Experimental and Clinical Experience with Small Composite Tissue Allotransplants and Cryopreservation Techniques

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ABSTRACT

Reconstruction surgery requires imagination, inventiveness, and creation—a quest that entails overcoming obstacles never before encountered. Over the past 25 years, with the advent in microsurgery of free revascularized transfers, it was believed that this fundamental breakthrough would be sufficient to resolve difficulties of whatever nature, and that little else would be necessary. Undoubtedly, the results obtained by using free autotransfers have been so remarkable that it is no longer possible to undertake reconstruction in plastic surgery without fully mastering these techniques. Nevertheless, limitations remain, especially with regard to form and shape, as there are areas where form and function merge. As a result, some clinical cases today continue to induce a sense of powerlessness, as was the case 25 years ago when surgeons were presented with large skin defects that are nowadays treated routinely and with a sense of confidence. The sense of powerlessness today clearly signals that another milestone needs to be reached; we believe this milestone should be allotransplantation. Yet allotransplantation should not remain within the realm of the exceptional; on the contrary, it should become routinely accessible. But in order for it to become so, cryopreservation, a pathway that has so far received little attention, and about which much remains to be learned, should be explored. Accordingly, in this article, we report our experience with xenotransplantation, a mainly clinical procedure in the area of hand surgery.

KEYWORDS: Allotransplantation, composite tissue transfer, tendon, xenotransplantation, cryopreservation, heterotopic revascularization

Surgical reconstruction is not easy. Whereas excellent technical solutions may often be envisaged, some cases remain difficult to treat despite recent technological progress in graft material and advances in microsurgery and island transfers. Thus the surgical shortcomings of years ago have remained largely unchanged. The main aim of reconstruction is to restore

sufficient and morphologically acceptable function; however, function and harmony of form are sometimes so closely associated in hand surgery that the slightest deviation from what is physiologically normal results in poor function. In such cases, allotransplantation may be justified. Since time immemorial, human structures have been used for replacement; however, progress in surgery

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has nevertheless been impeded by religious, social, and technological hurdles. Fortunately, philosophical and religious objections are retreating in the face of technical progress in microsurgery and antirejection medication. Kidney transplants, for example, are performed successfully every day and have saved thousands of lives. Yet they remain a last resort because taking medication on a long-term basis may prove harmful for the patient.

In the past decade, new technical advances and the overall interpretation of various transplant outcomes have progressively led to a change in thinking. Whereas the religious taboos have begun to vanish, ethical taboos remain because immunosuppressors are clearly not risk-free. Allotransplantation has therefore been indicated only in life-threatening circumstances, and the surgical indications of reconstruction to improve quality of life have been considered unfounded. However, in 1989 we launched a program to reconstruct finger flexor systems in patients who had previously undergone traditional procedures and in whom the outcome was unsatisfactory. Our approach, which has been enriched by studies of single tendon allografts stored in various liquids, is based on experience gained by performing numerous islanded vascularized tendon transfers. Better knowledge of collagen antigenicity and progress in microsurgical procedures led us to perform two tendon allotransplants^{1,2} where it was decided to administer immunosuppressive treatment only for a short 6-month period (Fig. 1). The results were very encouraging, and the program would have continued if it had not been for the governmental decision to stop such procedures while awaiting further research on the transmission of HIV by blood transfusion.

The major problem encountered was to first eliminate all possible sources of viral infection. Resolving this problem took time and entailed suffering for some patients because would-be recipients were somewhat frustrated by the diffidence of the general population at that time toward organ donation and transplants. However, the project was spectacularly relaunched in September 1997 by Professor J.M. Dubernard^{3,4} and his team when they undertook the *en bloc* transplantation of an entire hand, thus opening up an entirely new era of reconstructive surgery. Many other cases followed^{5,6} with exceptional results that were previously unthinkable. Such procedures provided very surprising and useful information and they led to the transplant of a hemiface, signaling new hope for patients hitherto suffering from untreatable defects or injuries.

SPARE PARTS PROGRAM

Nevertheless, these cases of entire amputations of limbs or extensive tissue destruction remain rare; routine reconstruction continues to involve partial repair of an existing structure. Our program therefore builds on our

Figure 1 (Top) Allotransplant of human flexion system with digital sheath. (Middle) Sketch of vascular connections and tendon sutures. (Bottom) Tendon allotransplant before placement in recipient (1991).

initial experience but with the objective of transplanting small organs that do not trigger large immune reactions, are difficult to reconstruct with traditional techniques, present frequently in routine practice, and in which prostheses provide little benefit for the patient.

In hand surgery, and especially in finger surgery, reconstruction of the articular surfaces, tendons, and sliding sheaths should aim at restoring a perfect shape and function, even if this involves using partial toe transfers or inserting prosthetic devices that do not offer full rehabilitation. Turning a finger into tendinous, articular, or composite transferable spare parts is therefore an exciting prospect (Fig. 2). Such spare parts could include the following: a flexion sliding unit composed of the flexor tendons, the pulleys, and sliding sheaths; the P1-P2 joint with the extensor; or a more complex composite transfer including the P1-P2 joint and the flexor and extensor systems islanded on the radial or cubital artery with a dorsal vein for venous return. Clearly such a program cannot involve the same requirements as hand or hemiface transplantation because the surgical indications in terms of joint reconstructions, extensor or flexor tendon transfers are frequent (Fig. 3); for example, during interventions whenever it becomes apparent that the outcome may be unsatisfactory. For these reasons, allotransplantation

Figure 2 (Top) Finger dissectible into spare parts. (Bottom) Sketch of all procurable parts (MP = Metacarpo-Phalangeal).

should be available when necessary. However, as we found in our program during the 1990s, even if the protocol is in place, circumstances may be such that allotransplantation is not performed because of the time required to establish it administratively and because of the legislative bureaucracy that comes into play. In addition, procurement sometimes has to be performed at night, which is tiring for the surgeon. Moreover, if the delay is too long, the patient's motivation may weaken.

Figure 3 Various spare parts that may be used. (Top) Flexor unit with flexor tendons, pulleys, and digital sheath vascularized by ulnar pedicle and dorsal veins. (Middle) P1-P2 articulation with extensor tendon system. (Bottom) Unit containing flexor, P1-P2 joint, and extensor system. PIP joint, proximal interphalangeal joint.

Finally, there is the issue of the effective procurement of only one organ per patient if other organs cannot be preserved. This regrettable waste of valuable tissue led to the idea that tissue should be stored under the best possible conditions while awaiting its use. We therefore decided to cryopreserve structures after procurement on brain-dead subjects, the objective being to institute organ banks from which it would be possible to select organs on the basis of size, side tissue compatibility, and other features. Because several batches have now been procured, it will become possible to use the most compatible tissue according to the receiver's requirements.

A CRYOPRESERVED, STORED SPARE PARTS PROGRAM

Why use cryopreservation? The idea arises from several sporadic reports over the past 20 years of fingers being reimplanted successfully after being placed in cold storage for several hours. Although few cases have been documented, circumstantial evidence obtained during informal discussions with other teams seems to suggest that far more cases have actually been treated in this way. Moreover, a few animal studies⁷⁻⁹ have demonstrated the feasibility of revascularizing cryopreserved structures while maintaining their functionality. For example, frozen ovaries subsequently revascularized were shown to produce viable eggs.¹⁰ In addition to these advantages, cryopreservation seems to promote immune tolerance, especially in such structures as the tendons and articulations, which are less subject to immune reactions.

Cryopreservation Protocols

Bone segments such as the iliac crest, long bone, or the head of the femur can be preserved at -80°C by freezing without recourse to a cryopreservative, and there are several techniques for using one or more cryopreservatives in liquid nitrogen at -196°C . These techniques are differentiated on the basis of the cryopreservative used and the speed of cryopreservation. To date, more than 50 cryopreservatives have been devised. They fall into two categories. First, there are the intracellular cryoprotectors such as glycerol, dimethyl sulfoxide (DMSO), methanol, ethanol, ethylene glycol and 1,2-isopropanediol, whose molecular weight is lower than 400. Second, there are the extracellular cryoprotectors like polyvinyl pyrrolidone and hydroxy ethyl with a molecular weight greater than 10,000. Their rates of cryogenization vary according to the tissue under treatment and the survival time required. Recent research into the specific protective gels found in certain fish is opening up new areas of interest.

CRYOPRESERVATION OF VARIOUS TISSUE TYPES

1. CARTILAGINOUS TISSUE

Cryopreserved chondrocytes have a survival of up to 75% when they are kept in a medium comprising Dulbecco's Modification of Eagle's Medium (DMEM), 10% human serum, and 10% DMSO and are cooled with the following protocol: cooling by 10°C/min to -40°C, then by 2°C/min to -60°C, then 5°C/min to -150°C, and final storage at -196°C. Interestingly, the potential for synthesis of type 2 collagen is not affected by warming chondrocytes.¹¹ Cryopreservation of whole cartilaginous tissue yields a $62 \pm 13\%$ survival when DMSO 10% is used with the following protocol: tissue maintained at -4°C for 60 minutes, then at -8°C for 30 minutes, then at -40°C for 10 minutes before final storage at -196°C. However, after transplantation of cryopreserved cartilage, lesions still remained detectable 1 year later, especially in the midlayer.¹²

2. BONE TISSUE

Osteoblasts in segments of iliac crest may survive when preservation is performed with Me₂SO 10% at -80°C for 24 hours. They recover normal activity after warming at 37°C and incubation in a specific medium containing DMEM, Fetal Calf Serum (FCS) 10%, air 95%, and CO₂ 5%.¹³ For periosteal tissue, the best protocol seems to be to use Me₂SO 10% at 10°C/min to -80°C. After thawing, the tissue recovers its vitality and osteogenic potential even after periods of storage exceeding 4 months.¹⁴

3. VASCULAR STRUCTURES

Segments of porcine iliac artery were immersed in M 199 at 4°C for 24 hours, then in a growth medium comprising M 199, fetal bovine serum, penicillin, streptomycin, L-glutamine, endothelial cell growth factor, and DMSO 10%. Next they were frozen at 10°C/min and were kept at -80°C, -145°C, or -196°C for 30 days. Provided that thawing was performed slowly, the structures returned to normal, with a perfectly intact endothelial layer.^{15,16}

4. SKIN

Dermo-epidermal rat allografts were immersed in glycerin 1.4 M at 4°C for 1 hour and then incrementally frozen to -70°C before storage at -196°C. Thawing was performed by immersion in water at 20°C, then rinsing abundantly to eliminate traces of glycerin. After the use of an immunosuppressor (FK506), allografts proved to be successful, without any sign of rejection. The same protocol was used on free abdominal alloflaps with intravascular infusion of glycerin. Survival was totally successful, without any sign of rejection after thawing of the alloflaps, elimination of the cryoprotector, and

microsurgical revascularization under cover of an immunosuppressor (FK506).¹⁷

From the results of studies examining the cryopreservation of various tissue types, it is possible to determine which cryoprotectors are most appropriate to be selected, and which freezing and thawing protocols should be designed for complex settings in which several tissue types are involved. All these advantages motivated us to begin a series of animal experiments. The aim of our xenotransplantation program was to demonstrate the feasibility of revascularizing segments of human fingers after several months of cryopreservation. The experimental protocol in rabbit comprised three phases: (a) procurement of the digital xenotransplant; (b) cryopreservation; and (c) thawing and revascularization.

MATERIALS AND METHODS

1. PROCUREMENT OF XENOTRANSPLANT

The digital segments were obtained in two kinds of surgical situations after informed written consent was obtained. One setting involved segments accidentally amputated and where reimplantation was not indicated. The other involved functionally bothersome segments that had to be amputated as Chase forefinger amputations. Any procurement is dependent upon two conditions being fully respected. First, a bar code must be assigned to each tissue segment for purposes of traceability. Second, the donor must be prepared to undergo a series of serologic tests to determine whether or not the tissue is contaminated. The tests include the following:

- Hepatitis E Virus (HeV) ELISA
- Hepatitis B Surface Antigen (HBs) and anti-Hepatitis B Core (HBc)
- HIV 2 tests ELISA and anti-HIV
- Human T-Lymphotropic Virus (HTLV) test ELISA
- Syphilis

Xenotransplants are handled at low temperature (4°C) to minimize tissue ischemia. After procurement, the dominant collateral artery is located and catheterized so that the necessary infusions for cryopreservation might be performed. Abundant rinsing with direct intra-arterial infusion is performed using CUSTODIOL[®] solution until a clear venous return is obtained. CUSTODIOL[®], (Essential Pharmaceuticals, PA), HTK (pH 7.4 to 7.45 at 4°C [39.2°F]; osmolarity 310 mOsm/kg) is a solution used to infuse organs such as the kidney, liver, pancreas and heart either before or immediately after procurement. Its composition is as follows:

1.000 mL CUSTODIOL[®] contains:
Sodium chloride 0.8766 g

Figure 4 (a) Part of finger amputated with Chase technique owing to functional hindrance. (b) Placement in cryopreservative. (c) Bag identification. (d) Cryopreservation.

Potassium chloride 0.6710 g
 Potassium hydrogen 2-ketoglutarate 0.1842 g
 Magnesium chloride · 6H₂O 0.8132 g
 Histidine HCL · H₂O 3.7733 g
 Histidine 27.9289 g
 Tryptophan 0.4085 g
 Mannitol 5.4651 g
 Calcium chloride · 2H₂O 0.0022 g
 In sterile water for injection
 Anion: CL 50 Mval

Washing is performed with two antibiotics (amphotericin B and vancomycin) to prevent any bacterial contamination. A sample of the washing solution undergoes bacteriologic analysis before the xenograft is placed in a Cryokit[®] (Cryokit, France) dual container. The latter, kept at 4°C, is rapidly transferred to the Blood Institute where the process of cryopreservation proper begins.

2. CRYOPRESERVATION OF THE XENOTRANSPLANT

All handling is done in strictly sterile conditions under a laminar flow hood. The container is opened and the tissue is removed. Intravascular rinsing is again performed with an albumin 4% solution diluted 1 to 3 v/v. The solution is sampled and tested for possible bacteriologic contamination, and a tissue fragment is placed in a tissue bank for purposes of traceability. The cryopreservative is then administered intravenously to impregnate the capillary circuit. The solution used is DMSO prepared as follows: 26.50% albumin 4%, 58.50% isotonic solution, and 15% DMSO. The catheter is then withdrawn from the collateral artery, and the xenotransplant is placed in a special bag into which

DMSO can be injected so that the xenotransplant is fully covered by a bubble-free solution. A sample of the solution is then taken in a tube to act as control during cryogenization. The bag is placed vertically in a computer-programmed freezer where the temperature is gradually decreased at predefined increments to -196°C. The bag is then placed horizontally in a storage chamber where the duration of storage is theoretically 10 years (Fig. 4).

3. THAWING AND REVASCULARIZATION OF XENOTRANSPLANTS¹⁸

First, the receiver site is prepared for revascularization of the xenotransplant. We used a rabbit model with the carotid artery and jugular vein as receiver vessels owing to the similarity between their diameters and those of the digital segment. The xenotransplant and the bag were placed in a bain-marie (technique where a large pan containing hot water can receive smaller pans to heat them or to keep them warm) at 37°C until the ice had finally disappeared. The bag was then opened and the tissue removed. Next it was abundantly rinsed in isotonic solution. The dominant collateral artery was again catheterized, and intravascular washing was performed with albumin 4% to eliminate all traces of DMSO, which would be toxic for the cells at ambient temperature. Washing was then done with heparin to prepare the capillary circuit and to avoid any thrombosis at the moment blood circulation was reestablished. After thawing and washing of the xenotransplant, the tissues recovered their normal texture and suppleness (Fig. 5).

The finger was then fixed into the cervical region, and microsurgical revascularization was performed with

Figure 5 (a) Bags after 6 months of cryopreservation. (b) Warming. (c) Digital segment recovers anatomic shape. (d) Digital segment recovers anatomic shape. (e) Tissue transplant becomes fully supple. (f) Preparation of cervical area in rabbit. (g) Jugular vein. (h) External carotid.

end-to-end anastomosis between the carotid artery and the dominant palmar collateral artery; or, alternatively, between the jugular vein and a dorsal digital vein. Before releasing the vascular clamps, we intravenously administered a fibrinolytic and an anticoagulant. The rabbit was then caged with protective dressing at a constant temperature of 37°C. Continuous intravenous infusion was set up, containing a fibrinolytic (Streptase, ZLB Behring GmbH), an anticoagulant (heparin), and a vasodilator (lidocaine 1%) for 15 days.

RESULTS

All segments were revascularized in a time lapse comparable with that required for auto-reimplant (Fig. 6). Cryopreservation was not a barrier to revascularization. However, this rabbit model is very diffi-

cult to manage, and results after 7 days could not be interpreted, either because of perioperative death due to mobilization of an arterial catheter near the carotid glomus or because of degradation of the revascularized segment by the animal. Postoperative monitoring was performed in strict accordance with the terms of the Helsinki Agreement, and special humane animal care measures were taken in compliance with the NIH "Guide for the Care and Use of Laboratory Animals." Further experiments are planned in animals, but it will be difficult to avoid the above-mentioned problems. These initial findings on tendon allotransplantation, revascularization, and xenotransplantation, together with our expertise in microsurgery, have led us to envisage performing the allotransplantation of cryopreserved human segments in hand surgery.

Figure 6 (a) Rinsing of finger. (b) Insertion in cervical area of rabbit. (c) Vascular sutures. (d) Release of clamps and immediate revascularization.

DISCUSSION

Surgical Indications

The indications will initially entail tendinoarticular reconstructions of burned hands. The sequelae frequently

encountered in such cases are often solved by arthrodeses. However, other types of indications are encountered in traumatology (Fig. 7). The skin problem will be resolved by using autotransfers. Nevertheless, solutions must be found to two problems.

Figure 7 (a) Rear of burned hand. Extensor system no longer present and articulations destroyed. (b) Articular destruction of flexor and extensor system. (c) Articular destruction and flexor system. (d) Rupture of three sublimis and profundus flexors.

Figure 8 (a) Sketch of typical allotransplant. (b) Caution taken with heterotopic connection on cubital pedicle. (c) Caution taken with heterotopic connection on cubital pedicle. (d) With retrograde technique, rotational point of cubital pedicle is more distal, providing more flexibility in transplantation.

1. How can the patient's existing structures be preserved during allotransplantation? To address this issue, we envision a two-step transfer. The first stage will concern a heterotopic implantation at the inferior third of the forearm at the level of the cubital pedicle.¹⁹ Then a few weeks later, the transplant will be positioned with a retrograde island technique at the recipient site. The strategy will be based on the ulnar vascular axis whose distal point of rotation is more useful than the radial point. Our experience with cubital transfers has shown that the suppleness of cubital pedicles makes them more suitable for retrograde transfers and that the procedure is as reliable as using radial transfers (Fig. 8).

2. There is also the issue of the duration of immunosuppressive treatment. In our initial cases, we decided arbitrarily on a period of 6 months of cyclosporine, a decision that proved to be well founded. Since then, other less aggressive therapies have become available, and more will be developed in the future. Moreover, our transplants contain only collagen and no cutaneous tissue; therefore, fewer immunologic problems arise. We believe that, with associated cryopreservation, immunosuppressive treatment for allotransplants could last for a maximum of 1 year while we await new therapies. Therefore, the continued progress of microsurgery and transfers that began in the 1980s is ensured. In sum, although temporarily

impeded by immunologic issues and rivaled by cloning and genetic engineering, allotransplantation coupled with cryopreservation is nevertheless opening a new era in human reconstruction.

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