both the MRSA and *S. epidermidis* inoculated grafts there was no significant difference between the grafts for the type of bacterial harvest. Similarly, no significant differences were detected between graft types for the macroscopic parameters of perigraft abscess, anastomotic disruption and or graft thrombosis. Despite Thoratec being associated with the greatest percentage of infected specimens for both MRSA *and S. epidermidis*, the Thoratec grafts were associated with zero graft thrombosis an unexplained finding in light of the propensity of the sheep carotid to thrombose in the presence of infection as postulated by Avramovic and Fletcher (1991).

Our findings were in contradistinction to Schmitt et al (1986b) who in an *in- vitro* model compared the bacterial adherence of four strains of bacteria (*S. aureus*, "mucin" and "non-mucin" producing *S. epidermidis* and *E. coli*) to ePTFE, woven Dacron and velour knitted Dacron. They found that bacterial adherence was greatest to velour knitted Dacron and least with ePTFE. Our findings would suggest that an overwhelming staphylococcal infection of the studied prosthetic grafts would result in universal graft infection with no graft type being less likely to be susceptible to infection.

Previous work investigating 1.2 mg/ml rifampicin soaked Gelsoft grafts interposed in the sheep carotid artery reduced in the prevention of subsequent infection following

challenges with high concentrations of *S. epidermidis* (Sardelic, et al. 1995) and *S.aureus* (Sardelic, et al. 1994) demonstrated a significant reduction in the total number of infected cultures. In view of these results and our *in-vitro* results it was our intention to see if increasing the concentration of rifampicin to 10 mg/ml would further reduce the incidence of graft infection for both methicillin resistant *Staphylococcus aureus* and *S. epidermidis*. Despite our *in-vitro* study demonstrating improved efficacy with 30 mg/ml, the crystallisation of the grafts at this concentration was a concern and as such, 10 mg/ml was chosen as the preferred maximal concentration.

For *S. epidermidis,* the rate of abscess formation, anastomotic disruption or thrombosis between control and treatment arms was reduced but this was not statistically significant. For MRSA, graft thrombosis was significantly reduced in the higher concentration group compared to both lower concentration and control groups ($p=0.002$ and $p=0.002$, respectively). Abscess formation and anastomotic disruption were significantly reduced with the 10 mg/ml group ($p=0.0004$ and $p=0.04$ respectively) when compared to the control group only.

The 10 mg/ml rifampicin group was associated with significantly fewer total cultures for both MRSA and *S. epidermidis* when compared with the control and the 1.2 mg/ml rifampicin groups.

When the 10mg/ml and the control groups were compared using logistic regression analysis it was evident that with increasing rifampicin concentration and adjustments for the type of staphylococcal species there was reduction in both macroscopic and bacteriological parameters in all recorded parameters. When comparisons for the staphylococcal species were made with adjustments for concentration statistical reductions were only evident for *S. epidermidis* when compared to MRSA for only the bacteriological and not the macroscopic parameters.

These findings would support the recommendation that Gelseal grafts used for arterial reconstructive surgery be impregnated with 10 mg/ml rifampicin for the prevention of subsequent graft infection.

The surgical management challenge to the vascular surgeon is not only the prevention of graft infection but also the management of established infection. Knowing that 10 mg/ml rifampicin impregnated Gelseal grafts were superior to 1.2 mg/ml and non-impregnated grafts for both MRSA and *S. epidermidis* it was the intention of the reinfection studies to establish the role of replacement of staphylococcal infected vascular grafts with 1.2 mg/ml or 10 mg/ml rifampicin impregnated Gelsoft grafts.

For MRSA, at the second harvest, there was no significant difference between the groups for any of the macroscopic or bacteriological parameters recorded.

With *S. epidermidis*, there were significant reductions in the number of perigraft, internal, and ground graft samples with the 10 mg/ml rifampicin treated group when compared to the controls. The 1.2 mg/ml rifampicin treated group was significantly better compared to the control group for perigraft cultures. All other comparisons between samples were insignificant. There was however, a statistically significant reduction in the number of total infected specimens in the 10 mg/ml group when compared to both the 1.2 mg/ml group and the control group.

From the results it is recommended that in-situ replacement of infected staphylococcal grafts be reserved for *S. epidermidis* and not MRSA proven graft infection. This is supported by Bunt (1994) who recommends that in-situ revascularisation be reserved in the setting of the bacterial pathogen being of low virulence and the infection localised to the site being treated.

Intimal hyperplasia is a pathological condition which is described by Quiñones-Baldrich (1993) "...is the disordered communication and loss of local control systems" as a consequence of vessel injury.

One of the interesting findings noted with increasing rifampicin concentration in the prevention of graft infection for both MRSA and *S. epidermidis* was the significant reduction in graft occlusion. It was on this background that the final *in-vivo* analysis performed to determine if rifampicin had any role in the genesis of intimal hyperplasia.

No significant differences were apparent in the average amount of intimal hyperplasia when comparison were made between no rifampicin, 1.2 mg/ml rifampicin and 10 mg/ml rifampicin impregnated grafts. In contradistinction, Lundell et al (1992) using the carotid arteries of sheep indicated that acute thrombogenicity and graft occlusion were increased in rifampicin soaked Dacron grafts compared with control Dacron grafts soaked in saline. Our findings would indicate that rifampicin alone is not contributory to the formation of intimal hyperplasia.

The limitations of the thesis relate to the use of historical controls. The inclusion of historical control data is of lesser value scientifically. The reasoning for the inclusion of such data is outlined in the following. Firstly, it would be difficult on ethical grounds to repeat a control study on an established animal model especially when a number of the experimenters are identical. Secondly, the costs to perform repeated identical procedures are prohibitive with limited research funding. Thirdly, the methodology for the historical control study was identical to the study performed in the thesis. Finally, the historical control groups have been published in peer-reviewed journals (Sardelic, Ao, & Fletcher 1994; Sardelic, Ao & Fletcher 1995; Sardelic, Ao, Taylor, & Fletcher 1996; Ao, Hawthorne, Vicaretti & Fletcher 2000).

The relevance of animal studies in the assessment of vascular graft infections is that these infections are associated with significant morbidity and/or mortality in the human

population. Profound differences have been reported in graft patency between species, especially sheep and dogs with sheep having a much higher graft occlusion rate (Örtenwall, Bylock, Kjellström, & Risberg 1988). This highlights the difficulty in finding an animal species that behaves in a similar manner to humans when confronted with vascular graft infections. At present our understanding of the disease process is insufficient to confine our experimentation to humans but as our understanding increases from knowledge obtained from both *in-vitro* and *in-vivo* experimentation our reliance on other animals species will dramatically reduced.

FUTURE DIRECTIONS

A number of questions relating to vascular graft infections remain unanswered. With a better understanding of the interplay between microorganism, immune system and prosthesis, more directed therapy to the prevention and management of graft infections will be possible. This may include more powerful antibiotics either administered parenterally or with increasing technology into textiles, incorporated into the prosthesis, acting as a local delivery system for prolonged periods of time. The role of the biofilm in the pathogenesis of graft infection needs further understanding from both a molecular and an immune level.

Rifampicin to a concentration of 10 mg/ml impregnated Gelsoft grafts have been shown to be protective against overwhelming methicillin resistant staphylococcal infection and effective management for the in-situ replacement of *S. epidermidis* graft infection at three weeks. Research needs to be continued with regards to the maximum rifampicin concentration attainable before rifampicin crystallisation and rifampicin resistance occurs. Experimentation in addition, needs to be extended beyond three weeks, especially for the insidious type organisms to determine the role of antibiotic impregnated prosthesis. The issue of mixed graft infection needs to be addressed as a single antibiotic impregnated graft may be insufficient management for local and or systemic microorganism control.

The grafts of the future may not only have delivery systems directed towards preventing infection in the short and long terms but may be non- thrombogenic available in varying sizes, lengths, and shapes, be protective against intimal hyperplasia formation, with viscoelastic properties similar to the host artery. Considering that reconstructive vascular surgery using prosthesis is relatively new with Voorhess, Jaretski, and Blakemore (1952) introduction of the Vinyon "N" cloth tubes, with countless numerous advances to date, one can only speculate that the ideal vascular conduit is within the sights of today's researchers.

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