Limb ischemia after iliac ligation in aged mice stimulates angiogenesis without arteriogenesis

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Abstract

Objective(s)—Older patients are thought to tolerate acute ischemia more poorly than younger patients. Since aging may depress both angiogenesis and arteriogenesis, we determined the effects of age on both angiogenesis and arteriogenesis, in a model of severe acute limb ischemia.

Methods—Young adult (3 month) and aged (18 month) C57BL/6 mice underwent right common iliac artery and vein ligation and transection. Data were collected on days 0, 7, and 14. Perfusion was measured with laser Doppler and compared to the contralateral limb. Functional deficits were evaluated with the Tarlov scale. Capillary density and endothelial progenitor cell (EPC) number were determined by direct counting lectin-positive/alpha-actin-negative cells and VEGFR2/CXCR4 dually-positive cells, respectively; angiography was performed to directly assess arteriogenesis.

Results—Young adult and aged mice had a similar degree of decreased perfusion after iliac ligation (young, n=15: 20.4±1.9%, vs. old, n=20: 19.4±1.3%; p=.72, ANOVA); however, young mice recovered faster and to a greater degree than aged mice (day 7, 35±6% vs. 17±4%, p=.046; day 14, 60±5% vs. 27±7%, p=.0014). Aged mice had worse functional recovery by day 14 compared to young mice (2.3±.3 vs. 4.3±.4; p=.0021). Aged mice had increased capillary density (day 7, 12.9±4.4 vs. 2.8±0.3 capillaries/hpf; p=.02) and increased number of EPC incorporated into the ischemic muscle (day 7, 8.1±0.9 vs. 2.5±1.9 cells; p=0.007) compared to young mice, but diminished numbers of collateral vessels to the ischemic limb (1 vs. 9; p=.01), as seen on angiography.
Conclusions—After severe hindlimb ischemia, aged animals become ischemic to a similar degree as young animals, but aged animals have significantly impaired arteriogenesis and functional recovery compared to younger animals. These results suggest that strategies to stimulate arteriogenesis may complement those that increase angiogenesis, and may result in improved relief of ischemia.

Clinical Relevance—The incidence of chronic limb ischemia increases with age as do the consequences of acute ischemia. We show, using a new model of severe acute limb ischemia that does not wound the ischemic limb, that aged mice increase angiogenesis in response to acute ischemia, but do not show arteriogenesis, i.e. large collateral formation. These results suggest why elderly patients develop large vessel disease such as claudication but can still heal small wounds. They also suggest that strategies to treat ischemia in elderly patients should focus on stimulating large vessel arteriogenesis, rather than solely small vessel angiogenesis.

Keywords
ischemia; angiogenesis; arteriogenesis; endothelial progenitor cell; collateral

Elderly patients with chronic lower extremity ischemia have reduced survival compared to younger patients.1, 2 Nevertheless, operative outcome for chronic ischemia, including graft patency and limb salvage, are similar in elderly patients and younger patients, with many series reporting excellent limb salvage in elderly patients.3–8 However, elderly patients have worse outcome after acute limb ischemia, with increased risk of mortality and amputation.9–12

Femoral artery ligation, without or with concomitant femoral vein ligation, is a common animal model used for investigation of acute limb ischemia.13, 14 This commonly used model has demonstrated that remodeling and angiogenesis in response to acute ischemia depends on endogenous endothelial-derived nitric oxide synthase (eNOS), nitric oxide (NO), vascular endothelial growth factor (VEGF)-A, and Akt1, at least in young animals.15, 16 In addition, bone marrow-derived endothelial progenitor cells (EPC) play a role in wound healing in the ischemic leg.13, 17 The femoral artery ligation model has also demonstrated that aged animals have reduced blood flow and limb salvage after acute limb ischemia compared to young animals.18 Reduced limb flow in aged animals is thought to be due to impaired angiogenesis, possibly due to age-related endothelial dysfunction or reduced NO or VEGF-A expression.18, 19 Interestingly, administration of exogenous VEGF-A can restore the angiogenic potential in aged mice and exercise increases VEGF-A expression, although these results have not been applicable to humans.20–23 In addition, the femoral artery ligation model has demonstrated that wound healing is impaired in the ischemic limb.17, 24

However, the femoral artery ligation model, especially in absence of femoral vein ligation, is not an especially severe limb ischemia model, compared to other models such as the femoral/popliteal/saphenous artery excision model.25 In addition, the femoral artery model necessarily involves an incision in the ischemic, but not control, leg, confounding easy interpretation. Since elderly patients often present with advanced ischemia, we developed a novel model of ischemia that is more severe compared to femoral artery ligation but does not require surgical manipulation of the ischemic leg. Using iliac artery and vein ligation, we test the hypothesis that aged animals have worse outcome after severe limb ischemia compared to young adult animals, and that worse outcome in aged animals is due to impaired angiogenesis.
Methods

Animal model

Young adult (3 month) and aged (18 month) male C57Bl/6 mice (National Institutes of Aging) underwent unilateral common iliac artery and vein ligation; the contralateral leg served as control. Anesthesia was performed using ketamine (100 mg/kg) and xylazine (5 mg/kg). A midline incision was performed in the abdomen and the right common iliac artery and vein were circumferentially exposed but not separated from each other. The artery and vein were doubly ligated with 7-0 silk and divided proximal to the internal iliac artery; the vein was ligated both to increase the severity of the ischemia as well as to increase the technical reproducibility of the model, as isolation of the iliac artery alone often results in tearing of the iliac vein resulting in fatal hemorrhage. The abdomen was closed in 2 layers and the mouse allowed to recover. Animal care and experimental procedures complied with “Principles of Laboratory Animal Care” (Guide for the Care and Use of Laboratory Animals, National Institutes of Health publication 86-23, 1985), and the Institutional Animal Care and Use Committee approved all experimental protocols.

Flow measurement

In some animals, blood flow was measured in anesthetized animals, both in the ischemic leg and the control leg, preoperatively, immediately postoperatively, and at 1 and 2 weeks after induction of hind limb ischemia, using the PeriFlux Laser Doppler Perfusion Measurement (LDPM) unit with a “deep probe” configuration (Perimed, North Royalton, OH). Access to the soleus muscle was obtained through a 3 mm skin incision on each hind limb that was closed after measurement; the same incision was used for all four measurements. This incision was only made for mice having flow measurements and not for any other experiments. This method allows for reproducible measurements directly in the muscle bed, avoiding cutaneous blood flow and impaired wound healing, and it is not significantly temperature dependent. Blood flow values were expressed as the ratio of ischemic to control leg perfusion.

Functional scoring

Mice were examined preoperatively, immediately postoperatively but after recovery from anesthesia, and at 1 and 2 weeks after induction of hind limb ischemia. Functional grading was performed according to the Tarlov scale, a standardized mouse limb ischemia grading scale, and a modified ischemia scale to detect less severe levels of ischemia (Table).

Viability index

Mice were sacrificed preoperatively, immediately postoperatively, and at 1 and 2 weeks after induction of hind limb ischemia. The soleus muscle was harvested and dessicated over night. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay was performed as previously described, and is an index of tissue viability as well as mitochondrial function.

Capillary density analysis

Mice were sacrificed at 0, 1 or 2 weeks after surgery, and the soleus muscles of the legs were harvested, methanol fixed, and paraffin embedded. 5 micron sections were used. Muscle fiber number and size were examined in sections stained with hematoxylin and eosin, averaging the counts of 5 separate fields in 4 distinct areas in each specimen. Capillary density in the thigh muscle was assessed by immunofluorescence using FITC-conjugated mouse monoclonal anti-alpha-actin (Sigma) and TRITC-labeled lectin (Sigma), with capillaries identified as vessels that stained positively for lectin but not for alpha-actin. After overnight incubation at 4°C, sections were mounted, and observed under a microscope equipped with the appropriate filters (Axioimager A1; Carl Zeiss). Capillary densities, i.e. number of capillaries per number of

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muscle fibers, were measured using image analysis (MetaMorph software) in 5 different randomized fields of each slide. The ratio of capillary density in the ischemic leg compared to the control leg was recorded.

**Endothelial progenitor cells**

EPC were detected in the soleus muscle as previously described, i.e. as cells that were detected as both VEGFR2 and CXCR4 positive.\(^{30}\) CXCR4 is the receptor for SDF-1, a key regulator of stem cell trafficking between the peripheral circulation and the bone marrow. CD34+ cells express CXCR4; 66% of EPC express CXCR4 and respond to CXCR4 ligands in a dose-dependent fashion.\(^{31}\) Slides were washed in xylene and graded EtOH prior to incubation with Target Retrieval Buffer (Dako) at 96 °F for 20 minutes. After cooling, nonspecific targets were blocked with 10% goat serum. Slides were incubated overnight with primary antibodies (goat anti-mouse CXCR4, 1:300, Capralogics; rabbit anti-mouse VEGFR2, 1:100, Abcam) and washed prior to incubation with secondary antibodies (donkey anti-goat AgG-FITC, sheep anti-rabbit IgG-rhodamine) for 1 hour. Counterstaining was with DAPI (Vector).

Immunofluorescence was used for detection, and positive staining and total numbers of cells were directly counted by a blinded observer, averaging the counts of 10 separate fields in 4 distinct areas in each specimen.

**CT Angiography**

At 1 week after surgery mice were anesthetized with isoflurane and the left internal jugular vein cannulated. In vivo CT scanning was performed using an X-SPECT machine (GammaMedica-Ideas), X-ray energy 75kV/265\(\mu\)A, zoom 2.0, and spatial resolution approximately 75 \(\mu\)m; in vivo scans were performed without and with intravenous contrast (FenestraVC (0.2cc) and Omnipaque (0.1cc)), but without heparin or vasodilators. In scans to be used for densitometry, barium was used as an alternative contrast agent. The descending aorta was cannulated and vessels were flushed and fixed with 4% paraformaldehyde followed by infusion with contrast (barium sulfate 60% emulsion) and then CT scanning. Collaterals were counted in the 3D reconstructions by densitometry above and below the femur, with a threshold of 50 (pixel value range 0–255), and confirmed by direct counting.\(^{32}\) CT scans were restricted to the arterial phase by injection of contrast agent until the foot blanched, with care to cease additional injection of contrast upon blanching; as the contrast agent induces death, no venous phase can be obtained.

**Direct Injection Angiography**

At 1 week after surgery mice were anesthetized and given intravenous heparin. The aorta was perfused with PBS containing vasodilators (papaverine, 4 mg/L; adenosine 1 g/L) for 3 minutes at physiological pressure. Vessels were fixed with 2% paraformaldehyde for 5 min, flushed with PBS for 2 min, and then infused with contrast (bismuth oxychloride in saline, and 10% gelatin in PBS, 1:1) under pressure (120 mmHg) controlled by a sphygmomanometer; contrast injection simultaneously induces death. Mice were then placed in ice to solidify the contrast. Microangiography was then performed using a Faxitron x-ray machine (Hewlett-Packard) at 25 kV and 3.25 mA for 3 minutes.\(^{15}\)

**Statistics**

Statistical analysis was performed using StatView (SAS Institute). Comparisons between multiple groups were performed using one-way ANOVA. P-values 0.05 were considered statistically significant.
Results

Reduced function in aged mice after limb ischemia

To develop a novel model of acute limb ischemia, young adult (3 month) and aged (18 month) male C57Bl/6 mice underwent common iliac artery and vein ligation (Figure 1A), with the contralateral leg serving as control; no incision was made on the ischemic leg as part of the model, unlike the femoral ligation model that requires a leg incision (Figure 1B). Leg swelling was not observed in any ischemic limb. Young adult and aged mice had similar amounts of blood flow in the leg and functional status at baseline (Figure 1). In young adult mice, blood flow dropped rapidly after arterial ligation and returned slowly over 4 weeks, achieving 60% of pre-procedural flow by 2 weeks (Figure 1C). In aged mice blood flow was reduced by a similar amount by the operative procedure, but, in contrast, flow remained approximately 25% of baseline and did not recover (Figure 1C). Similarly, functional status returned to baseline in young adult mice, whereas old mice remained impaired in functional deficits (Figure 1D; supplementary movies #1 and #2).

Although aged mice exhibited reduced blood flow and less recovery of function after hind limb ischemia compared to young adult mice, we observed that distal foot ischemia did not appear more severe in aged mice. Using a standard mouse limb ischemia score, aged mice did not exhibit more severe degrees of gangrene or ulceration compared to young adult mice (Figure 1E). To determine if a more sensitive scale could detect more subtle differences in ischemia, we examined young adult and aged mice on our own modification of the ischemia score (Table); nevertheless, young adult and aged mice exhibited similar degrees of limb ischemia (Figure 1F).

Since distal limb ischemia was similar among young adult and aged mice (Figures 1E and 1F), we examined deep muscle histology to determine whether there were any differences that could account for the reduced function of aged mice after acute limb ischemia. At baseline, aged mice had increased number of smaller muscle fibers compared to young adult mice (Figures 2A, 2B, and 2C). Two weeks after induction of limb ischemia young mice demonstrated loss of mean muscle fiber area (Figures 2A and 2C), with preservation of fiber number (Figures 2A and 2B), consistent with muscle fiber atrophy after ischemia. In contrast, aged mice did not show decreased mean muscle fiber area, but had diminished number of muscle fibers and replacement with connective tissue after ischemia (Figures 2A, 2B, and 2C). These results suggest different patterns of response to ischemia in young adult and aged mice and are consistent with diminished function in aged mice (Figure 1B). However, in spite of these different responses, the mean viability index in ischemic limbs was not reduced in aged mice compared to young adult mice (Figure 2D), consistent with similar degree of limb ischemia in aged and young adult mice (Figures 1E and 1F).

Limb ischemia stimulates angiogenesis in aged mice

To determine whether reduced function after limb ischemia in aged mice reflects reduced angiogenesis, we directly examined angiogenesis in ischemic limbs by counting capillary density (Figure 3). After limb ischemia, young adult mice demonstrated approximately 3-fold increased angiogenesis compared to the control leg. In contrast, aged mice demonstrated approximately 7-fold increased angiogenesis compared to the control leg, to a greater degree than young adult mice (Figure 3). There were no differences in number of collaterals in the control legs of young adult or aged mice.

Since direct measurement of angiogenesis demonstrated that aged mice have the capacity for angiogenesis after hind limb ischemia (Figure 3), we confirmed these results by measuring the endothelial progenitor cells (EPC) that incorporated into the ischemic muscle tissue.30 EPC
were noted adjacent to the muscle fibers of both young adult and aged mice; however, young adult mice demonstrated 2–4-fold increased numbers of EPC in ischemic tissue, whereas aged mice demonstrated 5–8-fold increased numbers of EPC in ischemic tissue (Figure 4). Since EPC may directly or indirectly stimulate angiogenesis, these results are consistent with increased angiogenesis in aged mice compared to young adult mice.

**Limb ischemia stimulates arteriogenesis in young adult but not aged mice**

Since aged mice do not exhibit reduced angiogenesis in response to hind limb ischemia, we determined whether there were any differences in arteriogenesis between young adult and aged mice that could account for diminished function in aged mice in response to limb ischemia. CT angiography was used to visualize collaterals (> 75 μm diameter) and confirmed that fewer collaterals developed in the ischemic pelvis and limb of aged mice compared to those seen in young adult mice (Figures 5A–C). Direct contrast angiography was used to confirm the reduced number of proximal collaterals in aged mice, compared to young adult mice, in response to hind limb ischemia; direct puncture of the aorta for contrast angiography consistently showed a large proximal collateral vessel supplying the ischemic limb of young adult mice, but no collaterals were seen supplying the ischemic limb of aged mice (Figure 5D; 0±0 vs. 1±0, n=3; p>.99). These results suggest that limb ischemia stimulates arteriogenesis in young adult but not aged mice.

**Discussion**

We demonstrate, using a novel model of severe limb ischemia that aged mice maintain the ability to undergo angiogenesis, perhaps to a greater degree than young adult mice; however, reduced blood flow and impaired function in aged mice correlates with reduced arteriogenesis, i.e. large vessel collateral formation. These results demonstrate the importance of arteriogenesis in the development of, and potential therapies for, limb ischemia.

Our model of severe acute limb ischemia uses ligation of the common iliac artery and vein to induce ischemia. Since the internal iliac artery, which supplies the pelvis, is the only major branch between the femoral and common iliac arteries, it is not surprising that young adult mice regain blood flow to a similar degree and with similar kinetics as the more severe femoral limb ischemia models (Figure 1C). However, ligation of the iliac artery is technically simpler to perform than excision of the femoral, popliteal, and saphenous arteries and reduces the confounding variable of a large wound in the ischemic limb. The utility of this model is clear as it shows that aged mice have reduced blood flow and function in the ischemic leg compared to young adult mice (Figure 1), similar to previous results reported in aged rats. Additional studies that directly compare this model to other severe ones will help clarify the degrees of ischemia that can be achieved; however, our use of iliac vein ligation to increase the severity of the ischemia may prevent direct comparison with other models of ischemia that do not perform concomitant venous ligation. In addition, the effect of venous ligation may be different in young adult and aged mice. Our limiting the examination period to two weeks postoperatively, rather than 3–4 weeks, reflects the poor postoperative survival of aged mice. As such we limited our studies to the minimal possible to detect differences between young adult and aged mice; it is possible that differences in timing, such as in EPC recruitment or incorporation into tissue, may account for our findings, and that these differences might be minimized over time.

Nevertheless, similar preservation of distal tissue in the ischemic limbs of young adult and aged mice was surprising and contrary to the study hypothesis (Figures 1E and 1F); we interpret limb preservation in aged mice to be accounted for, at least partially, by preservation of...
angiogenesis in aged mice (Figures 3 and 4). Our finding of increased angiogenesis in aged mice is also surprising and contrary to the hypothesis of this study, as well as a previous report of decreased angiogenesis after femoral/saphenous artery resection in aged rabbits and mice. On the other hand, our finding of increased angiogenesis in aged mice is consistent with higher serum VEGF levels in aged animals with limb ischemia compared to younger animals. It is possible that this discrepancy suggests that our iliac artery ligation model is not as severely ischemic as the arterial excision model. However, we believe that other studies performing surgery in the ischemic leg are confounded by the unilateral healing surgical wound in the ischemic leg, possibly diverting EPC away from the distal foot into the more proximal wound. A recent study has demonstrated that EPC homing to the ischemic leg is dependent on phosphorylation of Akt, induced by VEGF and stromal derived growth factor (SDF)-1, both of which can be secreted by a healing wound. The source of these EPC is controversial but it is possible that they are derived from the bone marrow, as suggested by several other studies.

Our finding that aged mice have diminished ability to form large vessel collaterals was demonstrated by CT angiography as well as direct angiography (Figure 5). Although direct angiography confirmed the results of CT angiography, we note that direct angiography was less sensitive than CT angiography and was only able to detect more proximal collaterals, near the area of iliac ligation. In addition, we noted greater numbers of doubly-actin-positive/lectin-positive arterioles in the distal limb muscles of the ischemic limbs of aged animals compared to those in younger animals (data not shown), although the few number of doubly-positive cells in the field is subject to sampling error precluding reliable statistical analysis. Diminished capability for arteriogenesis is consistent with the functional outcome of reduced blood flow and function in ischemic legs of aged mice (Figure 1) and is also consistent with the increased angiogenesis in aged mice compared to younger mice as a potential compensatory mechanism (Figure 3). Arteriogenesis, dilation of preexisting arterioles to increase functional blood flow, is distinct from microvascular angiogenesis as well as embryonic vasculogenesis. Limb ischemia in both human patients and animal models stimulates VEGF and other angiogenic cytokines, such as stem cell factor, SDF-1, angiopoietin-1 and -2, and PLGF; however these factors, after stimulating c-kit, CXCR4, tie-2, and VEGFR-1 and -2 receptors, stimulate angiogenesis that is insufficient to overcome atherosclerotic, or animals models of, limb ischemia. The clinical observation of robust collateral formation allowing asymptomatic limb perfusion and function in many patients confirms the importance of arteriogenesis as a necessary component of revascularization. It is not surprising that trials of therapeutic “angiogenesis” are now focusing on factors that stimulate multiple pathways, including factors that stimulate arteriogenesis, such as monocyte chemoattractant protein, colony-stimulating factors, and cell-based approaches. These approaches capitalize on the observations that circulating cells, perhaps monocytes or stem cells, may release multiple factors that stimulate multiple processes including angiogenesis and arteriogenesis.

In summary, we developed a novel model of severe limb ischemia that does not create a large unilateral surgical wound in the ischemic leg. This model demonstrates that aged mice have deficient arteriogenesis that, in spite of robust angiogenesis, results in diminished limb blood flow and function. These results suggest that strategies to simulate arteriogenesis may complement those that increase angiogenesis, and may result in improved relief of ischemia.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgements

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References


Figure 1.
Decreased function in aged mice after hind limb ischemia. A) Sketch of the model showing location of iliac artery and vein ligation. B) Comparison of skin incisions needed for the femoral (left panel) and iliac (right panel) ligation models. Note that the iliac model does not require an incision in the ischemic limb. C) Decreased flow in aged mice compared to recovery in young adult mice. Young adult and aged mice had a similar degree of decreased perfusion after iliac ligation (young adult, n=15: 20.4±1.9%, vs. aged, n=20: 19.6±1.3%; p=.72; however, young adult mice recovered faster and to a greater degree than aged mice (day 7, 35±6% vs. 17±4%, p=.046; day 14, 60±5% vs. 27±7%, p=.0014). D) Decreased functional status in aged mice compared to recovery in young adult mice. Aged mice had worse functional recovery by
day 14 compared to young adult mice (2.3±.3 vs. 4.3±.4; p=.0021). E) Similar ischemia score in young adult and aged mice. F) Similar modified ischemia score in young adult and aged mice.
Figure 2.
Altered muscle fiber morphology in young adult and aged mice after hind limb ischemia. A) Representative photomicrographs of control and ischemic muscle in young adult and aged mice (n=12). Magnification, 20X. B) Diminished mean fiber number after ischemia in aged mice (n=3 mice/group). C) Diminished baseline mean fiber area in aged mice (n=3 mice/group). D) Similar limb viability in young adult and aged mice. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium.
Figure 3.
Increased capillary density in aged mice after hind limb ischemia. A) Representative photomicrographs of capillary staining of ischemic limbs, day 7, in young adult and aged mice (n=18). Limb muscles were fixed in 10% formalin and embedded in paraffin. Sections were incubated with FITC-conjugated alpha-actin and TRITC-conjugated lectin antibody. Capillaries were identified by lectin staining (red) in absence of alpha-actin staining (green), and arterioles were detected by alpha-actin staining (green). B) Quantitative analysis of capillary density. Values are expressed as mean ± SEM (n=2–4). The difference between capillary density in young adult and aged mice is significant (ANOVA, p=0.01); the differences at day 7 and 14 are significant (p=0.007 and 0.02, respectively; post-hoc testing).
Figure 4.
Increased endothelial progenitor cells in aged mice after hind limb ischemia. A) Representative photomicrographs of immunofluorescence in ischemic tissue of young adult (n=10; left column) and aged (n=8; right column) mice at baseline and after 1 or 2 weeks of hind limb ischemia. Magnification, 60x. Red, VEGFR2; green, CXCR4; blue, DAPI; yellow, colocalization of VEGFR2 and CXCR4. Arrows demonstrate representative colocalizing cells; all these cells were noted to be adjacent to the muscle fibers, not within the fibers themselves. B) Bar graph depicts mean number of cells colocalizing for VEGFR2 and CXCR4. The differences between mean number of cells in young adult and aged mice is significant (control leg, p<0.0001; ischemic leg, p=0.008; ANOVA); the differences at day 7 and 14 are significant.
(control leg, p=0.0002 and 0.04, respectively; ischemic leg, p=0.007 and 0.01, respectively; post-hoc testing).
Figure 5.
Decreased arteriogenesis in aged mice after hind limb ischemia. A) Representative CT angiographic images of young adult (left panel) and aged (right panel) mice, 1 week after hind limb ischemia (n=4). Yellow arrows show collaterals; *, point of ligation; B, bladder. B) Bar graph showing number of collaterals in CT angiograms as determined by pixel densitometry (*, p<.0001). C) Bar graph showing number of collaterals directly counted in CT angiograms (*, p=0.0129). D) Representative direct angiographic images of young adult (left panel) and aged (right panel) mice, 1 week after hind limb ischemia (n=3). Arrows point to lumbar collaterals present (yellow arrows) or absent (red arrows) near the area of iliac ligation (*).
## Table

Functional and ischemic scales.

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