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The morphological and functional examination of the autologous internal rectus fascia sheath vascular graft in dogs

Theses of PhD dissertation

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1. INTRODUCTION

Vascular substitutes are widely used in vascular surgery. There are several different types including synthetic vascular prostheses, autologous vessels, heterologous donor vessels, xenografts of bovine or ovine origin and cryopreserved vessels but none of them is ideal in every circumstances. The potentially useable autologous vessels are limited in a patient and in many cases their qualtiy is also poor mainly because of systemic vascular diseases like atherosclerosis. The synthetic materials are widely used and the results are good in larger diameters, but the small diameter vessel grafts are often obstructed because of neointimal hyperplasia. The septic complications in immunsupressed patients are also more common in case of synthetic materials, than with the autologous vessels.

Kóbori and his co-workers started to use tubular grafts made from the posterior rectus fascia (RF) sheath in their experiments on dogs in order to find an ideal autologous vascular graft that would meet the needs of liver transplantation surgery. Most of the grafts remained patent and septic complications did not occur even under immunosuppressive treatment. (Kóbori 2000; 2003; 2005; 2008).

We performed further clinical experiments with the ARFS graft as a venous patch-graft and we repeated the experiment of Kóbori to perform more detailed morphological examinations, because thorough morphological evaluation and verification of the histological arterialisation of the graft are lacking. Beside the extended histomorphological evaluation we performed a functional test, - isometric tension recording -, of the ARFS graft and control arteries too. We hypotethised that the ARFS graft will act similair as the control arteries on the test as an evidence of the presence of a healthy and phisiologically functioning endothelial layer.

2. AIM OF THE STUDY

We had 3 hypotheses:

1. The ARFS graft is suitable for vein reconstruction.
2. The ARFS graft morphologically changes and becoming similair to the host vessel after the implantation.
3. The morphologically changed ARFS graft will act similair to the control arteries upon vasoactive substances with contraction and relaxation.

We performed 3 experiments:

1. Implantation of ARFS grafts as venous patch-grafts, in vivo follow up for 3 and 6 month and after removal morphological examination (histopathology and immunohistochemistry).
2. Implantation of ARFS grafts as tubular arterial grafts, in vivo follow up for 3 month and after removal morphological examination (histopathology and immunohistochemistry).
3. Isometric tension recording of the ARFS grafts and control arteries after stimulation with different vasoactive substances..

3. STUDY I.: EXPERIMENTAL RESULTS OF USING AUTOLOGOUS RECTUS FASCIA SHEATH FOR VENOUS PATCH GRAFTS IN DOGS

3.1 Materials and methods

The experiment included four Beagle dogs (authorisation number: 73/2008). We used the grafts created from the rectus fascia (RF) sheath for covering artificial wounds made on the common iliac and jugular veins. A total of 8 iliac and 7 jugular grafts were prepared. The surgical interventions were performed in general anaesthesia. During the operation, from the post-umbilical area of the internal rectus sheath we excised a 2 × 3 cm piece of rectangular shape, from which we created a 1 cm long and 0.5 cm wide patch. The patch was positioned in such a manner that its peritoneal surface covered with mesothelial cells faced the lumen of the vein. Subsequently, we secured the patch to the surface of the vein with continuous sutures. In the postoperative period, the dogs received meloxicam (0.2 mg/kg s.c. SID) for 5 days, and heparin (200 IU/kg s.c. BID) as anticoagulant therapy for two weeks.

The common iliac vein grafts were monitored for 6 months, while the functioning of the jugular vein grafts was followed up

for 3 months. The morphology and patency of the venous segments containing the graft were monitored by colour Doppler Ultrasonography and by CT angiography.

At the end of the follow-up period the patch grafts were removed for histopathology and immunohistochemistry. The following antibodies were used for the immunohistochemical reactions: anti-claudin-5 and anti-CD31. The positive controls were endothelial cells of microvessels in canine mammary gland carcinoma for claudin-5 (Jakab et al., 2008*b*), and canine haemangiosarcoma for CD31 (Jakab et al., 2009*a*).

3.2 Results

Obstacles related to the surgical technique did not occur during the creation and implantation of the vascular grafts, and after the start of recirculation the blood flow through the grafts was undisturbed. Macroscopic signs indicative of perioperative complications such as vascular suture dehiscence, septic complications, infection or necrosis were not observed. Occlusion of the grafts did not occur and all surgical areas remained patent in the postoperative period. Signs indicative of complications did not occur during the sampling operations either. Adhesions or signs indicative of other morphological damage were not seen.

According to the results of colour Doppler ultrasonography and CT angiography, the experimental veins were patent in all cases.

The surgically excised samples were subjected to routine histomorphological examination, which revealed an intact, single-layer endothelial lining in the inflammation-free, non-thrombotised and non-stenotic grafts. Neither areas devoid of endothelium nor those showing endothelial hyperplasia were seen. The subendothelial tissue layers proved to be free from infiltration by inflammatory cells. In the subendothelial areas, thick collagen fibres were predominantly seen, with

elongated, spindle-shaped fibrocytes (mature connective tissue cells) among them, and with the cross-sections of capillaries and arterioles present in multiple foci. Histopathological signs indicative of rejection were not seen in the vascular grafts either in the endothelial and subendothelial or in the deeper tissue layers. Endothelialisation was studied further by the use of immunohistochemical staining. Immunohistochemical tests performed with the use of humanised anti-claudin-5 and anti-CD31 antibodies confirmed the results of routine histopathological examinations, namely that a differentiated, single-layer endothelial layer devoid of inflammation and degenerative changes had developed on the internal surface of the vascular grafts. The immunohistochemical tests demonstrated an intense positive linear membrane reaction for claudin-5 in the membranes of endothelial cells in all vascular grafts. Tests performed with anti-CD31 antibodies also demonstrated a positive CD31 membrane reaction in the endothelial cells. Areas covered by the graft had undergone complete vascular transformation, and there were no notable differences in histological results obtained 3 and 6 months after graft implantation.

3.3 Discussion

We studied in our experiment the possible clinical use of the rectus fascia sheath as an autologous venous patch graft. The rectus fascia graft was already used in experiments as an arterial patch graft (Cousar 1952, Pacholewicz 1998) and tubular vascular graft (Kóbori 2003, 2008; Németh 2005). Reconstruction of large veins is indicated in veterinary medicine in case of vessel compressing or destroying adrenal gland masses, thymomas and thyreoid gland carcinomas (Holsworth 2004, Tobias 2012).

The experimental protocol was designed using the method elaborated and successfully used for arterial tubular grafts by Kóbori and it was adopted to the veins.

Surgical exposure of the veins and preparation of the patch grafts did not present any technical problem. Grafts implanted into the jugular vein and into the common iliac vein were removed 3 months and 6 months after implantation, respectively. This allowed us to perform histomorphological examinations by the use of two different follow-up periods.

By the use of colour Doppler ultrasonography and CT angiography, the morphological and flow conditions of the experimental veins could be monitored in the postoperative

period. We used the protocol known from the human medical literature (Sun, 2006; Kock et al., 2007) for the CT angiography, which proved to be perfectly suitable for the examination of dogs as well. Abnormalities (stenosis, obstruction, dilatation) did not occur, and the two diagnostic imaging modalities provided identical results.

In addition to haematoxylin-eosin staining, immunohistochemical examinations using humanised endothel-specific markers (CD31, claudin-5) were also used for morphological examination of the graft-covered venous segments removed at the end of the follow-up periods. We laid especially great emphasis on examination of the endothelium, as low thrombogenicity is one of the fundamental criteria necessary for ensuring good haemodynamic properties. According to the histological and immunohistochemical results, the rectus sheath had become incorporated into the vein and it was covered by a single endothelial layer. Histological signs indicative of rejection or inflammation were not observed.

The 15 venous grafts implanted in this study represent a relatively low number of cases. However, the results obtained with these grafts are consistently favourable. In conclusion, the patch graft created from the RF sheath can be used in the reconstruction of large veins.

4. STUDY II.:

MORPHOLOGICAL EVALUATION OF EXPERIMENTAL AUTOLOGOUS RECTUS FASCIA SHEATH VASCULAR GRAFTS USED FOR ARTERIAL REPLACEMENT IN A DOG MODEL

4.1 Materials and methods

This study has been approved by the competent authorities acting upon the recommendation received from the Scientific Ethics Committee on Animal Experimentation (authorisation number: 73/2008).

Four purpose-bred Beagle dogs were utilised to create 8 arterial internal rectus fascia sheath (ARFS) grafts. The experimental protocol was designed using the method elaborated and successfully used for arterial tubular grafts by Kóbori (Kóbori 2003, 2008).

The surgical interventions were performed in general anaesthesia. After a ventral midline abdominal incision, both external iliac arteries were dissected from the surrounding tissues. A piece of internal rectus fascia sheath (3 × 4 cm) was tailored. A standard glass rod (5 mm in diameter) was used to create the ARFS graft as a 20-mm-long tubular graft and it was end-to-end anastomosed to the bisected a. iliaca externa

with its peritoneal layer inside.. Immediately before clamping the iliac artery, 200 IU/kg heparin sodium injection (Heparin inj., Richter Gedeon) was given intravenously. The morphological characteristics of the graft were documented after re-establishment of circulation in the artery. Heparin sodium (200 IU/kg BID) was given subcutaneously as a postoperative anticoagulant for two weeks. Meloxicam (0,2 mg/kg sc. sid) was used as a postoperative analgesic for 5 days.

The morphology and patency of the segments containing the graft were regularly monitored by Doppler ultrasonography

At the end of the 3-month follow-up period, ARFS tubular grafts were surgically removed and morphological examination (histopathology and immunohistochemistry) was performed. The following antibodies were used for the immunohistochemical reactions: anti-*claudin-5,* anti-*desmin,* anti-*α-smooth muscle actin* (α-SMA) and anti-*pancytokeratin AE1-AE3*.

4.2 Results

Four out of the 8 ARFS grafts were patent and viable with intact luminal surface and a thick and rigid wall after 3 months. Stenosis and obstruction of the ARFS graft occurred in two cases during the first week and in 2 cases during the first month. The dogs did not show any clinical signs related to the stenosis or obstruction of the iliac artery. Well developed collateral vessels were seen around the stenotic graft during the surgical revision. Gross examination of the patent ARFS grafts seemed viable with intact luminal surface and a thick and rigid wall.

The morphology of ARFS grafts was compared to that of the intact arteries and rectus sheath samples. The HE staining showed a vessel-like layered wall of the grafts. The inner surface of the graft was covered with elongated endothel like cells and the hypocellular wall contained spindle shaped mesenchymal cells. Azan staining revealed large amounts of collagen fibres and orcein staining elastic fibers within the graft. Based on immunohistochemistry, the endothelial lining of the intact artery samples showed claudin-5 positivity and pancytokeratin negativity, the mesothelial lining of the intact IRFS samples was positive for pancytokeratin and negative for claudin-5, and the luminal surface of the ARFS graft was covered by flat and fusocellular cell lining showed a linear

claudin-5 positivity and pancytokeratin negativity (**Table 1**). Endothelial cells of the vasa vasorum were positive for claudin-5 (internal positive control) in the ARFS grafts. Smooth muscle cells of the tunica media of the intact arteries showed α-SMA and desmin positivity, the wall of the intact IRFS was α-SMA negative and desmin positive, and the spindle-shaped cells of the wall of the ARFS grafts showed moderate α-SMA and desmin positivity.

Table 1. Results of the immunohistochemistry

	Control artery	**Rectus fascia**	**ARFs graft**
CLD-5	+++	-	++
α-SMA	+++	-	++
Desmin	+++	+++	+/++
Pancytoceratin	-	+++	-

4. 3 Discussion

In the previous studies made by Kóbori the patency rate of these grafts were 90%. In our study only 50%. The difference between the results may be due to technical failures (different graft and host vessel diameter, unexpereince). A part of this can be the damage of the sensitive mesthel cell layer on the luminal surface of the grafts. It is presumed that the mesothel cell layer plays an important role in the prevention of the thrombosis in the grafts. Another reason for the different patency results can be an inproper anti-coagulant therapy. Altough Heparin sodium (200 IU/kg BID) was given subcutaneously as a postoperative anticoagulant for two weeks we did not use low-dose acetyl-salicylic acid antiplatelet therapy.

Based on our histomorphological examinations, the structure of rectus sheath used as a vascular graft has changed after the implantation. Although the inner surface of the intact internal rectus fascia sheath (IRFS) is originally covered by claudin-5-negative and pancytokeratin-positive mesothelial cells, the internal cells became claudin-5 positive and pancytokeratin negative. Based on this, a complete re-endothelisation process took place during the 3-month follow up period. Spindle-shaped cells of the wall of ARFS grafts were α-SMA positive like smooth muscle cells of the intact

arteries, but α-SMA immunoreactivity was negative in the intact IRFS. The change in the immunoreactivity of the ARFS grafts shows that fibroblast cells of the IRFS have changed into myofibroblast cells, which are transdifferentiated fibroblast cells containing smooth muscle actin (Nagamoto 2000; Meng 2001). This morphological change may increase the elasticity of the ARSF grafts, enabling them to accommodate to the new circumstances as a vascular substitute.

In conclusion, the ARFS autograft may be used as an alternative in arterial replacement, since the graft becomes morphologically and functionally similar to the host vessel via arterialisation enabling it to behave as a blood vessel, and furthermore, rejection is not a possible complication, but the wall of the grafts becomes thicker and less elastic than that of an intact artery. Nevertheless, in our experiment only half of the grafts remained patent, and the obstructed 4 grafts suffered from stenosis. Further investigation of the long term clinical behaviour is recommended before the ARFS graft is routinely used in clinical cases as a tubular vascular substitute.

5. STUDY III.

ISOMETRIC TENSION RECORDING OF THE AUTOLOGUS RECTUS SHEATH VASCULAR GRAFT

5.1 Materials and methods

At the end of the 3-month follow-up period the experimental dogs were subjected to another surgical intervention, during which the implanted tubular grafts were removed in such a manner that the sample taken should contain the implanted grafts and also a control sample originating from the intact arterial segment surrounding it. The vascular graft samples were stored in Tyrode-solution for 60-100 minutes before the myographic examinations. The method of the tension recording was based on the method of Högestatt (Högestatt 1983) and Hardebo (Hardebo 1986). One of the eight implanted grafts were used for the tension recording. A 30 mm long segment was cut from the femoral artery, containing the graft in the middle surrounded by the original femoral artery proximal and distal from the graft. This segment was immersed in room temperature Krebs solution and fat and connective tissue were removed under an operating microscope. 2 ring segments of 5 mm were prepared from the middle part of the graft and 1-1 segments from the proximal and distal femoral artery. The segments were mounted on

stainless steel vessel holders (200 µm in diameter) of a conventional myograph setup. Special care was taken to preserve the endothelium. The segments were exposed to 124 mmol/L K^+ Krebs solution made by isosmolar replacement of Na^+ by K^+ to determine whether the transplanted graft had any contractile activity. Thirty minutes later the vessels were subjected to increasing concentrations of phenylephrine (PE, 0.01 to 10 µM), a well known constrictor of canine femoral arteries. To investigate the functional integrity of the endothelium, increasing concentrations of acethylcholine (Ach, 0.001 to 10 µM) was added after a stable plateau of contraction had been reached. Thirty minutes after washing out the ACh, the vessels were exposed to the thromboxane A_2 mimetic U-46619.

5.2 Results

The 124 mM K^+ induced contraction is presented in **Figure 1**. The normal femoral artery had 80 mN increase of tension while the transplanted graft contracted only 20 mN

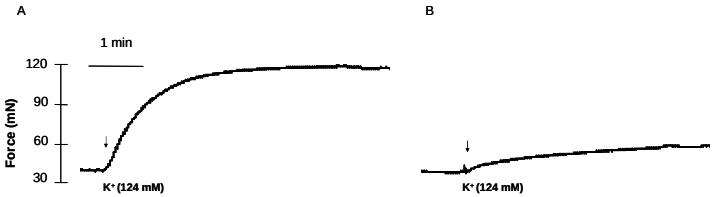

Figure 1. Recording of the 124 mM K^+ induced contraction in the femoral artery (A) and in rectus fascia graft (B). Vertical bar = tension (mN), horizontal bar = time (min)

Both tissues were then exposed to the contractile agonist phenylephrine (PE) from 0.01 to 10 µM. When the contraction reached the peak, acetylcholine (Ach) from 0.001 to 10 µM was added to test NO mediated relaxation. In contrast to the femoral artery rings, the dose dependent contraction effect of PE was decreased in the rings prepared from the transplanted graft, as well as the cumulative relaxation effect of Ach (**Fig. 2.**).

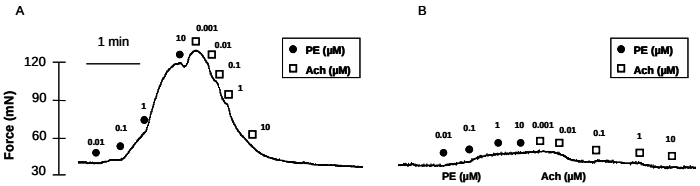

Figure 2. Recording of the PE induced contraction and Ach induced relaxation in the femoral artery (A) and in rectus fascia graft (B). Filled dots represent administration of PE, empty squares Ach, respectively. Vertical bar = tension (mN), horizontal bar = time (min)

Interestingly, the contractile force evoked by the thromboxane receptor agonist U-46619 had comparable amplitude in the graft and vessel rings (**Fig. 3.**).

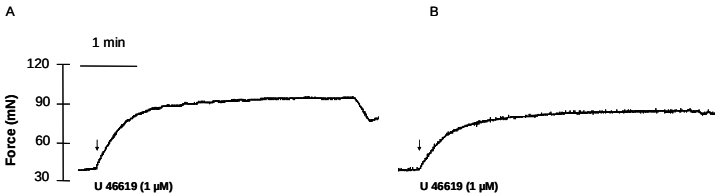

Figure 3. Recording of the U-46619 induced contraction in the femoral artery (A) and in rectus fascia graft (B). Vertical bar = tension (mN), horizontal bar = time (min)

5.3 DISCUSSION

The evaluation of the tension recording measurment results show that the RF sheath grafts reacted to K^+, though this contraction was far less strong then in the control arteries. The cause of this difference may be explained by the morphological difference between the graft and the arteries, mainly beause of the lack of proper smooth muscle cell layer. The RF sheath grafts reacted with contraction to PE too, but the contraction was far less strong then in the control arteries. The RF sheath grafts reacted with Ach which connects to the muscarin receptors of the endothel cells. Ach cause NO release from the endothel cells and relax the smooth muscle cells. The relaxation was close to 100%, but this data's interpretation is difficult considering that the precontraction was very week. Based on this we do not have information about the possible reaction in case of strong precontraction. The RF sheath graft reacted with the thromboxan agonist U-46619. The contraction was similair with the contraction of the control arteries. If we compaire this result with the reference K^+ caused contraction, we could see that the contraction in the control arteries is about 60% and in the graft about 200%. The thromboxan caused very strong contraction in the graft.

In conclusion the used myographic method is suitable for isometric tension recording of canine arteries and the implanted RF sheath vascular grafts. The reactions of the graft were much weaker than same reactions of the control arteries, except the thromboxan caused contraction. Based on this the RF sheath graft reacts in physiological circumstances similair to the normal arteries but for exact statistical analysis the experiment should be repeated on several more grafts too.

6. LIMITATIONS

The results of our studies are limited by several factors.

The ARFS graft was used in venous environment only as a patch-graft. We do not know how could it function as a tubular graft. This could have relevance in the reconstructin of large vein defects, but further experimental work is needed.

We had only a 50% patency rate in our arterial experiment which heavily influences our opinion about it's use in clinical cases. Previously Kóbori has described much better patency rates on larger number of grafts, but based on our less promising results, further experimental work is recommended.

We could clearly demonstrate the presence of the healthy and functioning endothelial layer on the surface of the graft by the myographic examination, but we could not do a proper statistical analysis due to the low case number. Further experiments are needed for the numerical results.

7. NEW SCIENTIFIC RESULTS

1) It was proved that the autologous internal rectus sheath venous patch-graft had been incorporated in the veins without rejection signs suggestive of inflammation.

2) It was immunohistochemistry confirmed that the internal rectus sheath venous patch-graft's internal surface was covered with claudin-5 and CD31 positive intact endothel cell layer three months after implantation.

3) It was experimentally confirmed that the autologous internal rectus sheath as a venous patch-graft is suitable for vein reconstruction.

4) It was demonstrated that the internal rectus sheath arterial tubular vascular graft morphologically transformed after implantation and the immunohistochemistry pancytoceratin positive mesothelial cell layer was occupied by a claudin-5 positive endothelial cell layer.

5) It was immunohistochemistry demonstrated that fibroblasts in the internal rectus sheath arterial tubular vascular graft were converted to α-SMA positive myofibroblast cells after implantation.

6) It was demonstrated that the miogrophic method was suitable for measuring the isometric tension of dog artery and the internal rectus sheath arterial tubular vascular graft. It was found that the vascular graft was capable to react with contraction and relaxation upon vasoactive substances.

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