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Variations in Surgical Procedures for Hind Limb Ischaemia Mouse Models Result in differences in Collateral Formation

A.A. Hellingman [a,b], A.J.N.M. Bastiaansen [a,b], M.R. de Vries [a,b], L. Seghers [a,b], M.A. Lijkwan [a,b], C.W. Löwik [c], J.F. Hamming [a], P.H.A. Quax [a,b,*]

[a] *Department of Vascular Surgery, Leiden University Medical Center, Leiden, The Netherlands*
[b] *Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands*
[c] *Department of Endocrinology and Molecular Imaging, Leiden University Medical Center, Leiden, The Netherlands*

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Abstract *Objective:* To identify the optimal mouse model for hind limb ischaemia, which offers a therapeutic window that is large enough to detect improvements of blood flow recovery, for example, using cell therapies.
Materials and Methods: Different surgical approaches were performed: single coagulation of femoral and iliac artery, total excision of femoral artery and double coagulation of femoral and iliac artery. Blood flow restoration was analysed with Laser Doppler Perfusion Imaging (LDPI). Immuno-histochemical stainings, angiography and micro-computed tomography (CT) scans were performed for visualisation of collaterals in the mouse.
Results: Significant differences in flow restoration were observed depending on the surgical procedure. After single coagulation, blood flow already restored 100% in 7 days, in contrast to a significant delayed flow restoration after double coagulation (54% after 28 days, $P < 0.001$). After total excision, blood flow was 100% recovered within 28 days. Compared with total excision, double coagulation displayed more pronounced corkscrew phenotype of the vessels typical for collateral arteries on angiographs.
Conclusion: The extent of the arterial injury is associated with different patterns of perfusion restoration. The double coagulation mouse model is, in our hands, the best model for studying new therapeutic approaches as it offers a therapeutic window in which improvements can be monitored efficiently.

* Corresponding author. Leiden University Medical Center, Postal zone K6-P, PO box 9600, 2300 RC Leiden, The Netherlands. Tel.: +31 71 5264005; fax: +31 71 526 6750.
E-mail address: p.h.a.quax@lumc.nl (P.H.A. Quax).

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Symptoms of ischaemia in patients with peripheral arterial disease (PAD) are dependent on several factors. For instance, the extent and level of stenosis or occlusion are important. Furthermore, factors affecting the development of collaterals such as haemodynamic factors as good antegrade flow and peripheral runoff vessels play a role.[1] These factors, among others, make it challenging to develop a good animal model for studying collateral formation in PAD. Animal models of hind limb ischaemia have been developed in mice,[2–10] rats[11,12] and rabbits.[2] Ischaemia-induced collateral artery formation has been mostly studied in mouse models. Surgical procedures range from a single ligation of the femoral or iliac artery[8,10] to a complete excision of the artery[5,13] and sometimes even the vein and nerve are dissected too.[14,15] Besides, the level of vascular occlusion, which is a determinant of the amount of ischaemia, ranges from a proximal ligation of the iliac artery[12] to a distal ligation just proximal to the bifurcation of the saphenous artery and the popliteal artery of the lower limb of mice.[6] These variations hamper the comparison of the outcomes of hind limb ischaemia induction. Another issue is that mice rapidly form collaterals, which limits the therapeutic window for potential arteriogenic agents.

The aim of this study was to develop a hind limb ischaemia mouse model with a therapeutic window large enough for testing new therapeutic approaches such as cell therapy. The effect of different surgical techniques and levels of vascular occlusion was compared for repair of blood flow, collateral artery formation and capillary formation in the ischaemic hind limb. First, a single electrocoagulation of the femoral artery in C57Bl6 mice, which is the most traditional model of hind limb ischaemia, is discussed. Second, a more proximal electrocoagulation was studied. Third, a total excision of the femoral artery with all their side branches as an often-used model of hind limb ischaemia was studied.[5,13] Finally, an alternative model of double electrocoagulation of both femoral artery and iliac artery, more closely resembling multi-level PAD, was developed.

Materials and methods

Experimental animals

For testing different surgical approaches to induce hind limb ischaemia, male C57Bl6 mice (Jackson) were used, aged 10—12 weeks. In addition, we performed a double coagulation in immune-deficient NOD-scid IL2Rgamma (null) mice. Experiments were approved by the committee on animal welfare of our institute.

General aspects of the surgical procedures

Before surgery, mice were anaesthetised with an intraperitoneal injection of a combination of midazolam (5 mg kg^{-1}, Roche), medetomidine (0.5 mg kg^{-1}, Orion) and fentanyl (0.05 mg kg^{-1}, Janssen). In all models, the femoral vein and nerve were preserved. After surgery, the skin was closed with 6/0 Ethilon sutures.

Technical details of different surgical procedures for inducing hind limb ischaemia (Different surgical procedures are also illustrated in Fig. 1)

- Single electrocoagulation of femoral artery:

A small skin incision was made in the left inguinal region. Directly after incision, the subcutaneous fat pad in the

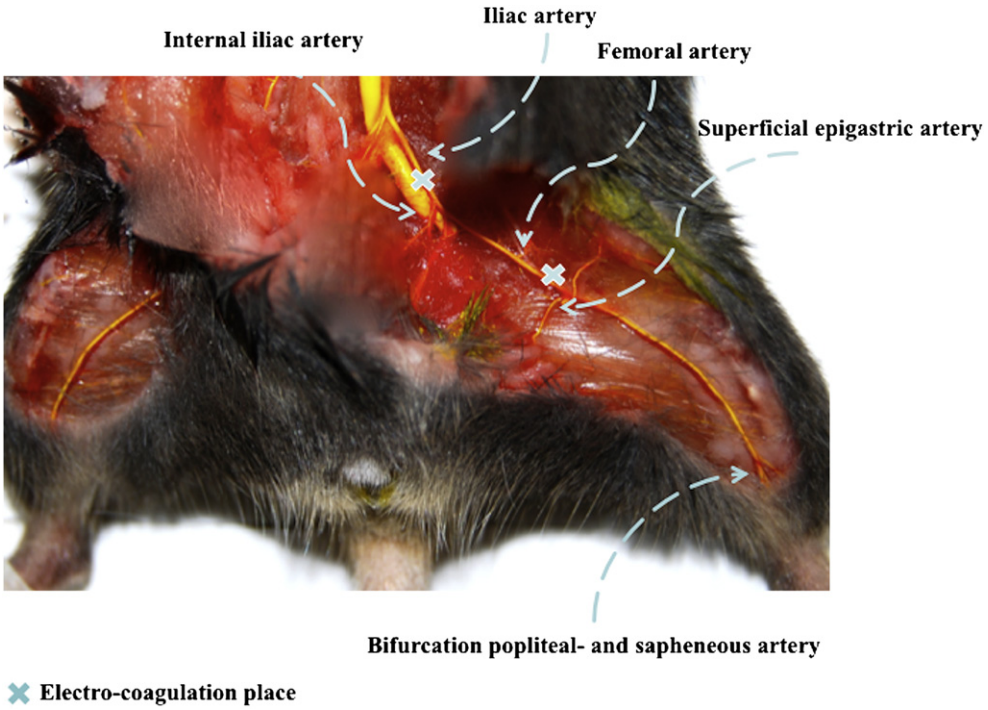

Figure 1 Illustration of the anatomical levels of electrocoagulation placed in different models of hind limb ischaemia. Crosses represent electrocoagulation places.

thigh was visible. It was not necessary to cleave the fat pad, but just pull it distally. After dissection of the artery from the nerve and vein, ischaemia was induced by electrocoagulation of the left femoral artery, proximal to the superficial epigastric artery. Electrocoagulation resulted in complete transaction of the artery. After electrocoagulation, the proximal end of the artery is moving proximally into the surrounding tissue and the distal end is moving distally, so there is a distance of a few millimetres between both ends after the surgical procedure.

- Single electrocoagulation of iliac artery:

A bigger skin incision in the inguinal region is made now. Further, there is no need to cleave the fat pad. For exposure of the iliac artery, we used a retroperitoneal approach. By carefully moving the peritoneum proximally with a cotton swab, a good exposure of the iliac artery was possible. Further, preparation of the artery from the vein was necessary. The internal iliac artery serves as a landmark; direct proximally of the internal iliac artery was an electrocoagulation of the common iliac artery performed.

- Total excision of femoral artery:

After incision of the skin from the inguinal region till the knee, we cleaved the subcutaneous fat pad for a better exposure. First, preparation of the common femoral artery took place (proximal excision site). Two 8/0 ties were placed around the artery, in the direction of the inguinal ligament as much as possible. Then, dissection of the whole artery from the vein and nerve in the distal direction was performed. All side branches of the artery were carefully dissected free and coagulated. Before excision, preparation of the distal level was performed and again two 8/0 ties were placed around the artery. The distal ligation level is at the popliteal artery level, just distal from the bifurcation of the saphenous artery and the popliteal artery. After cutting the artery between the two ligatures proximal and distal, the whole artery was removed from the surrounding tissue.

- Double electrocoagulation of both femoral artery and iliac artery

For a double coagulation model, both common iliac artery and femoral artery were electrocoagulated. First, an electrocoagulation of the common iliac artery was performed and subsequently an electrocoagulation of the femoral artery was performed. These coagulations are at the same anatomical levels used in the single electrocoagulation procedures of the femoral artery and the iliac artery. Similar techniques were used as described above.

Laser Doppler Perfusion Imaging (LDPI)

Measurements of perfusion were performed of the mouse hind limb before, directly after and weekly over 4 weeks after the surgical procedure with Laser Doppler Perfusion Imaging (LDPI) (Moor Instruments). To control for temperature variability during measurements, all animals were kept in a double-glassed jar filled with 37 °C water, keeping environment temperature at a constant level during the LDPI-measurements. Since LDPI-outcomes are sensitive for temperature changes, it is very important to control environment temperature during LDPI-measurements. Each animal served as its own control. Eventually, perfusion was expressed as a ratio of the left (ischaemic) to right (non-ischaemic) paw. Before LDPI, mice were anaesthetised with an intra-peritoneal injection of midazolam (5 mg kg^{-1}, Roche) and medetomidine (0.5 mg kg^{-1}, Orion).

Imaging

Post-mortem angiography of both hind limbs was performed using polyacrylamide—bismuth contrast (0.1gr ml^{-1}).[9] After thoracotomy, contrast fluid was injected into the left ventricle of the mouse heart. About 5 min before contrast injection, mice were intravenously injected with papaverine (50 mg ml^{-1}) for vasodilatation. The skin of both hind limbs was removed and X-rays were made. For CT scans, the same contrast and injection procedures were used. A SkyScan 1076 micro CT scan with a resolution of 18 micron was used. Angiographs and CT scans were solely used to illustrate collateral formation in the post-ischaemic hind limb. Quantification of collaterals was performed using immunohistochemistry.

Immunohistochemistry

About 5-μm-thick paraffin-embedded sections of skeletal muscle fixed with 3.7% formaldehyde were used. These were rehydrated and endogenous peroxidase activity was blocked for 20 min in methanol containing 0.3% hydrogen peroxide. For CD31 staining, sections were pre-incubated with trypsin for 30 min at 37 °C and incubated overnight with primary antibody (rat anti-mouse CD31Ab, BD Biosciences, dilution 1:200). Anti-rat immunoglobulin antibody was used as secondary antibody (goat anti-rat, AbCam, dilution 1:300). For an anti-α smooth muscle actin staining (mouse anti-human, DAKO, dilution 1:800), no antigen retrieval was necessary. Rat anti-mouse HRP (rabbit anti-mouse, DAKO, dilution 1:300) was used as secondary antibody. Negative controls were performed by using isotype controls. Stainings were quantified from randomly photographed sections using image analysis (Qwin, Leica).

Statistical analysis

Results are expressed as mean ± SEM. Comparisons between means were performed using an independent *t*-test or one-way analysis of variance (ANOVA). *P*-values < 0.05 were considered statistically significant. All calculations were performed using Statistical Package for Social Sciences (SPSS) 16.0.

Results

Impact of two different anatomical levels of electrocoagulation on blood flow restoration

After single electrocoagulation of the femoral artery and single electrocoagulation of iliac artery, the LDPI-ratios were significantly decreased immediately after coagulation. For

both procedures, blood flow dropped to <10%. No significant differences in blood flow restoration were observed, despite differences in the anatomical level of coagulation used to initiate ischemia measured with LDPI (Fig. 2).

Different patterns of blood flow restoration after total excision of femoral artery

Like a single electrocoagulation of the femoral artery, a total excision of the femoral artery resulted in a decline of blood flow perfusion. However, perfusion in the mouse hind limb restored considerably slower after a total excision (Fig. 2). After total excision of the femoral artery, C57Bl6 mice just had 100% recovery after 28 days, whereas C57Bl6 mice already had 100% blood flow recovery within 14 days after a single electrocoagulation of the femoral artery.

Thus, total excision of the femoral artery in C57Bl6 mice showed a more attenuated blood flow recovery compared with a single electrocoagulation.

Magnitude of impaired blood flow recovery and paw necrosis after a double electrocoagulation approach

After double electrocoagulation of both femoral and iliac artery, blood flow restoration was significantly impaired to 54% after 28 days compared with 100% blood flow restoration in 7 days after single electrocoagulation of femoral artery or iliac artery ($P < 0.001$) (Fig. 2). Although this is an extensive ischaemic model and there was slow blood flow recovery after the surgical procedure, only three out of 10 mice had necrosis of one or more toenails. There was no necrosis of the foot or limb. After single electrocoagulation of the femoral artery or iliac artery, we could hardly see any necrosis of toenails.

Imaging of collateral artery formation and capillaries in different surgical approaches of hind limb ischaemia

At 28 days after single electrocoagulation of the femoral artery or double electrocoagulation of both left femoral artery and iliac artery in C57Bl6 mice, angiographs showed normal arterial anatomy at the right side (non-operated side) and an increased number of collateral arteries in the left hind limb (operated side). Typical corkscrew-like collaterals can be observed in the (post-) ischemic hind limb (Fig. 3(A) and (B)). Angiographs made 28 days after a total excision of the femoral artery in C57Bl6 mice also showed more neovascularisation in the (post-) ischaemic hind limb compared with the non-operated hind limb. However, vessels formed after total excision of the femoral artery seem to have a different aspect on angiographs, that is, a very disturbed pattern of vasculature, with little or no

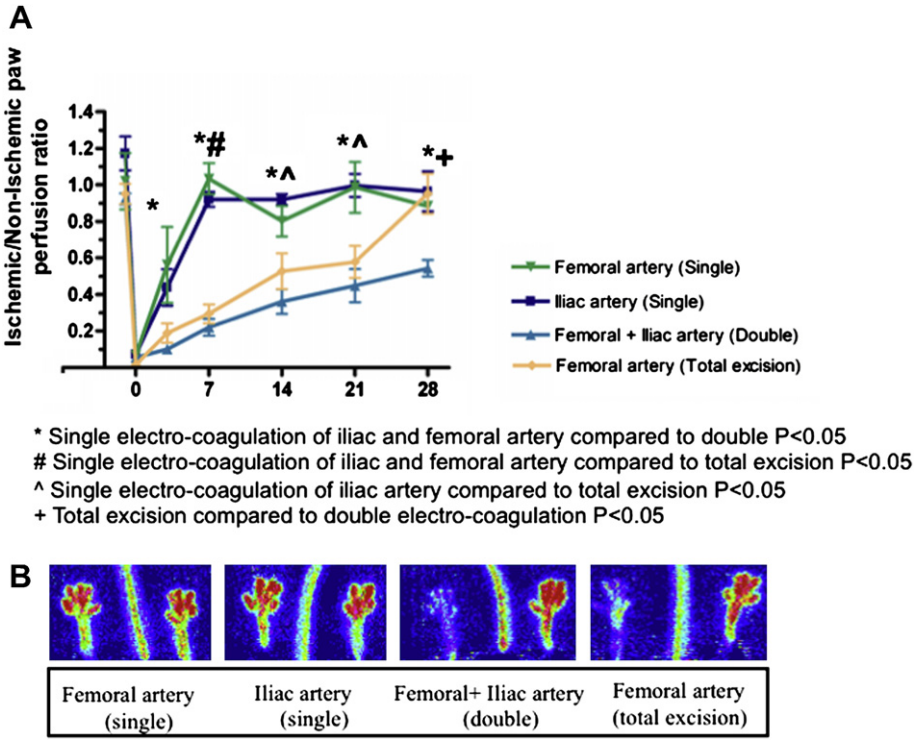

Figure 2 Blood flow restoration in hind limb of C57Bl6 mice. **A.** Blood flow recovery after a single femoral artery (distal anatomical level) electrocoagulation ($n = 3$, green line) or a single iliac artery (proximal anatomical level) electrocoagulation ($n = 9$, black line) or double electrocoagulation of both femoral artery and iliac artery ($n = 9$, blue line) or total excision of the femoral artery ($n = 6$, orange line) as monitored by Laser Doppler Perfusion Imaging (LPDI) and expressed as ratio between coagulated and non-coagulated limb. Data are presented as mean ± sem. $*\#\hat{}+P < 0.05$ (ANOVA-test) **B.** LDPI images of the paws at day 7 after different surgical procedures.

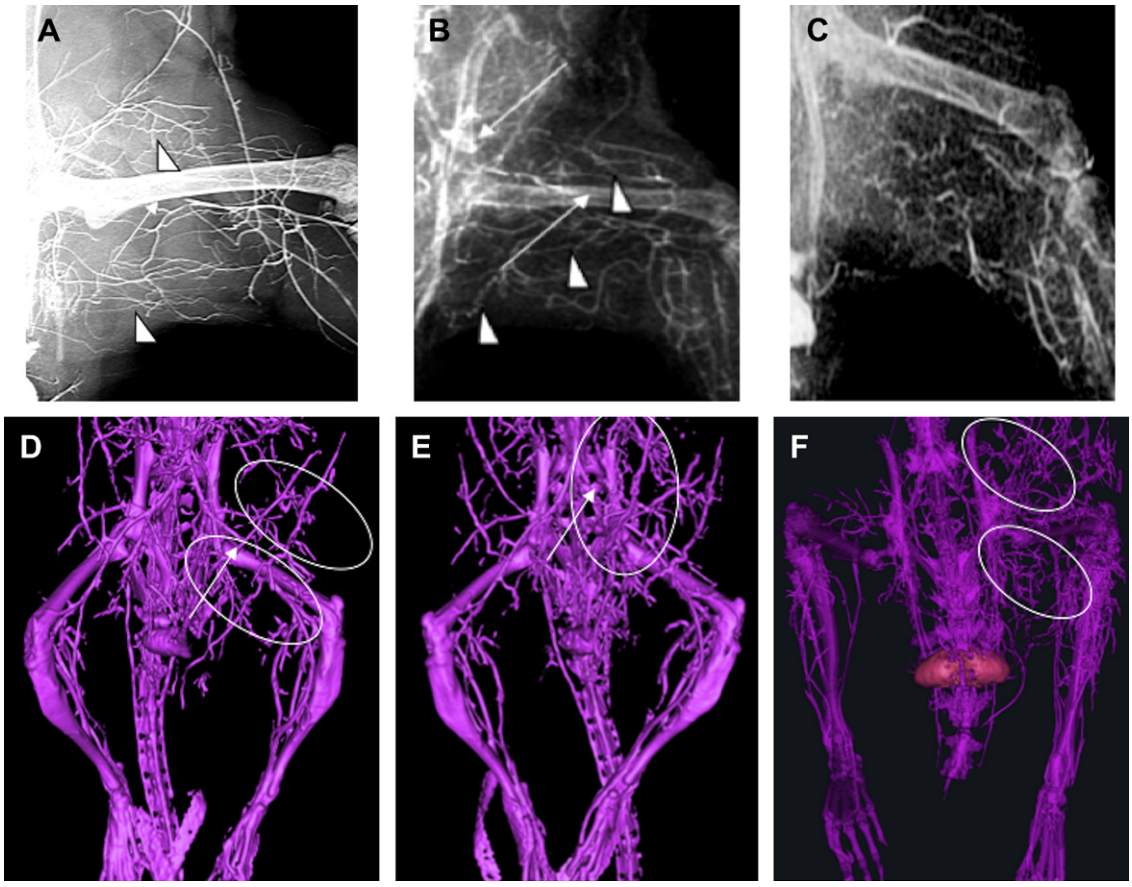

Figure 3 Angiographs of hind limbs of C57Bl6 mice made 28 days after induction of ischaemia by different surgical procedures. Angiograph made after **A.** single electrocoagulation of femoral artery, **B.** double electrocoagulation of both femoral artery and iliac artery and **C.** total excision of the femoral artery. Arrows indicate electrocoagulation places in the single electrocoagulation model (note that the artery retracts after coagulation) and double electrocoagulation model. Arrowheads show numerous typical corkscrew collaterals formed in (post) ischemic hind limb. After a total excision of the femoral artery and all side branches, a disturbed pattern of small vessels is formed in the adductor muscle. Micro CT scans of hind limbs made 28 days after, **D.** single electrocoagulation of the femoral artery, **E.** single electrocoagulation of the iliac artery, **F.** double electrocoagulation of both femoral artery and iliac artery. Numerous collateral arteries are formed around the iliac artery after single electrocoagulation of the iliac artery. Moreover, after single electrocoagulation of the femoral artery, collaterals are formed solely at femoral level. Double electrocoagulation showed numerous collaterals at both levels. Arrows indicate electrocoagulation places. Circles represent collateral zone.

typical corkscrew collaterals as we observed after single or double coagulation (Fig. 3(C)).

The increase in collaterals in the (post-) ischaemic hind limb was also confirmed by CT scans of these mice made 28 days after coagulation of both femoral artery and iliac artery (Fig. 3 (D)−(F)). These CT scans illustrate very nicely the formation of new vessels both on the iliac level after iliac coagulation as well as on the femoral level after femoral coagulation.

Increased collateral and capillary density in the ischaemic muscle after double coagulation of femoral artery and iliac artery

Collateral density in the adductor muscle of the (post-) ischaemic hind limb was higher compared with the adductor of the non-ischaemic hind limb, although not significant (respectively, 1.73 and 0.97; $P = 0.246$) (Fig. 4(A)−(C)). In addition, in the lower limb, a significant increase in capillary density was observed in the ischaemic as compared with non-ischaemic calf muscle 28 days after surgical procedure ($P = 0.020$) (Fig. 4(D)−(F)).

Double electrocoagulation of both femoral artery and iliac artery in immune-deficient mice

To validate the double electrocoagulation model of hind limb ischaemia for testing human cell therapies, we performed a double coagulation in immune-deficient NOD-scid IL2R-gamma(null) mice. Similar to double electrocoagulation in C57Bl6 mice, blood flow restoration after double electrocoagulation in NOD-scid IL2Rgamma(null) mice was significantly decreased to 31% after 7 days compared with 104% after a single electrocoagulation of the femoral artery ($P = 0.002$) (Fig. 5(A)−(C)). Nine out of 10 mice had necrosis of one or more toenails in this model of extensive ischaemia. There was no necrosis of the paw or limb in these mice.

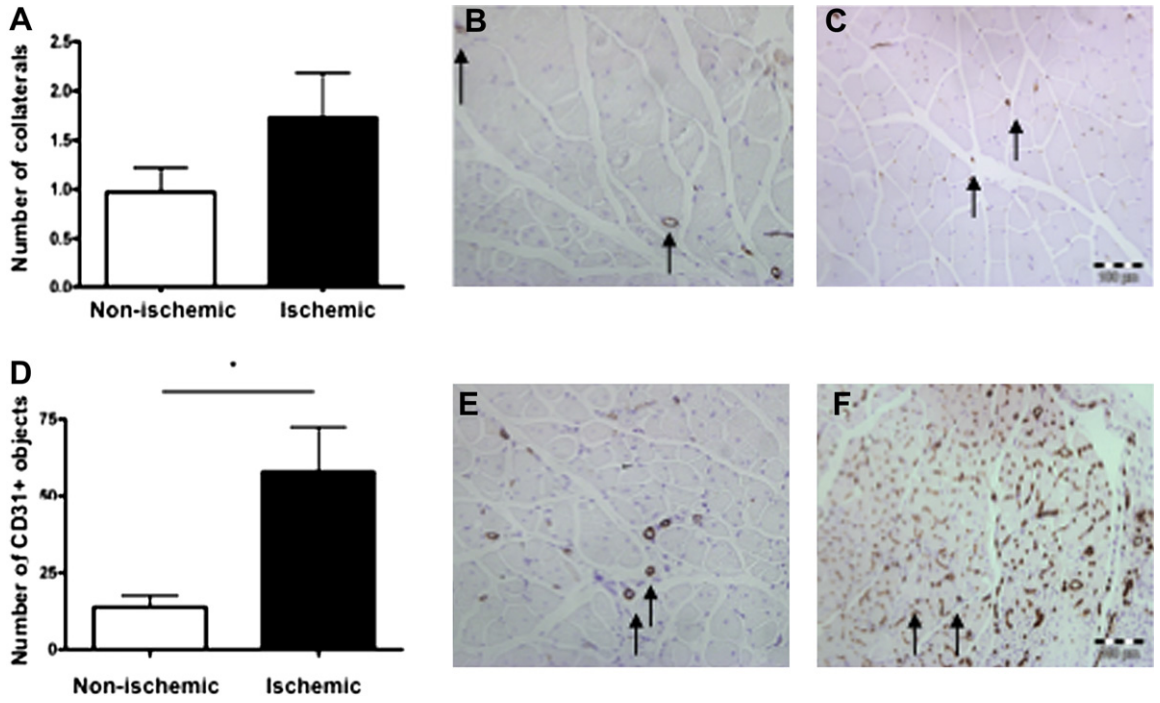

Figure 4 Immuno-histochemical staining of skeletal muscle after 28 days of double coagulation with anti-α-smooth muscle acting antibody and anti-CD31 antibody for detection of collaterals and capillaries. **A.** Quantification of anti-α-smooth muscle actin stained adductor muscle sections comparing ischemic hind limb with non-ischemic hind limb (9 section per mouse were analyzed to get obtain the mean per animal, next the mean of $n = 9$ animals was determined). Although the number in collaterals seems to increase, the differences between the number of collaterals are not statistically significant; $P = 0.246$. Data were presented as mean $\pm$ sem. Representative photographs of anti-α-smooth muscle actin stained **B.** non-ischemic adductor muscle sections and **C.** ischemic adductor sections. **D.** Quantification of anti-CD31 stained calf muscle sections comparing ischemic hind limb with non-ischemic hind limb (9 section per mouse were analyzed to get obtain the mean per animal, next the mean of $n = 9$ animals was determined). Number of CD31 + blood vessels in ischemic hind limb differs significantly from non-ischemic hind limb. $*P = 0.020$. Data were presented as mean $\pm$ sem. Representative photographs of anti-CD31 stained calf muscle sections after double electrocoagulation of both femoral artery and iliac artery of the **E.** non-ischemic hind limb and **F.** ischemic hind limb.

Discussion

In the present study, it is demonstrated that the extent of the arterial defect (single ligation of artery, total excision of artery or double ligation of artery) is associated with different patterns of perfusion restoration in the mouse hind limb. Blood flow recovery was substantially impaired in a mouse model of double electrocoagulation of both femoral artery and iliac artery compared with single electrocoagulation of one of these arteries. This results in an increase of the therapeutic window to study improved restoration of blood flow after experimental therapeutic approaches as cell therapy.

The anatomical level of occlusion of the artery (single electrocoagulation of femoral artery or iliac artery) had a similar effect on blood flow recovery in the hind limb ischaemia mouse model. These results resemble studies of Shireman et al.[16] They showed similar patterns of blood flow recovery after transection of the proximal femoral artery, compared with transection of the distal femoral artery.

Angiographs made 28 days after total excision of the femoral artery showed a disturbed pattern of new small vessels formed in the (post-) ischaemic hind limb. In contrast, angiographs made after single or double coagulation of the vascular tree showed more profound collateral arteries with the typical corkscrew phenotype in the (post-) ischaemic hind limb. Some technical and physiological differences between these models could explain the disturbed pattern of vessels on angiographs after a total excision of the femoral artery. First, after a single ligation of the femoral artery, all side branches of the artery were kept intact. However, after total excision of the femoral artery all the connections to the pre-existing collateral bed were likely to be disrupted completely. For restoration of the blood flow, not only pre-existing distant vessels need to enlarge their diameter to become collaterals, but also the disrupted connections need to be repaired in this model. Accordingly, in the profound ischaemic model of total excision, it is not very likely that a process of arteriogenesis will solely appear. The process of angiogenesis will be most likely involved too, because all pre-existing connections of arterioles to the vascular tree are disrupted and need to be repaired. Sprouting of new capillaries (angiogenesis)[17] is a distinct process from collateral artery formation (arteriogenesis).[18] Formation of new capillaries is mainly triggered by ischaemia.[17,19,20] Arteriogenesis refers to the remodelling of pre-existent arterial collaterals that interconnects the vascular networks lying proximal and distal to the arterial

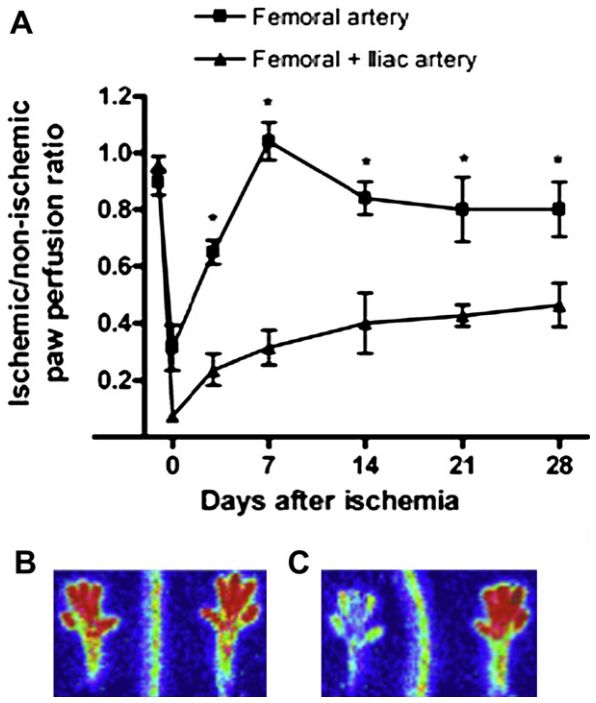

Figure 5 Blood flow restoration in hind limb of NOD-SCID IL2Rgamma(null) mice. A. Blood flow recovery after a single femoral artery electrocoagulation ($n = 5$) or a double electrocoagulation of both femoral artery and iliac artery ($n = 10$). After a double electrocoagulation of femoral artery and iliac artery, perfusion remained significantly impaired until 28 days after the surgical procedure. $*P = <0.014$. B. LDPI images of ischemic paw perfusion (left) and non-ischemic paw perfusion (right) 7 days after single electrocoagulation. C. LDPI images of ischemic paw perfusion (left) and non-ischemic paw perfusion (right) 7 days after double electrocoagulation.

obstruction and is triggered by increased shear stress.[21–23] Despite the fact that a disturbed pattern of blood vessels is formed in the adductor muscle, mice though can restore blood flow restoration to 100% after total excision. Since all pre-existing connections of arterioles to the vascular tree are disrupted and need to be repaired (angiogenesis) in this model, blood flow restoration takes longer compared with single electrocoagulation of the artery. Oses et al.[24] recently demonstrated very elegantly significant differences in ischaemia-induced vascular growth mechanisms between the tight (mostly attributable to arteriogenesis) and the tibiofibular region (angiogenesis predominated in the tibiofibular region). Therefore, the model of total excision of the femoral artery seemed not to be recommendable for studying arteriogenesis, solely. One has to keep in mind that technical variations in hind limb ischaemia mouse models do have physiological consequences, although the impact of these variations is often underestimated.

The impact of the use of technical variations in hind limb ischaemia models on the outcome can be illustrated with conflicting outcomes of several experiments on VEGF-mediated gene therapy. Several research groups[25,26]reported an enhanced revascularisation after arterial gene transfer of VEGF in the ischaemic hind limb models, whereas others did not see any effect.[27] Takeshita et al.[25] showed a significant increase in angiographic score of developed collaterals after VEGF administration. On the other hand, van Weel et al.[27] did not see any effect of VEGF in the hind limb ischaemia model on angiographic rentrop score and blood flow restoration measured with LDPI. This difference in outcome could be explained by the fact that Takeshita et al. tested VEGF administration in a model of total excision of the femoral artery with all their side branches, whereas van Weel et al. used the model of single electrocoagulation of the femoral artery.

Models of hind limb ischaemia in immune-deficient mice have been established to investigate the role of human cells in arteriogenesis. Kalka et al.[28] reported impaired blood flow restoration in nude mice after resection of the femoral artery. However, our results showed that after single electrocoagulation of the femoral artery of immune-deficient mice, blood flow recovery was 100% within 7 days, once again, underscoring the impact of the different surgical procedures. The extremely fast blood flow restoration makes our model difficult for testing the potential stimulating role of different human cells in collateral artery formation. In this study, validation of the double electrocoagulation model was also performed in immune-deficient mice too. Although this is a more severe model of ischaemia, blood flow gradually recovered after a double electrocoagulation and no abundant paw necrosis was developed in these mice. Furthermore, the therapeutic window for stimulation of blood flow restoration is considerably enlarged in a double coagulation hind limb ischaemia model in immune-deficient mice (31% blood flow recovery within 7 days in NOD-scid IL2Rgamma (null) mice). This illustrates that the double coagulation model in immune-deficient mice is a useful model for testing new human cell therapies for patients with PAD.

Although the study was designed to identify the most optimal model for testing strategies to improve blood flow restoration, we realise that there are some limitations. The first relates to the degree of ischaemia that is inflicted. As it is not possible for us to quantify the differences in ischaemia that occurs after the surgery, we can only assume that inducing the different extents of arterial defects (single electrocoagulation, total excision or double electrocoagulation) is associated with climbing amounts of ischaemia. Therefore, we mainly focussed our analyses on differences in collateral artery formation, which is triggered by increased shear stress and not directly by ischaemia. A second limitation is that we have performed our studies on healthy mice, whereas most patients with severe PAD have risk factors such as diabetes and hypercholesterolaemia. To resemble clinical situation, one could consider using hypercholesterolaemic or diabetic mice for the hind limb ischaemia model. However, for comparison of the surgical procedures, we decided not to include these factors. The double electrocoagulation model was only tested in immune-deficient mice, in which human cells can be evaluated as candidates for cell therapy.

In conclusion, there is a variety of surgical approaches for inducing ischaemia in the mouse hind limb. The results of the present study show that the amount of injury to the vascular tree (single ligation of artery, total excision of artery or double ligation) does have consequences for the pattern of blood flow restoration, while the level of

vascular occlusion (femoral or iliac) does not. For testing new therapeutic approaches for patients with PAD, the double coagulation model might be the optimal model, because it provides a substantial therapeutic window to stimulate blood flow restoration.

Conflict of Interest

None.

Acknowledgements

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the on-line version, at 10.1016/j.ejvs.2010.07.009

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