A New Murine Model of Sustainable and Durable Chronic Critical Limb Ischemia Fairly Mimicking Human Pathology

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WHAT THIS PAPER ADDS
Critical limb ischemia (CLI) is frequent and associated with a very poor local and general prognosis. An experimental model fulfilling all CLI characteristics is lacking. This is the first study to analyze not only clinical and perfusion parameters, but also cellular and molecular mechanisms involved in CLI up to 30 days postoperatively. Clinical and scintigraphic scores confirmed CLI, together with impaired mitochondrial respiration, calcium retention capacity, and biogenesis. Ischemic muscles also demonstrated increased production of reactive oxygen species, decreased antioxidant enzymes, and myopathic features. Sequential femoral and iliac arteries ligations closely mimic human functional, clinical, scintigraphic, and skeletal muscle mitochondrial impairments, and could allow testing of therapeutic approaches needed in view of the gravity of CLI in humans.

Objective: To establish a chronic mouse model of critical limb ischemia (CLI) with in vivo and ex vivo validation, closely mimicking human pathology.

Methods: Swiss mice (n = 28) were submitted to sequential unilateral femoral (day 0) and iliac (day 4) ligatures. Ischemia was confirmed by clinical scores (tissue and functional damages) and methoxyisobutylisonitrile (MIBI) scintigraphies at days 0, 4, 6, 10, 20, and 30. At days 10, 20, and 30, muscle mitochondrial respiration, calcium retention capacity (CRC), and production of reactive oxygen species (ROS) were investigated, together with transcripts of mitochondrial biogenesis and antioxidant enzymes. Histological analysis was also performed.

Results: Clinical and functional damage confirmed CLI. MIBI scintigraphies showed hypoperfusion of the ischemic limb, which remained stable until day 30. Mitochondrial respiration was impaired in ischemic muscles compared with controls (V_max = 7.93 ± 0.99 vs. 10.09 ± 2.87 mmol/L O2/minute/mg dry weight [dw]; p = .01), together with impaired CRC (7.4 ± 1.6 mmol/L/minute/mg dw vs. 11.9 ± 0.9 mmol/L/minute/mg dw; p < .001) and biogenesis (41% decrease in peroxisome proliferator-activated receptor gamma coactivator [PGC]-1α, 49% decrease in PGC-1β, and 41% decrease in nuclear respiratory factor-1). Ischemic muscles also demonstrated increased production of ROS under electron paramagnetic resonance (0.084 ± 0.029 vs. 0.051 ± 0.031 mmol/L minute/mg dw; p = .03) and with dihydroethidium staining (3622 ± 604 arbitrary units of fluorescence vs. 1224 ± 324; p < .01), decreased antioxidant enzymes (32% decrease in superoxide dismutase [SOD]1, 41% decrease in SOD2, and 49% decrease in catalase), and myopathic features (wider range in fiber size, rounded shape, centrally located nuclei, and smaller cross-sectional areas). All defects were stable over time.

Conclusion: Sequential femoral and iliac ligatures closely mimic human functional, clinical, scintigraphic, and skeletal muscle mitochondrial characteristics, and could prove useful for testing therapeutic approaches.

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Article history: Received 26 August 2014, Accepted 4 December 2014, Available online 8 January 2015

Keywords: Critical limb ischemia, Scintigraphy mitochondria oxidative stress myopathy

INTRODUCTION
Critical limb ischemia (CLI) defines an advanced stage of chronic arterial insufficiency describing patients with typical ischemic rest pain, or patients with ischemic skin lesions, either ulcers or gangrene.1–3 The term CLI should only be
used in relation to patients with chronic ischemic disease, defined as the presence of symptoms for >2 weeks. Diagnosis of CLI should also only be made in patients with symptoms attributable to objectively proven arterial occlusive disease, and verified by ankle or toe pressure. The diagnosis of CLI is thus a clinical diagnosis of chronic arterial disease, but, inevitably, has to be supported by objective diagnosis of CLI.

The pathophysiology of lower-limb ischemia—reperfusion has been greatly improved with both skeletal muscle dysfunction and increased oxidative stress appearing as major components. All of these alterations should be observed in relevant experimental models of CLI. Furthermore, notwithstanding that ischemia induces neoangiogenesis and formation of collateral vessels—showing that even after femoral artery excision the perfusion of the limb returns to normal within a few days—stability of the lesions over time should be obtained.

Models already exist, but result in progressive blood flow restoration, with complete restoration 7 days after surgery. Further, these models have not always been fully characterized on a clinical, functional, and pathophysiological basis, particularly when considering oxidative stress and mitochondrial functions that are now considered as key factors in CLI, including in human pathology.

The main objective of the present study was to develop and comprehensively characterize, both in vivo and ex vivo, a chronic, stable, and long-lasting established CLI experimental model, closely mimicking human functional, clinical, and skeletal muscle mitochondrial pathology. Indeed, such a durable model is currently lacking and may prove useful for testing therapeutic approaches.

MATERIALS AND METHODS

Animals

Male Swiss mice (n = 28) weighing 30–35 g were handled according to French laws for animal use and care, and in accordance with the guidelines of the European Community Council.

CLI model

Surgery was performed under general anesthesia. Induction was conducted in an airtight ventilated chamber with a mixture of 3% isoflurane (Aerrane; Baxter Healthcare, Maurepas, France) and air. Maintenance of anesthesia was ensured by spontaneous ventilation through a mask delivering a mixture of 2% isoflurane and air.

Ligation of the right femoral artery was performed midway between the superficial epigastric artery and the bifurcation of the popliteal and saphenous arteries under microscope. Three collateral vessels were also ligatured. Four days later, ligation of the right common iliac artery 0.5 cm distal to its origin, after visualization of the origin of internal iliac artery, was performed by laparotomy. In this unilateral ischemia model, the contralateral limb can be considered as a control. The duration of ischemia was counted from the first operation onwards.

Animals were divided into three groups: one group to be sacrificed 10 days after surgery (group 1; n = 10); one group to be sacrificed 20 days after surgery (group 2; n = 8), and one group to be sacrificed 30 days after surgery (group 3; n = 10) in order to study in vivo perfusion, as well as ex vivo muscle damage over time. Of these 28 mice, one from group 1 died at day 1 and one from group 3 died at day 4. Nine mice were then allocated to ex vivo tissue harvest at day 10, eight at day 20, and nine at day 30.

Chosen outcomes

In order to mimic as closely as possible human pathology and according to Trans-Atlantic InterSociety Consensus (TASC II) guidelines, in vivo chosen outcomes were functional signs objectified by clinical scores—scanographic arguments proving the hypoperfusion of the ischemic limb, for >2 weeks. Ex vivo outcomes were alteration of mitochondrial function, by decreased mitochondrial respiratory chain complex activities and calcium retention capacity (CRC), increased production of reactive oxygen species (ROS), and alteration of biogenesis and antioxidant system.

In vivo follow-up of clinical and functional damages and of limb perfusion

Using already established clinical scores, clinical tissue damage was graded as follows: normal or white aspect of the limb, toe cyanosis or necrosis, and spontaneous amputation of a toe (attributed 1, 2, 3, 4, or 5 points, respectively). Functional damage was graded as follows: normal function of the limb, plantar flexion without toe flexion, no plantar flexion, and dragging the limb (attributed 0, 1, 2 or 3 points, respectively). Clinical scores were assessed at days 0, 4, 6, 10 (n = 26), 20 (n = 17), and 30 (n = 9).

Tissue perfusion was assessed with gamma camera scans (Gaede MedizinSysteme GmbH, Freiburg im Breisgau, Germany) under general anesthesia, the animals being placed in a dedicated heating cell (Minerva, Esternay, France). Scans lasting 15 minutes were performed 30 minutes after injection of the tracer (methoxyisobutylisonitrile [MIBI]) at the tail vein, at day 0 (before femoral ligation), day 4 (before iliac ligation), and at days 6, 10 (n = 26), 20 (n = 17), and 30 (n = 9).

Ex vivo muscle analysis

Nine mice were allocated to ex vivo tissue harvest at day 10, eight at day 20, and nine mice at day 30. Ischemic and contralateral gastrocnemius tibialis muscles were collected. Gastrocnemius muscles were harvested and immediately
used for measurement of mitochondrial respiration, CRC, and production of free radicals. Tibialis muscles were frozen in order to perform histological sections and transcripts analyses.

**Mitochondrial respiratory chain complex activities.** The mitochondrial respiratory chain is made of four complexes generating electron transfers in order to produce energy. This activity requires oxygen. The study of mitochondrial respiratory chain complex activities is a technique based on the measurement of oxygen consumption in skinned fibers in order to determine the functional oxidative capacity of the skeletal muscle in its cellular environment. Oxygen concentration is measured with a Clark electrode, and substrates are then added in order to activate or inhibit the different complexes of the respiratory chain. Adenosine diphosphate (2 mM) was added in order to study complex I, III, and IV activity. Succinate (25 mM) was then added to study complex I, II, III, and IV activity, which determines the maximal oxidative capacity ($V_{\text{max}}$). The addition of amytal (0.02 mM) subsequently inhibited complex I, allowing determining vamytal (complex II, III, and IV activities). The addition of N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD; 0.5 mM) and ascorbate (0.5 mM) specifically activated complex IV ($V_{\text{tmpd}}$).

**CRC.** The CRC of gastrocnemius muscle fibers measured by spectrofluorometry is the amount of calcium required to enable the opening of the mitochondrial transition pore, leading to apoptosis.\(^{17}\) Calcium pulses (20 mmol/L) were applied to the skinned gastrocnemius muscle fibers until calcium release. The number of calcium additions needed to trigger mitochondrial permeability transition provided the CRC.

**Production of ROS using electron paramagnetic resonance.** Gastrocnemius muscles (1-mm\(^3\) fragments) were incubated with a 1-hydroxy-3-methoxycarbonyl-2, 2, 5, 5-tetramethylpyrrolidine HCl (CMH) molecular probe, which is oxidized in the presence of unpaired electrons of ROS. The amount of oxidized CMH, and thus the amount of free radicals produced, was measured by the intensity of the resonance signal.

**Histological analysis: muscle structure and ROS production.** Tibialis muscles were immersed in liquid nitrogen and stored at –80 °C. Muscles were then embedded in paraffin, and 10-mm-thick sections were prepared using a cryostat microtome, and mounted onto glass slides. Two types of analyses were subsequently performed: images of slide specimens stained with hematoxylin and eosin were acquired under bright-field microscopy, while other slides were incubated with 2.5 mM dihydroethidium (DHE). DHE produces red fluorescence when oxidized to ethidium bromide, mainly by superoxide anion. After staining, sections were examined under epifluorescence microscope (Nikon Eclipse E800; Nikon, Tokyo, Japan) and emission signals recorded. Protein transcripts encoding for mitochondrial biogenesis and antioxidant defense. The main protein-encoding transcripts involved in mitochondrial biogenesis (peroxisome proliferator-activated receptor gamma coactivator [PGC]-1α, PGC-1β, and nuclear respiratory factor [NRF]1) and antioxidative enzymes (superoxide dismutase [SOD]1, SOD2, catalase) were analyzed. RNA (2 µg), isolated with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), was converted to cDNA with SuperScript II reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA, USA) and hexamer primers according to the manufacturer’s instructions. Quantitative reverse transcription polymerase chain reaction was performed using the Quantitect™ SYBR Green PCR kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as mean ± SD. Comparison were assessed using the nonparametric Mann–Whitney test. p-Values <0.05 were considered as indicative of statistical significance.

**RESULTS**

**Follow-up of clinical and functional parameters and of limb perfusion**

Clinical and functional damages. Clinical examination showed tissue damage: toe necrosis in 22 (85%) mice and cyanosis in 4 (15%) at day 10; toe necrosis in 15 (88%) mice and cyanosis in 2 (12%) at day 20; and autoamputation in 2 (22%) mice, toe necrosis in five (56%), and cyanosis in 2 (22%) at day 30. Clinical examination also showed functional damage: 20 (77%) mice dragged the ischemic limb and 6 (23%) mice could not achieve planter flexion at day 10, day 20, or day 30 (Fig. 1A, B).

Limb perfusion. MIBI scans showed hypoperfusion of the ischemic limb, in comparison with control limb and such hypoperfusion remained stable until day 30 after surgery (Fig. 1C).

**Ex vivo muscle analysis**

Mitochondrial respiratory chain complex activities. Mitochondrial respiration was significantly impaired in ischemic muscles compared with control muscles at days 10, 20, and 30 after surgery. This was also true for all mitochondrial respiratory chain complexes. At day 10 ($n = 9$), $V_0$ was 2.05 ± 1.01 versus 3.57 ± 1.02 mmol/L O$_2$/minute/g dry weight (dw) ($p < .01$) in ischemic and control legs, respectively. $V_{\text{max}}$ was 6.97 ± 2.41 versus 10.29 ± 2.02 mmol O$_2$/minute/g dw ($p < .01$). Complex II, III, and IV activity ($V_{\text{amytal}}$) was 4.32 ± 1.84 versus 6.87 ± 1.46 mmol/L O$_2$/minute/g dw ($p < .01$). Finally, complex IV activity ($V_{\text{tmpd}}$) was 8.27 ± 2.47 versus 11.01 ± 2.60 mmol/L O$_2$/minute/g dw ($p < .01$) in ischemic and control legs, respectively.
At day 20, $V_0$ ($n = 8$) was $1.90 \pm 0.70$ versus $3.26 \pm 0.41$ mmol/L O$_2$/minute/g dw ($p < .01$); $V_{\text{max}}$ was $7.30 \pm 1.59$ versus $9.86 \pm 1.44$ mmol/L O$_2$/minute/g dw ($p < .01$); $V_{\text{amytal}}$ was $4.64 \pm 1.23$ versus $6.03 \pm 1.05$ mmol/L O$_2$/minute/g dw ($p < .01$) and $V_{\text{tmpd}}$ was $8.07 \pm 1.25$ versus $10.25 \pm 0.93$ mmol/L O$_2$/minute/g dw ($p < .01$) in ischemic and control legs, respectively.

At day 30 ($n = 9$), $V_0$ was $2.33 \pm 0.70$ versus $3.34 \pm 0.84$ mmol/L O$_2$/minute/g dw ($p = .01$) in ischemic and control legs, respectively. $V_{\text{max}}$ was $7.93 \pm 0.99$ versus $10.09 \pm 2.87$ mmol O$_2$/minute/g dw ($p = .01$). Complex II, III, and IV activity ($V_{\text{amytal}}$) was $4.99 \pm 0.58$ versus $6.45 \pm 2.39$ mmol/L O$_2$/minute/g dw ($p = .06$). Finally, complex IV activity ($V_{\text{tmpd}}$) was $8.68 \pm 1.18$ versus $10.36 \pm 2.02$ mmol/L O$_2$/minute/g dw ($p = .04$) in ischemic and control legs, respectively (Fig. 2A).

CRC. CRC was also impaired in ischemic muscles compared with control muscles: $7.3 \pm 1.2$ versus $10.6 \pm 1.2$ mM/mg dw for the nine mice sacrificed at day 10 ($p < .01$).

At day 20 ($n = 8$), CRC was $20 \pm 6.8$ mmol/L mg dw versus $11.4 \pm 0.7$ mmol/L mg dw ($p < .01$).

At day 30 ($n = 9$), CRC was $7.4 \pm 1.6$ mmol/L mg dw versus $11.9 \pm 0.9$ mmol/L mg dw ($p < .01$) in ischemic and control legs, respectively (Fig. 2B).

Production of ROS using electron paramagnetic resonance and DHE staining. Production of free radicals was increased in ischemic gastrocnemius compared with control muscles: $0.089 \pm 0.033$ versus $0.080 \pm 0.022$ mmol/L min/mg dw for the nine mice sacrificed at day 10 ($p = .46$); $0.102 \pm 0.024$ versus $0.083 \pm 0.034$ mmol/L min/mg dw for the eight mice sacrificed at day 20 ($p = .01$); and $0.084 \pm 0.029$ versus $0.051 \pm 0.031$ mmol/L min/mg dw for the nine mice sacrificed at day 30 ($p = .03$). Fluorescence after DHE staining was also higher in ischemic tibialis fibers: $3622 \pm 604$ arbitrary units of fluorescence (AUFs) versus $1224 \pm 324$ AUFs ($p < .01$) (Fig. 3A, B).

Histological analysis: muscle structure. Chronically ischemic tibialis muscle exhibited myopathic features, as established by hematoxylin and eosin coloration with a wider range in fiber size, a more rounded shape, centrally located nuclei, and smaller cross-sectional areas than control fibers (Fig. 3C).

Stability of the model over time. As shown in Fig. 1, clinical, functional, and scintigraphic parameters were similar in the control leg at days 10, 20, and 30 after surgery. Importantly, impairments in ischemic legs were also stable during the study follow-up.

Mitochondrial respiration, CRC, and production of free radicals showed a similar evolution, demonstrating the stability of the model (Fig. S1; see Supplementary Information).

Transcripts encoding proteins involved in mitochondrial biogenesis and antioxidant defense. RNA analysis revealed a decrease in biogenesis, with a decrease in PGC-1α of 63% ($p = .01$), 44% ($p = .01$), and 41% ($p = .04$) at days 10, 20, and 30, respectively; a decrease in PGC-1α of 60% ($p = .01$), 36%
A. Mitochondrial respiration

Figure 2. (A) Mitochondrial respiration and (B) calcium retention capacity are decreased after induction of ischemia. Note. \( V_0 \) = basal mitochondrial oxidative capacity; \( V_{\text{max}} \) = maximal mitochondrial oxidative capacity; \( V_{\text{anymyta}} \) = complex II, III, and IV activity; \( V_{\text{tmpd}} \) = complex IV activity. *\( p < .05 \); **\( p < .01 \); ***\( p < .001 \).

\( p = .03 \), and 49% \( p = .01 \) at days 10, 20, and 30, respectively; and a decrease in NRF1 of 19% \( p = .04 \), 35% \( p = .02 \) and −41% \( p = .02 \) at days 10, 20, and 30, respectively.

The antioxidant system was impaired at days 10, 20, and 30: SOD1 was decreased by 39% \( p = .01 \), 44% \( p = .01 \), and 32% \( p = .04 \), respectively; SOD2 by 59% \( p = .01 \), 38% \( p = .04 \), and 41% \( p = .02 \), respectively; and catalase by 60% \( p = .01 \), 36% \( p = .04 \), and 49% \( p = .03 \), respectively (Fig. S2; see Supplementary Information).

B. Calcium retention capacity
A sustainable and stable-over-time model of CLI, according to TASC II guidelines, has been developed with clinical signs, confirmation of hypoperfusion by objective measures, lasting for more than 2 weeks, and using sequential artery ligations. Models already used in order to induce ischemia.

**A. Dihydroethidium stain** (a Control limb, b Ischemic limb)

**B. Production of free radicals**

![Graph showing production of free radicals over time](image)

**C. Hematoxylin-eosin stain** (a Control limb, b Ischemic limb, x40)

--- 100 μm

**Figure 3.** Oxidative stress is increased after induction of ischemia and chronic limb ischemia leads to myopathic features. (A) Dihydroethidium stain. (B) Production of free radicals. (C) Hematoxylin and eosin stain.

**DISCUSSION**

A sustainable and stable-over-time model of CLI, according to TASC II guidelines, has been developed with clinical signs, confirmation of hypoperfusion by objective measures, lasting for more than 2 weeks, and using sequential artery ligations. Models already used in order to induce ischemia.
are based on a single arterial ligation. In rodents, ligation of the femoral artery just distal to the origin of the profunda femoris is most commonly used. However, this method leaves most of the collateral circulation to the lower limb intact and, consequently, blood flow to the limb is fully restored within 7 days. Another method consists of excision of the femoral artery, removing the collateral bed. However, blood flow is restored progressively, with a third of the original blood flow restored only 7 days after excision of the femoral artery. In fact, collateral vessels in the mice arise mostly from the internal iliac artery. By performing sequential ligations in the CLI model presented herein, a sustainable ischemia was obtained, likely because the second ligation performed on the common iliac artery reduces the collateral perfusion provided by the internal iliac artery.

Herein, a new CLI model is proposed, which fulfills all criteria defining CLI. In order to diagnose CLI, current recommendations require that both clinical signs and symptoms are present for >2 weeks along with objective measurements of arterial perfusion. Using already-established clinical scores and MIBI scintographies, it has been demonstrated that the present model fully complies with these recommendations. While clinical scores are not routinely used when characterizing animal models, they can, nevertheless, be easily transposed as animals also present with several degrees of lower limb impotency and, moreover, the observed tissue lesions follow the same pattern as in humans, ranging from a cyanotic aspect of the limb to necrosis. Also, rather than using invasive arteriography, scintigraphy was preferentially used, as it has already been proven to be a good approach for cardiac or skeletal muscle perfusion studies. Indeed, such a noninvasive technique enables potential perfusion changes to be followed over time. Furthermore, Tc-99m MIBI scintigraphy can detect hypoperfusion earlier than with laser Doppler imaging, as the uptake of this radiopharmaceutical compound is dependent on the distribution of regional blood flow, an early indicator of hypoperfusion. Currently, MIBI scintigraphies are under consideration for the study of peripheral arterial disease (PAD). Finally, in the present study, particular care was taken to follow the animals until 30 days after the first surgical ligation, thus allowing assessment of the observed symptoms and hypoperfusion for a period of >2 weeks.

The pathophysiology of lower limb ischemia—reperfusion has been largely improved in recent years and, as a result, key mechanisms, such as muscle mitochondrial function and oxidative stress, were further investigated. Given that mitochondrial dysfunction and increased oxidative stress are largely involved in acute lower limb ischemia, whether these elements are also key factors in CLI was investigated. Accordingly, in our CLI model, ischemic murine muscles displayed impaired mitochondrial respiration involving all mitochondrial respiratory chain complexes. Furthermore, early Ca$^{2+}$ release in the ischemic muscle was also observed herein. These data support that chronically ischemic muscles are prompt to apoptose. Finally, mitochondrial dysfunctions were associated with a reduced mitochondrial biogenesis, which also probably participated in the chronic alteration of ischemic muscles. Mitochondria may potentially be both actors and targets in chronic ischemia—reperfusion-related injuries as they both enhance and are impaired by ROS production. A reduction in antioxidant capacities was also observed, as mRNA encoding SOD1 and SOD2, and catalase, the main enzymes involved in ROS detoxification, were significantly decreased in ischemic muscles. Pipinos et al. observed that ischemic human muscles presented mitochondrial alterations and exhibited myopathic features, with ischemic myofibers demonstrating a broader range in terms of size, a more rounded shape, centrally located nuclei, and smaller cross-sectional area than control fibers. In the CLI model presented herein, mitochondrial alterations and myopathic features of ischemic myofibers also mimic human pathology.

However, despite its wide similitude with human pathology, this model has limitations. First, human CLI generally occurs on already pathological arteries linked to cardiovascular risk factors and comorbidities associated to PAD. Thus, CLI is a result of a chronic process associated with the build-up of atherosclerotic plaque over many years leading to arterial stenosis. Here, sequential ligations are performed on healthy arteries. Further, CLI pathogenesis in humans develops gradually at multiple and different locations (iliac arteries, superficial femoral arteries). These obliterations are progressive, and not acute, as were the ligations done in the model. However, as in PAD, sequential ligations likely allowed neoangiogenesis and collaterality, leading mainly to hypoperfusion rather than to complete acute ischemia of the limb. Thus, sequential ligations fairly mimic the progressivity of PAD, until a threshold of hypoperfusion causing CLI.

In conclusion, the present study shows that sequential artery ligations lead to a valid CLI model based on both in vivo (clinical, functional and perfusion scores) and ex vivo (mitochondrial respiration, biogenesis, CRC and oxidative stress) impaired functions. Although needing to be tested in other mice strands demonstrating, for instance, cardiovascular risk factors, this experimental model would likely help to better investigate efficient therapeutic strategies to be later proposed in patients with CLI.

ACKNOWLEDGEMENTS

We thank Fabienne Goupilleau, Isabelle Bentz, and Anne-Marie Kasprowicz for their expert biological and secretarial assistances.

CONFLICTS OF INTEREST

None.

FUNDING

This work was supported by a grant from the French Society of Vascular Surgery and from the ADIRAL.
APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejvs.2014.12.010.

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