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ALLOTRANSPLANTATION OF

FREE VASCULARISED SKIN FLAPS

Submitted for the Degree of Master of Medicine Plastic and Reconstructive Surgery with the University of Cape Town by

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INTRODUCTION

The Plastic Surgeon is frequently confronted with the problem of large soft tissue defects which cannot be covered by skin grafts or local skin flaps. The examples are limb injuries, defects resulting after release of burn scar contractures and in tumour surgery. Another problem is the situation where skin grafts can cover the defects but where the resulting deformity severely affects the quality of life, for instance in children who sustained severe facial burns. The possibility of a composite reconstruction of such a defect by a one stage readily available reproducible method is quite appealing. Even more appealing would be some means of obtaining an unlimited supply of donor tissues which do not require a surgical assault upon the patient or require additional operative time during the reconstructive procedure. A composite allograft of skin and subcutaneous tissue transferred with microvascular anastomosis for vascularisation would meet these criteria if it were not rejected later. The problems to date have been primarily technical ones associated with the microvascular vessel anastomosis and the persistent immunological problems of allograft rejection.

FREE FLAP ALLOTRANSPLANTATION

Skin has been the organ most used in studying transplantation immunobiology but in nearly all cases it has been used as a free graft. A review of the literature revealed only two studies dealing with the immunologic properties of skin when transferred as an immediately vascularised composite allograft (Salyer 1973, Egerszegi 1984). Salyer in 1973 showed that the graft versus host reaction could be diminished by pretreatment of the recipient with anti lymphocyte serum

and of the donor with radiation or anti lymphocyte serum. He concluded that passenger leucocytes from the donor play a role in the early sensitisation of the host but that the near elimination could only delay but not prevent the subsequent graft versus host reaction. Egerszegi and Daniel 1984/1986 used Cyclosporin A to reduce the immunogenicity of free neuro-vascular flaps and complete hand transplants in baboons. Some of these transplants have been successful and at the time of publishing, been followed up at 211 days for the flaps and 304 days for the hand. Complete skin survival was noted as well as reinnervation. However, the side effect of Cyclosporin A such as nephrotoxicity, hepatoxicity and malignant lymphomata have to be accepted at present for such lifesaving operations like heart or liver transplants but are unacceptable in the case of limb or skin transplantation.

Heck et al 1985, successfully transplanted composite skin grafts consisting of previously frozen split thickness allograft which were overgrafted after removal of the epidermal layer with auto-epidermal cells. No signs of rejection were seen at follow up, up to 12 months post-operatively. No immunosuppressive agents have been used.

AIMS OF STUDY

Based on this idea an experimental study was designed to :

- discover whether frozen vascularized skin flaps could be transplanted without using immunosuppressive drugs.
- establish a technique of vascularized free flap replantation and transplantation
- determine the effect of freezing upon skin. Subcutaneous tissue and vessels using cryoprotective agents.
- determine to which extent freezing could reduce the antigenicity of skin in combination with autograft of the epidermis.

The overall question was whether skin flaps could be transplanted without using immunosuppressive drugs.

HISTORY OF ORGAN TRANSPLANTATION

The birth of organ transplantation took place in Vienna in 1902. Emerich Ullmann reported the first transplantation of dogs' kidneys to the neck with the aid of Payr's Cannulas for vessel anastomosis of which one allotransplant functioned for five days. In 1912 Alexis Carrell of Chicago, transplanted not only kidneys but the spleen, the small intestine and the thyroid and also made attempts at heart transplantation. In 1912, he received the Nobel Prize in recognition of his work on vascular suture and the transplantation of blood vessels and organs. In the same year Guthrie, the man who had some years earlier in Chicago helped Carrell to perfect the vascular suture technique, published his book on blood vessel surgery and its application. This world-wide recognition that the technical problems could be solved should logically have been a prelude to a more general use of organ transplantation but, was in reality an end. The experiments had shown that autogenic organs survived after vessel anastomosis and were able to function despite denervation and lymphatic interruption but they had also shown that an unknown biological factor frustrated the technical successes in all cases of allo-transplantation. In this same year the German surgeon Georg Schone published conclusive evidence that this unknown process was an immune reaction. In his paper published in the Munchner Medizinische Wochenschrift he stated that there is a transplantation immunity and which is different from that which regulates the growth of tumour tissue but which determines also the fate of normal tissue. Most scientists disregarded this work and it was only thirty years later that Medawar finally proved that Schone was correct.

In further studies over the next 15 years a few surgeons (notably, Sauerbruch's assistant Schmidt) tried to solve the problems of transplantation by surgical means only. However Schmidt had to admit that after his experiments with parabiosis there was very little hope of achieving the success with skin transplants using this means. Bittner in 1932 showed that susceptibility to tumour transplants was transmitted and that this transmission took place strictly according to Mendelian laws. Once the genetic basis was clarified there was one major question remaining - by what mechanism does the body cause the rejection of tissue of a divergent genetic constitution? Among the many different hypotheses advanced and tested at this time, two predominant concepts emerged, namely, a primary local toxicity of the transplant and a general immunisation against foreign tissue. The second view was supported by Gorer's observation that in certain strains of mice after tumour transplantation an agglutinin against the donor cells circulated in the recipient. But here also in the thinking of research workers immunity meant serological immunity and the concept of antibodies was inseparably linked with the concept of humoral, serologically defined antibodies. Serological tests however, continued to be mostly negative in transplantation and therefore the problem remained undecided until Medawar began his work. On the basis of precise observations of a skin graft recipient he concluded with Gibson in 1942 that time factors, the absence of a local cellular reaction under certain conditions and the more rapid rejection of secondary transplants could be explained only by active immunisation of the recipient. One of the important results of these ideas was that the presence or absence of humoral antibodies was no longer considered to be the decisive criterion of an immune-mechanism

but rather the more rapid rejection of secondary transplants. Finally, thirty years after Schone, the immunological origin of allotransplant rejection was clearly and definitely proved. Further experimental work continued but was interrupted by World War II. However, in 1952, one of the pioneers of clinical kidney transplantation David Hume published his first experiences. The development of the artificial kidney which had made temporary treatment of renal insufficiency possible made the replacement of irreversibly diseased kidneys an urgent problem. Although this period therefore saw the first major efforts at kidney allotransplantation in man, it remained impossible to combat the immune reaction and failure was therefore the logical consequence. In the same year however, Baker found that cortico steroids could prolong the life of allotransplants but it took many years before the cortico steroids finally were introduced into clinical practice.

The publication by Neptune and collaborators(1952) on lung transplantation shows that the second technical phase of organ transplantation had started. For nearly forty years from 1912 to 1950 technical work on whole organ transplants had been abandoned and now it was taken up again. It was advances in anaesthesia, treatment of shock, antibiotics, the use of heparin and later extra corporal circulation that stimulated and made possible a new phase of surgical activity. Kidney, lung and heart transplants were performed experimentally with increased enthusiasm in the following years. In 1962, renal transplantation in man finally became a reality. Both Kuss for the group from Paris and Murray from Boston reported on their first

long term surviving allotransplant recipients. To achieve these results, total body irradiation was used. This was a very dangerous procedure which was abandoned later.

In the early 1960's three events occured which changed the course of clinical transplantation. The first was the development of tissue typing and these early methods gave the confidence that close matching could eventually be obtained. The second was the development of methods for regular dialysis treatment in therapy which could be integrated with transplantation and the third and most important was the appearance of new potent immunosuppressive drugs.

Karl Landsteiner in his Nobel Prize lecture of 1931 had suggested a search for tissue groups analogous to the blood groups which he had been first to describe. P.A. Gorer obtained the first evidence for such a system in mice and showed that the system was genetically determined as did George Snell in America who first used the term "histocompatibility". The hunt for human white cell agglutinins and hence the sera which could be used for tissue typing was rewarded when Jean Dausset in 1952 identified such antibodies in the serum of multiply transfused patients, work for which he shared a Nobel Prize in 1980. In 1958 J J van Rood and Rose Payne independently detected such antibodies in multiparous patients and thereafter increasing numbers of antigens were identified by such sera using Paul Terasaki's method of testing. Starting in Paris in 1962, these methods were increasingly used to match donors and recipients. These methods could also be used to test for

preformed antibody in the recipient and thus hyper-acute rejection could be avoided.

The second innovation in the early 60s was the invention of Scribner and Quinton of the arterio venous shunt. Their finding that patients with chronic renal failure could be kept in surprisingly good health by regular dialysis profoundly altered the status of transplantation treatment.

Last and most important was the finding by Schwartz and Dameshek in 1959 that one of the newly derived anti-mitotic agents, 6-Mercaptopurine, had potent effects on immune responses while being less toxic than the drugs from which it was derived. Roy Calne in a series of 104 canine renal allotransplants came to the conclusion that azathioprine was at least as effective but less toxic than 6-Mercaptopurine. In 1963, Starzl added steroids to azathioprine therapy a combination which remains the standard regimen to this day.

HISTORY OF SKIN TRANSPLANTATION

The technique of replacing skin as a free graft has been known for centuries. For example, Sushruta who practised in Northern India almost 2500 years ago is credited with using skin grafting in the repair of noses lost in battle or mutilated for punishment of crimes. However, the emphasis that surgeons continued to place on the use of skin flaps for repair of such defects emphasised that the healing of free grafts was probably unsatisfactory. This was presumably due to rejection of any allografts which might have been utilised or perhaps the use of autografts which were too thick to permit satisfactory revascularisation. The recruitment of slaves or other persons not in a position to object as donors for skin allografts was frequently reported (Rogers 1959). The long-term success of these allografts is unclear but what must have been the rejection phenomenon certainly received various interesting explanations. Reports of the sympathetic nose for example, which seemed to die when the person from whom it had been taken died, indicated that the expected period of survival of the unfortunate donor was about equal to the allograft rejection time. By the beginning of the 19th century, techniques were developed before free grafting that finally assured a consistent rate of success. Reverdin in 1871 is attributed with the initial discovery that split thickness skin provided a perfectly satisfactory graft without injuring the donor site. Shortly thereafter Thiersch further advanced the knowledge of skin grafting with the first histological study of graft healing. As early as 1880 sheep skin and soon thereafter, porcine skin had been utilised for coverage of burn wounds. However, anti-vivisection law significantly discouraged further progress into xenografting until a

reintroduction of porcine skin as a temporary dressing by Bromberg in 1965. The major disadvantage of xenograft skin is its limited survival. The grafts, whether fresh, irradiated, or lyophilized usually adhered to the underlying tissues without evidence of revascularisation. Thus the porcine xenograft acts really as a temporary biologic dressing whose survival is ended by a process of desiccation rather than one of rejection. The first allografts of burn wound coverage were performed by Pollock in 1870. He applied his own skin and that of a black man next to autografts on a patient's burns. Subsequently he observed ulceration and loss of the black man's skin and his own but continued survival of the autografts. He thus unknowingly, provided a description of the importance of histocompatibility for graft survival. A true appreciation of the survival to be anticipated following placement of allografts or xenografts did not occur until the early 20th century being stimulated by the observations of Schone. His work correctly demonstrated that prolonged survival was not to be expected although the fundamental dependence of graft survival upon the immunogenetic relationship between donor and recipient remained unknown. It was not until the early 1940s that the most important monumental work of Gibson and Medawar describing skin transplants in man and rabbits finally placed the knowledge of the laws of transplantation on a firm scientific basis. This work finally provided a solid foundation for the subsequent rapid development of cellular immunology and immunogenetics. The first observations which were made held till today viz. that when skin allografts are exchanged between healthy volunteers including closely matched siblings mean graft survival time is usually 12-16 days.

It has become evident however, that patients suffering severe thermal injuries especially when their course is complicated by prolonged convalescence and malnutrition have major alterations in the immune competence. This is manifested as a non-specific immunological deficit which partially accounts for the recurrent and often fatal infections they suffer. This altered immune response may be exploited to allow prolonged skin allograft survival as has been demonstrated both clinically and experimentally (Alexander and Montgrief 1966). Randomly selected allografts generally reject during the second or third post-transplant week. But it was observed that in the non-septic well nourished child even grafts obtained from ABO compatible parents survived no more than three weeks (Whelchel et al). Allografts as a means of wound coverage, therefore, have also been of limited usefulness in treating burns except where used as repeatedly replaced biologic dressings. This has been an effective approach for patients suffering moderate-sized burns which then can be fairly rapidly closed with autograft skin from uninjured donor sites. In the more massively injured patient however, this approach with its hazards of repeated anaesthetic exposures, blood and fluid losses and recurrent infections has been almost universally unsuccessful. Immunosuppressive agents have been used to prolong skin allograft survival until the wounds which were primarily and continuously closed with allografts could gradually resurface with sequentially harvested autografts. Today temporary allografts are the basis of salvaging massively burned patients. The small amount of useable autograft is intermingled with allografts.

ANTIBODY STRUCTURE AND FUNCTION

The injection or introduction of a foreign tissue into an animal or a human such as by transplantation can elicit the ability to react against that specific foreign substance. Immunologists term the foreign invader an immunogen or antigen with the protein formed against it being known as an immunoglobulin or antibody. Antibodies have various biologic properties including the following.

(1) The ability to bind specifically with antigen.

(2) The ability to fix (bind) and thus activate serum complements - these are a series of proteases that, once activated, can mediate lysis of cells and

(3) The ability to enhance phagocytosis by a process known as opsonization. In opsonization, immunoglobulin first binds with antigen and then subsequently attaches to a phagocytic cell. (Hutchinson et al 1977) The foreign particle remains bound to one end of the immunoglobulin molecule so that the phagocyte can then ingest the foreign material and destroy it.

Five different classes of immunoglobulin molecule exist. These are IGG IGM IGA IGD and IGE.

Cellular interactions in the immune response.

Lymphocytes

Lymphocytes become activated upon binding a specific antigen to a receptor on the cell surface. If the lymphocyte is a B-cell (bone marrow derived lymphocyte) then differentiation will occur following antigen binding. The receptor on the B-cell consists of an immunoglobulin that is specific to a particular antigen. The B-cell then ultimately becomes a plasma cell which produces and secretes immunoglobulin molecules (Janeway and Jason 1980). T-cells (thymus-derived lymphocytes) participate in the immune response by modulatory and effector functions. The receptor for specific antigen on T-cells appears different from the immunoglobulin receptor presente on the surface of B-cells. Yet, it seems that the antigen binding sites of both types of receptors function similarly (Achauer 1985).

T-cells include the following:

- those with modulatory functions
- Helper T-cells which amplify the immune response
- Suppressor T-cells which inhibit the immune response

These regulate both effector T-cells and B-cells (Steinmuller 1978).

- Killer T-cells (cytotoxic T-cells) mediate target cell destruction.

Macrophages

Activation of helper T-cells (amplifier T-cells) requires the synthesis of a soluble factor termed Interleukin-1 by macrophages. This occurs either simultaneously or following the presentation of antigen to the T-cell by the macrophage. Thus macrophages also participate in the

Interleukin 2 is then produced by T- amplifier cells concomitantly with the synthesis for receptors of Interleukin-2 on the surface of these cells. Full activation of T-amplifier cells can then proceed as a result of production of Interleukin 2 (Bunjes et al 1981). Activated lymphocytes can produce other soluble mediators which can activate macrophages to become more phagocytic with enhanced bactericidal effects. Soluble factors produced by lymphocytes are collectively termed lymphokines (Guttmann 1981).

Major histocompatibility complex

A great deal has been written on this subject, but the following short summary of its role in transplantation biology suffices for this dissertation.

The major histocompatibility gene complex is known as the HLA complex in humans and is located on Chromosome 6. The major histocompatibility gene complex in the mouse is termed the H2 complex. In the mouse it has been shown that cell interaction genes located in the H2 complex regulate T-cell, B-cell and macrophage interactions. Immune response genes also located in the H2 complex regulate the ability of an individual to respond against antigenic challenge. Transplantation antigens are major histo-compatibility complex gene products and participate in inducing allogenic immune responses.

IMMUNOSUPPRESSION IN TRANSPLANTATION

A. Non-specific Immunosuppression

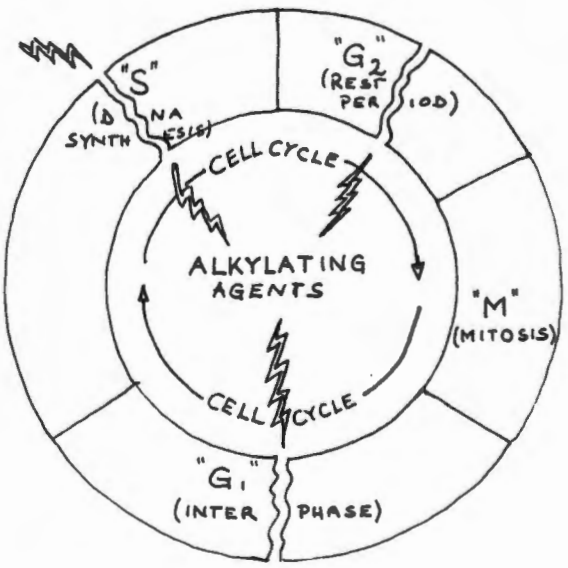
The immune system of an individual can be readily depressed by a large number of drugs and other agents. The disadvantage of all the immunosuppressive agents however, has been the non-specific way in which they act in an environment teeming with potentially pathogenic micro-organisms. Man requires his immune system as a defence and any impairment of that function will necessarily increase his susceptibility to infection. Fortunately it is often possible with conventional drugs to reach a half-way situation where rejection is suppressed yet the patient's ability to fight infection still retained. Just how this is accomplished at an immunological level is not well understood., for the interaction of drugs on the various mediators of the immune response is extremely complex (Berenbaum 1974). It would seem that under an umbrella of non-specific immunosuppression the immune system becomes adapted to accept the transplant thereby allowing the doses of drugs to be reduced. Such adaptation is essential since without it, high doses of drugs or other agents would be required indefinitely and their severe toxic effects would be inevitable. Although some form of adaptation undoubtedly occurs in successfully transplanted patients, drug therapy even at very low doses is still required.

Pharmacological immunosuppressive agents

Transplant rejection starts with the recognition of foreign material by the immune system and it is thought that macrophages have an important role in this step. Agents that immobilise macrophages such as gold, silica and anti-macrophage serum can help to prolong the survival of tissue allografts. Soon other members of the lymphoid system are involved with proliferation of those cells with specificities of the foreign antigens. B-cells develop into plasma cells, and K-cells and macrophages invade the graft to bring about its destruction. It is because cell proliferation forms part of the immune response that many cytotoxic drugs are immunosuppressive. The action of these drugs is better understood if one considers the metabolic events that occur in cell division.

The cell cycle

Lymphoid cells like other cells in the body replicate by cell division.



THE CELL CYCLE

The metabolism of such cells enters a cycle which comprises the G1 (gap phase 1) (the state at interphase), passes through a DNA synthesising or S phase to premitotic resting phase G2. Then follows the M or mitotic phase, and the cell once again enters interphase. In a non-stimulated lymphocyte population many of the cells are not in cycle but in a prolonged G1 or G0. Some immunosuppressive drugs are phase specific in that they only act during certain parts of the cycle. Anti-metabolites which include azathioprine are active during the S-phase and consequently are only effective against dividing cells. Alkylating agents act at many points of the cycle and some agents such as nitrogen mustard, cyclophosphamide and irradiation are also effective against resting G0 cells.(Hill and Baserga 1975)

Anti-inflammatory effects of immunosuppressive drugs

The immune process that brings about rejection of a foreign tissue is usually accompanied by a local inflammatory response. Lymphocytes, macrophages and polymorphs are attracted to the area in a non-specific way. Therefore drugs which act as anti-inflammatory agents will also depress to some extent the expression of an immune response without necessarily modifying the specific lymphocyte sensitisation which underlies it.

Anticoagulants and rejection

Fibrin deposition and thrombosis and small arteries are common features of rejection especially when there is antibody mediated damage. Unfortunately anticoagulants have not been very helpful in preventing this.

Immunosuppressive compounds

A vast range of different compounds seem capable of depressing the immune response but it has been particularly disappointing that so few of these have shown potential as immunosuppressive agents in man.

Anti-metabolites

These compounds interfere with protein synthesis by competing for and blocking specific receptors. They include the purine antagonist 6-mercaptopurine and azathioprine, the pyrimidine antagonist 5 fluoro-uracil and the folic acid antagonist methotrexate.

Since these agents are cycle-specific and only effective against proliferating cells they are most effective when given after, rather than before the exposure to antigen. Goldwyn et al studied the feasibility of canine limb homotransplantation using 6-Mercaptopurine and azathioprine as immunosuppressive drugs. In the untreated group by the sixth post-operative day the limb transplant showed active tissue destruction and started to lose its viability. Survival could be prolonged to a mean of 18 days by the administration of azathioprine. Thus the immunosuppressive therapy only postponed the rejection process but did not significantly alter its outcome.

Alkylating agents

These compounds possess an alkyl radical with active terminal end groups which can bind two or more different molecules causing them to become cross-linked. The alkylating agents are mostly cycle-specific but their activity is in general not confined to just one phase. Some agents such as Nitrogen mustard, Sulphurmustard and Cyclophosphamide are also active against resting cells. With most alkylating agents DNA synthesis is inhibited to a greater extent than is RNA synthesis but the alkylation of DNA does not necessarily lead to cell death since repair is possible. Although alkylating agents have been useful in treating malignancies they have on the whole been of little value as immunosuppressants.

Steroids

In organ transplantations steroids are frequently administered in high concentrations as a prophylaxis against rejection or for treatment of rejection after it has occurred. The side effects of such treatment are well known and it is surprising that the dosage is still largely empirical with different kidney transplant centres using very contrasting regimes (McGeown, 1973). Steroids have a large number of actions at the biochemical level. They bind to specific cytoplasmic receptors which transport them to intranuclear receptors where, at toxic levels they inhibit a variety of enzymes, with a resulting depression of protein, RNA and DNA synthesis. There is extensive death of small lymphocytes both in the blood and in the thymus, lymphnodes and spleen although the mechanism for this last effect is not well understood. It

is often assumed that high dose steroid therapy must be started immediately rejection has been diagnosed if the graft is to be saved and yet this may not be true. Salaman and Couhig (1980) found, using a rat heart allograft model that a single pulse of methylprednisolone is more effective in prolonging graft survival when given late than when given early in the rejection process. In organ transplantation it has been the usual practice to administer high doses of steroids immediately after transplantation as a prophylaxis against rejection.(Salaman 1982) The complications of such treatment have led to the realisation that heavy steroid dosage during the first month following transplantation is probably not required. Studies in England (Chan et al 1980, Kauffman et al 1977) showed that patients on low dose therapy with 25mg prednisolone daily showed even better results with less complications than those treated with high doses (150mg for three days that was reduced to 30 mg over the following three weeks). Steroids seem to be the only agent which can reliably reverse rejection episodes.(Chan et al 1980) It is usual practice to treat a rejection episode with high doses of steroids for 3 to 5 days after which the graft will often recover. (Bell et al, 1973; Salaman & Couhig, 1980) The treatment is repeated if there has been no response but most transplant centres would not treat for a third time since the patient would be very likely to develop complications which might prove fatal.(Mussche et al, 1976; Gray et al, 1978; McGeown et al, 1977)

Complications of non-specific Immune suppression.

The administration of azathioprine and prednisolone can be associated with a great variety of toxic effects.

(i) Infections - New or reactivation of latent, for instance TB.

(ii) Impaired growth and wound healing

(iii) Bone disease - osteoporosis, avascular necrosis, marrow suppression

(iv) Cataracts and other ophthalmic complications

(v) Diabetes, obesity, cushingoid appearance

(vi) Peptic ulceration, colonic perforations

(vii) Pancreatitis, liver dysfunction

(viii) Hypertension

(ix) Psychiatric disturbances

(x) Malignancy

Infection is the greatest threat to the patient and many transplant centres adopt a policy of barrier nursing their patients during the first few weeks after transplantation. A few years ago patients were at great risk of dying from bizarre fungal and viral infections but with a more cautious use of immunosuppression nowadays these life-threatening infections are seen much less often.(U.K. Transplant Service 1980) Of the other non-fatal complications perhaps the most disabling is avascular necrosis which usually affects the femoral head. This appears to afflict patients quite randomly and has been unrelated to the total dose of steroid administered.(Salaman 1982) Some form of prophylaxis is available against the more serious side effects. Continuous medication with antacids and/or cimetidine should prevent

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ulceration and long term anti- tuberculose therapy may be needed for patients who have contracted this disease in the past.

Blood transfusion effects and immunological enhancement

In 1973 Opelz et al demonstrated that the success of cadaver renal transplants one year following surgery has significantly improved in patients, who had received nonspecific random blood transfusions prior to transplantation, compared with non-transfused patients. In a more recent study these investigators reported a strong dose effect of prior blood transfusion on kidney allograft survival (Opelz et al 1981). Many other reports have supported the beneficial effect of blood transfusions on human renal allograft survival (Solheim 1979, Persijn et al 1977, Williams et al 1979).

The beneficial effect of previous administration of blood has also been demonstrated in animal studies. Skin allograft survival has been enhanced in minimally immunosuppressed mice by pretransplant administration of donor strain white blood cells (Wood et al, 1981) or multiple non-specific blood transfusions (Okazaki et al 1980).

The mechanisms underlying immunologic enhancement remains unclear. Several possibilities have been advanced.

1. Induction of enhancing antibody, which has been theorized to interfere with antigen recognition (Morris 1971; Snell et al 1960).
2. Opsonization of antigen-reactive cells by antigen-antibody complexes which may serve to inhibit host cellular migration to graft tissue (Hutchinson 1977).

3. Induction of anti-idiotypic antibodies, which would block lymphocytic proliferative responses through the bindings of receptor idiotypes (Binz & Wigzell 1975).
4. Loss of donor passenger leukocytes carried in donor allogenic tissue (Billingham, 1971) which have been demonstrated to be powerful immunogens (Steinmuller 1967).
5. Induction of suppressor T-lymphocytes which would inhibit developing alloimmune responses (Dorsch & Roser 1977; Rilshaer & Brent 1977; Steinmuller 1978).
6. Inactivation of T-helper lymphocytes in the continued presence of major histoincompatible antigens which has been speculated to induce specific immune non-responsiveness (Batchelor 1978, 1979).

Cyclosporin A

The Cyclosporins A and C were initially isolated from the fermentation broth of a cell fungus Tricodermapolysporum (Borel et al 1976). Various other cyclosporins have now been identified but only cyclosporins A, C and G show strong immunosuppressive activity. Cyclosporin A has been markedly effective as an immunosuppressant in a variety of species and in the rabbit prolonged survival of kidney allografts has been observed after relatively short periods of treatment (Green and Allison 1978). This led the authors to suggest that cyclosporin A had helped to induce a state of specific immunological tolerance. It is now known that although Cyclosporin is particularly effective in the rabbit, the mechanism of its action is non-specific. In rats and mice Cyclosporin A will readily prolong the survival of skin and heart allografts but its action does not persist in these animals and rejection has occured

within a short time of stopping treatment. (White 1980, Kim et al 1984, Black et al 1985) It was found unexpectedly when Cyclosporin first came to be used in man that the drug was nephrotoxic and some of the patients with kidney transplants had prolonged periods of unexplained oliguria (Calne et al 1978). This is discussed further later. It was also found that a combination of Cyclosporin A, Prednisolone and a Cyclophosphamide analogue was dangerous and was associated with serious infections (Calne 1981). The current practice in Cambridge is to treat all transplanted patients who have immediately functioning kidneys with Cyclosporin A alone but to add short courses of steroids when rejection is diagnosed (Calne et al 1981). This treatment was also given to patients receiving liver and pancreas transplants with good results (Calne et al 1981). Most patients were commenced on a dose of 17 mg per kg. of Cyclosporin A per day reduced by 2mg per kg. each month to 6-8mg/kg per day. The Minnesota/Pittsburgh and Houston groups use moderate doses of steroids during induction therapy with a lower incidence of allograft rejection (17-30%) opposed to the English groups (62-70%). Interestingly only those centres that have noted a lower incidence of rejection have reported the benefit in Cyclosporin treated transplant patients of a lower risk of infection compared with conventionally treated allograft recipients. Long term follow-up for steroid complications will indicate which strategy using steroid during induction of immunosuppressives or steroid intervention for treatment of rejection will be the best to gain the benefit from Cyclosporin treatment.

Toxicity of Cyclosporin

The major concern regarding Cyclosporin therapy has focussed upon its nephrotoxic and hepatotoxic side effects. At Stanford, in two of seventeen cardiac transplant patients an end stage renal disease occurred. The biopsies revealed interstitial fibrosis and focal sclerosis of glomeruli in many of these patients (Myers et al 1984). Similar reports were published in patients with bone marrow transplants treated with Cyclosporin (Shulman et al 1981). Rare incidences of a haemolytic uraemic like picture have been reported with the use of Cyclosporin in renal transplant recipients (van Buren et al 1984). Clinical evidence suggests that much of the nephrotoxicity is reversible (Salvatierra et al 1983). Significant decreases in serum creatinine levels following elective changing of Cyclosporin treated patients to azathioprine therapy have been found but the incidence of rejection is increasingly high following conversion (19-70%) (Rocher et al 1984). At Houston 50 of the 380 renal transplant patients have demonstrated intractable nephrotoxicity. Those patients were give azathioprine, prednisone and low dose cyclosporin (2-4mg/kg per day) with good results. Hepatotoxicity appears to be less of a problem in Cyclosporin treated patients. The incidence ranges from 20-40% with hyper-bilirubinaemia being the primary pertubation. Most cases can be successfully managed by reduction of cyclosporin doses (Flechner et al 1985). Other side effects are hypertrichosis, fluid retention, hypertension and gingival hypertrophy.(Laupacis 1983)

Antilymphocyte globulin

The immunosuppressive property of anti-lymphocyte serum lies in its gammaglobulin and it is the gammaglobulin that is usually administerd to patients. It was first shown by Woodruff and Anderson in 1963 that ALG was an inhibitor of graft rejection (skin homografts in rats). It is prepared from the serum of animals that has been immunised with lymphoid tissue from another species. In experimental procedures it has been shown to have a profound suppressive effect upon cellular immunity and to prolong survival of allografts and even xenografts. It probably works by severely depleting both the circulating T-lymphocytes and those within the lymphoid organs. The problem with ALG is that it is a biological product and its potency can vary enormously depending upon the type of animal used , the type of antigen injected, the route of administration, the purification of the globulin and the mode of administration to the patient. The most effective ALG has been raised in rabbits that have been injected with human lymphoid cells which have been grown in culture or obtained from the thoracic duct or thymus. It should be given IV at a high dosage for at least two weeks starting on the day of transplantation or the day before.(Thomas et al 1977) It is an expensive procedure as is the whole process of ALG manufacture and purification.(Thomas et al) Nonetheless, a few commercially manufactured preparations of ALG are available and some centres use these routinely as part of their prophylactic anti-rejection treatment. Although active ALG preparations are capable of suppressing rejection, the effect may cease as soon as the ALG treatment is stopped. ALG has earned a place for itself in the management of rejection episodes.(Wechter et al 1979) In patients who have failed to respond

to a course of steroids it may be used successfully as a second line of treatment without subjecting the patients to hazards of heavy steroid therapy.(Shield et al 1979, Light et al 1981)

Irradiation

Whole body irradiation was one of the first forms of immunosuppression used in human recipients of kidney transplants. Although it was not particularly effective and caused considerable morbidity as well as mortality a few patients survived with well-functioning transplants (Hamburger et al 1962). Much more commonly radiation has been used to treat the transplant itself principally at the times of rejection. Woolf et al in 1969 showed that irradiation of a dog kidney allograft would delay its rejection. Godfrey and Salaman (1977) however, performed a controlled clinical trial of 600 rad of graft irradiation to patients whose cadaver renal transplants were undergoing rejection. They saw no benefit from irradiation, in fact the irradiated group went on to have more rejection episodes subsequently. Total lymphoid irradiation has been found to be more successful. Here the supra and sub-diaphragmatic fields are irradiated simultaneously and all the major lymphnodes, the spleen and the thymus are involved. Slavin et al 1976,1977 gave 200 rad in 17 fractions to mice which caused a profound fall in the level of circulating lymphocytes and although numbers of B-lymphocytes began to rise after two weeks, the T-lymphocytes remained undetectable for at least a month and were still at a reduced level a year later. Lymphocytes from these animals responded poorly if at all to mitogens or allogenic cells and antibody production was severely depressed. Skin allografts survived on these animals for about 50

days. A more lasting tolerance has been seen when TLI has been followed by an injection of donor bone marrow. When mice were treated this way approximately half accepted allogenic skin grafts for more than 100 days.(Slavin et al 1978) Similar results were obtained in dogs, monkeys and baboons.(Slavin et al 1978, 1979) A few transplant centres have begun to use TLI for preparing certain patients for kidney transplantations and the early results appear to be promising.(Najarian 1981) It may have a place as a form of pre-treatment for patients who have demonstrated strong immunity to previous kidney transplants or blood transfusions .

Specific immunosuppression

Specific immunosuppression may be defined as treatment which directly or indirectly selectively suppresses the action of the lymphocyte clones responsible for the rejection of the particular graft the patient receives i.e. it is antigen specific immunosuppression directed at the histocompatibility antigens of the graft donor.(Fabre 1982) One of the most important advantages of specific immunosuppression is its safety. It is free from the immunological complications of generalised immunosuppression (the development of infection and malignancy) and free also from non-immunological complications such as the bone marrow depression seen with azathioprine. Although the long term aim is that specific immunosuppression be used alone for the prevention of graft rejection this ideal seems unlikely to be realised in the immediate future. To date two major approaches to donor specific immunosuppression have been studied .

(i) Antigen induced suppression (active enhancement) which refers to specific immunosuppression induced by treatment of the recipient with preparations containing donor histocompatibility antigens.(Billingham et al 1963; Fabre 1976; Little et al 1975; Winearls et al 1979)

(ii) Antibody induced suppression (passive enhancement) where specific immunosuppression is induced by the administration to the recipient of antibodies directed against donor histocompatibility antigens.(Batchelor & Welsh 1976; Gallico et al 1979) Active enhancement offers the best hope for the use of specific immunosuppression. However, until the factors are better understood, which determine whether exposure to antigen results on the one hand in sensitisation or on the other in specific suppression active enhancement remains too dangerous for clinical use. Passive enhancement has not found any clinical application to date as the stimulus necessary for clinical trials has been lacking. Other methods being investigated are T-lymphocytes suppressor factors (Kohler & Milstein 1975), induction of anti-idiotypic immunity (Weinberger et al 1980), and antigen suicide experiments.(Myburgh & Smith 1973; Weinberger et al 1979) There is no currently studied approach likely to be of clinical value in the near future but a new and more thorough knowledge of basic immunology accummulated in recent years is opening up new avenues.

Immunosuppression in skin transplantation

The strong antigenic properties of the epidermis make skin graft prolongation a particularly exacting test of immunosuppressive agents.

The cortico-steroids which have been so effective with azathioprine in reducing rejections of renal and cardiac transplantation have had only limited success in skin graft recipients. When administered in conventional dosages they have not provided significant prolongation of skin allograft survival. Azazthioprine also has limited effect.(Burke 1975) More specific immunosuppression can be achieved with anti-thymocyte globulin which is also an attractive choice as an immunosuppressant for infection prone patients.(Diethelm et al 1974) The rather selective T-cell depletion which it produces can be readily monitored by the sheep red blood cell rosetting technique.(Bishop et al 1975) Using this assay the recipient's remaining level of T-lymphocytes can be sequentially monitored in order to estimate the degree of immunosuppression being achieved and thus subsequent therapy can be adjusted appropriately.

Clinical trials of patients with massive burns who have been treated with allografted skin and temporary immunosuppression with cyclosporin have shown encouraging results. (Achauer et al 1985, 1986). With low dose cyclosporin A (less than one half of the initial dose used for an organ transplant) and no concomitant steroids, rapid primary coverage with autografts and allografts was achieved. Once the patient had healed and suitable donor sites were available, the allografts were replaced with autografts and the cyclosporin discontinued.

THE EFFECTS OF FREEZING ON TISSUES

In proposing the hypothesis that freezing alters the antigenicity of the transplanted skin (Roels et al 1982; Heck et al 1985; Pegg & Taylor 1984), it is of importance to note the effects of cooling and freezing on the membrane structure.

Cell membrane structure

Cell membranes are complex heterogenous structures predominantly composed of glycerol phosphatides, cholesterol and proteins. The phospholipids are arranged in bi-layers with the polar groups at the surfaces whereas cholesterol and proteins are randomly distributed. At normal physiological temperatures the phospholipids are highly fluid and because of the regular bi-layer arrangement this state is known as liquid crystalline.

Effects of cooling on cell membrane structure

When it is cooled the lipid bi-layer undergoes a phase change to the gel state in which phospholipids are arranged in an hexagonal crystalline lattice and fluidity is vastly reduced. The phase change usually occurs at an abrupt transition temperature, the value of which varies widely with the chemical nature of the lipid. Natural membranes contain a complex mixture of many phospholipids with different individual thermotropic properties: in these circumstances phase separations occur- that is, different phases exist simultaneously in the plane of the membrane. Cholesterol has the property of reducing membrane fluidity above the transition temperature but it inhibits crystallisation and therefore increases fluidity below the transition temperature.

Intrinsic membrane proteins also affect the thermo-tropic behaviour of membrane lipids in a manner similar to cholesterol and produce packing faults radiating outwards from the inserted protein. However, the phase behaviour of the phospholipids also has a marked effect on protein distribution and function. Lateral phase separations occur at low temperatures producing regions of phospholipid crystals and regions of high protein concentration. Quinn in 1985 proposed a sequence of events in which those lipids with the greatest tendency to crystallise as hexagonal structures also have the highest transition temperatures. During cooling these lipids phase-separate first, producing gel phase domains that exclude intrinsic membrane proteins. On rewarming, it is proposed that these domains form inverted micelles sandwiched between the lipid bi-layer, thereby destroying the barrier properties of the membrane. Quinn has made the prediction that hydrophilic neutral solutes such as glycerol and Dimethylsulphoxide will reduce the transition temperatures of those lipids that tend to separate first during cooling and raise the transition temperature of those that normally separate at lower temperatures. This would reduce the segregation of protein from lipid and prevent the formation of inverted micelles. One additional mechanism of membrane damage that is well established involves oxygen free radicals. These highly toxic agents are generated by the step-wise addition of the four electrons that are normally added to oxygen in a single step by cytochrome aa3. The enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase are normally responsible for converting these radicals to harmless water and many compounds including mannitol, glycerol and dimethylsulphoxide for example, have free radical scavenging properties.

Free radicals attack membrane lipids releasing lipid peroxides and seriously damaging the cells. Since free radical reactions have a low activation energy they are depressed to a lesser extent by decreasing temperatures than the enzymatic reactions that normally remove them and it is therefore entirely possible that oxygen free radicals are important in hypothermic injury.(Stuart et al 1985)

Effects of freezing

By further lowering the temperature the chemical reactions are not merely retarding but arresting with the probability that lower temperature membrane damage will be increased. However, the major difficulties are due to the formation of ice and effective cryopreservation requires means of preventing freezing injury.

Mechanism of freezing injury

When aqueous solutions freeze, only sufficient ice is formed at each temperature to concentrate the solutes in the remaining liquid in order to produce a solution with that freezing point. The question therefore arises: "are cells damaged by the fall in temperature, the ice, or the increase in solute concentration?"

Effects of increasing salt

The studies of Lovelock (1953) showed that the injury to erythrocytes that was produced by slow cooling to relatively high sub-zero temperatures could be accounted for quantitively by the increase in salt concentration in the remaining liquid phase. He showed that cells exposed to high salt concentrations whether by freezing or by direct

exposure to strong salt solutions without freezing were made susceptible to damage by further cooling or by returning them to an isotonic environment (osmotic shock). These changes were attributed to damage by high electrolyte concentrations to the cell membranes. This mechanism has been termed solution effects and is dominant at low cooling rates. As the cooling rate is increased cell recovery first increases, then reaches a maximum and then decreases. The rate giving maximum recovery varies from cell to cell so the meaning of slow cooling in this context also varies from cell to cell. This characteristic response to cooling rate has been explained as the consequence of two distinct damaging mechanisms that are oppositely dependent on cooling rate. Solution effects are presumed to be both time and temperature dependent and therefore to be reduced by increasing the cooling rate thus producing an increase of survival with more rapid cooling. Mazur in 1977 showed that the fall in cell survival after still more rapid cooling is due to the occurence of intracellular freezing. The dependence of intracellular freezing on the cooling rate rises because the cell contents have a greater tendency to super cool than the external fluid. Hence, ice forms first in the extracellular space and the consequent rise in solute concentration produces an osmotic pressure gradient across the cell membrane. If cooling is sufficiently slow, equilibrium will be maintained by the loss of cell water and ice will never form intracellularly. However, as the rate of cooling is increased, water will eventually be unable to leave the cells fast enough to maintain equilibrium. An intracellular freezing will be progressively more likely. Mazur has examined and analysed these phenomena extensively. He has shown that the critical cooling rate for

intracellular freezing differs widely from cell to cell and is primarily dependent on two parameters. These are the water permeability of the cell membrane and the surface area to volume ratio of the cell such that optimal cooling rates for red cells, Hela cells and mouse-ova as examples are approximately 3000, 50 and 1^0 C per minute respectively.(Mazur 1977) The fundamental mechanisms of cell damage by solution effects and intracellular freezing are still a matter of debate. Intracellular freezing is generally supposed to damage by a directly destructive effect of the ice crystals upon intracellular structures although other mechanisms have also been proposed. Whatever the fundamental mechanism, it is at least clearly established that there is an optimum cooling rate for each cell type, that these optimum rates vary very widely and that the peak recovery and that optimum also vary from negligible to almost 100% with different cells.

Properties and effects of cryoprotectant agents (CPA)

Cryoprotectant compounds are able to increase the absolute survival at the optimum cooling rate but they also change the optimum cooling rate (Pegg 1984). On the basis of his electrolyte theory of freezing injury, Lovelock (1954) proposed, that the addition of any compound that has a high solubility, a low molecular weight, and will permeate cells without toxicity, will reduce freezing injury. Compounds that need these requirements are cryoprotectant, the best known being glycerol and dimethylsulphoxide. The additon of these compounds simply increases the total concentration of solutes and therefore reduces the amount of ice formed at any sub-zero temperature and hence limits the rise in salt concentration. For this to be effective, high molar concentrations of

the added solute are needed (1-5 molar) and this is the reason for requiring high solubility and low molecular weight.

The ideal cryoprotective agent should penetrate rapidly and uniformly into cells without undue osmotic stresses, should control the size and rate of ice crystal formation by binding intracellular water, and should be non-toxic. Glycerol has little toxicity but penetrates slowly, while dimethylsulphoxide penetrates rapidly, but is nephrotoxic. High concentrations of the agents are needed for adequate cryoprotection but the concentrations required are usually associated with severe organ toxicity and severe vascular damage. (Marshall 1982)

Cryoprotectants must be non-toxic but every compound is harmful if its concentration is raised sufficiently. There is always an optimal initial concentration for cryopreservation which is a compromise between cryopreservation and toxic action. It is commonly in the range of 1- 2 molar with glycerol or dimethylsulphoxide but the precise nature of toxicity at higher concentrations is unclear. Since most cryoprotectants do penetrate the cells, osmotic lysis occurs if the external medium is diluted too rapidly and the so-called toxic limit is therefore very sensitive to the means used to remove the cryoprotectant. The interactions of temperature, liquid volume, salt concentration and cryoprotectant concentration are complex and only incompletely understood at the present time. Not only does the cooling rate and the cryoprotective agent concentration interact in determining cell survival but also the warming rate and cooling rate interact in determining cell survival. Akhtar et al in 1979 could show that cooling L-cells at 1°

per minute was associated with an optimal warming rate of 200°C per minute but the same recovery could be obtained by cooling at 0.2° per minute and warming at 0.3°C per minute.

The changes with rewarming

The rate of rewarming has several fundamental effects on phenomena occuring in and around the cells at low temperatures. If cooling has been too rapid for all the ice to form extracellularly there will be small intracellular ice nuclei that can be prevented from growing during thawing only by rapid warming. On the other hand, very slow cooling may permit substantial net solute loading of cells and this could lead to osmotic lysis during rapid warming, since rapid thawing of ice will cause rapid dilution. Slow rewarming will enable the excess solute to leave the cells gradually. Slow warming, however, permits extensive recrystallisation of external ice with pronounced changes in the dimensions of the unfrozen liquid channels. When the melting of ice is complete, these cells and their surrounding medium still contain the initial concentration of cryoprotective agent and dilution must therefore be continued in order to return them to a physiological environment. As this is done, an osmotic gradient is again generated across the plasma membrane and this is relieved both by solute loss and since it is a more rapid process, by the entry of water leading to cell swelling. If the cells are to survive the degree of swelling must be controlled and limited. This can be achieved by diluting the cryoprotective agent slowly or in a sequence of stepped decreases in concentration. Alternatively the cells may be placed in a cryoprotective agent free medium that contains a non-penetrating

substitute for the cryoprotective agent such as Mannitol which enables the cells to lose cryoprotective agent without a corresponding influx of water.

Specific difficulties with freezing solid tissues

The precise arrangement of many different types of cell and of extracellular structures that comprise a whole organ introduces important additional difficulties . For example, the sheer size of an organ may render it impossible to achieve the desired rates of temperature change. The closed packing of the cells may invalidate extrapolation from dilute cell suspension. The presence of many different cell types might mean that no one cooling and warming procedure can satisfy them all. Also damage to extracellular structures from extracellular ice may be sufficient to destroy the organ even though the cells survive. There are a number of reports of attempts to cryopreserve whole organs using techniques derived from methods that were effective with single cell systems but these experiments have served only to emphasise the extreme difficulty of the enterprise (Pegg & Jacobson 1983). It very soon became clear that the introduction and removal of CPA had to be done very gently if injury was to be avoided and that even in the presence of one or two molar CPA moderate freezing produced severe damage. True cryopreservation (storage at -80°C or below) at least for several days has never been achieved. It seems that the major cause of freezing injury in whole organs is extracellular ice and that while cooling alone does cause damage, substantial progress will require techniques that will

completely prevent freezing or at least reduce the quantity of ice drastically.

New approaches

Two approaches are available. The first is to increase the concentration of CPA progressively during cooling relying upon the fall in CPA toxicity with decreasing temperature to make it possible to reach concentrations of CPA that will prevent freezing altogether. Attempts have been made to apply this approach experimentally so far without success, probably because of the technical difficulties of perfusion and diffusion at very low temperatures. The other approach is to employ conditions that favour vitrification rather than freezing. Vitrification is the amorphous solidification of solutions without the separation of ice crystals, and normally requires very high concentrations of appropriate solutes and rapid cooling. There are no studies in the literature on the cryopreservation of vascularised skin flaps. The method used for cryopreservation of the skin flaps which will be discussed later is mainly based on the experience of freezing skin. According to Berggren (1974) either a 1.4 mol glycerol or 1.4 mol dimethylsulphoxide solution as a cryoprotectant is recommended in a balanced salt solution. The soaking time in dimethylsulphoxide should not exceed 1 hour whereas the skin should be exposed to glycerol for at least 2 hours. In both instances the soaking should be carried out at 4^{o} - $7^{o}C$. The cooling rate is not critical but it appears to be most satisfatory when it is slow. An acceptable range is $1^{o}C$ per minute - $10^{o}C$ per minute. Storage for long periods should be at temperatures less than $-120^{o}C$ for maximum storage times and the preservation of viability.

Storage at dry ice temperatures (-79°C) will give good results up to 5 months but no reliable data are available for storage beyond this point at this temperature.

Techniques of thawing

Various techniques of thawing have been used but it is best achieved by immersing the frozen skin in a bath of sterile saline or Ringer's solution of 37°C.(Billingham & Medawar) Lehr 1964 found using Dimethylsulphoxide as a cryoprotectant agent for skin, that thawing had to be faster than 50°C per minute for maximum survival. Microwave thawing has also been studied but the use of domestic microwave ovens is to be avoided because of the difficulty of controlling energy absorption and the consequent risk of burning (Lehr 1971). Saline solution is not a balanced tissue solution and is therefore fairly damaging to tissues.

Vascular damage

A review of the literature on organ cryopreservation shows that a prominent feature of organs transplanted after freezing and thawing has been a rapid cessation of blood flow suggesting that vascular damage is an important factor in their functional failure.(Goodman et al 1977, Jacobson 1979, Jacobson et al 1984) It is assumed that this vascular damage is caused by the formation of ice in the lumina of the vessels. In the case of the investigated kidneys, it was particularly the glomerular capillaries. Jacobson et al found that both the glomerular and peritubular capillaries were distended which would not be expected once the perfusion pressure had been removed. Vascular distention was not observed either in immersion fixed or perfusion fixed unfrozen

organs. They also found that more ice was formed when the cooling rate was rapid than when it was slow. The shrunken and condensed vascular endothelium of their frozen kidneys was very similar to appearances that have been correlated with preservation of structure and function in other cells. While no confident conclusion can be drawn, the uniform appearance of the endothelium and only minor focal detachment from the basement membrane suggested that it had not been disrupted by cooling to -80°C at any of the rates used in their study. Jacobson et al found an optimal cooling and warming rate of 1°C per hour for kidneys. However, even in these transplants all the renal veins were found to be thrombosed when the grafts were removed and pronounced ischaemic damage was seen in the histological studies. They concluded that even a near normal and even pink colour of the kidneys was not a sufficient condition for post-transplant function. Goldman et al, investigating arterial interposition allografts showed that dimethylsulphoxide cryoprotected vein grafts showed evidence of aneurysm and thrombotic changes suggestive of early failure. They concluded that these grafts contained enough endothelium and cell debris to initiate thrombosis and resulting vascular injury. It has been assumed that the major site of the injury was the vascular endothelium and they suggested that the total stripping of vascular endothelium in conjunction with protection of organ function from cryoinjury would be an alternative approach to organ preservation.

In conclusion one can say that cryoprotection of vascularised skin flaps has not been attempted yet, and it appears upon review of the literature that a vascular damage must be expected whatever techniques are used.

Comparing advantages and disadvantages of glycerol and dimethylsulphoxide it appeared that glycerol was less toxic, caused less vascular damage and required a slower thawing rate than dimethylsulphoxide.

For the present study, slow cooling and rapid thawing was chosen as an appropriate procedure to use for the vascularized skin flaps using glycerol at a concentration of 1.6 mol.

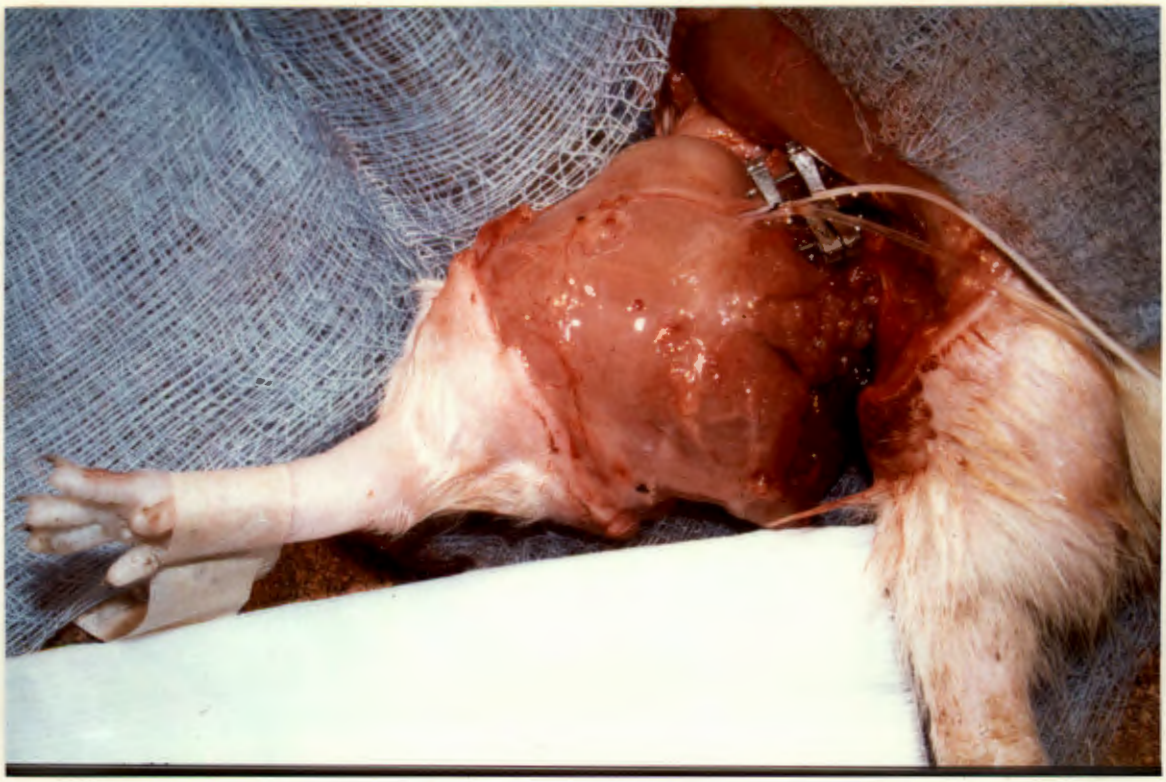


Fig 1 Perfusion of rat hind limb with glycerol catheters are placed into femoral artery and vein

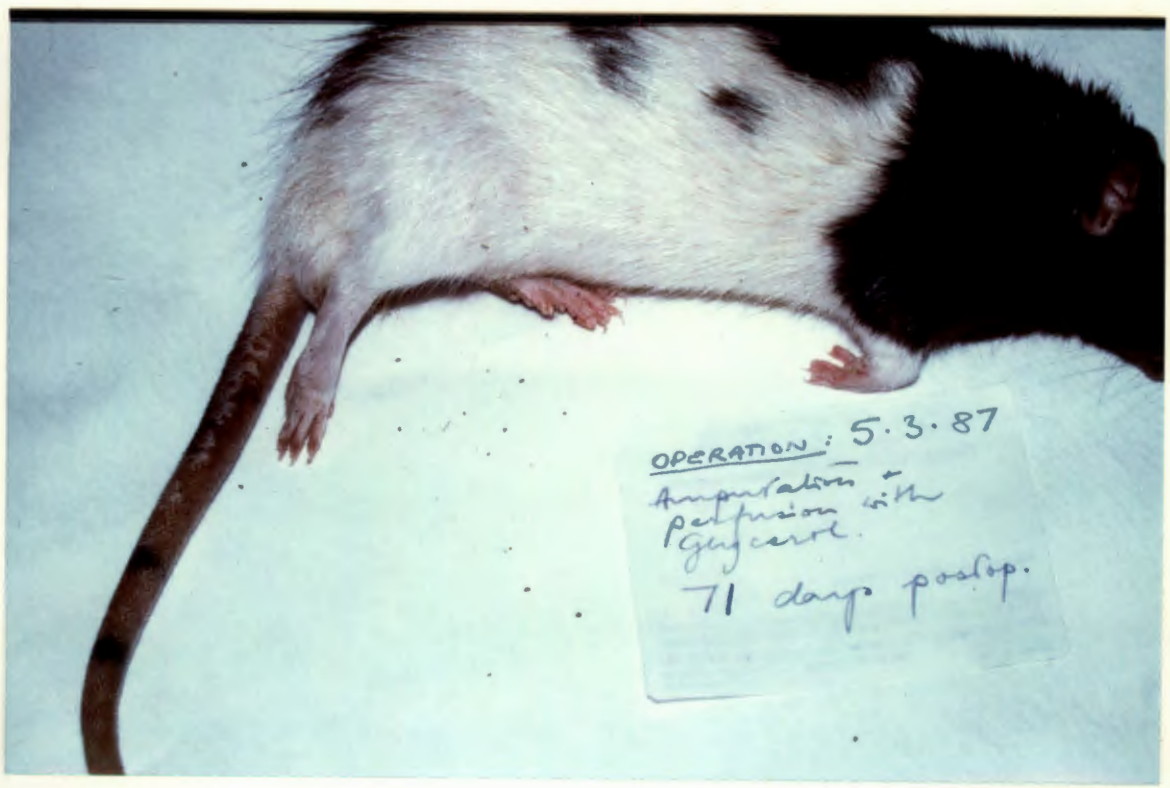


Fig 2 The amputated, perfused and replanted leg 71 days postoperative.

MATERIALS AND METHODS

Long Evans outbred rats with an average weight of 300gm were used throughout the studies.

Experiment 1

- to show whether the cryprotective agent glycerol at the concentration of 1.6M would cause any localised tissue damage.

Method: the hind limb of the rat was chosen. The limb was sub-totally amputated, leaving the femur and the sciatic nerve intact but dividing in a circular fashion the skin, subcutaneous tissue, vessels, and muscles. The bone was left intact to have a better post-operative stability and the sciatic nerve was left intact to leave sensitivity in the limb in order to prevent it from being chewed off. A catheter was inserted into the femoral artery and the limb was perfused in slowly increasing concentrations of glycerol, diluted in Ringer's solution up to a concentration of glycerol of 1.6M.(Fig.1) The glycerol was then washed out in the same procedure in slowly decreasing concentrations of glycerol again diluted with Ringer's solution. The femoral artery and vein were then anastomosed with 10/0 nylon interrupted sutures to regain a normal blood perfusion of the limb.

Results:

The first rat operated in this way died several hours after the operation for an unknown reason. The second rat died also after the replantation of the limb due to excessive blood loss. In the third rat, a thrombus formed at the site of the anastomosis and the rat had to be sacrificed three days post-operatively due to limb necrosis. In rat

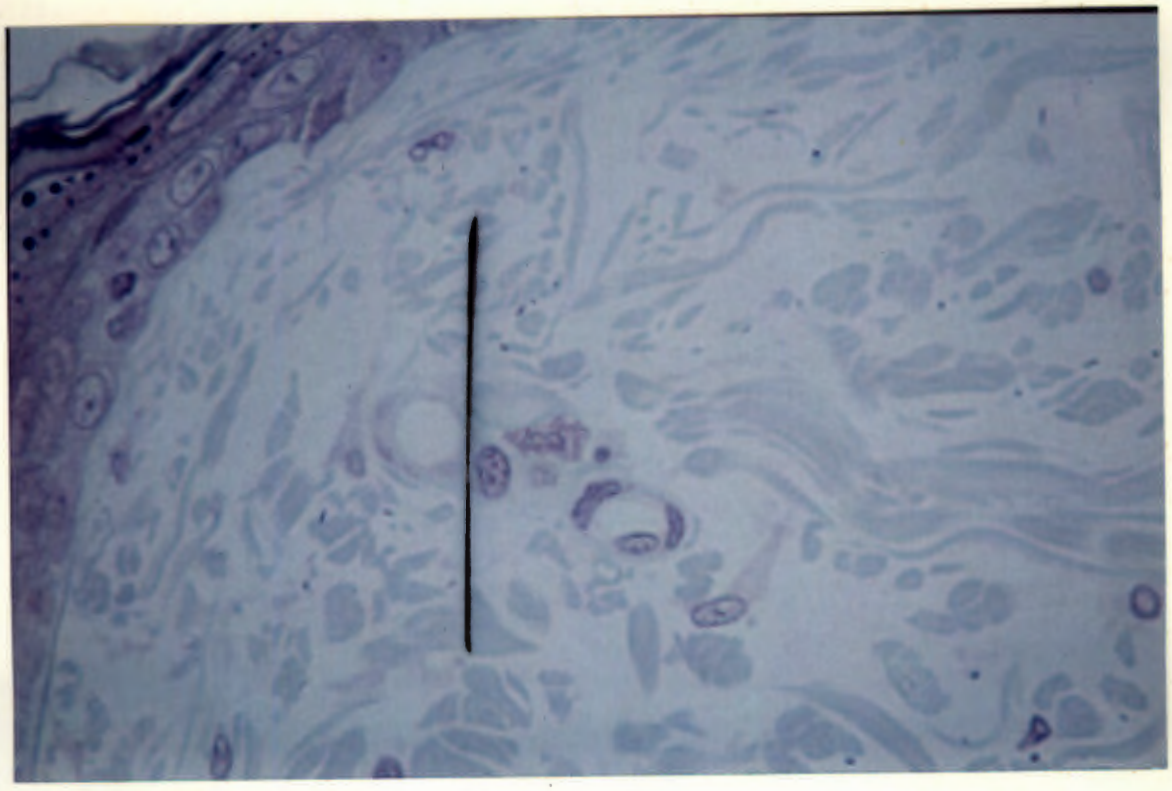


Fig 3a Histology skin

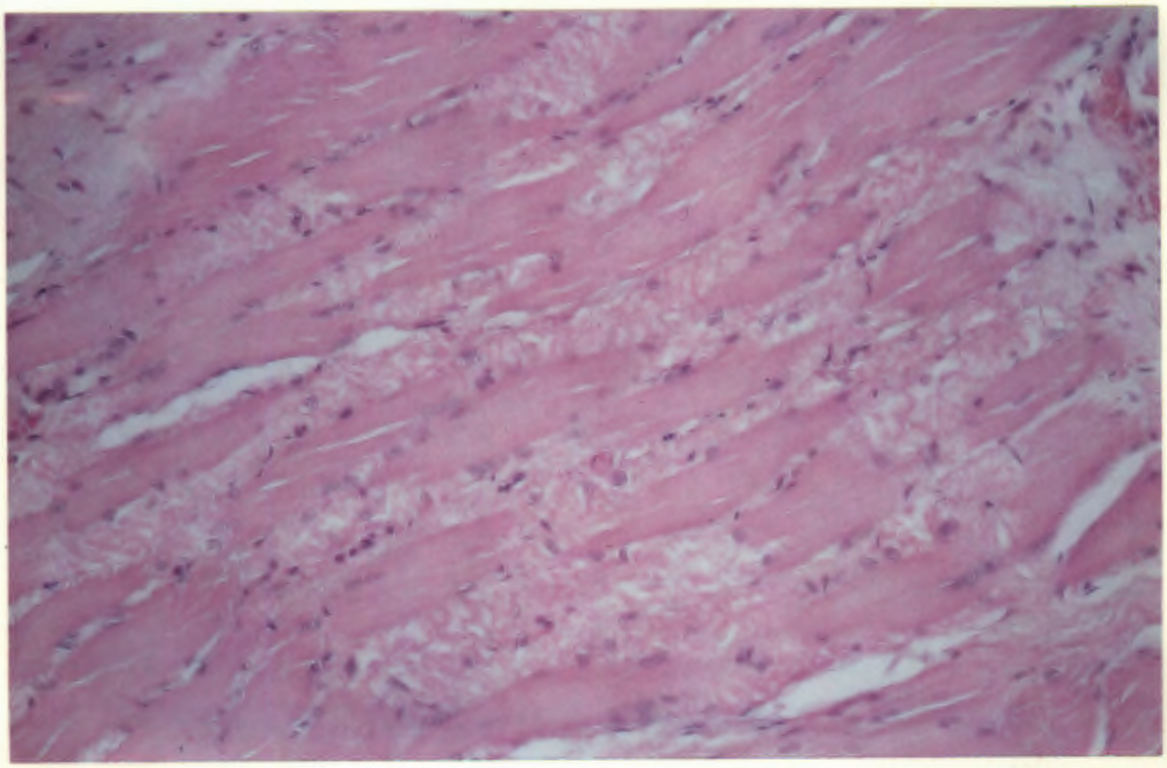


Fig 3b Skeletal muscle
Focal muscle atrophy
and blood vessel
proliferation

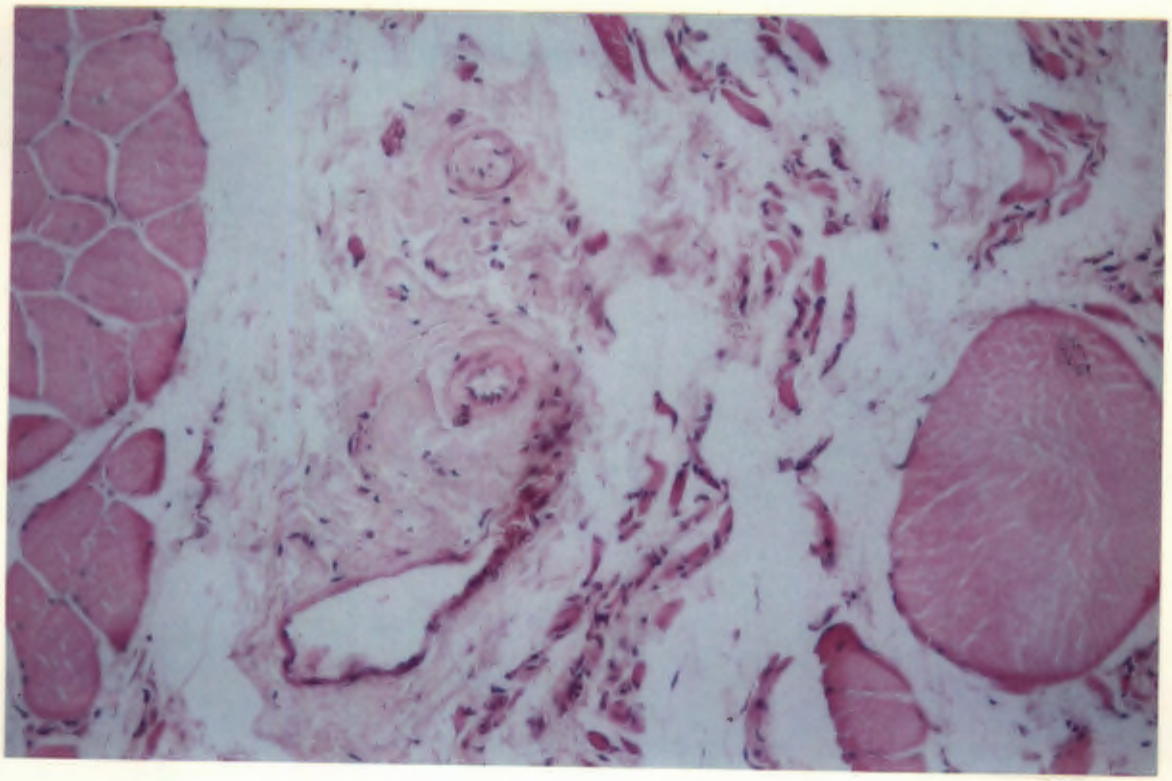


Fig 3c New blood vessels

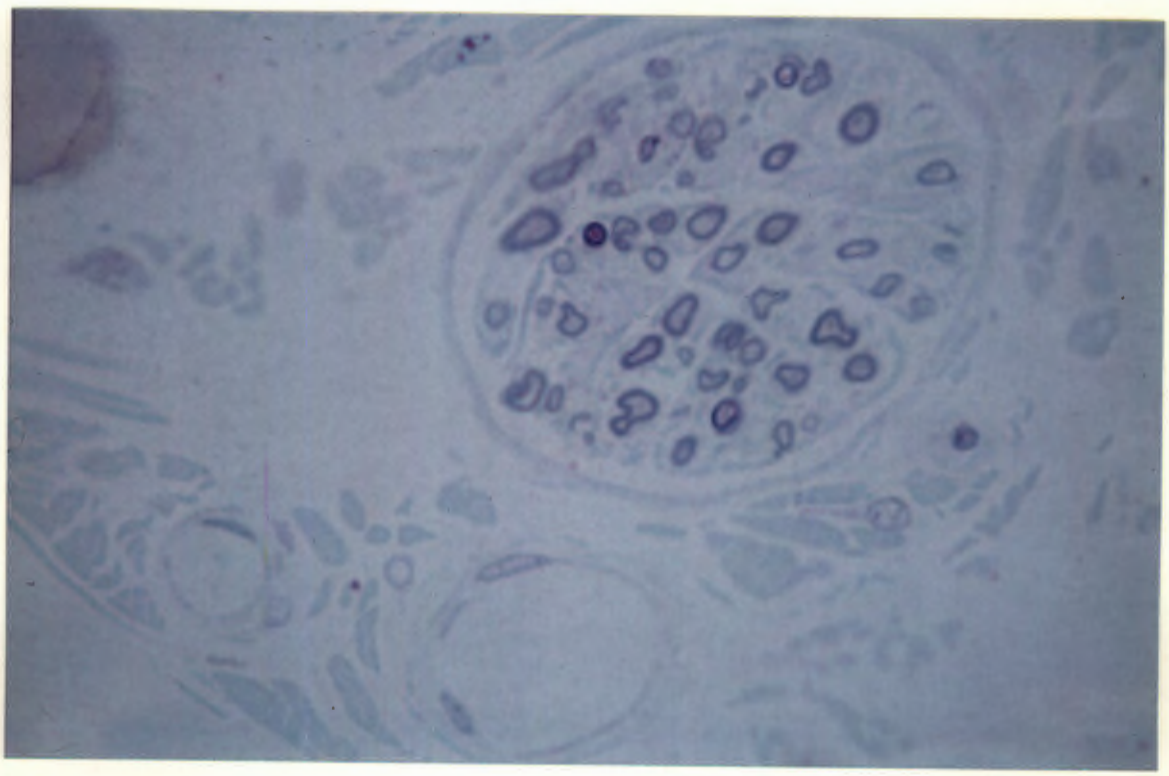


Fig 3d Peripheral regenerating nerves

number four, the procedure was technically successful. The limb was well perfused and there was no significant post-operative oedema. Eight days post-operatively the fur of the operated leg became scarce and thin and on the eleventh post-operative day, the rat was found dead in the cage. No obvious reason could be found for this death. A fifth rat was done in the same way and in this case, a complete survival of the limb was noted. Three months post-operatively, the rat was walking on the replanted leg although it was not using it completely normally. (Fig. 2) Seven months after the operation the rat was walking normally on this leg. The rat was sacrificed, the leg amputated and sent for histological assessments.

Histology findings:

The general histological structure of the skin (Fig. 3a), subcutaneous tissue, and skeletal muscle (Fig 3b) was preserved. Focal proliferation of the connective fibrous tissue was seen between skeletal muscle fibres, which showed focal signs of atrophy or degeneration.(Fig 3b & 3c) New blood vessel proliferation between muscular fibres and occasionally regenerating peripheral nerves were found.(Fig 3c & 3d)

Experiment 2-

Aim: to develop a suitable groin flap for transplantation.

A groin flap was designed +- 3x6 cm based on the superficial inferior epigastric artery and vein. The technique in the first three rats consisted of raising the flap incising the margin of the flap and dissecting it off the abdominal wall in the clear fasciral plane which is found just superficial to the muscle. Every bleeding point was cauterized, dissecting out the vascular pedicle, dividing the inferior

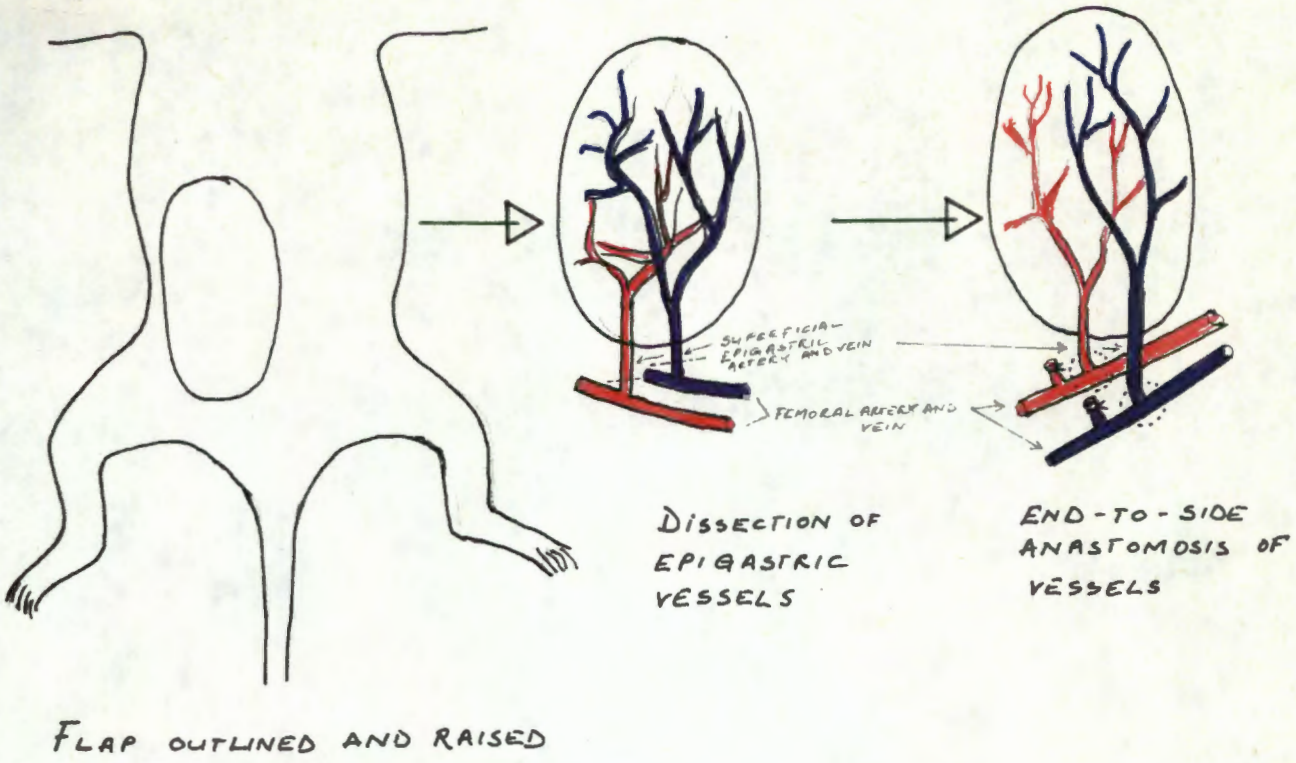
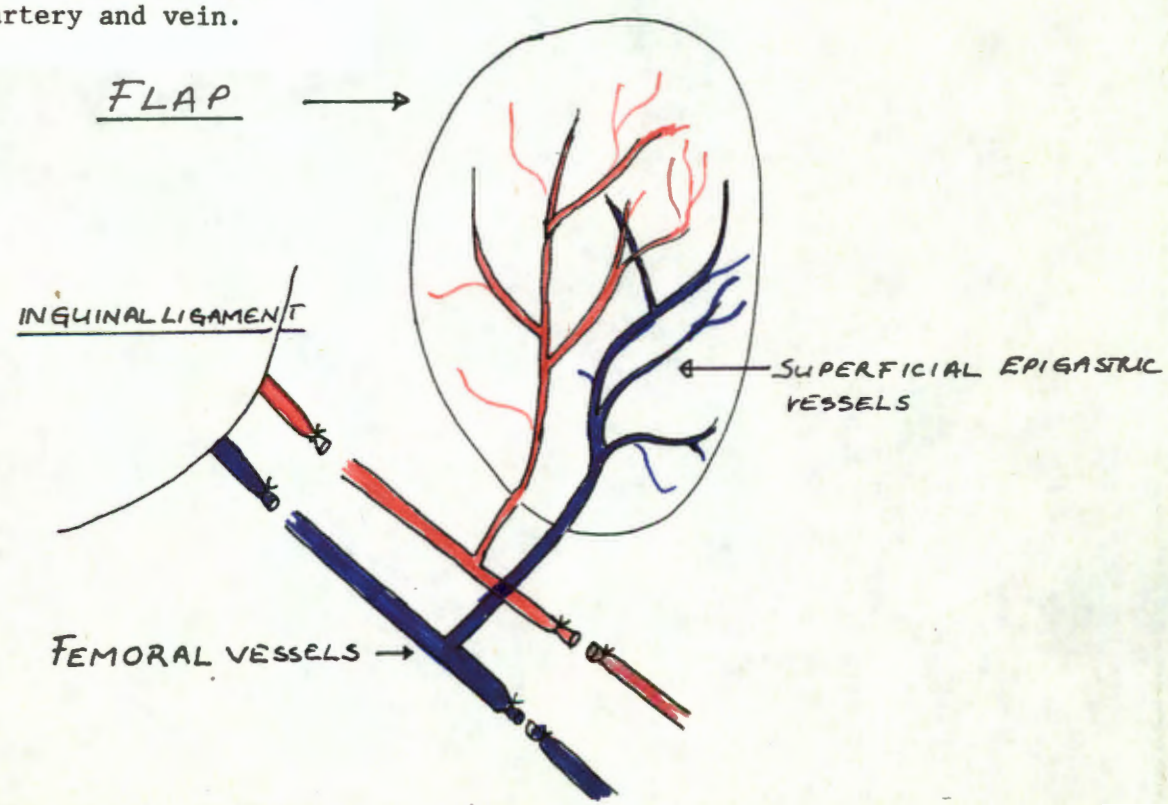


Diagram 1 End-to-side anastomosis of inferior epigastric artery and vein to femoral artery and vein

Diagram 2 Raising the flap including the femoral artery and vein.



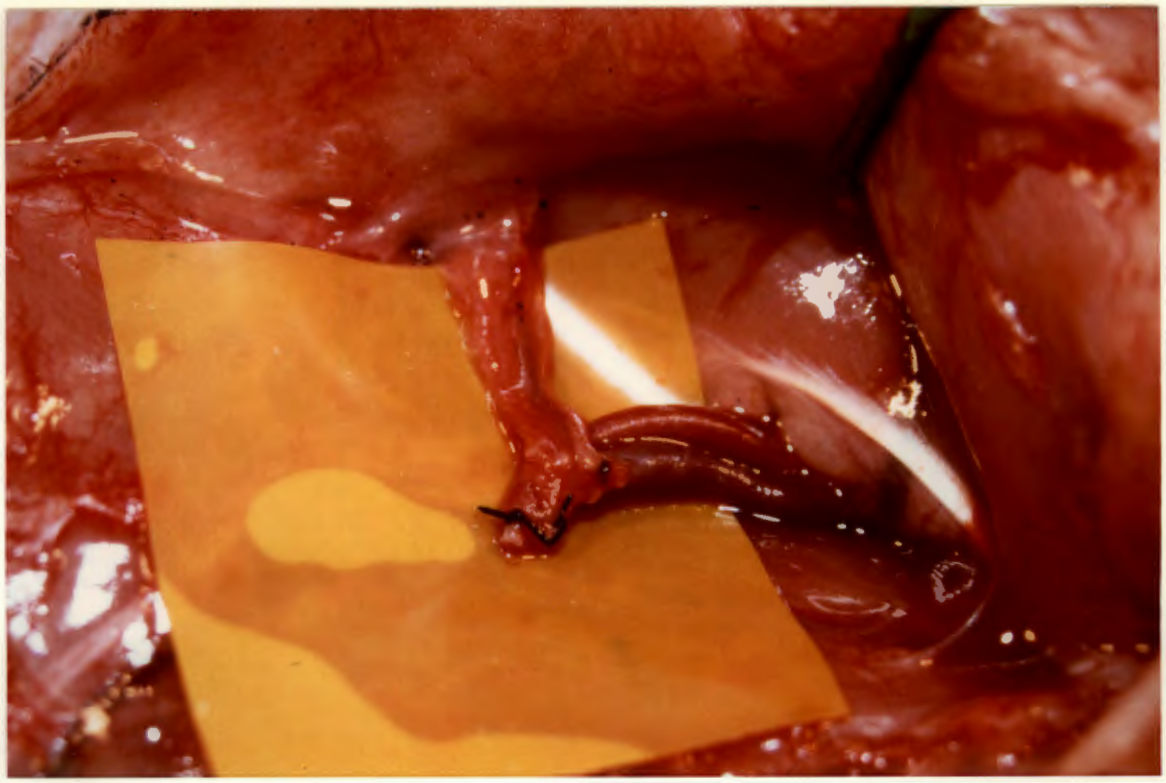


Fig 4 The vascular pedicle of the inguinal flap including the femoral and epigastric vessels

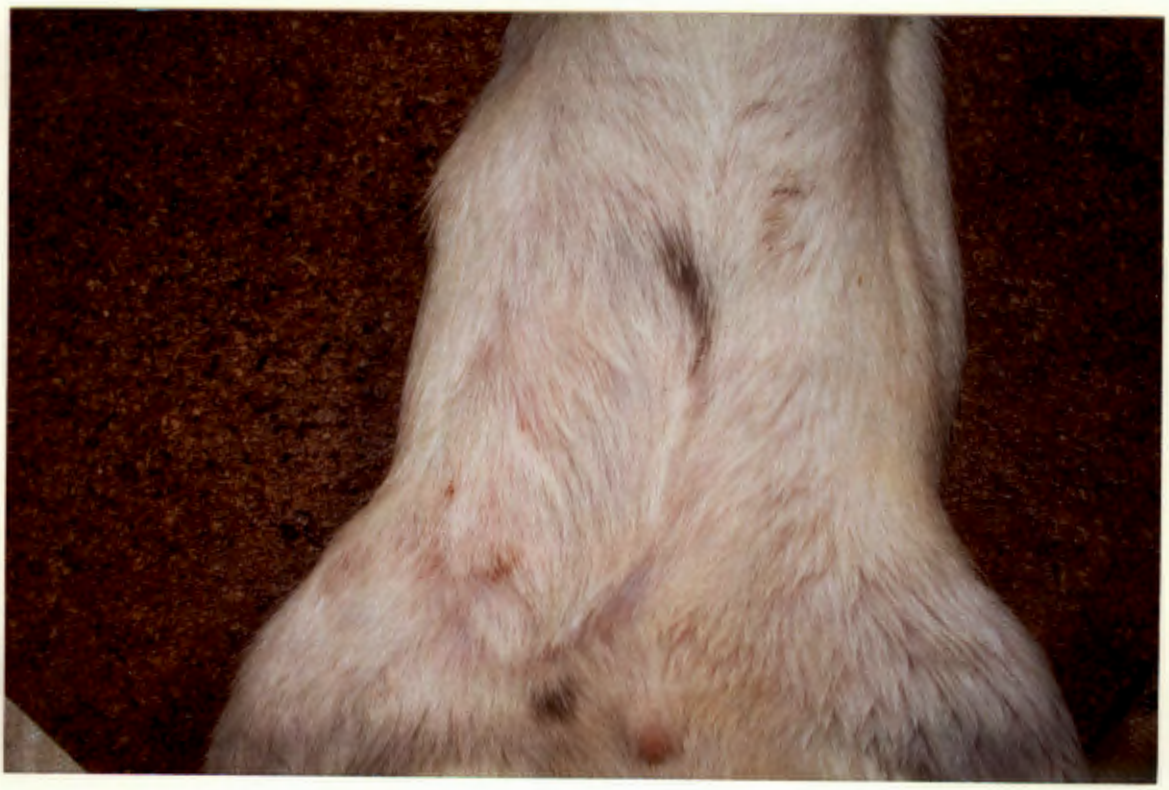


Fig 5 A replanted inguinal flap 2 months postoperatively

epigastric artery and vein and anastomosing both vessels in an end-to-side fashion to the femoral artery and vein (Diagram I). In all three cases, the anastomoses was patent post-operatively and there was good post-op perfusion of the flaps but all flaps became necrotic within four to thirteen days post-operatively. Because of the minimal size of the superficial inferior epigastric artery of which the outer diameter is +- 0,6 mm, this technique was abandoned and in all further experiments a segment of the femoral artery and vein were included into the vascular pedicle in order to achieve an easier and safer anastomosis. Distally the femoral artery and vein were ligated.(Diagram 2 & Fig. 4)

Methods

Ten free groin flap replantations were done.

Results

Five of these survived completely and those flaps which did not survive technical problems with the anastomosis, leading to occlusion, were the reasons for failure. The problems encountered occured mostly in the first flaps of the series; of the last six which were done, five survived. (Fig.5) The rats were sacrificed after 2 months.

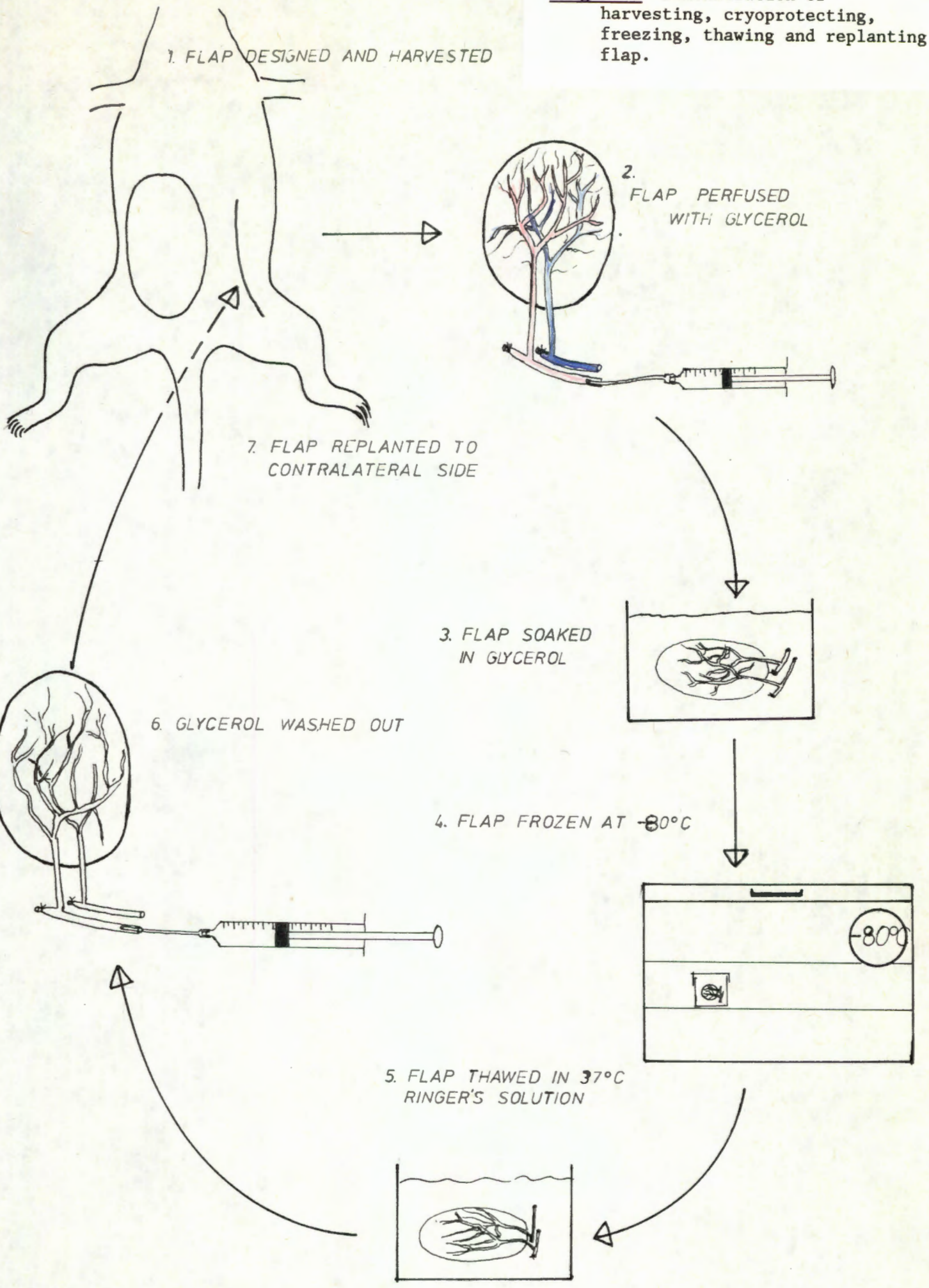
Experiments 3:

Aim - to use a cryopreserved frozen flap in a transplant

Method

An inferior epigastric flap was dissected and raised using the same technique (Diagram 3) as described above including a segment of the femoral artery and vein. A small sized catheter with a diameter of 0,7 mm was inserted into the femoral artery and the blood was then washed

Diagram 3 Demonstration of harvesting, cryoprotecting, freezing, thawing and replanting flap.



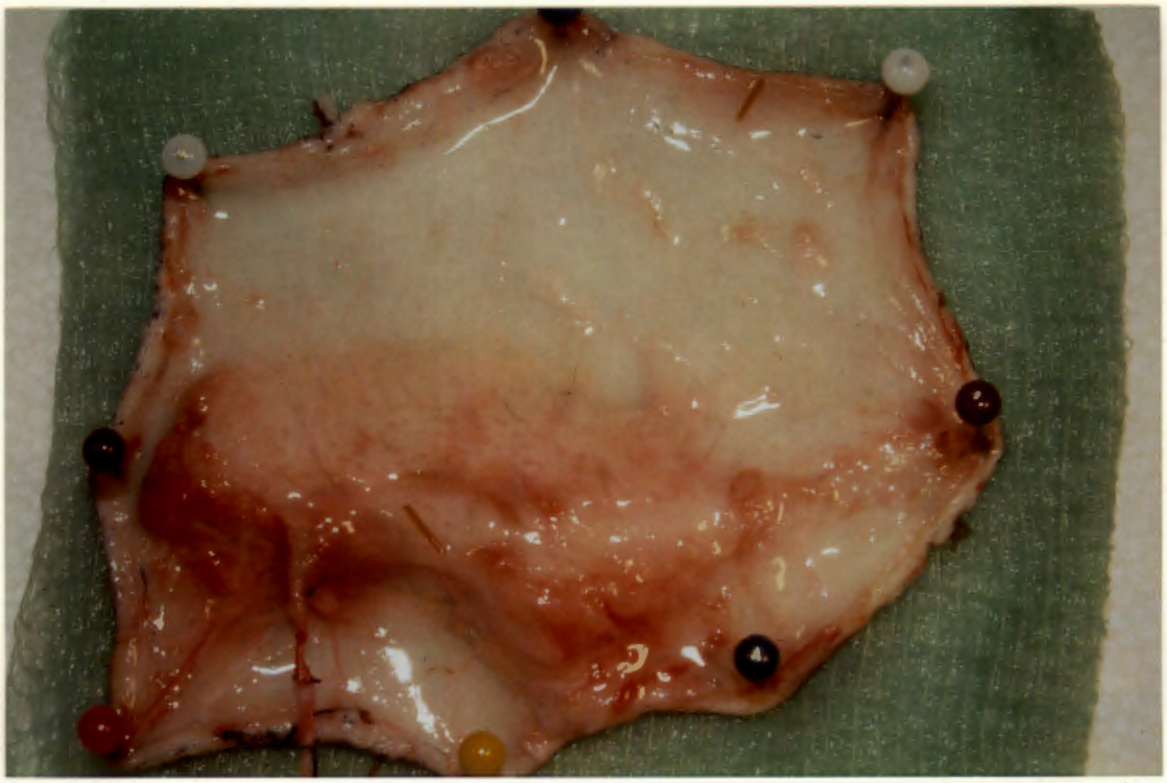


Fig 6 The harvested flap is stretched out and perfused with glycerol. Most of the blood has been washed out at this stage.

out with Ringer's solution adding continually glycerol of 1.6M until eventually only glycerol of 1.6M concentration was injected.(Fig.6) During the procedure, the flap was held outstretched with pins and cooled till +- 4°C (Fig 6). The flap was then put into a small container which was filled with glycerol of 1.6M concentration. This container was then placed into a deep freeze with a temperature of -80°C. The cooling rate was +- 0,5°C per minute. The flaps were frozen for between 1 and 10 days. At the time of replantation, the recipient bed was prepared on the contra-lateral side by excising skin in the left abdominal area and demonstrating the femoral artery and vein. The flap was then thawed by immersing it into a water tank of 37°C resulting in a thawing time of 3-4 minutes. The flap was then washed out with slowly decreasing concentrations of glycerol and eventually pure Ringer's solution. The flap was then replanted applying end-to-end anastomosis to the femoral artery and vein. The rats were heparinised with 20 units of heparin during both the period of raising the flap as well as replanting the flap.

Results

Seventeen flaps were raised for this procedure of which 14 were cryoprotected, frozen and replanted. Three flaps were not frozen because of insufficient perfusion with glycerol. The perfusion after harvesting the flap was a major problem and could not be solved satisfactorily in any of the cases. During the dissection of the vascular pedicle the vessels, specifically the superficial inferior epigastric artery, became so spastic that only a minimal amount of blood or none could pass this vascular segment. Various drugs have been used

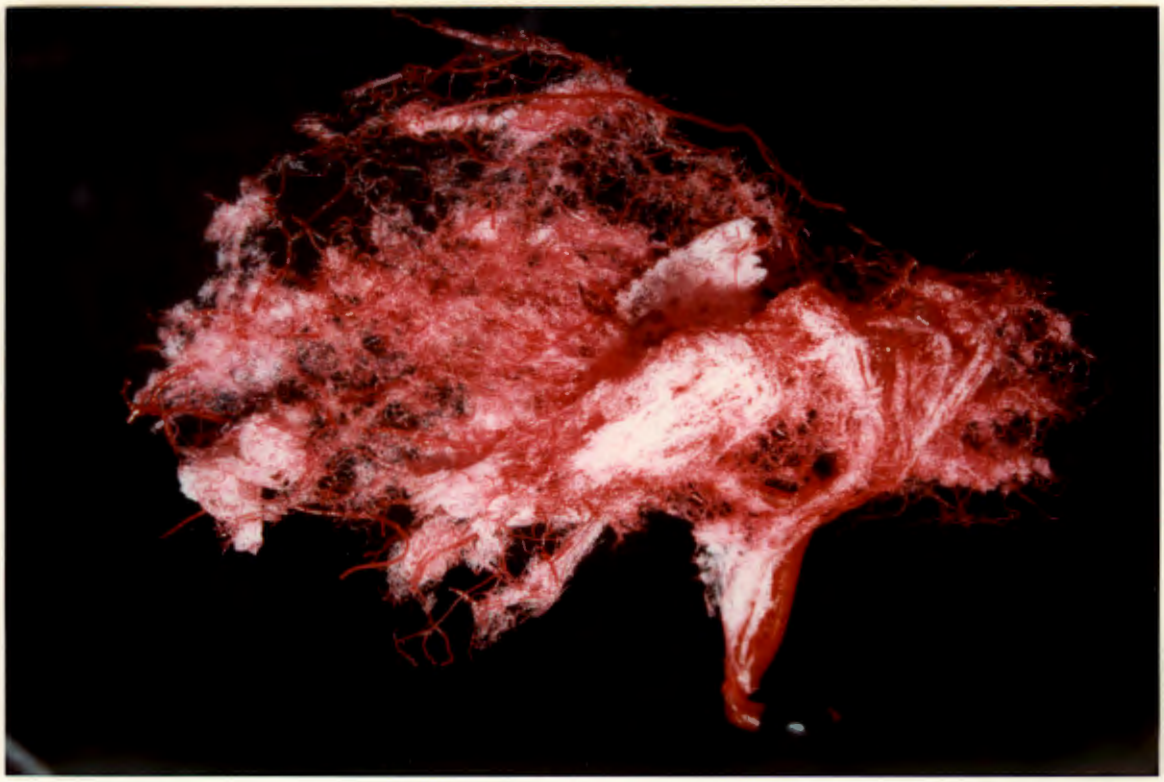


Fig 7 Resin corrosion cast study R inguinal area without raising the skin flap. Note the thickness of the vessels.

Fig 8 Another resin corrosion cast study after the flap has been raised. The diameter of the vessels is significantly smaller.

like Chlorpromazine and Lignocaine but only the latter in a concentration of 10% could solve this problem to some extent. With the external application of 10% Lignocaine the visible part of the vascular tree dilated significantly but it had no effect on the more distal branches within the flap. The most effective way, but by far not satisfactory to overcome this problem was to raise the flap and to dissect the vessels without transsecting them, tacking the flap back in place with a few sutures, covering the area with a moist swab, and then exposing it to a warm light source. A comparative resin corrosion cast study demonstrated the different diameter of the vessels when the resin was injected into the femoral artery without raising the flap and after the flap had been prepared in the way as described above.(Fig.7&8) Only in four flaps was there a good post-operative perfusion with blood. All these rats died in the early post-operative phase. Two of them probably died because of increased blood loss but the other two died for an unknown reason. In these two rats no significant amount of blood was found after their death neither in the dressings nor underneath the flap.

There were no deaths in those rats where the retransplantation of the flap had been unsuccessful. Four flaps became necrotic at an earlier or later stage (Fig 9), two flaps were eaten by the rat itself. One flap had an immediate thrombosis of the vessels. In two flaps the vessels were accidentally injured during the procedure and in one case the rat died from an overdose of ether at the end of the procedure.

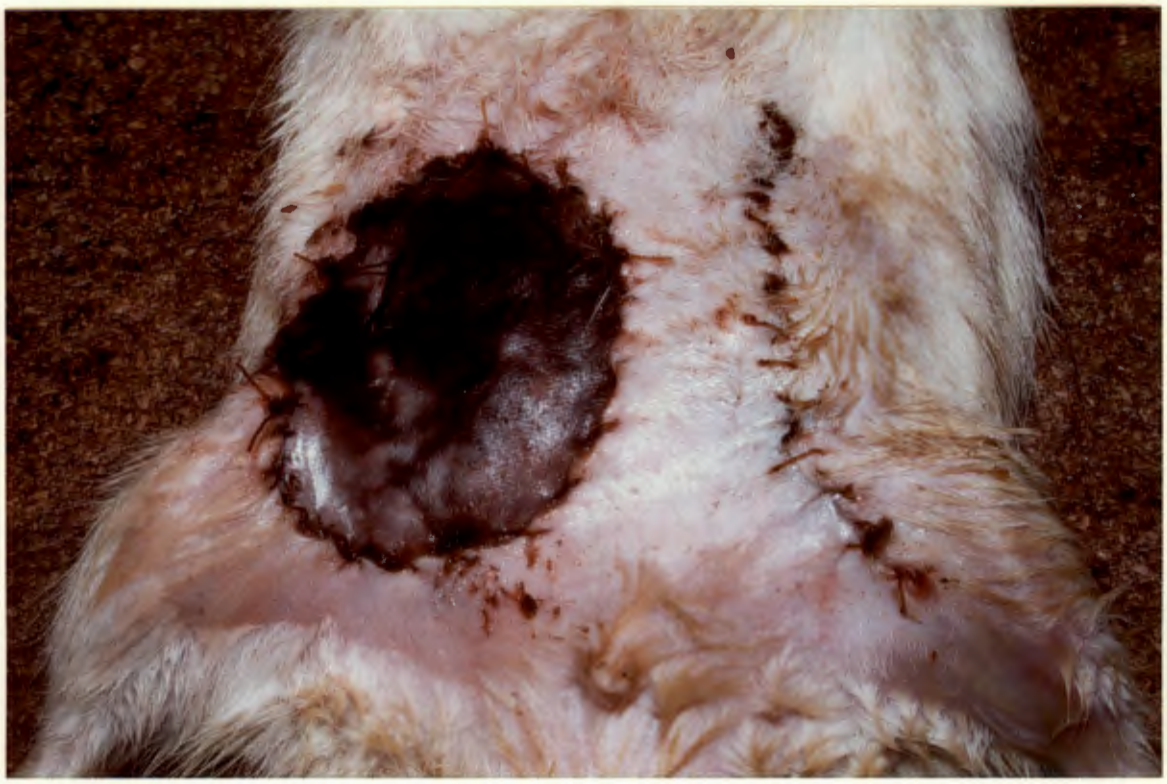


Fig 9 Necrotic transplanted flap

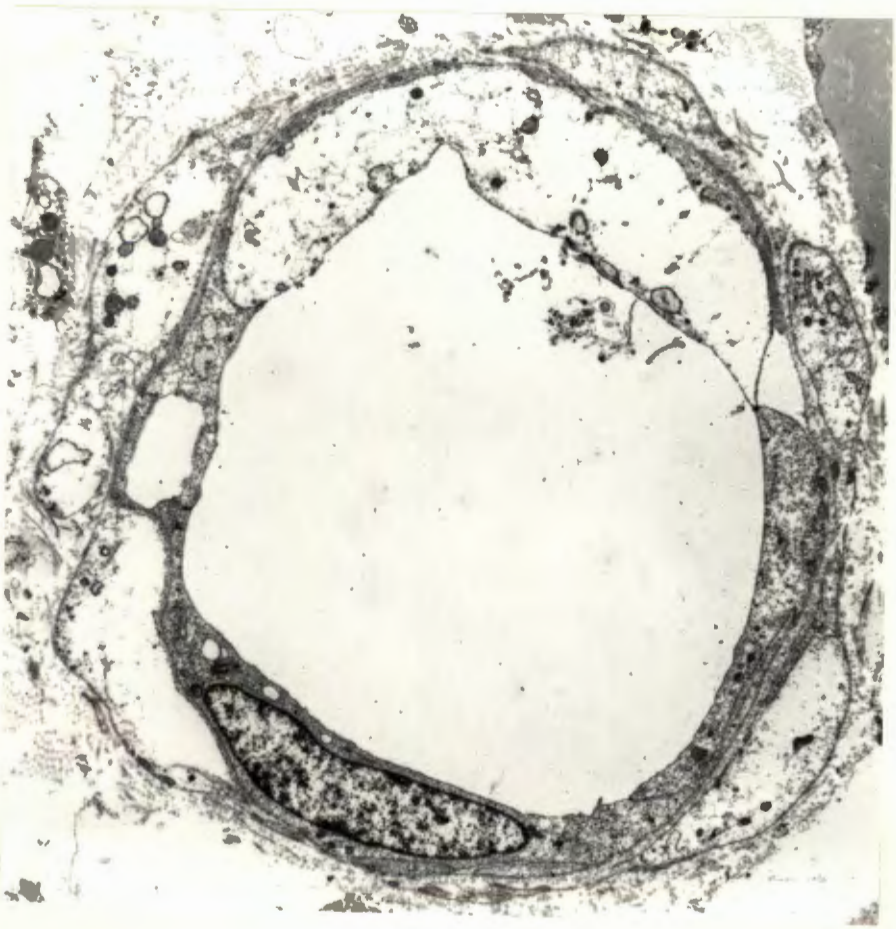


Fig 10 Vascular changes seen in electronmicroscopy

As there was no success in any of the frozen and replanted flaps a transmission electronmicroscopy study was used to show the extent of damage caused by the whole procedure. A groin flap from a rat was harvested in the usual manner. It was perfused in increasing concentrations with glycerol up to 1.6Mol, frozen for three weeks at -80°, thawed in Ringer's solution at 37°C. The glycerol was washed out in decreasing concentrations with Ringer solution and the flap was then immediately prepared for the electronmicroscopy study. The flap was perfused with formalin and four samples were taken including a proximal part of the inferior epigastric artery, two sections from the skin and one from the subcutaneous tissue. The specimen consisting of a fragment of rat skin represented the findings: slightly edamatous rat skin covered by squamous epithelium which show mild focal vacuolar changes in endoplasmic reticulum. More advanced lesions were observed in capillaries of the skin. Some capillaries contained cellular debris in the luminal space and dilated endoplasmic reticulum of endothelial cells. Rarification and deformation of endothelial cytoplasmic processions and flattened endothelial cells were noted. Some capillaries contained relatively preserved or dying endothelial cells. Vacuolar degeneration was seen in pericytes, mild degenerative changes in the peripheral nerve myelin detected.(Fig 10) These changes confirm that the failure of the replanted frozen flaps are not due to a technical problem of the anastomosis but rather due to tissue damage in the flap itself.

DISCUSSION

Many attempts of allotransplantation of composite tissues have been made with various methods of immunosuppression. By parabiosis and splenic tissue grafts from the donor Schwind induced immunological tolerance in non-inbred rats which later received a leg transplanted to their backs. In 75% of the animals this limb survived and grew. Lapchinsky has produced a functioning homotransplanted limb in a dog in which blood had been replaced six days after birth with that of an older dog later serving as the extremity donor. Goldwyn et al in 1966 in a study on canine limb homotransplantion transplanted hind limbs of dogs and used 6-Mercaptopurine and azathioprine. With this treatment the limb rejection was definitely postponed in comparison to a non-treated control group but always occured and was qualitatively different from that observed in the untreated group. Poole et al used immunologic enhancement. In their study rats with successfully enhanced long term surviving kidney grafts subsequently underwent limb transplantation. These rats demonstrated significantly prolonged survival of the limb grafts compared with control rats that underwent limb transplantation only. Black et al felt that the administration of blood before transplantation would be the next logical choice in the attempt to enhance the survival of limb grafts. With this technique they could prolong the limb survival in average to five days to the control group. Salyer (1973) transplanted free vascularised skin flaps in which the recipients were pre-treated with goats' anti-rat lymphocyte serum and the donor animals were treated with whole body irradiation in order to destroy the passenger leucocytes. This resulted in a delay but not in a prevention of the subsequent graft versus host reaction. Doi in 1979

used non-specific immunosuppressive drugs (azathioprine, prednisolone and 6-Mercaptopurine in various concentrations and combinations) and achieved a delay of the rejection by an average of 7 days. Lance et al in 1971 used beagles as experimental animals and a similar regimen of immunosuppression. Their grafted limbs remained intact for 24, 30, 53, 60, 71 and 112 days respectively and then the experiments had to be terminated for various reasons. A major breakthrough was achieved with the introduction of Cyclosporin A. Kim et al (1984), Black et al (1985), Press et al (1986), Hewitt et al (1983) used Cyclosporin A either alone or in combination with prednisolone and concentrations ranging between 5 and 20 mg per day. Indefinite survival was achieved by Black et al with 8mg per kg. a day Daniel et al transplanting limbs and free vascularised flaps in baboons needed very high Cyclosporin A levels for long-term survival of these transplants. Twenty mg per kilogram were injected twice daily to achieve serum levels between 800 and 1000 microgram per millilitre. In the clinical situation a similar regimen would be prohibited by the known side-effects of Cyclosporin A.

In this study a new method of reducing the antigenicity of free vascularised skin flaps was examined. Groin flaps from a rat were harvested, perfused with increasing concentrations of glycerol, frozen at -80^{o}, before replantation thawed in 37^{o} Ringer solution, the glycerol was washed out slowly decreasing the concentration and then transplanted. None of the 17 flaps transplanted this way survived any significant length of time. There are several factors contributing to this failure.

Firstly, the ischaemic time of the flap is considerably longer in the case of perfusion and freezing in comparison to a simple immediate replantation. During the period of perfusion with increasing concentrations of glycerol, the flaps were cooled but on average the procedure of perfusion took approximately one hour. The flap then had still to be kept at a slightly below zero temperature to allow the glycerol to penetrate into the tissues for another two hours. Only then was the flap gradually frozen to -80°. During the thawing procedure again the flap was immersed in Ringers solution of 37° and although the flap was just thawed and not allowed to warm up to 37°, a warm ischaemic time could be expected as the more superficial structures would thaw earlier than the central structures. The cryoprotective agent then had to be slowly washed out and during the time of replantation it was technically impossible to continue the cooling of the flap, thus another warm ischaemic time was added averaging 90 minutes.

Secondly, the procedure of perfusion of the flap with glycerol in itself causes damage to the intima of the vessels. Despite raising the flaps and exposure to a warm light source for three hours prior to injection, no means could be found by which the vessels could be dilated to the original size. As the perfusion following the transsection of the vessels had to be carried out through at least slightly constricted vessels, an increased pressure had to be applied in order to achieve a complete wash out of the blood and a good perfusion of the whole flap. This in itself may have caused damage to the intima, of the vessels which in addition to the trauma of freezing could be the reason of failure of the flaps to survive after the replantation. Harashina and

Buncke did a study of washout solution for microvascular replantation and transplantation. In this study they compared the results of rat hind limbs replanted after thirty minutes at room temperature. In one group no irrigation was used; in a second group the limbs were perfused with heparinised saline for 30 minutes and in a third group perfused with a C3 (C3+Collins solution which is used for kidney perfusion, consists of: sodium 10, potassium 115, magnesium 30, sulphate 30, phosphate 50, chloride 15, bicarbonate 10, glucose 139, mMol/L, Osmolality 300 mosmol/kg) solution for 30 minutes with an average total ischaemic time of one and a half hours. In the second series, the limbs were replanted after 30 minutes at room temperature and 4 hours at 4°C. The groups were as described above, but under these circumstances the average total ischaemic time was five and a half hours. With the immediate replants in the first series, 92% of limbs that were not perfused, 50% of the group perfused with heparinised saline survived and 55% of the group perfused with C3 solution survived. The second series was identical to the first series except that the amputated limbs were subjected to four hours of anoxia. In the first group 73% survived, in the second group 47% survived and in the third group 33% survived. In the discussion the authors comment that the hydrostatic pressure required to flush out the part itself damages the intima of the arteries causing subsequent thrombosis. In most instances, loss of a replant in their series was of gradual onset four to five days after replantation. This delayed loss might be attributed to disturbances in the micro-circulation due to the harmful effects of the washout solution. These disturbances seem to start with multiple areas of micronecrosis and then proceed to a total loss of the leg. They

discuss further that possible causes for the loss of the replants might be the pushing of micro-crystals or micro-organisms into the tissue by perfusion. If the solutions which these authors used caused such considerable damage, how much more damage can be expected if glycerol is added?(Marshall 1982)(See chapter on cryoprotectant agents)

Thirdly cryopreserved vascularised whole organs have never been transplanted successfully and as such the vascularised skin flaps have to be regarded. Only tissues such as skin, cornea, parathyroid and pancreatic islets can be frozen and preserved. The reason why whole organs can not be cryopreserved yet is that each cell type has its own optimum conditions for preservation.(Mazur 1977) This means they require different cooling rates, warming rates different type and concentration of cryoprotectant which is specific for each cell or tissue. It appears that vessels are particularly susceptible to damage caused by the formation of ice in the lumina and particularly the capillaries (Jacobson et al 1984). This was confirmed in this investigation by the electronmicroscopic changes, where advanced lesions were observed in the capillaries of the skin. Some of the capillaries contained cellular debris in the luminar space and dilated endoplasmic reticulum of the endothelial cells. Also a rarification and deformation of endothelial cytoplasmic processes and flattened endothelial cells were noted. This confirms the findings of Jacobson et al in the case of investigated kidneys where the glomeruli and peritubular capillaries were distended which would not be expected once the perfusion pressure had been removed. This vascular distension was not observed either in immersion fixed or perfusion fixed unfrozen organs.

The fact that only four flaps were perfused sufficiently immediately after the transplantation could mean that the damage caused by the perfusion with Ringer solution and glycerol and the freezing, was so severe that the blood immediately clotted when it came into contact with the vessel walls. In those four cases where a sufficient perfusion was observed post-operatively, the rats died within the immediate post-operative period. One reason for this could be that during the perfusion of the flap and the later wash out procedure, glycerol would diffuse into the extracellular space and would only be slowly resorbed from there after the transplantation of the flap was successful. It could well be that the glycerol in these amounts would have a toxic effect on the rats. Although hind limbs were perfused with glycerol in experiment 1 and no systemic problems have been observed, it could be possible that the glycerol with its slow perfusion rate has been washed out before it could enter the interstitial space. Miehl et al report that with replanted dog legs severe acidosis and death can occur once circulation is re-established. This is due to the pyruvate and lactate radicals, and potassium ions which enter the system from the extracellular fluid.

CONCLUSIONS

In this study I investigated the possibility of using freezing of tissues to diminish their immunogenicity. If this were successful, the transplantation of free vascularised skin flaps might be accomplished without the use of immunosuppressives which would be a great advantage. In this short study using perfusion of the flap with glycerol and subsequent slow freezing and thawing, it was observed that very severe damage was caused. Electronmicroscopy suggested that the principal region of damage was the vascular system and it is proposed that the use of glycerol, the long ischaemic time and the effects of freezing were the causes.

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