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A Rat Model for Severe Limb Ischemia at Rest

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Key Words
Limb ischemia · Rat model · Inflammation · Metabolism · Angiogenesis

Abstract
We sought an animal model able to discriminate metabolic and angiogenic processes in limb ischemia. For that we modified and evaluated a rat model of severe unilateral limb ischemia at rest. A two-stage surgical procedure entailing left femoral artery ligation preceded by interruption of collateral vessels originating from the infra-renal aorta and left iliac arteries was performed in Sprague-Dawley rats. The model was evaluated for up to 8 weeks with a transit-time flow meter, a laser Doppler perfusion imager, microspheres, arteriography and histology. It was found to be well tolerated with low mortality and perfusion in the foot skin was reduced up to 8 weeks, while collaterals were visible after 2 weeks. Histologic signs of ischemia were seen for up to 4 weeks. This rat model of severe limb ischemia at rest lasts up to 8 weeks and seems well suited for longitudinal studies of the pathophysiology of limb ischemia and healing mechanisms like angio- and arteriogenesis.

Introduction

Peripheral arterial disease (PAD) is a common problem with a prevalence of 5% among persons over 65 years of age [1]. The pathophysiological process leading to the end stage condition of PAD, critical limb ischemia, is not fully understood. The complexity of developing ischemia may explain the lack of efficacy of some clinical trials aiming to stimulate collateral growth and improving microcirculation for treatment [2–4]. Unfortunately, the pathogenesis is hard to study in patients because of this complexity and its slowly progressing nature.

If appropriate animal models of the ischemic event, and especially the healing process were available, understanding of the pathogenesis could be improved and introduction of new treatment concepts facilitated. Animal models have been proposed previously, most for acute limb ischemia and studies of the ischemia-reperfusion syndrome [5]. This limitation is due to the fact that most experimental animals rapidly develop efficient collaterals with early and fast normalization of perfusion. We sought an animal model of limb ischemia at rest with sufficiently long duration to allow studies of temporal variation in the different processes occurring in limb tissue subjected to an ischemic event. Examples of such are metabolism, inflammation, atrophy and angiogenesis and collateral vessel development.

The aim of this study was to identify and evaluate a rat model for such severe limb ischemia at rest.

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Methods

Animal Model

The model we used is based on a two-stage procedure in rat suggested by Seifert in 1985 that creates ischemia in the left hind limb whereas the right serves as control [6]. The rats are sedated by inhalation of methoxyflurane (Metofane®, Schering-Plough Animal Health Corp., Union, N.J., USA) dispersed in a glass cylinder and anesthetized with a combination of Hypnorm (fentanylcitrate 0.315 mg/ml and fluanisone 10 mg/ml, 0.05 ml/100 g BW) and pentobarbital (60 mg/ml, 0.05 ml/100 g BW) intraperitoneally.

The first operation is performed through a midline laparotomy. By the aid of a microscope all branches originating from the left side of the aorta distal to the renal arteries and all branches from the left iliac artery are visualized, ligated with 6-0 resorbable suture and divided (fig. 1). The nomenclature of rat vascular anatomy varies between authors and we have consistently used that proposed by Greene [7]. The internal spermatic artery – often originating from the left renal artery – is divided first. Moving distally one or two small lumbar arteries and the large iliolumbar artery are ligated. The inferior mesenteric artery arising from the ventral part of the distal aorta and the middle caudal artery are also cut. Subsequently, the left iliac artery is isolated from all its branches from the bifurcation to the level of the inguinal ligament. Typically, this entails ligation of 4–5 minor branches and the large hypogastric trunk. The incision is then closed and the rats are monitored for the following days.

After a week the rats are again anesthetized and a second operation is performed. Through a left inguinal incision, the femoral artery is ligated close to the origin of the superficial epigastric artery. The latter is also ligated and divided. From the femoral artery between the level of the inguinal ligament and its division arises the superficial circumflex iliac artery. Cutting the superficial epigastric artery and preserving the superficial circumflex artery differs from the original procedure suggested by Seifert and is done to achieve slightly less severe ischemia and to further simplify and standardize the operative procedure. Moreover, the spared artery serves as an ideal model of a developing collateral vessel and is easily harvested at a later time point.

An analgesic (Temgesic®, buprenorphin 0.4 mg in 200 ml water, Reckit & Colman, Hull, UK) is mixed into the drinking water after both the first and second operation. Operative mortality is less than 5%.

Protocol

Forty male Sprague-Dawley rats (mean weight 245, range 50 g) (B&K Universal, Sweden) prepared as described above were divided into five groups and kept for different time periods after the second operation: 1 day, 1 week, 2 weeks, 4 weeks and 8 weeks. These rats were used for perfusion scan with a laser Doppler Imager (LDI) and for histology. Three different rats were subjected to arteriography and another 14 were used to measure volume blood flow and blood flow in muscles using fluorescent microspheres. All protocols were approved by the Institutional Animal Care and by the Ethics Committee at Stockholm County (Dnr 56/97 and 284/98).

Perfusion, Blood Flow and Angiography

Laser Doppler Imaging

A moorLDI-VR (Visible red laser Doppler imager, Moor Instruments Ltd., Axminster, UK) was used to assess limb perfusion [8]. The laser Doppler source is mounted on a desktop stand and a laser beam scans the tissue using a moving mirror. The laser beam reflected from moving red blood cells in nutritional capillaries as well as in arterioles and venules is detected and processed to provide a flux value. The information is color coded to provide a map of tissue perfusion. In the present study regions of interest corresponding to the plantar and dorsal aspects of the hindlimb and the exposed anterior tibial muscle were marked manually using the Moor LDI Image Processing V 3.01 software. Mean flux values within the regions were then calculated to allow comparisons between the two sides.

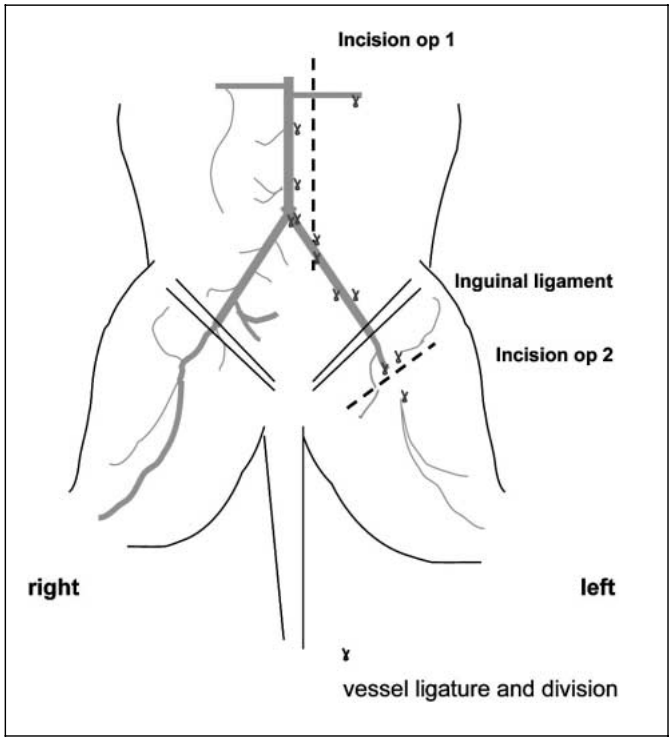

Fig. 1. Schematic drawing of the surgical procedure.

Measurements were done under anesthesia as described above. The rats were breathing room air spontaneously. The hemoglobin oxygen saturation was measured with a pulse oximeter (Nonin 8500V, Nonin Medical, Inc., Plymouth, Minn., USA) using a flexible sensor (Sensor 2000 SA, Nonin) on a forepaw. Supplementary oxygen was delivered on demand through a facemask, maintaining the saturation at or above 90%. To reduce heat loss during measurements the rats were kept on a 37°C heating plate. Repeated doses of Hypnorm and pentobarbital i.p. were given on demand.

Volume Blood Flow

Volume blood flow was measured with transit-time technique (Transonic Instruments, Seattle, Wash., USA). Through skin incisions in both groins 1-mm probes were applied to the distal femoral arteries. Volume blood flow in ml/min was simultaneously measured in both limbs for ten minutes and the values recorded on a strip chart. The mean value during the last minute from the recording was used for comparisons.

Microspheres

Six rats at 1 day of ischemia, 6 at 4 weeks and 2 at 8 weeks were used for injection of microspheres. The rats were anesthetized and both carotid arteries cannulated (Angiocath 22 gauge, Becton Dickson, Franklin Lakes, N.J., USA) through a midline neck incision. The right carotid was isolated from the vagus nerve, cannulated and connected to a pressure transducer by use of Silastic tubing filled with heparinized 0.9% NaCl and advanced until the pressure tracing showed that the catheter's tip reached the left ventricular cavity. The catheter was then secured with a suture, disconnected from the transducer and connected to the infusion port of reciprocal infusion/withdrawal pump (Model 55-1382, Harvard Apparatus, Holliston, Mass., USA) and used for injection of microspheres. The left catheter was advanced to aorta using the same technique and secured with a suture. It was used to draw the reference sample and to measure arterial blood pressure between sphere injections. For blood pressure measurements the catheter was connected via polyethylene tubing filled with heparinized 0.9% NaCl to a pressure transducer (model 1270A, Hewlett-Packard, Palo Alto, Calif., USA). The left carotid artery was used to conserve the leg arteries and distal aorta from a cannulation that might have interfered with perfusion of the legs. Around 400,000 fluorescent microspheres (Fluospheres, Molecular Probes Inc., Eugene, Oreg., USA), with a diameter of 15 μm were mixed vigorously and injected during 60 s. The spheres were suspended in stock solution diluted 3:1 with 0.9% NaCl. The reference blood sample was drawn at a constant rate of 0.39 $ml \cdot min^{-1}$ for 90 s starting before the injection of spheres. The procedure was repeated twice using spheres with different colors (blue-green and red) and the mean value was used for analyses. The samples were weighed wet and organic tissue digested in 5 ml 2 *M* KOH in 100% ethanol for 10 h. The samples were then washed and agitated in ethanol and the supernatant removed. Finally, 3.5 ml of 2-ethoxyethyl acetate was added to dissolve the microspheres, centrifuged at 1,500 *g* for 10 min to sediment any remaining material. The fluorescence intensity in the supernatant from each sample was measured using a spectrophotometer (Perkin-Elmer LS 50). The fluorescent signal from each tissue sample is proportional to the number of microspheres dissolved and blood flow in each sample was calculated by multiplying the fluorescent intensity and the reference sample withdrawal rate and dividing with fluorescent intensity in the reference sample.

For flow determination with microspheres the whole anterior tibial, soleus and gastrocnemius muscles and the proximal half of the excised adductor magnus and semimembranosus muscles (called proximal adductor) and vastus lateralis were used.

Angiography

Three rats (1 day, 2 weeks and 4 weeks) were anesthetized and the proximal abdominal aorta was ligated through a midline incision. The aorta was cannulated (PE_{50}) distal to the ligature and the tip of the catheter was placed just above the bifurcation. The rats were positioned supine on an image plate (AGFA), directly on the collimator of a mobile X-ray system. First 0.5 ml of papaverine (4 $mg \cdot ml^{-1}$) was injected followed by 0.3 ml/100 mg body weight of Omnipaque® (325 $mg \cdot ml^{-1}$) diluted to 75% in saline. Immediately after contrast injection, an image was obtained. The developed films were blinded and then examined by one of the authors (B.K.) to identify missed branches, but also to compare differences in the presence of collateral vessels between the control and the ischemic side.

Muscle Weight and Histology

The anterior tibial muscles from both sides were carefully dissected en bloc and weighed wet. In a subset of 3 rats at 1 day, 1 week, 4 weeks and 8 weeks this muscle was used for histologic examination. For comparison, samples were also taken from the adductor muscles (adductor magnus and semimembranosus). The samples were snap frozen in liquid nitrogen and stored at –80°C. From each specimen, three 5-μm-thick sections were cut using a cryostat (Miles) and placed on a glass slide. The sections were stained (hematoxylin and eosin) and three muscle specimens from each side were randomly selected and used for descriptive histology performed manually under a light microscope at high-power magnification by one of the authors (H.B.) in a blinded fashion. The findings were subjectively graded (0–3) regarding presence of ischemic signs, edema, inflammation and necrosis (0 = no changes, 3 = pronounced changes), by the examiner. An example is displayed in figure 3.

In 1 rat at each time point the femoral artery, including the spared collateral, was dissected free proximal to the ligature. The same was excised from the control leg. These were stained (hematoxylin and eosin) and used to examine the appearance of the vessel wall.

Statistics

Mean and SD was used to describe the data unless otherwise indicated. Wilcoxon signed-rank test was used to compare results from the ischemic and the control side. For comparison of data over time ANOVA was used. For all analyses, $p < 0.05$ was interpreted to denote statistical significance.

Results

Clinical Appearance

None of the rats died during the observation period or showed signs of gangrene or limb loss. Rat weight also increased over time (table 1). During the early postoperative days the rats seemed to favor the non-ischemic leg and at the first day after the second operation the plantar aspect of the left foot appeared cyanotic. This was not found at 1 week, but it presented pale and cool upon inspection and palpation compared to the control side up until 4 weeks. At 8 weeks both limbs appeared normal. Dark spots on the prominent plantar pads on the sole of the ischemic limb were noted in most rats between the second and fourth postoperative weeks.

Perfusion and Blood Flow

Volume Blood Flow

Median volume blood flow was 3.7 (range 2.9–4.2) $ml \cdot min^{-1}$ in the distal right femoral artery and higher ($p = 0.014$) than in the left side 0.06 (0.05–0.12) $ml \cdot min^{-1}$ 1 day after the second procedure. The difference persisted also at 4 weeks ($p = 0.021$), control leg 4.2 (3.6–4.4) and ischemic 0.9 (0.6–1.2) $ml \cdot min^{-1}$, respectively. The rise in volume blood flow in the ischemic leg over time tended to be significant ($p = 0.068$).

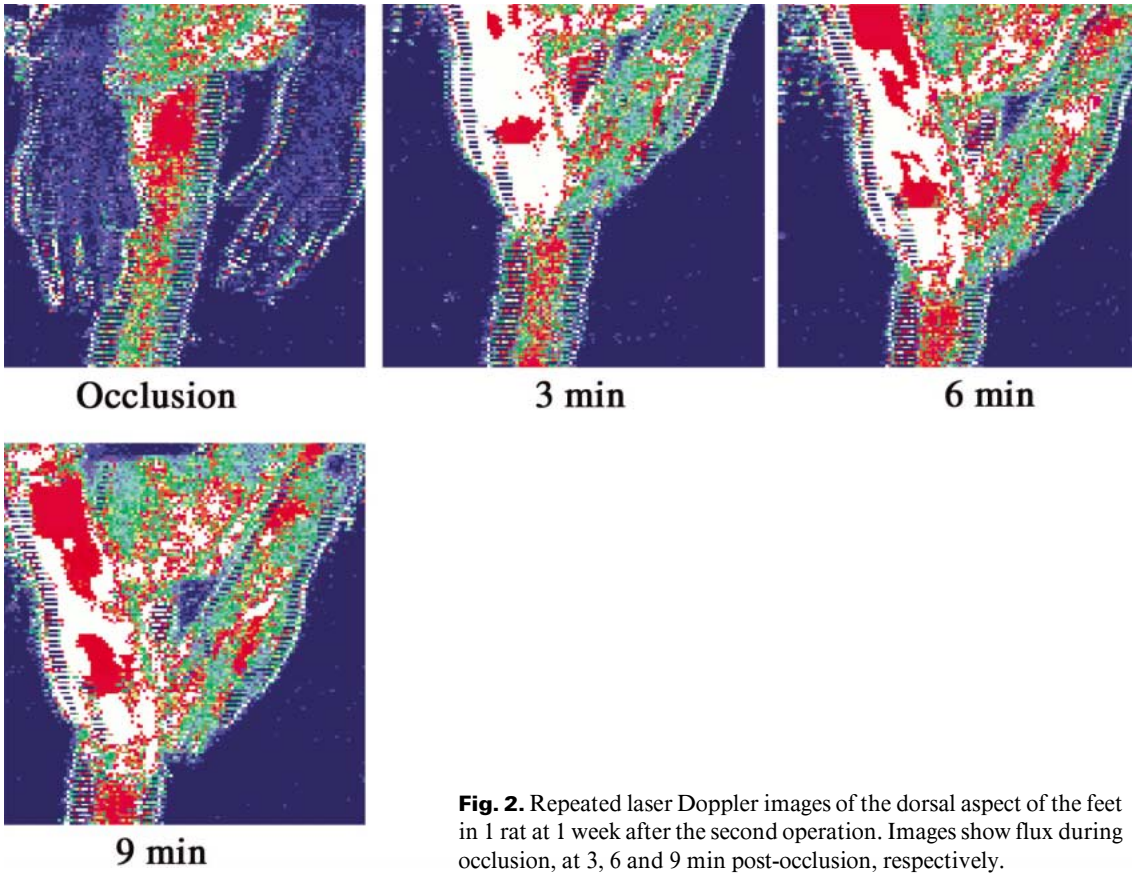

Fig. 2. Repeated laser Doppler images of the dorsal aspect of the feet in 1 rat at 1 week after the second operation. Images show flux during occlusion, at 3, 6 and 9 min post-occlusion, respectively.

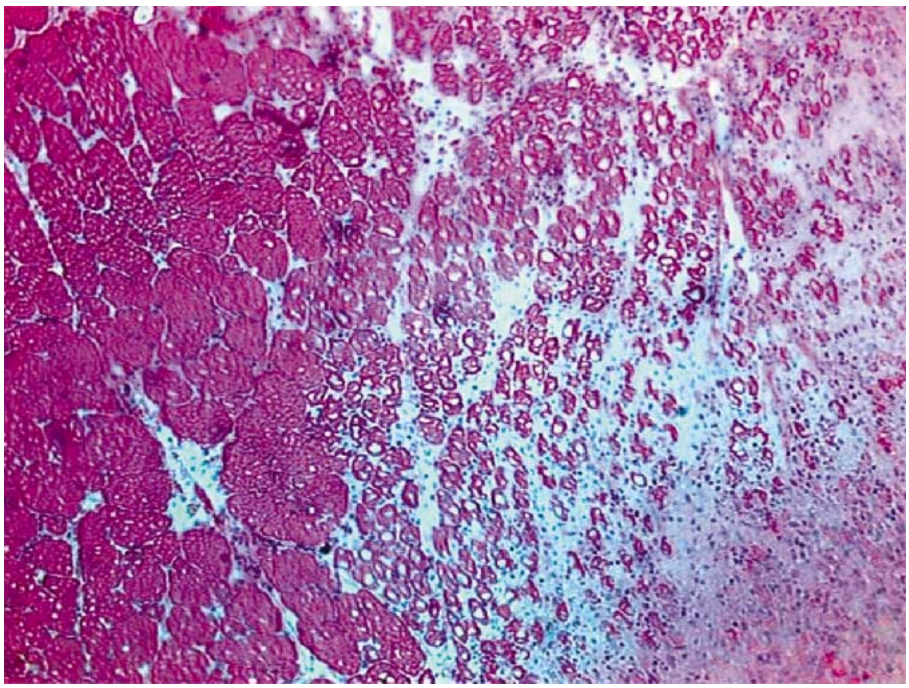

Fig. 3. Light microscopy of HE-stained section of anterior tibial muscle at 1 day. Normal muscle fibers are found in the left side of the picture, necrotic muscle fibers in the center and widespread infiltration of inflammatory cells in the center and in the lower right corner. (This slide was given score 3 for inflammation and 2 for necrosis by the examiner.)

Perfusion-LDI

LDI values are given in figure 4. For the dorsal aspect of the foot differences between the two limbs were significant ($p = 0.012$) at all time points. Perfusion in the ischemic leg increased between 1 day and 1 week ($p < 0.001$), and tended to increase also in the control leg ($p = 0.061$). The plantar aspect of the foot showed reduced ($p = 0.012$) perfusion compared to control in the operated limb until 4 weeks postoperatively, and the values increased ($p < 0.001$) between 1 day and 1 week, and 2 weeks. Also in the control leg the values increased ($p = 0.040$) between 1 day and 1 week. In the exposed anterior tibial muscle perfusion was significantly ($p = 0.012$) reduced only after 1 day. At 4 weeks two outliers with paradoxically high flow on the ischemic side were noted while the others had lower perfusion in the ischemic leg as compared to control. Perfusion improved between 1 day and 1 week ($p = 0.007$), and 1 week and 2 weeks ($p = 0.002$). Repeated laser Doppler images of the dorsal aspect of the feet in 1 rat at 1 week after the second operation are shown in figure 2. Images show flux during occlusion at 3, 6 and 9 min post-occlusion, respectively.

Microspheres

The microsphere method was attempted in 12 rats. Four died during catheter insertion in the carotid arteries (two at each time-point). The results from the remaining eight rats are shown in table 2. The variability in blood flow was considerable, possibly due to low numbers of spheres in the distal specimens (<400) and the limited number of rats in each group. It was significantly lower in all specimens on the ischemic compared to control side at one day of ischemia for skin, soleus proximal and distal gastrocnemius and distal adductor muscles. After 4 weeks the difference was significant only for skin and distal muscle in the leg while thigh muscle blood flow was similar between the sides.

Angiography

Examples of arteriograms are shown in figure 5. There were no visible side branches arising from the left side of the infrarenal aorta or the left iliac artery at any time point. At 1 day, smaller vessels in the left iliac region were filled with contrast while no vessels below the groin were visualized. At 2 weeks, the common iliac arteries were visualized bilaterally, though thinner on the left side. The left femoral artery was occluded below the groin and no large vessels were seen on either thigh or calf. At the level of the occluded femoral artery several small branches were filled from the iliac region.

At 4 weeks the left common and external iliac arteries were normal, the femoral artery was occluded in the groin. There were numerous visible small vessels both in the thigh and in the calf representing collateral vessels.

Histology

Anterior tibial muscle weights are shown in table 1. At 1 day, there was no difference between the two sides while at all other time points anterior tibial muscle mass was significantly lower on the ischemic side. Light microscopy of the anterior tibial muscle of the ischemic limb showed high rates (score 2–3) of edema and inflammation at 1 day and 1 week, moderate rate of necrosis (1–2) at 1 day while at 4 weeks only low rate of these parameters as well as low rate of fibrosis (0–1). An example is shown in figure 3. Specimens from the control limb showed no or only mild alterations (score 0–1). At 8 weeks there was no difference between the sides. In adductor specimens from the isch-

Table 1. Rat and tibialis muscle weights (median and range)

Time point	Body weight, g	Tibialis muscle weight, g	
		control (right)	ischemic (left)
One day	290 (255–325)	0.54 (0.45–0.66)	0.59 (0.41–0.71) n.s.
One week	300 (280–315)	0.60 (0.50–0.67)	0.48 (0.36–0.57)**
Two weeks	400 (355–450)	0.77 (0.69–0.87)	0.68 (0.57–0.82)*
Four weeks	470 (435–505)	0.90 (0.78–1.10)	0.74 (0.30–0.92)*
Eight weeks	560 (455–635)	0.99 (0.84–1.15)	0.89 (0.68–0.99)**

* $p < 0.05$ and ** $p < 0.01$ according to Wilcoxon signed-rank test.

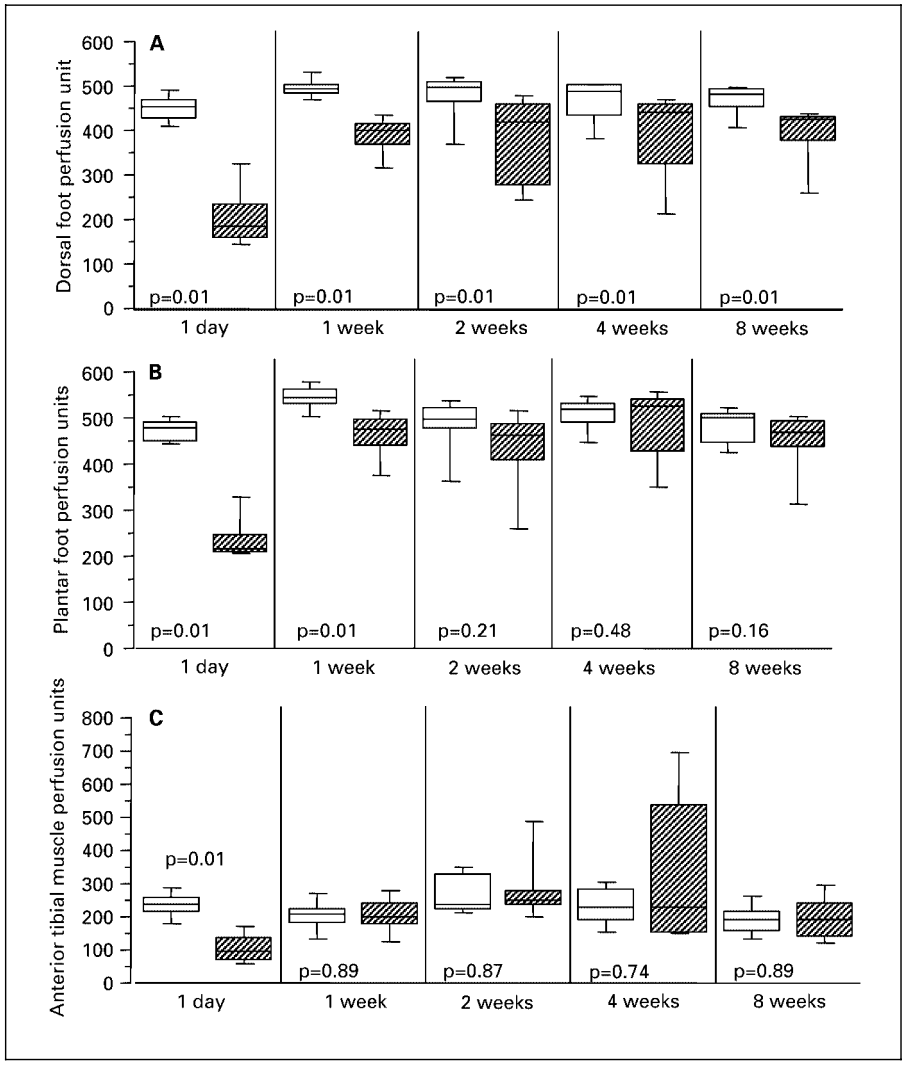

Fig. 4. LDI perfusion in the dorsal aspect of the foot (**A**), the plantar aspect of the foot (**B**) and the exposed anterior tibial muscle (**C**) in the control (□) and in the ischemic (▨) limb at different time points after the second operation. Arbitrary perfusion units, box plot showing median, 25th and 75th percentiles (box), and 10th and 90th percentiles (whiskers).

Table 2. Blood flow in muscle samples at 1 day and 4 weeks of ischemia – mean value of two injections of fluorescent microspheres (median and range)

Blood flow	1 day (n = 5)		4 weeks (n = 4)		8 weeks (n = 2)	
	right	left	right	left	right	left
Kidneys ($ml \cdot min^{-1} \cdot g^{-1}$)	10.6 (7.6–12.7)	11.4 (7.0–14.9)	8.8 (6.9–12.0)	8.0 (5.8–9.3)	14.9 (14.6)	13.9 (13.1)
Samples ($ml \cdot min^{-1} \cdot 100\ g^{-1}$)	Control	Ischemic	Control	Ischemic	Control	Ischemic
Proximal vastus	44.6 (29.7–74.7)	4.1* (0.5–7.6)	29.2 (21.9–54.3)	19.0 (9.2–6.0)	37.1 (86.5)	26.3 (35.3)
Proximal adductor	46.3 (22.1–66.0)	6.0* (0.3–10.7)	16.7 (9.0–23.4)	26.0 (3.9–41.0)	39.5 (85.9)	28.0 (34.4)
Anterio-tibial	39.8 (35.5–43.1)	2.8* (0.1–4.7)	21.1 (10.9–46.3)	7.6 (4.0–11.8)	82.9 (66.3)	31.5 (53.0)
Soleus	61.3 (43.0–88.2)	6.7* (0.2–20.2)	41.5 (33.7–70.1)	9.0* (1.2–15.5)	79.0 (75.8)	43.0 (73.3)
Gastrocnemius	78.6 (36.5–115.0)	9.5* (3.9–21.0)	46.6 (24.9–77.3)	13.1* (6.9–23.8)	57.0 (71.8)	38.5 (39.2)

* $p < 0.05$ according to Wilcoxon signed-rank test.

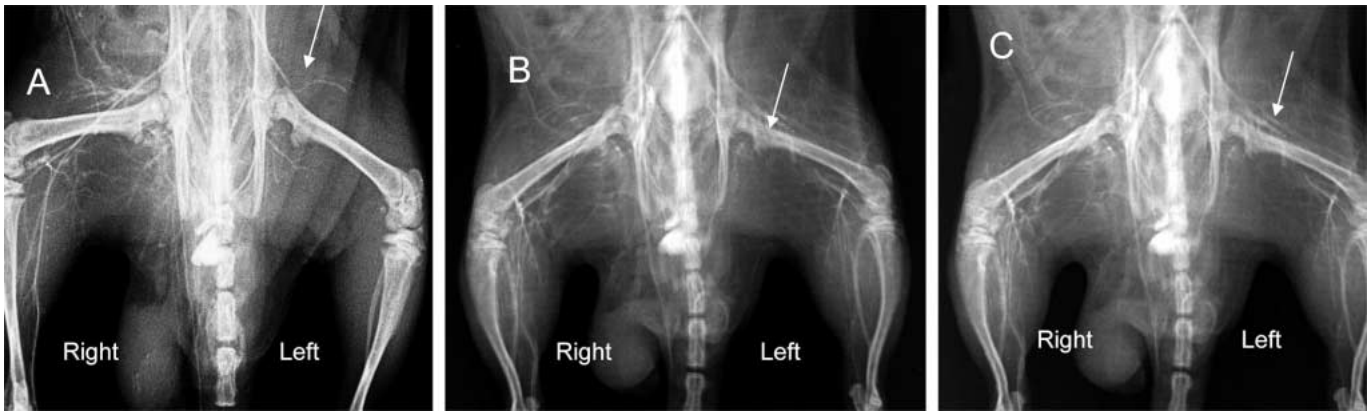

Fig. 5. Arteriograms obtained at 1 day (**A**), 2 weeks (**B**) and 4 weeks (**C**). Arrows mark the place for interruption of the femoral artery at the second operation.

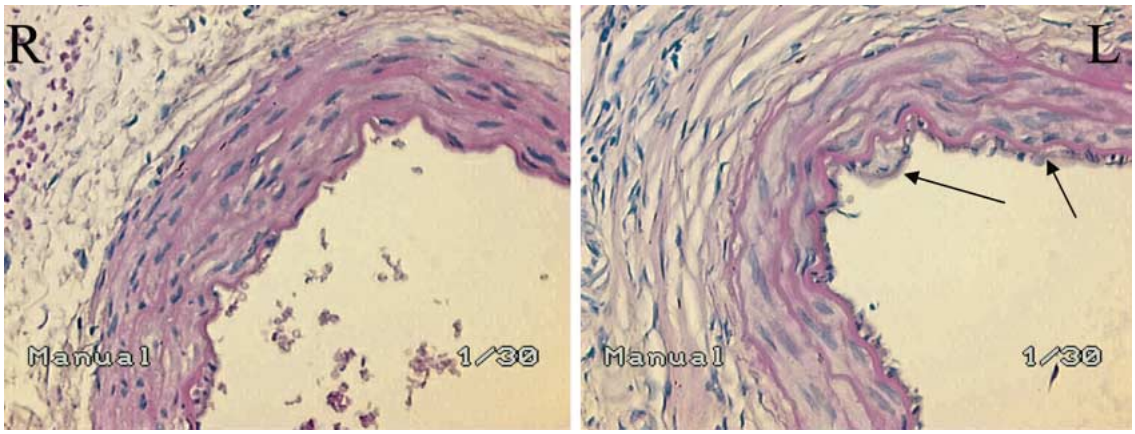

Fig. 6. Examples of femoral artery sections (HE) obtained at 4 weeks. L = Left, ischemic leg; R = right, control leg. Arrows show neointima formation.

emic side mild-to-moderate changes were seen at 1 day in 1 of the rats while at later time points and in the other 2 rats there were no differences between the two sides.

Femoral artery specimens were similar at 1 day, with the exception of one rat which had a thrombus proximal to the ligature. At 1 week none of the arteries were occluded and a neointima had formed in samples at 4 and 8 weeks (fig. 6).

Discussion

We have described and evaluated a rat model suitable for long-term studies of mechanisms involved in limb ischemia development. Considering the complexity of this disease any attempt to mimic it in an animal model can be regarded as futile. We propose that this ischemic rat limb could serve as a model of what takes place locally in the part of the much more heterogeneous human limb that is subjected to a local ischemic event. Acute metabolic disturbances, inflammation as well as collateral development through arterio- and angiogenesis and gradual adaptation could thus be studied in great detail.

To our knowledge, the only other rat model of severe leg ischemia that has been described besides the one suggested by Seifert [6] consists of excision of the entire femoral and external iliac artery combined with embolization of the internal iliac with microspheres [9]. The severity of ischemia in this model was similar to the present, creating a 90% reduction in plantar foot perfusion in the ischemic leg compared to control as assessed by LDPI at 1 day. The perfusion was normalized at 2 weeks. The surgical procedure appears to be more complicated than our modification of Seiferts model.

In the original paper by Seifert follow-up was limited to 5 days so it has not been known to what extent the model was suitable for studies of ischemia of longer duration. The model involves a two-stage procedure and was reported to be rather complex and time-consuming (2 h for the first operation). 10% mortality was also reported in the original article. It did, however, seem to have several obvious advantages. Besides producing satisfactory ischemia it leaves the entire leg to be used for subsequent analyses. Collaterals also seem to appear in the thigh. Accordingly, the present rat model is based on Seifert's, but modified to produce slightly less severe ischemia, saving one vessel to be spared for later analyses, and the model has now been evaluated for up to 8 weeks. In the present study we have found a profoundly reduced perfusion and intramuscular inflammation and necrosis early on after the ischemic event. As in other models, the perfusion of the ischemic foot was reduced still after 8 weeks. The observation of a rapid restoration of perfusion in anterior tibial muscle in our model was unexpected. Especially since the perfusion measurements with microspheres at 4 weeks showed marked decreased perfusion. The explanation to this discrepancy may be, at least partly, that the perfusion assessment with LDPI in anterior tibial muscle was inaccurate. Wasting of this muscle was substantial at 1, 2 and 4 weeks and the laser Doppler beam may have penetrated the muscle and scanned underlying vessels. Such explanation is supported by the observation that the 2 rats with the highest perfusion at 4 weeks also had the lowest muscle weight.

Overall, our microsphere data cannot be regarded as reliable. The variability in muscle blood flow in surviving rats were substantial and the low number of remaining rats did interpretation of the results difficult. Blood flow values obtained at 1 day were in agreement with LDPI data and around 10% of control limb in both proximal and distal samples. We could not identify previous publications on hindlimb ischemia giving absolute blood flow values measured with microspheres to allow comparison to our results.

Previous limb ischemia models proposed in the rat consist of arterial ligation, femoral [10, 11] or iliac [12] producing a degree of ischemia resembling claudication. It is often augmented by exercise or electrical stimulation [13, 14]. As an example, simple femoral artery ligation reduces gastrocnemius muscle blood flow at rest using xenon-133 injection technique with 50% at 1 week and less than 10% at 10 weeks following ligation [10]. Even though our model cannot be compared in detail to these claudicant models due to differences in the use of assessment methods, it is probable that our model creates more severe ischemia, but the duration of recovery, as well as the final deficiency in perfusion is similar.

Models in different species than rat have also been suggested, and the choice of laboratory animal has to be weighed considering costs, ethics and the purpose of the study. Pu et al. [15] suggested a rabbit model comprising ligation of the external iliac artery and excision of the entire femoral artery producing severe ischemia with little mortality. The duration of ischemia was limited to 40 days (venous lactate increase and limb blood flow reduction), while distal blood pressure was reduced up to 90 days. This model has been used extensively for studies of angiogenesis because collaterals in the thigh are clearly visualized by angiography [16]. Compared to the present model it may have the advantage of easier identification of collaterals but lack the possibility to excise the collateral vessels during sacrifice. It also only requires one surgical procedure but may not be as persistent. An identical femoral artery excision has been performed also in mice [17, 18]. Using either species the rather extensive dissection in the thigh may affect the surrounding tissue during the procedure and influence tissue analyses at early time points. In the mouse the small limbs make separation of different muscles difficult and invasive dynamic procedures such as electrical stimulation and microdialysis almost impossible to accomplish. Rats have an adequate size, they are inexpensive and have therefore been widely used to study limb ischemia.

In conclusion, this rat model is well tolerated by the animals and may be suited for longitudinal studies of events participating in the pathophysiology of limb ischemia, e.g. metabolism, inflammation and atrophy as well as healing mechanisms such as arterio- and angiogenesis.

Acknowledgements

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