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Particle-induced circulatory disturbances in transplanted rhesus macaque kidneys

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Abstract We have demonstrated the presence of crystalline particles in University of Wisconsin (UW) solution used to perfuse rhesus monkey kidney transplants. These particles were visible in obstructed blood vessels and associated with immediate graft thrombosis and necrosis. This occurred in 25.7% of kidneys perfused with UW solution and transplanted into young, unsensitized recipients. Two molecular species of crystals were defined by mass spectrometry. The particle size ranged from 3 to greater than 100 μm, with a preponderance of particles less than 25 μm in diameter. Such particles are not removed by 40-μm filtration, but can be removed by centrifugation. With extensive use of UW solution for organ storage, the potential for particle-induced damage in small vessels in both experimental and human transplants needs to be carefully scrutinized.

Keywords Rhesus · Primate · Kidney transplant · 15-Deoxyspergualin · Immunotoxin · UW solution · Particles

Introduction

The transport and storage of organs for transplantation has been greatly advanced by more effective organ preservation fluids [1]. Of the various preservation solutions, University of Wisconsin (UW) solution has generally been found to have superior performance when compared to other formulations for kidney preservation [2]. However, in rat organ transplant models, there are several reports describing post-perfusion problems associated with the presence of particles in UW solution [3, 4, 5]. This phenomenon has also been reported in clinical kidney transplants (reviewed in [4]). The particles have been shown to contain lipid salts derived from the plastics used in manufacturing of the bags. These particles have been associated with reduced, erratic blood flow and cyanosis early after reperfusion of the rat organ. Thus, when particle-laden UW solution is used to perfuse organs, circulation can be disrupted in sites where particles occlude small (3–100 μm) vessels.

In this report, we detail our experience with intraoperative circulatory disturbances in nine kidney allografts in nonhuman primates (NHPs). The occurrences were sporadic and appeared over a 4-year period, during which time UW solution was used in a rhesus monkey kidney transplant protocol related to tolerance induction [6]. These severe circulatory disturbances were observed intraoperatively and had catastrophic effects on the kidney allografts, requiring transplant nephrectomy in less than a week. In addition, three recipients died of disseminated intravascular coagulopathy. All of these kidney grafts were perfused with UW solution and, when examined by microscopy, grossly exhibited crystalline obstruction of small blood vessels. Furthermore, the presence of crystals in UW solution was demonstrated by microscopy and flow cytometry.

Materials and methods

Animal housing and care

Following 60 days of conditioning in quarantine, normal, specific pathogen-free, adolescent (3 kg) male rhesus macaques (*Macacca mulatta*) were maintained in a restricted-access facility. The

animals, reared in the USA and obtained from Covance (Alice, Tex.) and LABS (Yemasse, S.C.), were negative for high-titer anti-diphtheria toxin antibodies to accommodate use of diphtheria-based anti-CD3 immunotoxin as specified [7, 8, 9]. Procedures were performed in accordance with the NIH Guide for the Care and Use of Primates under supervision of the UAB Institutional Animal Care and Use Committee. For routine handling, animals were tranquilized with 10 mg/kg ketamine i.m.

Transplantation and supportive care

Heterotopic kidney allotransplantation was performed according to a method long established in our laboratory [10], with minor modifications [11]. After injecting ketamine for pre-medication, inhalation anesthesia was performed with isoflurane (average 0.9%) in a 50/50 mixture of nitrous oxide and oxygen delivered at 200 ml/min. Prophylactic antibiotic therapy consisted of cephazolin at 25 mg/kg per day. Post-operative analgesia consisted of butorphanol at 0.01 mg/kg for 2 days and aspirin at 81 mg/day for 5 days. Ultrasound analysis, to examine blood flow in the kidney transplants, was performed using an ATL5000 Doppler ultrasound instrument. Any kidney deemed a failed graft was nephrectomized and submitted to pathological examination. All failed grafts presented herein were nephrectomized within the first week after transplantation. Disseminated intravascular coagulation was confirmed grossly by the presence of large intravascular clots in small and large vessels, extending most of the length of the vena cava. Immmunosuppressive therapy in this protocol consisted of anti-CD3ϵ immunotoxin (IT) and 15-deoxyspergualin (DSG) as described. These kidneys were fixed in 10% formalin, and hematoxylin & eosin—as well as PAS-stained sections were obtained for analysis.

UW solutions

UW perfusion fluids were obtained from the Organ Procurement Center at the University of Alabama at Birmingham. Before 2000, the Organ Procurement Center purchased UW solution (ViaSpan) from Du Pont (Wilmington, Del.) and after 2000 from Barr Laboratories (Pomona, N.Y.). The fluids were not out of date and were stored according to the manufacturer's recommendations at 2–6 °C. Multiple lots (~26) of UW solution were used over the time period of this study. At the time at which we initiated the changeover to UW solution from Euro-Collins perfusion fluid (early 1997), it was not uniform policy at our institution to filter UW solution and, thus, filtration with a 40-μm filter did not become part of the protocol.

Tolerance induction protocol

Immmunosuppressive therapy in this protocol consisted of anti-CD3ϵ immunotoxin (IT) and 15-deoxyspergualin (DSG) as described [6]. IT, produced by Neville et al., was administered on day 0 and day 1 or 2 [12]. Among nine recipients with immediate transplant circulatory disturbances, four received i.v. IT (200 μg/kg) as the IgG conjugate of FN18-CRM9 and five received i.v. IT (200 μg/kg) as $F(Ab)_2$ FN18-CRM9. IT was filter-sterilized (0.22 μm) prior to use. DSG (a gift from Novartis, Basel, Switzerland) was a racemic mixture prepared according to a method based on the procedure described by Maeda et al. [13]. As per the protocol, DSG was administered to all recipients at 2.5 mg/kg i.v. daily up to 15 days after transplantation.

Physicochemical analysis of particles in UW solution

After centrifugation (1000×*g* for 10 min) of samples of UW solutions used for organ perfusion, insoluble pellets were noted. The insoluble material was washed three times in deionized water, resuspended in saline, and analyzed by flow cytometry (FCM) using an EPICS Elite Flow Cytometer (Beckman Coulter, Miami, Fla.). Particles were analyzed by forward and side scatter. In addition, particles were analyzed by mass spectroscopy at the University of Alabama at Birmingham shared facility on a Perkin Elmer API-3 electrospray instrument.

Results

Blood flow disturbances at the gross and Doppler ultrasound levels

Over a 4-year period (1997–2001), 9 of 35 kidney transplants (25.7%) were compromised as a conse-

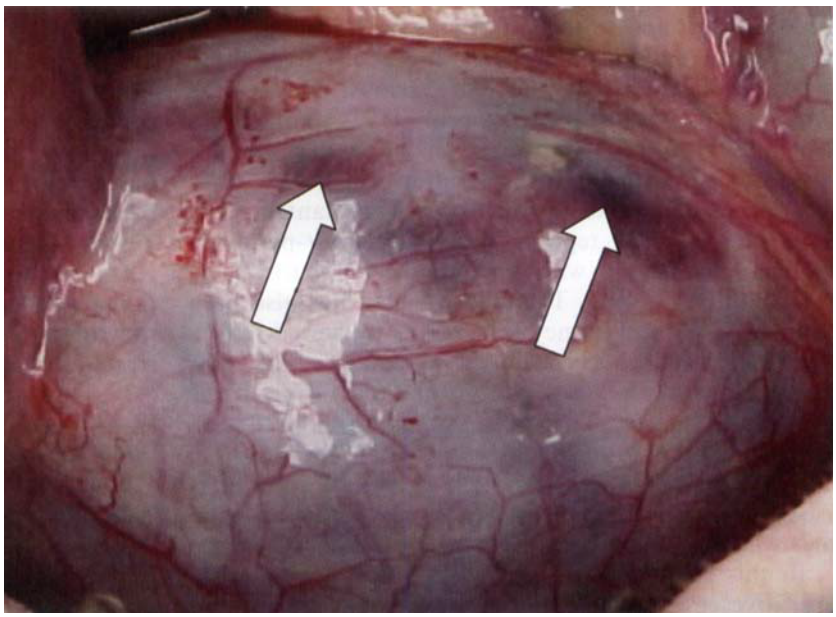

Fig. 1 Photograph of kidney transplant 5 min after reperfusion. The surface has numerous cyanotic areas. *Arrows* identify cyanotic spots

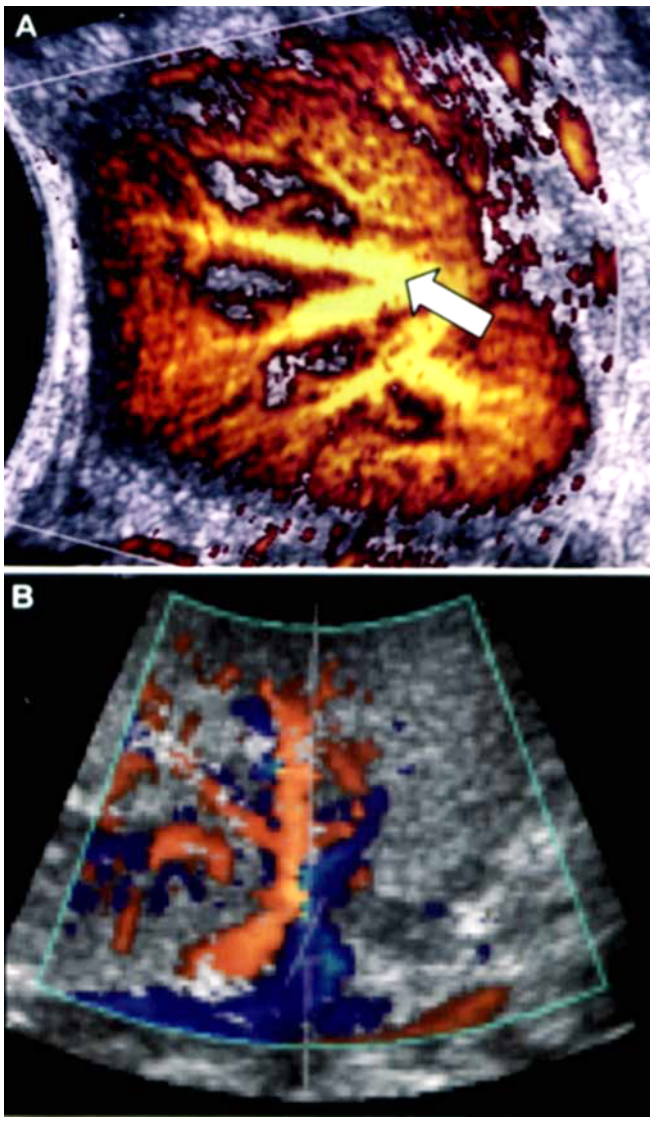

Fig. 2 Doppler ultrasound scan of (**A**) a control rhesus kidney allograft showing normal blood flow with *arrow* identifying robust blood flow; (**B**) obstructed blood flow in a kidney allograft after perfusion with UW solution containing crystalline particles

quence of vascular occlusion with particles from UW solution. The immediate gross manifestations of damage to reperfused kidneys ranged from dispersed cyanotic spots, as seen in Fig. 1, to a uniform cyanotic appearance. Intraoperative testing and Doppler ultrasonography on day 1 confirmed significantly reduced cortical perfusion of affected allografts (Fig. 2A) compared to normal perfusion in healthy allografts (Fig. 2B).

Of the nine affected transplants, all showed massive clots within the kidney graft, in three instances systemically with evidence of fibrin split products. All the grafts were lost in 1–7 days, an observation not previously encountered prior to this time in over 300 rhesus kidney allografts [11]. The transplants discussed here were performed by the same team using the same surgical protocol that previously resulted in a technical failure rate of greater than 3% [11]. In a large series with the IT and DSG tolerance protocol, rejection did not occur before 1 month. In the nine necrotic kidneys perfused with particle-laden UW solution, rejection was ruled out by the absence of mononuclear infiltrate, and the grafts showed massive thrombosis and necrosis. The excessively elevated serum creatinine kinase level on day 1 in this group of recipients ($>11{,}000$ to 79,000 CK U/ml vs <3000 in healthy kidney transplants given IT plus DSG) was consistent with the gross and histopathological findings of graft necrosis.

Presence of particles in kidney allografts

Micrographs (400×) of a representative kidney transplant removed on day 3 reveal vessels containing particles in tangential and cross sections, respectively (Fig. 3A, B). Multiple particles appear to be lodged in a blood vessel, resulting in complete blockade and leading to areas of blood stasis. Notably, the particles survived the strong organic solvents used in the processing of tissue sections (e.g., ethanol, xylenes). Kidneys were examined for the presence of host antibodies by fluorescence microscopy to rule out antibody-mediated rejection. No antibodies were found in multiple kidney sections examined ($n=4$, data not shown).

Isolation of particles from UW solution

Although the particles in UW solution were found to settle in unit gravity, we centrifuged the samples to obtain optimal yield and size distribution of the particles. The micrograph in Fig. 3C, also at 400×, shows various-sized (3 to >100 μm) and regularly shaped crystalline particles present in UW solution. Besides conventional microscopy, we examined these particles by FCM. In the forward-by-side scatter dot-plot shown in Fig. 4, the circular reference region corresponds to approximate diameters of 5–10 μm. The particles are heterodisperse, and most fall into a scatter region in which small lymphocytes and cellular fragments or organelles are usually seen. However, the size distribution of particles depicted in the FCM forward-by-side scatter plot (Fig. 4) reveals a pattern skewed quantitatively toward small particles (<10 μm in diameter) that would not be captured by the recommended 40-μm filter. Such small particles can be removed by 0.22-μm filtration or by centrifugation at 1000×*g*. An analysis by FCM of the UW supernatant from four different bags of UW solution after centrifugation showed no detectable particles (data not shown).

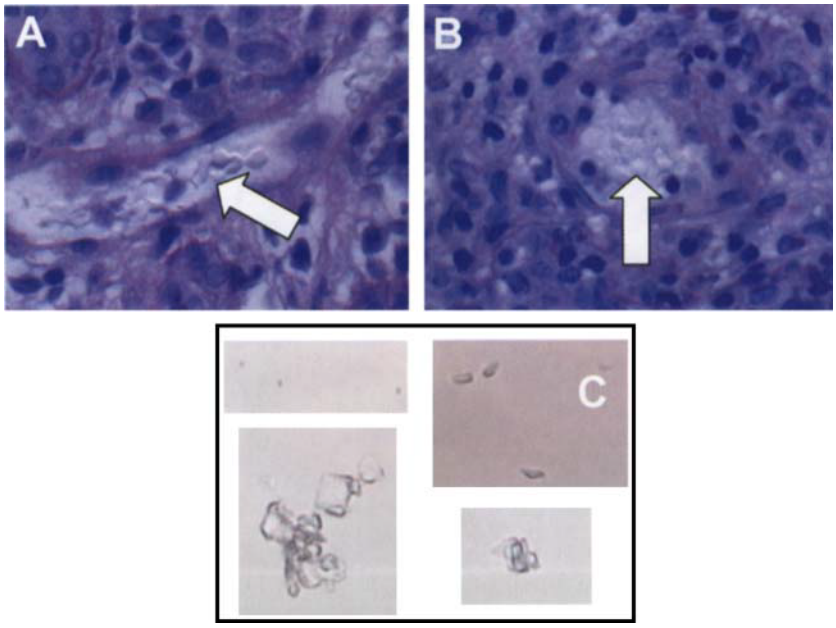

Fig. 3 (**A**) Section of kidney allograft at 400× stained with PAS showing crystalline particles, as indicated by *arrow*. (**B**) Cross-section of same kidney allograft at 400× showing complete blockage of vein as located by *arrow*. (**C**) Photomicrograph of isolated and washed crystalline particles from UW solution at 400×. The four panels depict representative particles of various sizes

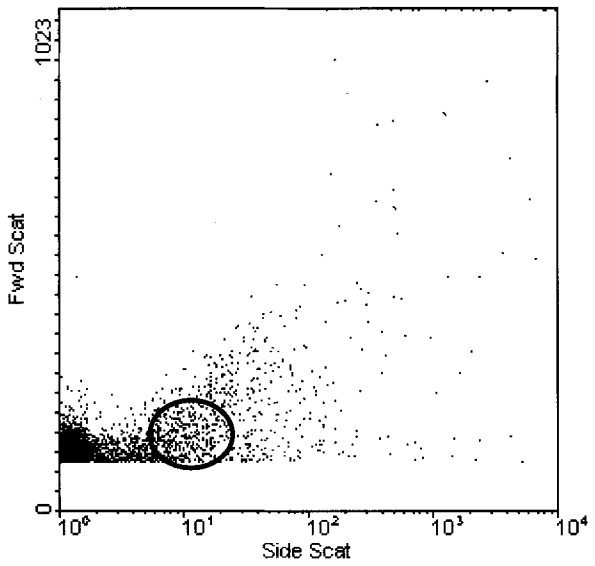

Fig. 4 Flow cytometric dot-plot of forward-by-side laser scatter. The *superimposed oval* corresponds to a region for particles ~5–10 μm in diameter (where lymphocytes localize). The majority of the particles appear to be relatively smaller than this region, with some very large sized particles

Isolated, washed particles were subjected to mass spectroscopic analysis. Mass analysis for the particles indicated prominent masses at 358 and 504, the identity of which remain unknown.

Discussion

These catastrophic events observed in nine recipients were not seen previously in over 300 rhesus monkey kidney transplants performed by the same group using similar and related immunosuppressive protocols [11]. Over a 20-year period, these unusual vascular abnormalities were not seen in our rhesus transplants, most of which were not perfused with UW solution. The circumstances of unusual pathology revealing widespread small vessel obstruction, thrombosis, and crystals along with the reports of crystals present in UW solution by other groups, promoted us to retrospectively study the problem in detail. It should be noted that, due to ethical and cost constraints, we did not feel injecting particle-laden UW solution into a transplanted nonhuman primate kidney justified to provide direct proof.

The clinical picture, intraoperative gross observations, and serial Doppler flow studies were most compatible with hyperacute rejection. However, the recipient-donor combinations were young naïve males without serological reactivity to mouse IgG or diphtheria toxin, both components of IT, or pre-existing alloreactivity. Thus, immune complexes and hyperacute rejection were ruled out as causes of the relatively immediate graft failure. Due to the nature and novelty of the immunosuppressive agents used in these studies, the problem presented a matrix of possibilities to explain the immediate graft failures. The episodic nature of the immediate failures, and the multiple batches of IT and DSG made it time-consuming to identify the causative agent(s). In our previous highly successful monkey tolerance series with IT and DSG, in which Euro-Collins was used, we never observed cyanosis, PNF, or immediate coagulation of kidney grafts. This suggested the problem was not due to the effects of the IT plus DSG tolerance protocol.

We demonstrated crystalline particles of 3 to greater than 100 μm in size in the UW solutions used for perfusion, and similar crystalline masses were found in the small vessels of the failed kidney allografts. As noted earlier, the UW solution was not filtered. In retrospect, despite a recommendation to filter UW solution with a 40-μm filter [14], it is uncertain that this would have been completely effective for our monkeys because the preponderance of particles we observed were under 40 μm. The recommended filter was found to be 99% effective only for particles of 25 μm and larger, with 44% of 5–10-μm and 29% of 10–25-μm particles passing the filter [15]. In contrast, the use of 0.2-μm filtration as performed by Walcher et al. [3] proved effective for removing crystal-induced circulatory disturbances in the rat.

The UW solution-associated circulatory disturbances observed in rhesus monkey kidney allografts are similar to those described by Walcher et al. [3]. These investigators reported grossly evident occlusion of vessels in rat livers perfused with UW solution and showed cyanotic spots on the surface of the liver within minutes of reperfusion. The UW solution used by Walcher et al. also exhibited insoluble particles that were crystalline in appearance and were of sufficient size and number to occlude small vessels and capillaries (20–150 μm).

A recent report by Tullius et al. noted that storage temperatures less than/equal to -3 ± 0.5 °C exacerbated particle generation in UW solution [5]. Under these conditions, insoluble, visible particles were substantial, with a yield estimated to be 700 mg/l of UW solution. In a series of confirmatory experiments, aortic perfusion of rats was performed with UW solution. Depending on the anatomical site and organ, the incidence of blocked vessels containing particles ranged from 10–35%. This occurred with only 20 ml of unfiltered UW solution per animal (S.G. Tullius, personal communication).

In a comparative study of several preservation fluids, Fischer and Jeschkeit also noted circulatory problems following perfusion of rat heart transplants with UW solution [4]. While the other fluids examined (Euro-Collins and Bretschneider's HTK) allowed for the functional and metabolic recovery of preserved hearts stored for 18 h, they observed no such recovery when using unfiltered UW solution. The hearts perfused with UW solution displayed a "spotty" appearance, and these authors subsequently identified crystalline particles in UW solution, with aggregates ranging to 100 μm and larger. In contrast to our findings of two prominent masses at 358 and 504, Walcher et al. identified the source of particles as lipid salts of stearic (mass 284.5) and palmitic acids (mass 256.4) derived from the plastic bags containing the UW solution [3]. Tullius et al. also found crystalline particles of composition other than palmitic and stearic acid in UW solution [5]. The particles they isolated featured multiple masses by mass spectrometry, with only adenosine (mass 267) being identified (A. Lun, personal communication). Thus, the chemical nature of the crystals in UW solution appears to be variable, and our particles seem to be different in terms of mass than those previously reported.

The manufacturer of UW solution (Du Pont Pharma, Bad Homburg, Germany) had acknowledged the presence of particles in ViaSpan (UW), but reports of the ViaSpan particle problem were only distributed to transplant centers in Germany, according to Fischer and Jeschkeit [4]. Thus, there may have been limited awareness of the problem of the potential for crystalline particles in UW solution. This presence can compromise the success of experiments in small animals. For rhesus monkey transplant tolerance experiments, a 25.7% primary nonfunction failure rate is financially and ethically insupportable. Prior to employing UW solution, we had used Euro-Collins perfusion fluid for more than 10 years without incident. For these reasons, our laboratory has switched to Euro-Collins perfusion without complication. It should be reiterated that filtration has been recommended as a means to remedy the problem of crystalline particles in UW solution. While this approach is straightforward and reliable, it poses an additional unknown in that the removal of particles (up to 700 mg/ml [5]) could also change the composition of UW solution.

The presence of crystalline particles in UW solution presents a dilemma. UW solution is arguably the current standard organ preservation medium, yet the variable presence of particles poses an unknown risk for small-caliber vessels of the perfused organ, which is already compromised by ischemia-reperfusion. Even if a 40-μm filter is employed, the residual (yet likely quantitatively larger) fraction of smaller particles (< 25 μm) could inflict damage to the microvasculature which may not be immediately appreciated. Given the known relationship of tissue damage to chronic rejection or chronic allograft nephropathy, it is possible that these crystals could unknowingly contribute to long-term failures, especially in small organs, such as found in nonhuman primates. Thus, the prospect of quantitative removal of all sized particles by the simple and inexpensive procedure of centrifugation should offer a solution worthy of serious consideration.

In summary, our report confirms the presence of crystalline particles in unfiltered UW solution. Our findings extend the prior experience with damaging UW solution particles from rat organ perfusion [3, 4, 5] to primates. This associates with vascular occlusion and thrombosis in renal allografts in young rhesus monkeys. Given the extensive use of UW solution for organ storage, the potential for particle-induced stress and focal damage at the microanatomical level is most likely to occur in pediatric and infant transplants, known to be

especially prone to thrombosis [16]. The potential for preservation solution particle-induced coagulation in small vessels in both experimental and human transplants needs to be carefully scrutinized.

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References

1. St Peter SD, Imber CJ, Friend PJ. Liver and kidney preservation by perfusion. Lancet 2002; 359: 604
2. Silva LA, Bertels IM, Paula FS, Falkenstein D, Figueiredo JF. The effect of Collins, Euro-Collins, and University of Wisconsin solutions on the function of isolated proximal straight tubules. Transplant Proc 2002; 34: 1108
3. Walcher F, Marzi I, Schafer W, Flecks U, Larsen R. Undissolved particles in UW solution cause microcirculatory disturbances after liver transplantation in the rat. Transpl Int 1995; 8: 161
4. Fischer JH, Jeschkeit S. Effectivity of freshly prepared or refreshed solutions for heart preservation versus commercial Euro-Collins, Bretschneider's HTK or University of Wisconsin solution. Transplantation 1995; 59: 1259
5. Tullius SG, Filatenkow A, Horch D, et al. Accumulation of crystal deposits in abdominal organs following perfusion with defrosted University of Wisconsin solutions. Am J Transplant 2002; 2: 627
6. Thomas JM, Hubbard WJ, Sooudi SK, Thomas FT. STEALTH matters: a novel paradigm of durable primate allograft tolerance. Immunol Rev 2001; 183: 223
7. Thomas JM, Neville DM, Contreras JL, et al. Preclinical studies of allograft tolerance in rhesus monkeys: a novel anti-CD3-immunotoxin given peritransplant with donor bone marrow induces operational tolerance to kidney allografts. Transplantation 1997; 64: 124
8. Contreras JL, Wang PX, Eckhoff DE, et al. Peritransplant tolerance induction with anti-CD3-immunotoxin: a matter of proinflammatory cytokine control. Transplantation 1998; 65: 1159
9. Thomas JM, Contreras JL, Jiang XL, et al. Peritransplant tolerance induction in macaques: early events reflecting the unique synergy between immunotoxin and deoxyspergualin. Transplantation 1999; 68: 1660
10. Thomas FT, Carver FM, Foil B, et al. Long-term incompatible kidney survival in outbred higher primates without chronic immunosuppression. Ann Surg 1983; 198: 370
11. Eckhoff DE, Haisch CE, Contreras JL, et al. Experience with renal transplantation in the nonhuman primate: a modified ureteroneocystostomy to prevent urologic complications. J Invest Surg 2000; 13: 247
12. Neville DM Jr, Scharff J, Srinivasachar K. In vivo T-cell ablation by a holoimmunotoxin directed at human CD3. Proc Natl Acad Sci U S A 1992; 89: 2585
13. Maeda K, Umeda Y, Saino T. Synthesis and background chemistry of 15-deoxyspergualin. Ann N Y Acad Sci 1993; 685: 123
14. Directions and preparation for use of ViaSpan (Belzer UW), 2002. (http://viaspan.com/viaspan.pdf)
15. Mueller BJ, Guessford SA, Chen TT, et al. Effect of inline filtration on ViaSpan cold-storage solution. Am J Health Syst Pharm 1998; 55: 266
16. Strey C, Grotz W, Mutz C, et al. Graft survival and graft function of pediatric en bloc kidneys in para-aortal position. Transplantation 2002; 73: 1095