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## Basic Control of Reperfusion Effectively Protects Against Reperfusion Injury in a Realistic Rodent Model of Acute Limb Ischemia

Florian Dick, MD\*; Jianhui Li, MD\*; Marie-Noëlle Giraud, PhD; Christoph Kalka, MD; Juerg Schmidli, MD; Hendrik Tevæarai, MD, eMBA

**Background**—Reperfusion injury is insufficiently addressed in current clinical management of acute limb ischemia. Controlled reperfusion carries an enormous clinical potential and was tested in a new reality-driven rodent model.

**Methods and Results**—Acute hind-limb ischemia was induced in Wistar rats and maintained for 4 hours. Unlike previous tourniquets models, femoral vessels were surgically prepared to facilitate controlled reperfusion and to prevent venous stasis. Rats were randomized into an experimental group (n=7), in which limbs were selectively perfused with a cooled isotone heparin solution at a limited flow rate before blood flow was restored, and a conventional group (n=7; uncontrolled blood reperfusion). Rats were killed 4 hours after blood reperfusion. Nonischemic limbs served as controls. Ischemia/reperfusion injury was significant in both groups; total wet-to-dry ratio was  $159 \pm 44\%$  of normal ( $P=0.016$ ), whereas muscle viability and contraction force were reduced to  $65 \pm 13\%$  ( $P=0.016$ ) and  $45 \pm 34\%$  ( $P=0.045$ ), respectively. Controlled reperfusion, however, attenuated reperfusion injury significantly. Tissue edema was less pronounced ( $132 \pm 16\%$  versus  $185 \pm 42\%$ ;  $P=0.011$ ) and muscle viability ( $74 \pm 11\%$  versus  $57 \pm 9\%$ ;  $P=0.004$ ) and contraction force ( $68 \pm 40\%$  versus  $26 \pm 7\%$ ;  $P=0.045$ ) were better preserved than after uncontrolled reperfusion. Moreover, subsequent blood circulation as assessed by laser Doppler recovered completely after controlled reperfusion but stayed durably impaired after uncontrolled reperfusion ( $P=0.027$ ).

**Conclusions**—Reperfusion injury was significantly alleviated by basic modifications of the initial reperfusion period in a new in vivo model of acute limb ischemia. With this model, systematic optimizations of according protocols may eventually translate into improved clinical management of acute limb ischemia. (*Circulation*. 2008;118:1920-1928.)

**Key Words:** inflammation ■ ischemia ■ peripheral vascular disease ■ prevention ■ reperfusion

Acute limb ischemia, one of the most common peripheral vascular emergencies, is associated with extensive morbidity, mortality, and costs.<sup>1-3</sup> Overall prognosis has not markedly improved over the decades despite major advances in surgical and pharmacological revascularization options;<sup>4,5</sup> all-cause mortality is still in excess of 25%, and survivors face a 20% risk of major amputation.<sup>3-7</sup>

### Clinical Perspective p 1928

Traditionally, clinical management has aimed at early tissue reperfusion without dedicated attention to imminent reperfusion-related injury.<sup>3,7,8</sup> The immediate and full restoration of blood flow, however, may paradoxically aggravate previous ischemic damage by setting into motion a host of

inflammatory responses that promote local tissue destruction and no reflow within the microcirculation,<sup>9-12</sup> whereas systemic washout of toxic metabolites may lead to life-threatening systemic complications, including renal, cardiac, and pulmonary failure.<sup>11,13</sup> Identified pathomechanisms include formation of reactive oxygen species, activation of complement system, increased leukocyte-endothelium cell adhesion, increased platelet-leukocyte aggregation, interstitial fluid accumulation, diffuse microthrombosis, and decreased endothelium-dependent vasorelaxation.

Decompressive fasciotomy currently represents the only generally accepted surgical salvage strategy after restitution of blood flow;<sup>3,7</sup> its therapeutic potential, however, is directed to secondary complications associated with established com-

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From the Department of Cardiovascular Surgery (F.D., J.L., M.-N.G., J.S., H.T.) and Division of Angiology (C.K.), Inselspital, and University of Berne, Berne, Switzerland; Imperial College Vascular Surgery Research Group, Division of Surgery, Oncology, Reproductive Biology and Anaesthetics, Charing Cross Hospital, London, UK (F.D.); and Department of Hepatobiliary Pancreatic Surgery, Key Lab of Combined Multi-Organ Transplantation, Ministry of Public Health, First Affiliated Hospital, Medical College, Zhejiang University, Zhejiang, China (J.L.).

\*The first 2 authors contributed equally to this work.

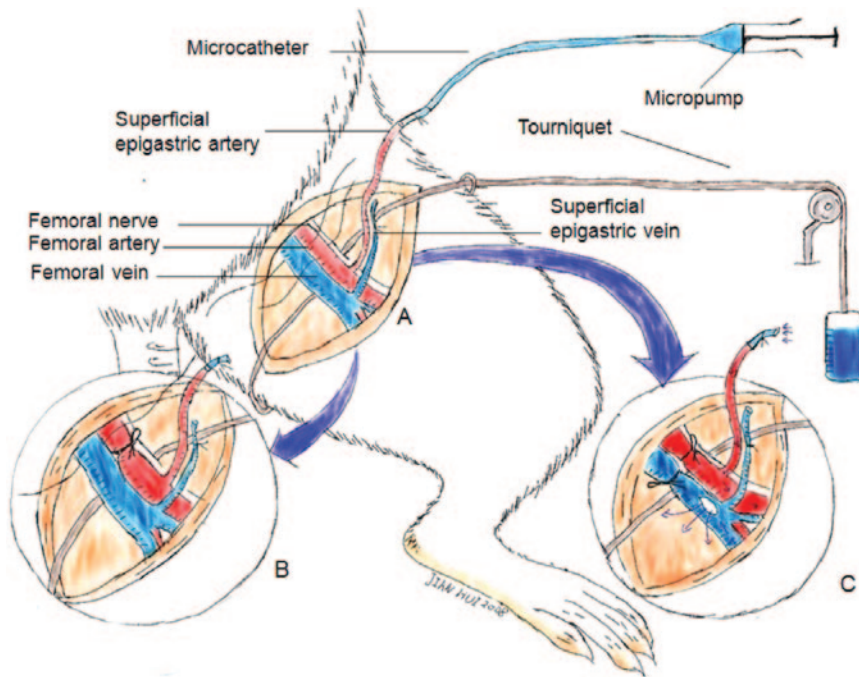
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Correspondence to Marie-Noëlle Giraud, PhD, Department of Cardiovascular Surgery, Inselspital, and University of Bern, 3010 Berne, Switzerland. E-mail [marie-noelle.giraud-flueck@insel.ch](mailto:marie-noelle.giraud-flueck@insel.ch)

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**Figure 1.** Induction of hind-limb ischemia by a modified controlled-tension tourniquet.<sup>28</sup> Femoral vessels are exposed up to the branching of the superficial epigastric artery. A, The tourniquet is passed underneath the neurovascular bundle. B, Ischemia is achieved by tension on the tourniquet and clamping of the common femoral and superficial epigastric arteries; venous drain is preserved. C, Controlled selective reperfusion is achieved with a microcatheter and the superficial epigastric artery as conduit. The femoral vein is clamped, and sequestered blood is washed out through a venous incision.

partment syndrome rather than to reperfusion injury per se. Ischemic preconditioning was found to effectively induce “ischemia and reperfusion tolerance” in a number of studies;<sup>14</sup> however, it is not applicable in clinical situations in which ongoing ischemia precludes preconditioning. A different strategy is thus needed to improve the outcome of acute limb ischemia.

Controlled reperfusion protocols aim to interfere with the course of reperfusion injury at various levels.<sup>15</sup> Limitation of initial blood flow and perfusion pressure, modifications of the reperfusate composition, and hypothermia all have been proposed for many years.<sup>16,17</sup> For instance, hypothermia and low initial flow rates have been shown to decrease the severity of reperfusion injury in skeletal muscle,<sup>18,19</sup> and flow-controlled reperfusion improved sinusoidal microcirculation in rat livers.<sup>20</sup> Filtration of leukocytes reduced reperfusion-related complications in lung and myocardium,<sup>21,22</sup> and use of a substrate-enriched reperfusate led to immediate recovery of contractile muscle function after prolonged warm ischemia.<sup>16</sup> Numerous additional modifications have been proposed,<sup>9,23</sup> including the administration of free radical scavengers, anticoagulants, and dedicated anti-inflammatory and vasodilative agents, as well as the disposal of sequestered blood. However, translation of such strategies to clinical management of acute limb ischemia has remained astonishingly sparse considering the considerable clinical potential that has been reported in preliminary case series.<sup>24–27</sup> Therefore, further applied research toward defining an optimized reperfusion protocol is clearly needed but has been hindered so far by the lack of a suitable experimental model.<sup>28</sup> Indeed, current rodent models either imitate acute limb ischemia insufficiently or do not allow selective reperfusion or assessment of *in vivo* processes, and larger animal models are cost intensive, are ethically challenging, and offer limited flexibility when several groups of various therapeutic options need to be compared.

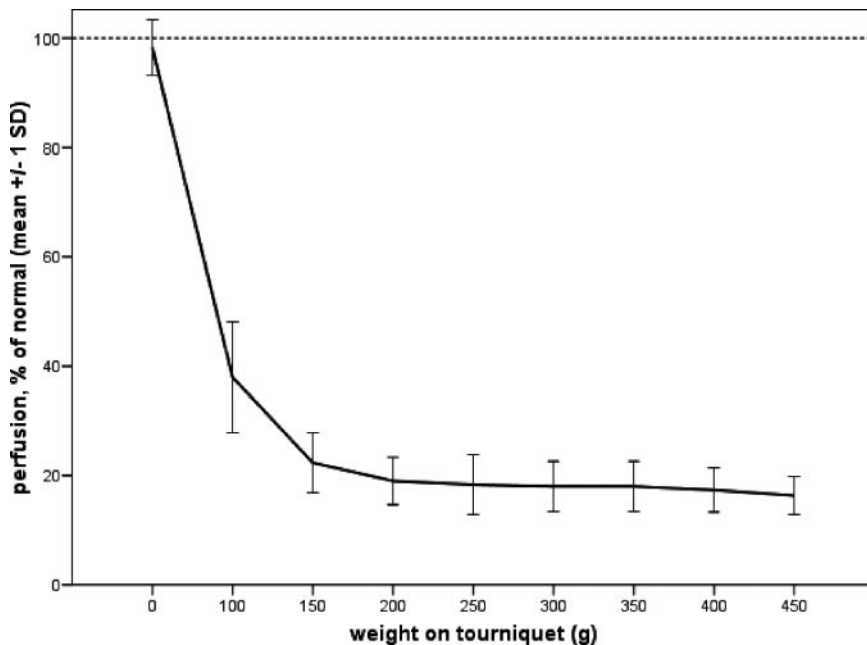
We developed a reality-driven rodent model of acute limb ischemia that allows selective limb perfusion and assessment of regional and systemic *in vivo* events. The aim was to test a basic reperfusion protocol against immediate restoration of blood flow after warm limb ischemia of critical duration (4 hours).<sup>9</sup> The protocol included flow-controlled application of an oxygen-free, acellular, and cooled heparin solution in combination with venous washout of sequestered blood, all of which could theoretically be easily applied in clinical practice. The results provide an important benchmark for more sophisticated protocols and may inspire further translational and clinical research.

## Methods

Animal care and all experimental procedures were approved by the Swiss animal welfare authorities and carried out in accordance with the *Principles of Laboratory Animal Care* (National Institutes of Health publication 86–23, 1985) and the position statement of the American Heart Association.<sup>29</sup> Wistar rats of either sex ( $305 \pm 75$  g body weight) were fed a standard diet ad libitum and transferred 1 hour before the experiment to the laboratory, where they were randomized into one of the study groups. The rats were anesthetized by continuous inhalation of isoflurane (2.5%). Facilities were temperature controlled (23°C to 25°C), and rats were restrained on heating pads in a standardized fashion to maintain their body temperature at 37°C.<sup>28</sup> Respiratory and circulatory parameters were monitored continuously. Animals were hydrated during experiments by intraperitoneal injection of 0.5 mL normal saline every 2 hours.<sup>28</sup>

## Ischemia Model

All animals were subjected to 4 hours of acute limb ischemia using an established tourniquet model of limb ischemia<sup>28</sup> that was modified as follows (Figure 1): Instead of placing the tourniquet just around the limb, we first exposed the femoral vessels over an oblique groin incision and prepared the superficial epigastric artery. The common femoral artery was temporarily ligated proximally (No. 6/0 Prolene); it is important to note that the femoral vein stayed open. The tourniquet (No. 2 Prolene loop suture) was then passed underneath the femoral vessels to spare them and placed around the thigh



**Figure 2.** Dose (weight on tourniquet)-response (limb perfusion) curve of the model (n=5). Interrupted bar delineates normal perfusion (100%).

as proximal as possible. Tension of the tourniquet was controlled by the amount of weight put on the loop suture. Dose-response testing had shown a reliable relationship between tourniquet pull and limb perfusion (n=5; Figure 2), and a weight of 450 g was chosen for all animals. Ischemia time began after temporary ligation of the superficial epigastric artery. All rats were given an intraperitoneal bolus of heparin (50 IU/kg body weight) after 3.5 hours of ischemia.

**Reperfusion Groups**

In the experimental group (n=7), a controlled reperfusion protocol was applied to ischemic limbs before blood circulation was reinstated (Figure 3). The prepared epigastric artery was used as a conduit for vascular access to spare the femoral artery any direct trauma. A polyethylene microcatheter (PE-10; inner diameter, 0.28 mm; outer diameter, 0.64 mm, thinned out by heat stretching; length, 20 cm) was inserted into the conduit and connected to a micropump (Single Syringe Pump, Harvard Apparatus, Holliston, Mass). The protocol included flow-controlled perfusion (0.3 mL/min) of the limb with a cooled heparin-containing crystalloid solution (15°C, ad 1000 IU heparin and Ringer’s lactate 1000 mL) for 20 minutes while the femoral vein was clamped and sequestered blood was washed out with the perfusion solution through a venous incision. The vein was repaired before blood flow was reinstated by release of tourniquet and arterial ligatures. In the control group (n=7), blood reperfusion was reinstated directly after ischemia (Figure 3).

Animals were kept under continuous anesthesia for standardized observation after blood reperfusion (4 hours) and were killed without recovering. Tissue samples were harvested from below the knee to

exclude a possible influence of the tourniquet along the thigh. In both groups, contralateral nonischemic limbs were used as intraindividual controls.

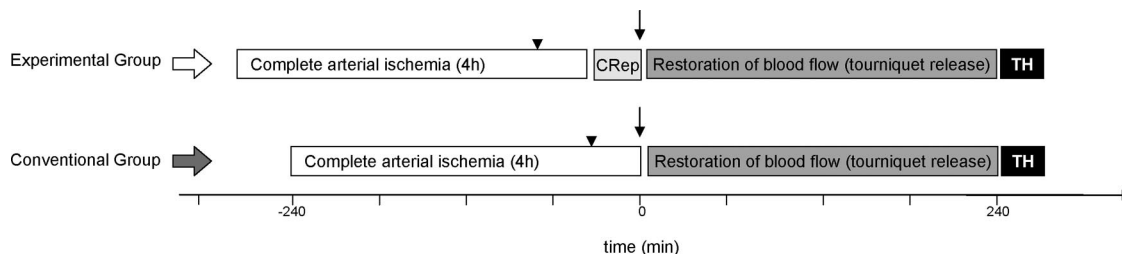
**Study End Points**

*Limb Perfusion*

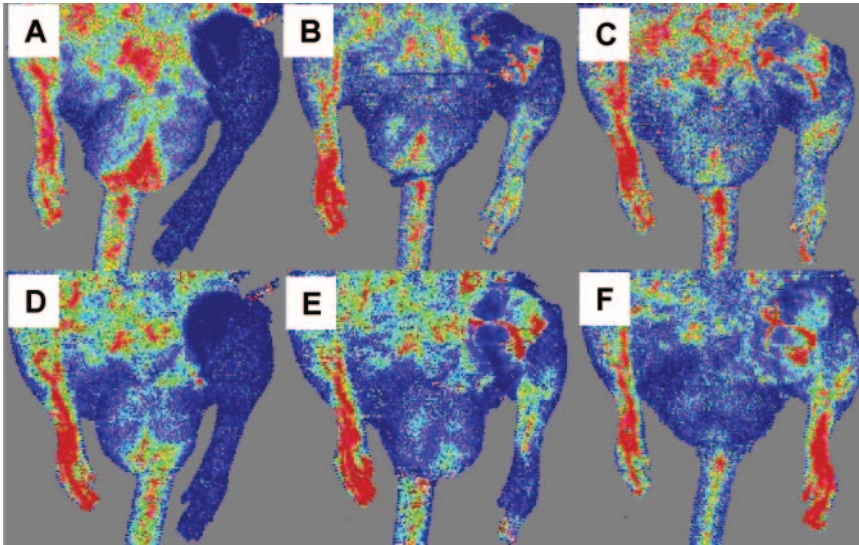
Perfusion was assessed simultaneously in both limbs for each measurement with a laser Doppler imager (Moor LDI-VR, Moor Instruments Inc, Wilmington, Del) as described previously.<sup>28</sup> The laser beam (780 nm) is reflected from moving red blood cells and processed by dedicated software (Laser Doppler Perfusion Measure, V51, Moor Instruments Inc). Baseline images were obtained 30 minutes after anesthesia. After application of the tourniquets, a second image confirmed limb ischemia (Figure 4). After release of the tourniquet, additional images were obtained immediately; after 5, 10, 15, 30, 45, and 60 minutes; and after every half hour up to 4 hours. Perfusion was measured in perfusion units and expressed as percent of the contralateral nonischemic limb.

*Tissue Edema*

Edema was quantified in lateral gastrocnemius muscles by measurement of the ratio of wet to dry tissue weight. Tissue samples were blotted and weighed immediately after harvest (wet weight) and placed in a drying oven (55°C) until a constant weight was obtained (dry weight; mean, 48 hours). Edema was indicated by an increase in the ratio of wet to dry tissue weight and expressed as percent of the paired control limb.



**Figure 3.** Timing of experiment stratified for the study groups. Rats in the experimental group underwent 4.5 hours of ischemia vs 4 hours in the control group. Vertical arrows indicate reinstatement of blood circulation to the limb. Arrowheads indicate an intraperitoneal bolus of heparin. CRep indicates controlled reperfusion protocol; TH, tissue harvest.



**Figure 4.** Synchronous record of perfusion of ischemic (left; animal limbs) and control limbs by laser Doppler directly after application of tourniquet, after reinstatement of circulation, and at the end of the reperfusion period (horizontal sequence). A through C, The control group; D through F, the experimental group.

### Tissue Viability

Tissue viability was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described in detail before.<sup>28</sup> Tetrazolium salt (MTT; Sigma, St Louis, Mo) is reduced to water-insoluble colored formazan crystals by mitochondrial activity. Samples of medial gastrocnemius muscles were incubated with 3 mL PBS (pH 7.4) and 0.3 mg MTT. After incubation, formazan crystals were extracted from samples, and their optical density was determined at 570 nm with a microplate reader (Infinite 200 spectrophotometer, Tecan, Männedorf, Switzerland). Viability index (arbitrary units) is expressed as an optical density at 570 nm after adjustment for dry tissue weight. In each analysis, a sample of dead tissue (obtained from rat muscle harvested >36 hours earlier and stored at room temperature) was included for background noise determination.

### Muscle Contractile Properties

Excitability and contractility were tested on soleus muscles as described.<sup>30</sup> Harvested muscles were bathed in oxygenated Tyrode's solution (NaCl 118 mmol/L, KCl 4.7 mmol/L, MgSO<sub>4</sub> 1.2 mmol/L, NaHCO<sub>3</sub> 24 mmol/L, KH<sub>2</sub>PO<sub>4</sub> 1.1 mmol/L, and glucose 4.5 mmol/L at pH 7.4 and 25°C) with increasing CaCl<sub>2</sub> concentrations up to 1.25 mmol/L. Association of maximal muscle twitch and muscle tension (preload) was assessed in a force transducer (muscle tester ORG, Heidelberg, Germany) by standardized stimulation (5 V for 0.5 ms at 2 Hz).<sup>30</sup> Force and speed of contractions were recorded at optimal preload. Cross-sectional area of muscles was calculated assuming the geometry of a cylinder with a specific gravity of 1.06 g/cm<sup>3</sup>.<sup>31</sup> Twitch amplitude and derivatives were corrected for cross-sectional area and are expressed as percent of paired control limbs.

### Statistical Methods

Investigators were blinded to study group for data analysis. Conventional descriptive statistics were used, giving mean±SD, medians (interquartile range), or percent of paired controls. Distributions were assumed to be nonparametric and compared by appropriate tests throughout. Paired tests were used for comparisons between ischemic and control limbs, and unpaired tests were used for comparisons between study groups. Proportions were compared by use of Fisher's exact test, and the difference in the kinetic of the 2 reperfusion curves (Figure 5) was assessed by repeated-measures Greenhouse Geisser ANOVA. All tests were 2 tailed. Statistical significance was accepted for values of  $P \leq 0.05$  and was alternatively adjusted according to Bonferroni (0.017) to appraise results under the assumption of multiple hypothesis testing. All analyses were performed with SPSS for Windows, version 15.0 (SPSS Inc, Chicago, Ill).

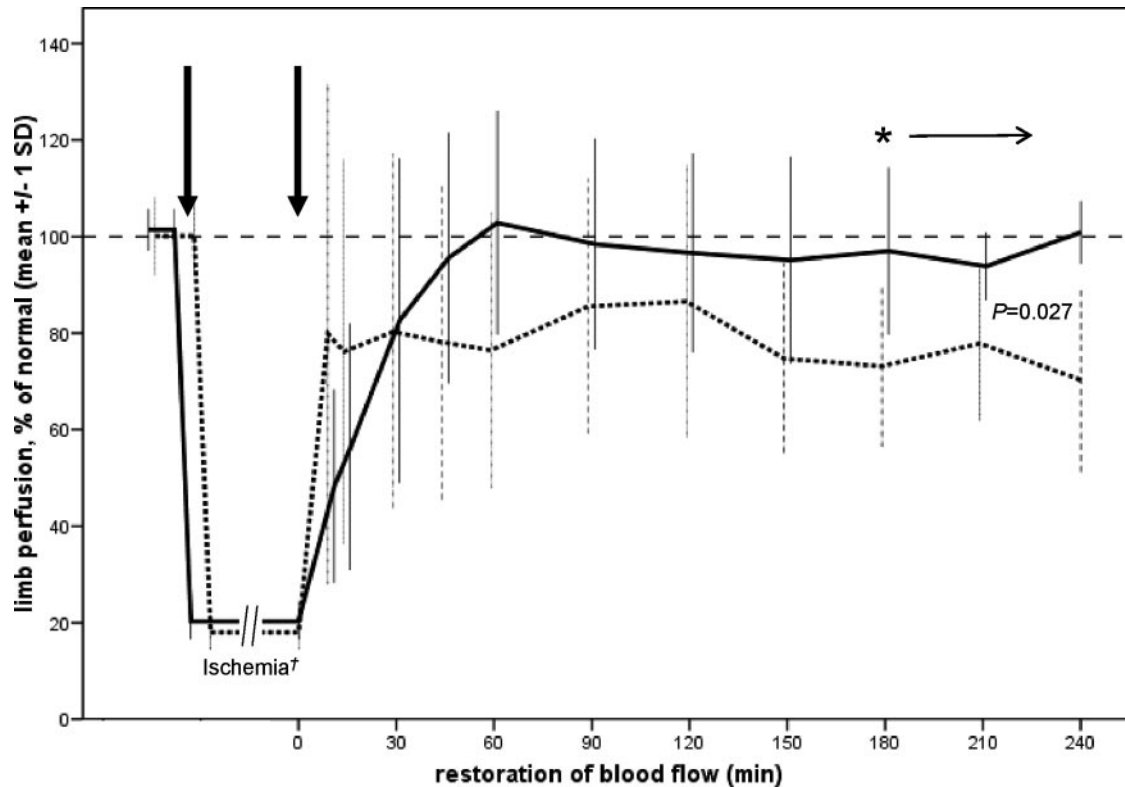
The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

### Results

All experiments were performed within 1 month after the methodology was established. All animals survived the experiments and were killed according to protocol. Induction of ischemia was surgically feasible in the described manner. Patency of the main venous outflow was ascertained by direct observation of moving blood in femoral veins through the operating microscope. Cannulation of the superficial epigastric artery was possible in all animals and added a mean of 10 minutes of operating time to the ischemic period of the experimental group. Preliminary experiments had demonstrated efficacy of arterial access by use of methylene blue and by observation of watery immersion from iatrogenic toe lesions during reperfusion. During the study, flow-controlled reperfusion circulated undisturbed and turned venous wash-out expeditiously watery and limbs anemic in all animals, whereas no leaks were observed at cannulation sites. The reperfusion protocol added 20 minutes to the experimental group, which eventually underwent 4.5 hours of ischemia as opposed to 4 hours in the control group (Figure 3).

Return of circulation followed a different dynamic in the 2 study groups after release of the tourniquet (Figures 4 and 5). Limb perfusion returned more promptly at first in the control group but stayed durably impaired, whereas circulation returned less rapidly in the experimental group but normalized completely over time. The total difference in the dynamic of the 2 reperfusion curves over time became statistically significant beginning at 180 minutes in repeated-measures ANOVA. Hence, recovery of limb circulation was significantly better after controlled reperfusion than after immediate restoration of blood flow over the first 4 hours ( $P=0.027$  by Greenhouse Geisser).

Main findings regarding severity of ischemia-reperfusion injury are given in the Table. The modified tourniquet model induced reliably significant ischemia/reperfusion injury in both study groups. Ischemic limbs across the groups present-



**Figure 5.** Blood-reperfusion curves after severe acute hind-limb ischemia (between vertical arrows) stratified for study group. Perfusion is expressed as percent of normal (horizontal interrupted bar). Differences regarding dynamic of restoration of circulation over time were assessed by repeated-measures ANOVA, which became statistically significant at and beyond 180 minutes (asterisk; horizontal arrow;  $P=0.027$ ). †Experimental animals ( $n=7$ ; solid line) underwent 4.5 hours of ischemia in total; control animals ( $n=7$ ; interrupted line) underwent 4 hours.

ed a total mean edema index of  $159 \pm 44\%$  ( $P=0.016$ ), a mean residual muscle viability of  $65 \pm 13\%$  ( $P=0.016$ ), and a mean residual contraction force of  $45 \pm 34\%$  ( $P=0.045$ ) compared with their nonischemic controls (100%) by paired testing. Paired differences also were statistically significant within individual groups except for contractility, which was not

significantly impaired after controlled reperfusion (the Table).

However, the type of reperfusion was clearly associated with the severity of ischemia/reperfusion injury. Whereas findings in nonischemic control limbs were very similar in both groups ( $P>0.5$ ), supporting the assumption of compa-

**Table. Characteristics of the Study Groups and Main Results of the Experiment**

	Experimental Group ( $n=7$ )	$P$	Conventional Group ( $n=7$ )	$P$	$P$ (Groups)
Median weight, g	350 (235–400)		250 (235–360)		0.441*
Male sex, n (%)	5 (71)		2 (29)		0.286†
Edema					
Ischemic limbs	4.8 (4.5–5.2)	0.016‡	6.3 (6.0–7.2)	0.016‡	0.002*
Control limbs	3.7 (3.6–3.9)		3.7 (3.2–3.9)		0.620*
Edema index, %	137 (118–144)		191 (156–204)		0.011*
Muscle viability					
Ischemic limbs	21.1 (17.8–24.2)	0.016‡	12.7 (11.6–23.2)	0.016‡	0.038§
Control limbs	29.5 (23.7–34.1)		24.9 (21.0–36.6)		0.805*
Muscle viability index, %	70 (65–82)		61 (52–62)		0.004*
Contractility force index, %	59 (33–107)	0.188‡	23 (21–31)	0.031‡	0.045*

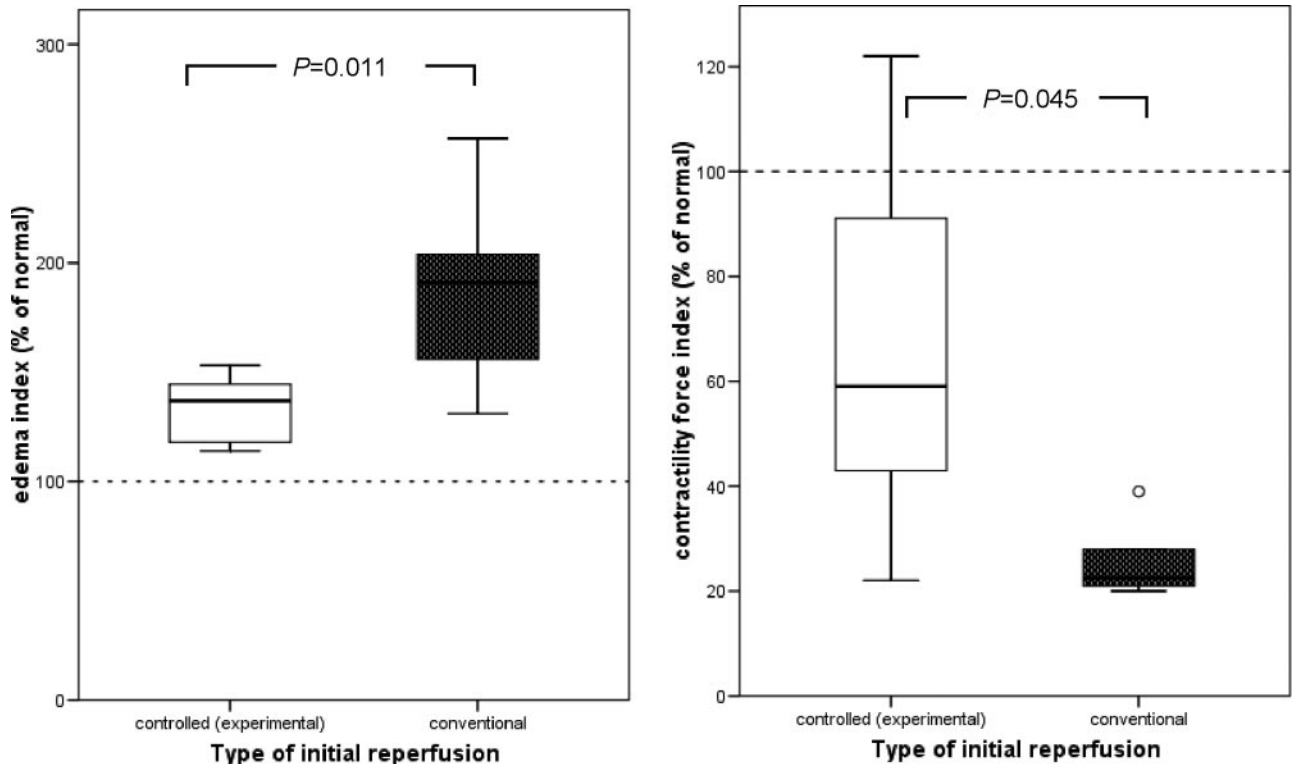
Values are given as median (interquartile range) when appropriate. Edema (ratio of wet to dry tissue weight), muscle viability (MTT assay), and contractility are calculated as indexes (percent of normal).

\*Nonparametric 2-sided unpaired test (Mann–Whitney  $U$ ).

†Two-sided Fisher's exact test.

‡Nonparametric 2-sided paired test (Wilcoxon).

§Multivariate ANOVA testing of intraindividual differences stratified for ischemia.



**Figure 6.** Comparison of edema (left) and contraction force (right) indexes of ischemic limbs after the controlled reperfusion protocol ( $n=7$ ; open boxes) and uncontrolled reperfusion ( $n=7$ ; gray boxes). Findings are expressed as percent of normal (horizontal interrupted bars).

ability, severity of reperfusion injury was significantly less pronounced in the experimental group than in the control group. Differences were statistically significant in direct comparisons of the raw results and stayed so after adjustment for individual variation by calculation of index values relative to control limbs (the Table). The controlled reperfusion protocol attenuated edema formation significantly (137% versus 191% in the control group;  $P=0.011$ ; Figure 6). Similarly, it preserved muscle viability (70% versus 61%;  $P=0.004$ ; Figure 7) and muscle contractility (59% versus 23%;  $P=0.045$ ; Figure 6) much better than early uncontrolled blood reperfusion. The adjustment for background noise of the MTT assay, however, confirmed residual mitochondrial activity in both study groups (Figure 7).

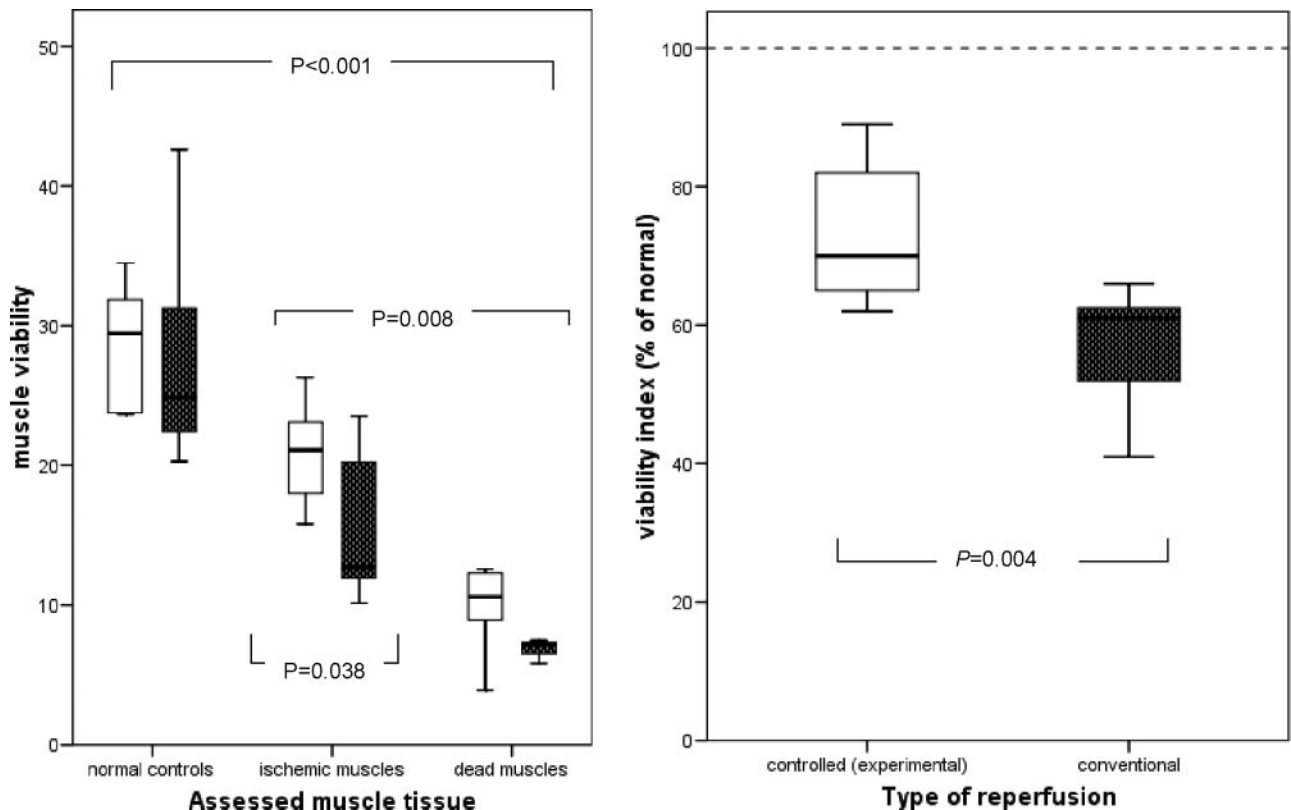
### Discussion

The present study aimed to explore the concept of controlled reperfusion in a new reality-driven rodent model of acute limb ischemia to support the transfer of this promising therapeutic concept to a more systematic clinical application. It emerged that the proposed model is suitable for representative in vivo studies and provides a missing link for structured translational research. Interestingly, very basic management of physical and chemical conditions of the initial reperfusion phase already conveyed effective protection against reperfusion-related injury.

Controlled reperfusion is increasingly being recognized as an effective approach to ongoing ischemia in various organs.<sup>15</sup> Originally described in ischemic myocardium,<sup>32</sup> it soon became established in transplantation and cardiac sur-

gery.<sup>15</sup> For acute limb ischemia, however, clinical data are scarce. Although small case series have reported striking results after up to 24 hours of ischemia<sup>24,25,33</sup> and a recent study claimed impressive benefits over simple embolectomy and immediate blood reperfusion,<sup>27</sup> a randomized controlled trial could not show any benefits regarding local outcome.<sup>26</sup> Nevertheless, experimental data are very promising,<sup>15</sup> and clinical success may depend mainly on a systematic optimization of an applicable reperfusion protocol worth being translated.<sup>34</sup> Essentially, our experiment reconfirmed the enormous clinical potential of controlled reperfusion and showed that simple and clinically available measures may already offer impressive protection against reperfusion injury (the Table). Moreover, the generic character of these modifications rendered this protocol a suitable benchmark for systematic preclinical refinements.

Reinstatement of blood perfusion is the obvious therapeutic goal in ischemia. However, perfusion is of use only if flow is actually brought to the suffocated cells. After acute ischemia, a no-reflow phenomenon may emerge as the consequence of a multifactorial vicious circle.<sup>12</sup> For example, immediate and full restoration of blood flow (shock reperfusion) has been shown to cause microvascular shutdown and congestion,<sup>12,20</sup> whereas increased hydrostatic forces enhance arteriovenous shunting and edema formation.<sup>17</sup> Conversely, flow control during initial reperfusion was effective in improving the microvascular stream<sup>20</sup> and consequently reduced peripheral vascular resistance and edema formation.<sup>19,35</sup> Other important factors promoting no reflow include inflammatory destruction of vascular beds and microthrombosis.



**Figure 7.** Comparison of residual muscle viability (left, absolute values; right, percentage of normal [dotted line]) in ischemic limbs after controlled reperfusion protocol ( $n=7$ ; open squares) and uncontrolled reperfusion ( $n=7$ ; gray squares). Dead muscle tissue (left) was used to determine the background noise of MTT assays.

Edema is directly associated with the compartment syndrome, the ultimate local complication of ischemia/reperfusion injury, and is therefore an independent therapy target. Like no reflow, its genesis is multifactorial. Besides hydrostatic shifts during shock reperfusion, ischemic endothelium is prone to leaking osmotic macromolecules, which is further promoted by ongoing inflammation. Indeed, eliminating the key initiators of inflammation from the initial reperfusion period such as oxygen,<sup>36</sup> complement factors, and leukocytes<sup>21,22</sup> has been found to be highly effective in alleviating local injury and tissue edema.<sup>8</sup> In our present study, we combined these basic mechanisms with the known cytoprotective effects of hypothermia<sup>11,19</sup> and supplemented heparin to interfere with propagation of microthrombosis and local inflammation.<sup>11</sup> Despite a prolonged net ischemic time, this protocol impressively improved local outcome compared with shock reperfusion (Figure 2).

Unlike the control group, limb perfusion recovered completely in the experimental group. Interestingly, the kinetic of perfusion recovery also was significantly different ( $P=0.027$ ; Figure 5) and resembled very closely the spontaneous reperfusion curve observed in a previous study after subclinical ischemia (1 hour).<sup>28</sup> Tissue edema was enormously reduced and tissue viability was highly increased, which certainly contributed to the marked preservation of contractility (the Table). These effects were more pronounced in earlier reports<sup>16,35,37</sup>; however, selective in vivo reperfusion has not been used in rodents before. Therefore, comparability was

limited because these data stemmed from isolated rat hind limbs or larger animal models with incomplete ischemia. Moreover, these reports used dedicated solutions to prevent edema<sup>37</sup> or to preserve cell viability by adapted supply of substrates.<sup>35</sup> With a generic protocol like ours used as a benchmark, however, such optimizations can eventually be systematized. Indeed, the modular character of our experimental protocol allows a structured and systematic evaluation of individually modulated perfusion parameters or an ever-growing spectrum of potentially effective pharmacological adjuncts.<sup>15,23,38</sup>

The explicit aim of the model design was to amend translational research. Earlier rodent models have used tourniquets<sup>28</sup> or rubber bands<sup>39</sup> and lacked vascular access for selective reperfusion. Venous stasis as a consequence of these approaches, however, is not a part of clinical limb ischemia and has been associated with severe damage to the microcirculation in earlier studies.<sup>40</sup> In our model, patency of the femoral vein was confirmed by direct observation, and the reperfusion dynamic in the control group strongly suggested that microcirculation had been relatively protected. In addition, blood circulation recovered remarkably better in our model after 4 hours of ischemia (80%; Figure 5) than in a recently reported 3-hour ischemia model involving venous occlusion (50%).<sup>28</sup> Together with a reliable vascular access, our model overcame a major limitation of earlier rodent models.

Reflection of clinically relevant outcome is crucial for an experimental model to qualify as translational platform.<sup>41</sup> Our



model consistently produced significant clinical signs of ischemia/reperfusion injury in all tested limbs compared with nonischemic controls (the Table), and the extent of injury was found to be in the same range as reported before for similar settings.<sup>28,35,38,42</sup> The timing of experiments is another important aspect because reperfusion models should reflect clinically relevant degrees of ischemia; however, any therapeutic benefit is being lost as soon as irreversible tissue damage occurs. Clinically, the critical threshold for acutely ischemic limbs is usually estimated to be  $\approx 6$  hours.<sup>9</sup> However, analogy with tourniquet ischemia is limited because clinical ischemia is normally not complete. Indeed, the critical acute ischemia window for traumatic amputations or ischemic surgical flaps is usually much shorter, ranging between 2 and 4 hours.<sup>9,11</sup> Hence, most models of complete peripheral ischemia settle for intervals between 2 and 4 hours, depending on the research question.<sup>28,35,38</sup> In our experimental setting, we chose an interval (4 hours) that ranged at the upper end of this critical window because we aimed to maximize reperfusion-related injury to facilitate the evaluation of reperfusion protocols in a clinically relevant setting.

The duration of the observation period after reperfusion is of similar relevance. Pathological investigations have shown that reperfusion-related changes occurred essentially within the first hour and that myonecrosis did not increase after that.<sup>9</sup> Another study investigated 3 hours of complete ischemia and reported that local injury was very similar after 4 and 48 hours of reperfusion.<sup>38</sup> In contrast, others found complete recovery of humoral markers of cell damage within 24 hours (2 hours of ischemia). Local injury, however, persisted in this study and was demonstrated by tissue assays.<sup>42</sup> In our study, we first aimed to standardize the experimental conditions to validate our approach and thus decided to keep the animals under continuous anesthesia. On the basis of the arguments above, we accepted a 4-hour observation period as sufficiently representative to assess local outcome.<sup>28,35</sup> Nevertheless, future experiments will obviously have to account for longer follow-up periods, particularly in the assessment of systemic outcome.

### Study Limitations

Some methodological limitations must be considered in the interpretation of the data of this series. First, because of organizational concerns in our unit, Wistar rats of different sexes and weights were used throughout the experiment. However, similar distributions of sex and weight were ascertained by randomization (the Table) and seem not to have influenced results, which were astonishingly consistent. Our study also may be criticized for the small number of animals. Given the primary goal of establishing and validating a new reality-driven rodent model, this study was conceived to challenge preliminary findings of a generic reperfusion protocol. Despite the small number of tested animals and the exclusive use of nonparametric tests, statistical separation of results was consistently significant throughout this randomized experiment and sustained not only by unpaired but also by paired testing. This reduced the risk of a type I error significantly; therefore, it is likely that the results will remain statistically significant in larger experiments. Although edema, viability, and contractility are likely to be understood

as parts of the same overall analysis, multiple hypothesis testing might be of some concern. Bonferroni adjustment (to  $\alpha=0.017$ ) appeased these concerns convincingly despite its conservative approach in 2 of 3 end points, and the equal direction of all results argued against random occurrence. For the same reason and because this study aimed to assess the translational capacity of a new model, end points were limited to clinical relevance. However, the model is equally suitable for molecular investigations<sup>28</sup> of local and systemic events, which will have to be taken into account in future studies. To suit this purpose, venous evacuation of sequestered blood was included in the baseline protocol because it has been associated with improved survival in human studies.<sup>26</sup>

### Conclusions

The present study proposed and validated an improved reality-driven rodent model of acute limb ischemia that, unlike earlier models, affords emulation of clinical management by controlled reperfusion. A basic set of modifications of the initial reperfusion phase, which could theoretically be applied easily in clinical practice, appeared highly protective and highlighted the clinical potential of controlled reperfusion. Moreover, it provided a methodological framework for structured translational research in this field. It remains to be seen how much additional local and systemic benefit can be achieved by pharmacological optimization of the reperfusate solution.

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### Disclosures

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### References

- Earnshaw JJ. Demography and etiology of acute leg ischemia. *Semin Vasc Surg*. 2001;14:86–92.
- Singh S, Evans L, Datta D, Gaines P, Beard JD. The costs of managing lower limb-threatening ischaemia. *Eur J Vasc Endovasc Surg*. 1996;12:359–362.
- Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *J Vasc Surg*. 2007;45(suppl S):S5–S67.
- Ljungman C, Holmberg L, Bergqvist D, Bergstrom R, Adami HO. Amputation risk and survival after embolectomy for acute arterial ischaemia: time trends in a defined Swedish population. *Eur J Vasc Endovasc Surg*. 1996;11:176–182.
- Ouriel K, Veith FJ, Sasahara AA. A comparison of recombinant urokinase with vascular surgery as initial treatment for acute arterial occlusion of the legs: Thrombolysis or Peripheral Arterial Surgery (TOPAS) Investigators. *N Engl J Med*. 1998;338:1105–1111.
- Jivegard L, Holm J, Schersten T. Acute limb ischemia due to arterial embolism or thrombosis: influence of limb ischemia versus pre-existing cardiac disease on postoperative mortality rate. *J Cardiovasc Surg (Torino)*. 1988;29:32–36.
- Hirsch AT, Haskal ZJ, Hertzner NR, Bakal CW, Creager MA, Halperin JL, Hiratzka LF, Murphy WR. ACC/AHA 2005 practice guidelines for the management of patients with peripheral arterial disease. *Circulation*. 2006;113:e463–e654.
- Collard CD, Gelman S. Pathophysiology, clinical manifestations, and prevention of ischemia-reperfusion injury. *Anesthesiology*. 2001;94:1133–1138.

9. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. *Cardiovasc Surg*. 2002;10:620–630.
10. Carden DL, Granger DN. Pathophysiology of ischaemia-reperfusion injury. *J Pathol*. 2000;190:255–266.
11. Khalil AA, Aziz FA, Hall JC. Reperfusion injury. *Plast Reconstr Surg*. 2006;117:1024–1033.
12. Rezkalla SH, Kloner RA. No-reflow phenomenon. *Circulation*. 2002;105:656–662.
13. Haimovici H. Proceedings: myopathic-nephrotic-metabolic syndrome associated with massive acute arterial occlusions. *J Cardiovasc Surg (Torino)*. 1973;14:589–600.
14. Pasupathy S, Homer-Vanniasinkam S. Ischaemic preconditioning protects against ischaemia/reperfusion injury: emerging concepts. *Eur J Vasc Endovasc Surg*. 2005;29:106–115.
15. Cryer HG. Therapeutic approaches for clinical ischemia and reperfusion injury. *Shock*. 1997;8:26–32.
16. Beyersdorf F, Matheis G, Kruger S, Hanselmann A, Freisleben HG, Zimmer G, Satter P. Avoiding reperfusion injury after limb revascularization: experimental observations and recommendations for clinical application. *J Vasc Surg*. 1989;9:757–766.
17. Okamoto F, Allen BS, Buckberg GD, Bugyi H, Leaf J. Reperfusion conditions: importance of ensuring gentle versus sudden reperfusion during relief of coronary occlusion. *J Thorac Cardiovasc Surg*. 1986;92:613–620.
18. Mowlavi A, Neumeister MW, Wilhelm BJ, Song YH, Suchy H, Russell RC. Local hypothermia during early reperfusion protects skeletal muscle from ischemia-reperfusion injury. *Plast Reconstr Surg*. 2003;111:242–250.
19. Wright JG, Belkin M, Hobson RW 2nd. Hypothermia and controlled reperfusion: two non-pharmacologic methods which diminish ischemia-reperfusion injury in skeletal muscle. *Microcirc Endothelium Lymphatics*. 1989;5:315–334.
20. Chun K, Zhang J, Biewer J, Ferguson D, Clemens MG. Microcirculatory failure determines lethal hepatocyte injury in ischemic/reperfused rat livers. *Shock*. 1994;1:3–9.
21. Byrne JG, Appleyard RF, Lee CC, Couper GS, Scholl FG, Laurence RG, Cohn LH. Controlled reperfusion of the regionally ischemic myocardium with leukocyte-depleted blood reduces stunning, the no-reflow phenomenon, and infarct size. *J Thorac Cardiovasc Surg*. 1992;103:66–71.
22. Halldorsson AO, Kronon M, Allen BS, Rahman S, Wang T, Layland M, Sidle D. Controlled reperfusion prevents pulmonary injury after 24 hours of lung preservation. *Ann Thorac Surg*. 1998;66:877–884.
23. Rubin BB, Romaschin A, Walker PM, Gute DC, Korthuis RJ. Mechanisms of postischemic injury in skeletal muscle: intervention strategies. *J Appl Physiol*. 1996;80:369–387.
24. Beyersdorf F, Mitrev Z, Ihnken K, Schmiedt W, Sarai K, Eckel L, Friesewinkel O, Matheis G, Buckberg GD. Controlled limb reperfusion in patients having cardiac operations. *J Thorac Cardiovasc Surg*. 1996;111:873–881.
25. Defraigne JO, Pincemail J, Laroche C, Blaffart F, Limet R. Successful controlled limb reperfusion after severe prolonged ischemia. *J Vasc Surg*. 1997;26:346–350.
26. Walker PM, Romaschin AD, Davis S, Piovesan J. Lower limb ischemia: phase 1 results of salvage perfusion. *J Surg Res*. 1999;84:193–198.
27. Wilhelm MP, Schlensak C, Hoh A, Knipping L, Mangold G, Rojas DD, Beyersdorf F. Controlled reperfusion using a simplified perfusion system preserves function after acute and persistent limb ischemia: a preliminary study. *J Vasc Surg*. 2005;42:690–694.
28. Bonheur JA, Albadawi H, Patton GM, Watkins MT. A noninvasive murine model of hind limb ischemia-reperfusion injury. *J Surg Res*. 2004;116:55–63.
29. Position of the American Heart Association on research animal use. *Circulation*. 1985;71:849A–850A.
30. Andrade FH, Hatala DA, McMullen CA. Carbonic anhydrase isoform expression and functional role in rodent extraocular muscle. *Pflugers Arch*. 2004;448:547–551.
31. Mellors LJ, Barclay CJ. The energetics of rat papillary muscles undergoing realistic strain patterns. *J Exp Biol*. 2001;204:3765–3777.
32. Buckberg GD. When is cardiac muscle damaged irreversibly? *J Thorac Cardiovasc Surg*. 1986;92:483–487.
33. Vogt PR, von Segesser LK, Pagotto E, Lijovic T, Turina MI. Simplified, controlled limb reperfusion and simultaneous revascularization for acute aortic occlusion. *J Vasc Surg*. 1996;23:730–733.
34. Walker PM. Ischemia/reperfusion injury in skeletal muscle. *Ann Vasc Surg*. 1991;5:399–402.
35. Beyersdorf F, Unger A, Wildhirt A, Kretzer U, Deuschlander N, Kruger S, Matheis G, Hanselmann A, Zimmer G, Satter P. Studies of reperfusion injury in skeletal muscle: preserved cellular viability after extended periods of warm ischemia. *J Cardiovasc Surg (Torino)*. 1991;32:664–676.
36. Walker PM, Lindsay TF, Labbe R, Mickle DA, Romaschin AD. Salvage of skeletal muscle with free radical scavengers. *J Vasc Surg*. 1987;5:68–75.
37. Matheis G, Beyersdorf F, Hanselmann A, Unger A, Wildhirt A, Kruger S, Zimmer G, Satter P. Reperfusion injury in skeletal muscle: interaction of osmotic and colloid-osmotic pressure in the initial reperfusate for oedema prevention. *Cardiovasc Surg*. 1994;2:725–736.
38. Hua HT, Albadawi H, Entabi F, Conrad M, Stoner MC, Meriam BT, Sroufe R, Houser S, Lamuraglia GM, Watkins MT. Polyadenosine diphosphate-ribose polymerase inhibition modulates skeletal muscle injury following ischemia reperfusion. *Arch Surg*. 2005;140:344–351.
39. Crawford RS, Hashmi FF, Jones JE, Albadawi H, McCormack M, Eberlin K, Entabi F, Atkins MD, Conrad MF, Austen WG Jr, Watkins MT. A novel model of acute murine hindlimb ischemia. *Am J Physiol Heart Circ Physiol*. 2007;292:H830–H837.
40. Hjortdal VE, Hansen ES, Hauge E. Myocutaneous flap ischemia: flow dynamics following venous and arterial obstruction. *Plast Reconstr Surg*. 1992;89:1083–1091.
41. Mankoff SP, Brander C, Ferrone S, Marincola FM. Lost in translation: obstacles to translational medicine. *J Transl Med*. 2004;2:14.
42. Arieli D, Nahmany G, Casap N, Ad-El D, Samuni Y. The effect of a nitroxide antioxidant on ischemia-reperfusion injury in the rat in vivo hind limb model. *Free Radic Res*. 2008;42:114–123.

### CLINICAL PERSPECTIVE

Clinical management of acute limb ischemia has not progressed fundamentally since safe and fast restoration of blood flow became possible with the introduction of balloon catheters. Although the restored circulation is absolutely vital, it may paradoxically also lead to exacerbated local injury and systemic complications, which essentially account for the poor prognosis. Controlled reperfusion with specific modifications of the initial reperfusion period, as routinely applied in transplantation or cardiac surgery, is thought to have great potential to improve clinical outcome. However, the lack of a suitable translational model has hindered the systematic development of an optimal reperfusion protocol for acute limb ischemia. The relative importance of physical and biochemical (perfusion pressure, flow rate, temperature, oxygen content, osmolarity), biological (filtration of leukocytes or inflammatory molecules), or pharmacological (free radical scavengers, anticoagulant and antiinflammatory agents,  $\text{Na}^+/\text{H}^+$  pump inhibitors) modifications still needs to be investigated. Our study validated a new reality-driven rodent model of controlled limb reperfusion that is suited for both structured translational research and molecular investigations because it allows in vivo studies of regional and systemic events during ischemia/reperfusion injury. Interestingly, a basic and, in clinical practice, theoretically easily applicable reperfusion protocol already conveyed major protective effects against reperfusion injury by flow-controlled application of a cooled isotone heparin solution. These findings highlight the enormous clinical potential of controlled reperfusion and, as a benchmark for refinements, provide the methodological framework for systematic research into an optimized and clinically applicable reperfusion protocol that may eventually translate into improved patient care.