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Establishment of a Standardized Rodent Model for Composite Tissue Allotransplantation (CTA): Improvement of Surgical Techniques, Anaesthesia and Monitoring of the Graft

To establish a valid and reproducible model of allogeneic vascularized limb transplantation different techniques of anaesthesia, transplant surgery, vascular anastomoses, ischemia time and the influence of common immunosuppressive therapy in an optimized rat model were analysed.
Transplantation of the forelimb employed Cuff Anastomosis Technique with a mean Warm Ischemic Time (WIT) of 30 minutes in IM anaesthesia with spontaneous breathing appeared to be the best model. To analyze the influence of immunosuppression, animals were evaluated in five groups: two isogeneic (with / without immunosuppression) and three allogeneic (no immunosuppression, Cyclosporin A and Tacrolimus monotherapy). Experiments were stopped when graft Functional Capillary Density (FCD) declined by 30% as a sign of microthrombosis of small vessels or at day 24. FCD was controlled by Cytoscan® technique at regular intervals. Allograft and Isograft transplant recipients were clinically evaluated daily for signs of rejection. Additionally, levels of Immunosuppressives and Interleukin 6 (IL-6) were analyzed and Cyto-Immunological Monitoring (CIM) was performed. Specimens were taken for histopathologic evaluation at completion.
All Allograft and Isograft transplant recipients without immunosuppression had to be terminated before day 24. Animals of the Isogeneic group with Immunosuppression did not have a significant reduction of FCD at day 24. This illustrates that in all groups with the exception of the Isografts with immunosuppression a graft rejection appeared. However, graft survival time was significantly different between the groups. Differences between immunosuppressive regimes were not found. Levels of immunosuppressants remained within the defined range. No correlation between rejection, CIM and IL-6- level was found. From this data we may conclude that CIM and IL-6 levels are not effective in monitoring early rejection in composite tissue allografts in rats.

Key words: composite tissue allotransplantation, standardized rodent model, anaesthesia, surgical technique

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Standardisiertes Modell für Composite Tissue Transplantationen beim Nager: Anästhesie, Operationstechnik und Transplant-Monitoring

Die Zielsetzung dieser Arbeit war die Entwicklung eines zuverlässigen und reproduzierbaren Nager-Modells für die allogene vaskularisierte Transplantation von Gliedmaßen. Dabei sollten verschiedene Aspekte zur Narkose, Operationstechnik und Ischämiezeit und Immunsuppression untersucht und optimiert werden.
Als günstigstes Modell erwies sich die Transplantation des Vorderlaufs der Ratte mittels der Cuff-Anastomosen-Technik mit einer durchschnittlichen warmen Ischämiezeit von 30 Minuten unter intramuskulärer Anästhesie und Spontanatmung. Um den Einfluss der Immunsuppression zu evaluieren, wurden 5 Versuchsgruppen gewählt: 2 isogene (mit/ohne Immunsuppression) und 3 allogene (ohne Immunsuppression/CyA-/FK506-Monotherapie). Die Versuche wurden beendet, sobald die funktionelle Kapillar-Dichte (FCD) der Transplantate als Zeichen einer Mikrothrombosierung der kleinen Gefäße um 30% abfiel, oder aber am Tag 24.
Die FCD wurde mittels Cytoscan in definierten zeitlichen Intervallen bestimmt. Isogene und allogene Transplantate wurden täglich auf klinische Abstoßungszeichen hin untersucht. Darüber hinaus wurden die Spiegel der Immunsuppressiva und Interleukin-6 (IL-6) bestimmt bei gleichzeitiger Durchführung eines Cyto-immunologischen Monitorings (CIM). Am Versuchsende wurden Gewebeproben aus den Transplantaten zur histopathologischen Untersuchung abgegeben.
Alle allogenen und jene isogenen Transplantatempfänger ohne Immunsuppression mussten vor dem 24. Tag euthanasiert werden. Dagegen zeigten die Tiere aus der isogenen Transplantatgruppe mit Immunsuppression keinen signifikanten Rückgang der FCD am Tag 24. Somit fand in allen Versuchsgruppen außerhalb der Isotransplantationen unter Immunsuppression eine Abstoßung statt. Dessen ungeachtet erwies sich die Transplantatüberlebenszeit zwischen den einzelnen Versuchsgruppen als höchst unterschiedlich. Unterschiede zwischen den einzelnen immunsuppressiven Medikamenten dagegen fanden sich nicht. Die Medikamentenspiegel der Immunsuppressiva bewegten sich stets innerhalb des vorgesehenen Schwankungsbereichs. Wir fanden keine Korrelation zwischen dem Zeitpunkt der Abstoßung, dem CIM und den IL-6-Spiegeln.
Aus diesen Ergebnissen müssen als Schlussfolgerung gezogen werden, dass das CIM und IL-6 keine zuverlässigen Parameter für ein akutes Abstoßungsmonitoring bei Composite Tissue Allotransplantationen am Nagermodell darstellen.

Schlüsselwörter: *Composite Tissue Allotransplantation, Nagermodell, Anästhesie, Operationstechnik*

Introduction

The reconstruction of extensive bone defects and destroyed joints following high-velocity trauma, infection or tumour is still a challenge in orthopaedic surgery. Recent methods such as total joint arthroplasty and biological transplants are subject to inherent restrictions. Autologous bone and cartilage grafts are limited in size and show disadvantages in biomechanics, comparable to allografts. Total joint arthroplasty has the risk of aseptic and septic loosening. In cases of a deficient extensor apparatus even total knee arthroplasty is no solution. Amputation of a limb with rehabilitation is effective, but is associated with increased risk of limp, dependence of walking aids, anxiety and loss of independence, e.g. the inability to drive. In a young patient, the medical condition is closely associated with psychological issues, loss of social status and income [4, 12, 13].
As an alternative, the concept of allogeneic vascularized bone and joint transplantation has been discussed for several years. Allogeneic vascularized transplantation of human femoral diaphyses with postoperative immunosuppression was published first by our group in 1995 [14]. One year later, we reported the first transplantation of a human knee joint in a similar technique [3, 15, 16, 20].
Young patients with bone malignancies would benefit greatly from the developments in bone and joint transplantation and multimodal combined therapy. With regard to current immunosuppressive therapy standards, transplantation in patients with malignancies is not viable. Immunosuppressive therapy for transplantation would potentially cause local tumour recurrence and a scattering of microfiliae, thus a progression to further malignant disease. However, there are combined transplant/tumour animal models which suggest that the combination of immunosuppression and chemotherapy has beneficial effects both on graft survival and tumour suppression [38, 39]. This may lead to a new therapeutic option: Is it possible to combine neo-adjuvantive chemotherapy and immunosuppression? Do cytostatic regimens have an immunosuppressive effect in a recipient of an allogeneic vascularized composite tissue allograft?

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The aim of this study was to establish a reproducible small animal model to evaluate the effect of standard immunosuppressive regimes in Composite Tissue Allotransplantation (CTA).

Material and Methods

Preliminary Trial

Part 1: Evaluation of Anaesthesia and Surgical technique. Male Lewis (L) rats were used as donors and recipients.
Part 2: Evaluation of Immunosuppressive Regimens. Male Lewis (L) rats served as donors and Brown-Norway (BN) rats as recipients. Control groups were BN rats as donors and recipients. Weights were between 250g and 270g in both groups.
Animals used were humanely treated in accordance with the National Institute of Health "Guide for the Care and Use of Laboratory Animals". Animals were caged individually under standard environmental conditions. They were maintained on commercial available balanced rodent food and unrestricted access to water.

Following groups were evaluated:
– Group O: preliminary trials / n = 44
– Group A: donor: BN; recipient BN, no immunosuppression (isogeneic) / n = 6
– Group B: donor: BN; recipient: BN, with Tacrolimus (isogeneic) / n = 6
– Group C: donor: L ; recipient: BN, no immunosuppression (allogeneic) / n = 8
– Group D: donor: L; recipient: BN with Tacrolimus (allogeneic) / n = 6
– Group E: donor: L; recipient: BN with Cyclosporin A (CyA) (allogeneic) / n = 7

Anaesthesia

General anaesthesia was used in all animals. Two methods of inhalative anaesthesia (halothane-nitrous oxide or ether) and one IM-anaesthesia (Fentanyl, Medetomidin and Midazolam) were compared.
In 20 animals Inhalation anaesthesia with Halothane (0.8 – 1.5%), Nitrous Oxide (2L) and Oxygen (1L) was used. Anaesthesia was introduced in a glass containment with 4% halothane, 2L Nitrous Oxide and 1L Oxygen. Suppression of the interdigital reflex indicated a sufficient level of anaesthesia. Animals were intubated and ventilated using a small animal ventilator (AZV 0.5ml, AF 30/min).
Inhalational Ether Anaesthesia was used (n=4) with spontaneous breathing. Anaesthesia was started in ether containment and oxygen was delivered continuously.
Intramuscular (IM) Anaesthesia was commenced with an initial sedation of the animals (n=20) using Medetomidin 0.15mg/kg, Midazolam 2mg/kg, Fentanyl 0.005mg/kg into a thigh muscle and repeated, if necessary. Spontaneous breathing was continued and supplemented with oxygen. Post surgery, IM-anaesthesia was reversed with Antipamezol (0.375mg/kg), Flumazenil (0.1mg/kg) and Naloxon (0.06mg/kg).
All animals were sacrificed by cardiac paracentesis and exsanguination at completion.

Surgical Technique

Limb transplantation was performed according to a modified technique described by Yaremckuk et al [36]. Five knee joints, one hind limb, 13 forepaws with a long vascular pedicle and 25 fore limbs were grafted heterotopically. A ventral approach was used with anastomosis to the carotid artery and jugular vein.
The donor limb was dissected with the vascular pedicle as proximally as possible. For knee joint transplantation the distal muscles were also resected using Diathermy, vessels legated with Vicryl® 5/0 and bone sealed with Tachocomb®.
In the case of knee transplantation no skin area with a constant blood supply via a defined vessel could be identified, so these grafts were de-epithelialized. This was unnecessary in total limb transplantation.
Vascular pedicles were rinsed with 5ml Heparin /NaCl [40].
Using an anterior approach the carotid artery and jugular vein were identified and ligated cranially. Both ends of the blood vessels were flushed with Heparin / NaCl solution [40].
Grafts were stored on ice after harvesting with a mean Cold Ischemia Time 205 min, including completion of anastomoses, and transplanted following preparation of the recipient site (n=23).
In other cases the vascular pedicle of the graft was dissected after preparation of the recipient site (n=21), therefore intermediate storage on ice was not required and mean Warm Ischemia Time was 39 min. End to End anastomoses were performed using 10/0 Vicryl® or by cuff-anastomoses, in a modified technique described by Goodrich et al [6] (Fig. 1). Small polymer tubes served as external stabilisation of the blood vessel (Abbocaths® 22, 24, 27G). Grafts were fixed by subcutaneous suture to the recipient (5/0 Vicryl®). In limb grafting the skin was sutured and toes fixed posterior to the neck with Ethibond® 5/0. For knee transplantation the de-epithelialized grafts were placed in a subcutaneous pouch.
Results of these preliminary trials indicated that IM-anaesthesia and grafting of the forelimb in the cuff-anastomoses technique was optimal for the principle study.

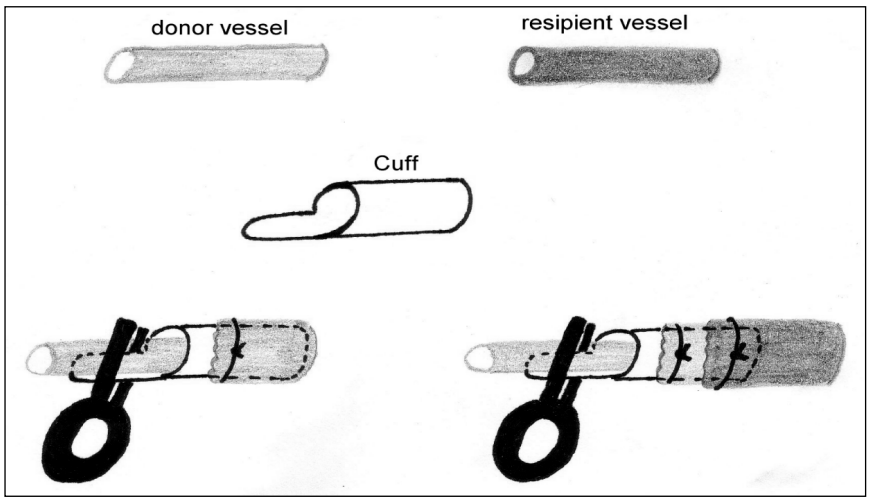

Fig. 1: Anastomoses in cuff technique, Kamada et al [19], Goodrich et al. [6]

Main Trial

Peri-Operative Monitoring and Medications

During surgery vital parameters such as respiratory rate, heart rate, body temperature, arterial blood pressure and oxygen saturation were monitored continuously. pO2, pCO2, bicarbonate, pH and base excess (BE) were analyzed intermittently via a catheter in a tail artery.

Blood and fluid losses intraoperatively were corrected with NaCl (0.5 ml/hr). Hypotensive episodes and/or tachycardia were managed with HAES 6 %. Heparin 200 IU commenced 12hr preop and continued daily. Immunosuppression with CyA (5mg/kg/d SC) or Tacrolimus (1mg/kg/d IM) was started the evening before transplantation.

Grafts were monitored post-operatively using Cytoscan® [22], an optical non-invasive orthogonal polarized light (wave length λ = 548nm) that visualizes the microcirculation by detecting red blood cells. The Cytoscan® was used every second day with a short anesthesia to assess the Functional Capillary Density (FCD). Non-grafted paws of the donors were evaluated as a control. Trial ceased if there was a 30% reduction in FCD as a sign of impaired microcirculation, or at 24 days. Transplant recipients were clinically evaluated on a daily basis for any signs of rejection including erythema, edema, scaling, hair loss, skin colour, capillary refill, exsudation and necrosis as described by Yaremchuk et al [36].

Pre-operative blood samples were taken from donors and recipients on the day of surgery. Recipients continued to be sampled every second day of the study period. Serum and Peripheral Blood Lymphocytes (PBL) were separated. Serum levels of Interleukin-6 (rat Il-6-ELISA), CyA and Tacrolimus (both by ELISA) were analyzed. PBL were used for the Cyto-Immunological Monitoring (CIM) [9] to distinguish between rejection episodes and viral and/or bacterial infection. Cells were examined microscopically using May-Grünwald-Giemsa staining. Histopathological examination of the graft and the contra lateral limb (control) involved removal, fixing in a phosphate-buffered formalin (4%) solution, embedding in paraffin, preparation and stain with Hematoxilin & Eosin. Rejection episodes were graded according to Muramatsu et al [29].

Results

Preliminary Trials

Anaesthesia

All three methods of anaesthesia were tested.

Recipient vital signs were measured at the following intervals and analyzed.

1. Induction of anaesthesia to start of surgery
2. Cessation of surgery to end of anaesthesia (i.e. extubation or awakening)
3. Duration of sleep post-anaesthesia

Technical problems during anaesthesia were documented. Post-operatively donor data was not included.

Ether anaesthesia was only used in four animals as the duration of sleep after the end of the anaesthesia was significantly increased. In addition, Ether in an open theatre system can lead to some adverse reactions in the staff.

Vital parameters of recipients were within the physiological range in all methods. The time intervals were significantly shorter in the IM anaesthesia group and no technical problems were noted, compared to Halothane-Nitrous Oxide. Thus, IM anaesthesia with spontaneous breathing was superior to the two other techniques trialled, especially with respect to application and handling. Consequently, in the main trial only IM anaesthesia was used.

Surgical technique

From a theoretical aspect four different models for CTA were available:

1. fore limb
2. fore paws
3. hind limb
4. knee joint

The lack of a sentinel skin graft as monitor of microcirculation excluded the knee-joint graft.

Hind limb transplantation seemed not to be applicable for our method. The placement of the hind limb at the recipient's neck is not possible because of the large size of the graft, which would handicap the recipient and limit the range of motion of the animal's head. Thus, only fore limbs or the forepaws remained as technically feasible grafts. Using the forepaw, the weight of the graft could be reduced, but only with a lengthening of the vascular pedicle (both significant), leading to more frequent venous or arterial thrombosis. Moreover, the vessels are often too small for a sufficient anastomosis (results were not significant, Fig. 2).

Ischemia times were compared between the groups. The coherence of ischemia time and reperfusion injury was described by Land and Meßmer [21].

Mean Cold Ischemia Time was 205 min if grafts were stored intermediately on ice after harvesting. This could be reduced significantly to 39 min if the graft was not separated from the donor site (thus keeping the blood flow intact)

Tab. 1: Histologic Grading of Rejection by Muramatsu [29]

Grade	Rejection	Bone	Cartilage	Bone Marrow	Muscle	Skin
0	None	Normal	Normal	Normal	Normal	Normal
1	Mild	Partial empty lacunae	Focal erosion	Increased marrow cell and partial extravasation	Mild round cell infiltration	Mono-nuclear focal infiltration
2	Moderate	Trabeculae thickening	Decreased staining of chondrocytes, rough surface	Decreased marrow cell	Severe extra vasation	Suprabasal bulla formation
3	Severe or total necrosis	Complete empty lacunae	Necrosis	Acellular marrow and fibrosis	Necrosis	Vasculitis and necrosis

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Tab. 2: Comparison of anaesthesia techniques (mean values)

	unit	Halothane-Nitrous Oxide (1) n = 20	ether (2) n = 4	IM anaesthesia (3) n = 20	Level of significance 1 vs. 3
1. induction of anaesthesia – start of surgery	min	9,65	3,5	3,33	< 0,001
2. cessation of surgery – end of anaesthesia	min	7,19	4,66	4,9	0,006
3. duration of sleep post-anaesthesia	min	9,38	25	5	< 0,001
4. mean arterial pressure	mm Hg	92	94	102	0,012
5. heart rate	1/min	178	154	196	0,022
6. oxygen saturation	%	95	94	97	0,003
7. p O2	mm Hg	142,23	127,75	195,53	0,001
8. p CO2	mm Hg	42,14	43,5	43,58	0,805
9. pH		7,344	7,286	7,293	0,009
10. BE	mmol/l	-2,79	-1,47	-0,31	0,095
11. technical problems		4	0	0	

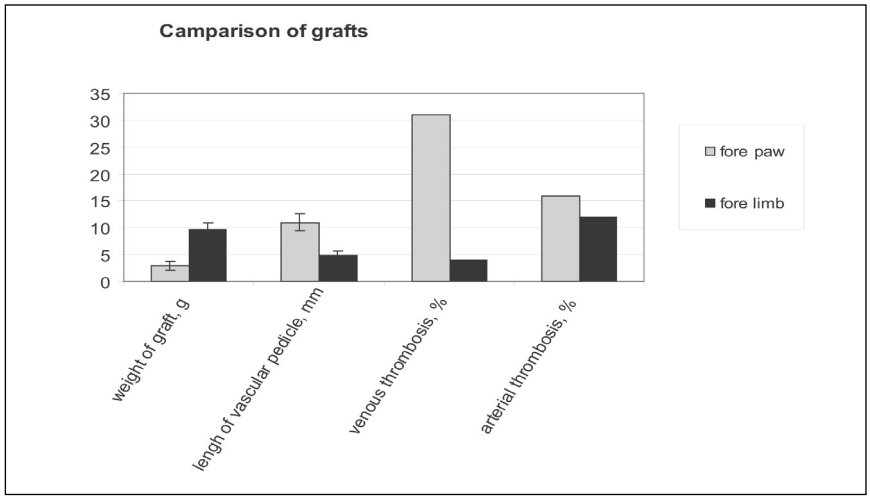

Fig. 2: Comparison of technical transplant parameters between fore paw and fore limb of rats

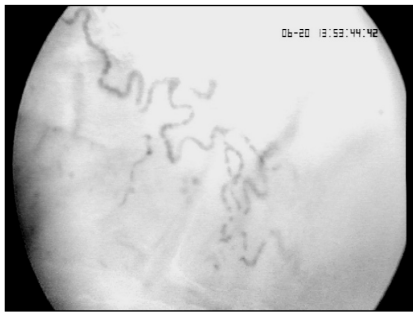

Fig. 3: Microcirculation (Red blood cell flow) on recipient's nail fold area by cytoscan detection

until the recipient site was prepared; hence intermediate storage on ice was unnecessary. In Cuff versus Suture technique there was a significant reduction in time (cuff technique: mean 30min, suture technique: mean 52min)

Main Trial

At 30 % reduction of FCD or on day 24, follow up of recipients was terminated. FCD was monitored by Cytoscan® analysis (Fig. 3) of the skin and nail fold area on the grafted forelimb. In the allogeneic groups mean graft survival was 4.5 days (L/BN without IS), 5.43 days (L/BN + CyA) and 5.71 days (L/BN + Tacrolimus). Graft survival was significantly longer in the group BN/BN without IS (mean 14 days). Only the recipients in group BN/BN + Tacrolimus showed no reduction of FCD and were routinely terminated on day 24 (Fig. 4). This increase of graft survival was significant compared to all other groups (Fig 5).

In clinical examination of the grafts capillary refill was visible at each time point. Monitoring for an early rejection episode, in the isogeneic BN/BN with Tacrolimus group no change of clinical parameters was found. As a reduction in FCD occurred there was simultaneous edema or erythema on the grafts. No loss of hair or exudation was observed. Histology confirmed all these results. In all cases a mild to moderate rejection (grade 1 or 2) was found (Fig. 6).

Established rat specific levels of immunosuppressive drugs were not available. Therefore Human Immunosuppressive Protocol serum level parameters were adapted to rats. CyA and Tacrolimus levels were within the defined treatment range.

No IL-6 was detected in the test or control groups, even when histology confirmed rejection appeared. Activation of mononuclear cells (MNC) is interpreted as a sign of rejection. In Cyto-Immunological Monitoring, Group B recipients showed no signs of cell activation. In all other groups an activation of MNC was found beginning at day two. However, this elevated cell count was inconsistent over the following days. The onset of rejection episodes did not correlate with CIM results.

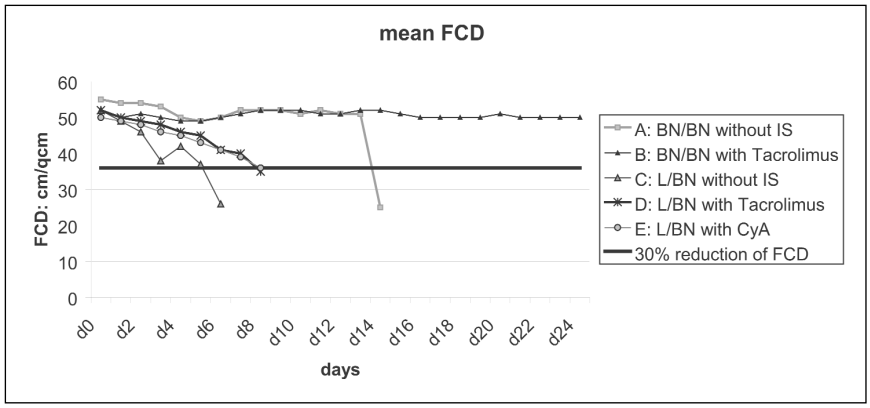

Fig. 4: Mean FCD by Cytoscan®

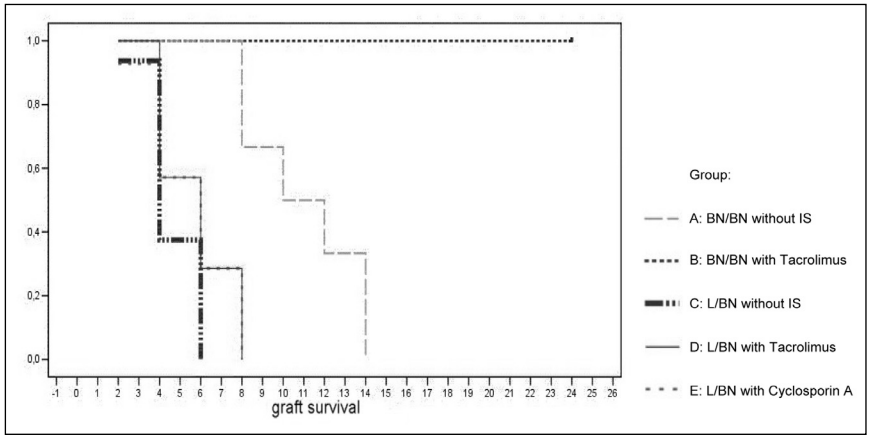

Fig. 5: Graft survival, Kaplan-Meier graphic

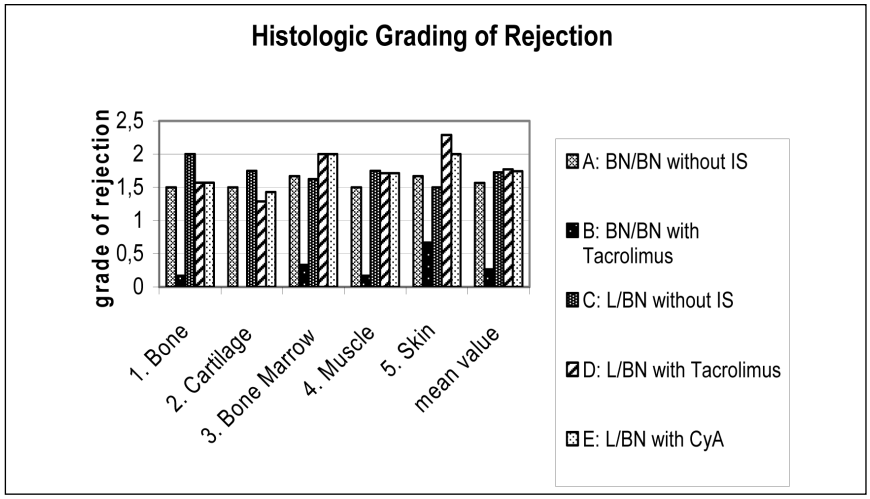

Fig. 6: Histological grading of rejection, mean values
Statistics of results in mean (Mann-Whitney Test):
p = 0,002 BN/BN without IS vs. BN/BN with Tacrolimus
p < 0,001 L/BN without IS vs. BN/BN with Tacrolimus
p < 0,001 L/BN with Cy A vs. BN/BN with Tacrolimus
p < 0,001 L/BN with Tacrolimus vs. BN/BN with Tacrolimus

Discussion

The first historical mention of composite tissue allotransplantation (CTA) dates back to the year AD 348, where legend has it that twin brothers from Arabia, Saints Cosmas and Damian (circa AD 286) posthumously transplanted a Ethiopian Moor's limb in place of an elder's amputated gangrenous limb. In the 20th century scientific work on this subject started. The first documented grafting of a human knee joint was performed by E. Lexer [25, 26]. Improved surgical technique of vascular anastomoses and the development of the first immunosuppressive drugs (Azathioprin, 6-Mercaptopurin, Corticosteroids) allow the study of graft rejection in different animal models. Dogs [4], pigs [32], mice [34] and mostly rats [2, 7, 8, 11, 17, 18, 24, 30, 31, 36] have been used. The most important trials have been published by Yaremchuk et al [36], Black et al [2] and Hovius et al [17] (Tab. 3).
The method of Yaremchuk was "fundamental" to establish a reproducible tool concerning type of graft, anastomosis and anaesthesia.

Surgical Technique

Yaremchuk [36] used two different allogeneic systems. Transplantations were performed against a minor histocompatibility barrier (L/wF) and against a major histocompatibility barrier (L/BN). Black [2] and Hovius [17] performed transplantation with hybrid rats in a minor barrier model. Hovius [17] described the problem of black BN rats as donors concerning the clinical evaluation of the grafts. All of them used end to end anastomoses. Only Black [2] performed orthotopic transplantation. If grafts can not be placed subcutaneously, they are in danger of being mutilated by the recipient animal, biting off the paw. This has been described by Black [2]. Therefore we grafted to the vessels of the recipient's neck.
Two further topics of discussion are the size of the graft and the length of the vascular pedicle. The fore limb was recognized as the most suitable graft in our model. The knee joint cannot be monitored because of the absence of a defined sentinel skin flap. The hind limb is too large for heterotopic transplantation and the vascular pedicle of the

Tab. 3: Comparison of trials in total limb transplantation

	Yaremchuk (1985)	**Black (1985)**	**Hovius (1988)**
rats	– Lewis rats – Fisher 344 rats (wF) – Brown Norway rats	– Lewis rats – Lewis/Brown Norway Hybrid rats	– Brown Norway/Bi Hybrid rats – Wag/Rij Hybrid rats
anaesthesia	– Pentobarbital 50mg/kg intraperitoneal	– no data	– ether
Surgical technique	– vascularized knee transplantation – transplantation of lower extremity – heterotopic subcutaneous transplantation in an abdominal pouch – end to end anastomosis	– orthotopic transplantation of lower extremity – internal fixation with K-wires – end to end anastomosis – reduction of warm ischemic time (45min)	– transplantation of lower extremity and anastomosis to the common femoral artery – long vascular pedicle with subcutaneous channel – end to end anastomosis – reduction of warm ischemic time (45 - 60 min)

forepaw is too long and therefore prone to thromboses of the anastomoses.
A further modification introduced to this model is the cuff technique for vascular anastomoses. The impact of ischemia / reperfusion injury to a graft is well known [21], resulting in the need to limit ischemia time. Black [2] and Hovius [17] described the simultaneous preparation of donor and recipient sites and achieved an ischemia time of 45 and 45 – 60 min, respectively. Using the cuff technique we were able to shorten the ischemia time to 30 min. In comparison, the mean cold ischemia time in human hand transplantation is 5.3 hrs [20]. Li [27] could show that intimal proliferation of the grafts vessels can be inhibited by using a heparin solution flush of the vascular pedicle.

Anaesthesia

Methods for anaesthesia of animals are not described in detail in the three quoted studies. Intraperitoneal injection of pentobarbital [36] and ether [17] are commonly used, both without mechanical ventilation. As most suitable method of anaesthesia, inhalation with controlled mechanical ventilation has been described so far. Advantages of this technique are absence of respiratory depression and no lack of oxygen distribution. Disadvantages are the demanding technique and the need of a ventilator [28]. Therefore this method is not regularly used in rodent models [28]. Mantel [28] compared different techniques of intramuscular anaesthesia. Easily antagonizable anaesthesia with Medetomidin, Midazolam and Fentanyl showed the best results taking respiratory depression, decrease of blood pressure and temperature into consideration.
Our aim was to combine easy handling with a maximum of safety for the recipients with best conditions for the grafts. IM anaesthesia [28] with spontaneous breathing and the possibility of simple reversal was superior to the inhalation methods.

Clinical and Technical Monitoring of the Transplant Recipients

Adequate blood perfusion of the graft is crucial for graft survival. Hence it is necessary to monitor the recipient's vital parameters during surgery.
Historically, investigators have relied on clinical impression and visual inspection to assess graft rejection. Yaremchuk [36] utilized an Ultrasound technique or fluorescence microscopy. Hovius [17] analyzed oxygen saturation in the toes of the graft in comparison to the normal lower extremity. Clinical examination detected the onset of rejection episodes, which were then confirmed by histology. Disadvantages of fluorescent microscopy and blood analyses are their invasiveness. Cytoscan®, as well as Doppler are non-invasive and should be preferred. The vascular pedicle in our model was unsuitable for Doppler analysis so the Cytoscan® was a useful complement to clinical examination for the evaluation of microcirculation.
Serum levels of CyA and Tacrolimus were evaluated by routine blood measures and allowed dosage adjustments. However this was not necessary in our trial.
Levels of IL-6 and CIM showed no correlation to rejection episodes.

Perioperative Medication and Fluid Management

In most trials data concerning perioperative medication and fluid management are not supplied [2, 29, 35, 36]. Hovius [17] used 2ml 0.9% NaCl during surgery and injected 50 IU Heparin in the vein of the graft. In addition, Lidocain 2 % was dripped on the anastomoses for prophylaxis of vascular spasm. We recommend the application of an established fluid management and anticoagulation regimen in animal models. This was realized in our model by continuous replacement of NaCl 0.9 % IV and HAES 6% IV as required. Anticoagulation (heparin 200 IE/d) was commenced with the reperfusion of the vascular pedicle. Prolongation of graft survival time by inhibition of thromboses was proven by Shapira et al [32].
CyA and Tacrolimus are used as immunosuppressants with MMF, antilymphocyte-globulin and Cortisone before [1, 2, 29, 30, 31, 33, 34, 36]. We used CyA (5mg/kg/d) or Tacrolimus (1mg/kg/d), respectively in a dosage published by Timmermann [34], as monotherapy. The dosage of CyA was reduced compared to the studies of Yaremchuk [36] (10mg/kg/d), Black [2] (25 or 8mg/kg/d) or Muramatsu [29] (15mg/kg/d) to reduce side effects. Tacrolimus was given in the equivalent dosage used by Muramatsu [29]. In contrast to Muramatsu [29], we were not able to show a difference in graft survival time between CyA and Tacrolimus.
In our studies the differences in graft survival time between allogeneic groups was not significant. Only in the isogeneic group with immunosuppresion (group B) was graft survival time significantly prolonged. This is in contrast to the results of Yaremchuk [36] and Hovius [17] who could show long

time graft survival in isogeneic groups without immunosuppression.

A possible explanation of this surprising result in isogeneic group A and B may be that the BN rats used were not strongly inbred. These standard strains had the composite profile of "alike" sublines and were not completely syngeneic [10]. Tacrolimus (1mg/kg/d) led to long-time graft survival.

In the allogeneic groups (C, D, E) transplantation was performed against a strong major histocompatibility barrier. By trying to reduce side effects of immunosuppression moderate doses of CyA and Tacrolimus were used. Obviously this low-level monotherapy was not able to inhibit acute graft rejection.

Nevertheless, we achieved an improvement of this CTA model in rats concerning the following aspects:

1. Method of anaesthesia and perioperative monitoring which is safe and easy to perform
2. Intraoperative fluid management and anticoagulation
3. Simplification of a surgical technique using the cuff anastomoses
4. Reduction of warm ischemia time
5. Reproducible monitoring of graft microcirculation using Cytoscan in addition to clinical examination.

An improvement of graft survival could be achieved by using a combination therapy as published by Ozer et al [27, 28], Siemionow et al [30] and Ayrout et al [1].

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