Reperfusion injury in skeletal muscle: controlled limb reperfusion reduces local and systemic complications after prolonged ischaemia

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Previous studies in isolated limbs using crystalloid perfusion solutions have shown that control of the initial reperfusion reduces postischaemic complications. However, no experimental study has been undertaken to evaluate the concept of controlled limb reperfusion experimentally in an *in vivo* blood-perfused model and to assess the local as well as systemic effects of normal blood reperfusion and controlled limb reperfusion. Of 20 pigs undergoing preparation of the infrarenal aorta and iliac arteries, six were observed for 7.5 h and served as controls; 14 others underwent 6 h of complete infrarenal occlusion. Thereafter, embolectomy was simulated in eight pigs by removing the aortic clamp and establishing normal blood reperfusion at systemic pressure. In six other pigs, the composition of the reperfusate and the conditions of reperfusion were controlled during the first 30 min, followed by normal blood reperfusion. Some 6 h of infrarenal aortic occlusion leads to a severe decrease in high-energy phosphates and muscle temperature, together with a slight increase in creatine kinase and potassium in the systemic circulation. Normal blood reperfusion resulted in severe reperfusion injury: massive oedema developed, the tissue showed a marked decrease in oxygen consumption, glucose consumption, tissue ATP, total adenine nucleotides, muscle pH and total calcium in the femoral vein. Furthermore, a massive increase was seen in plasma creatine kinase concentration and potassium, together with the development of muscle rigidity. In sharp contrast, initial treatment of the ischaemic skeletal muscle by controlled limb reperfusion resulted in normal water content, oxygen consumption, glucose consumption, flow and muscle rigidity. Furthermore, controlled limb reperfusion resulted in higher total adenine nucleotides content, less tissue acidosis, markedly reduced creatine kinase release, and potassium release as compared with that of normal blood reperfusion. This study shows that 6 h of acute infrarenal aortic occlusion will result in severe reperfusion injury (postischaemic syndrome) if normal blood at systemic pressure is given in the initial reperfusion phase. In contrast, initial treatment of the ischaemic skeletal muscle by controlled limb reperfusion reduces the metabolic, functional and biochemical alterations.

Keywords: limb ischaemia, reperfusion, skeletal muscle, acute ischaemia, postischaemic syndrome

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Revascularization of limbs after prolonged, severe ischaemia is associated with a high incidence of postoperative complications[1,2]. Mortality rates vary between 7.5%[1] and 41%[3]. Subsequent amputation rates range from 12% to 22%[4], and postoperative limb function is only approximately 60–70%[1]. The principal aetiology of these increased morbidity and mortality rates (postischaemic syndrome)[5,6] is the restoration of blood flow, which causes reperfusion injury in addition to the damage produced by the preceding ischaemic period[7], regardless of whether reperfusion is accomplished surgically[1,3,4] or medically[8]. The only treatment option is usually symptomatic management of each complication *after* the onset of the postischaemic syndrome. There is no strategy to *avoid* the complications.

The authors' experimental studies [7,9–13] in a crystalloid-perfused isolated rat hindlimb model, together with the results of others[14–21], have shown that the deleterious consequences of reperfusion after prolonged ischaemia in skeletal muscle can be substantially reduced by modifying the initial reperfusate. However, a controlled study in an *in vivo*, blood-perfused model to evaluate the local and systemic effects of the authors' new technique of controlled limb reperfusion compared with those of normal blood reperfusion has not been conducted.

The present study in an *in vivo* pig model was undertaken to: (1) investigate the effects of reperfusion in a blood-perfused, *in vivo* system, which resembles the clinical situation; (2) evaluate the sequelae of normal blood reperfusion at systemic pressure after severe ischaemia (i.e. 6 h of infrarenal aortic occlusion); (3) investigate the effect of a 30-min controlled limb reperfusion phase followed by normal blood reperfusion; (4) study the practicability of the authors' new technique of controlled limb reperfusion for future clinical application; and (5) examine the consequences of controlled limb reperfusion on systemic biochemical changes following revascularization.

Materials and methods

Twenty adult domestic pigs of both sexes (mean(s.d.) weight 63(13) kg) were anaesthetized with ketamine (10 mg/kg), metomidate (2 mg/kg), and pentobarbitone (45 mg/kg per h). The pigs were intubated and the lungs ventilated by positive-pressure with 30% oxygen at a rate of 12–15 cycles/min. Physiological saline (0.9%) was continuously infused at a rate of 3–5 ml/kg per h to maintain hydration.

The electrocardiogram (ECG) was recorded with an eight-channel ECG recorder (Gould; Cleveland, Ohio, USA). The right carotid artery was cannulated to monitor arterial pressure continuously with a strain gauge transducer. A thermodilution catheter (Hoyer, Bremen, Germany) for cardiac output monitoring was also inserted in the right carotid artery and connected to a cardiac output computer (HMV 7905; Hoyer Bremen, Germany). The right external jugular vein wa cannulated to administer saline and heparin, and t sample central venous blood. A Swan–Ganz cathete was inserted into the left internal jugular vein t monitor haemodynamics. A catheter was also place into the left carotid artery to obtain arterial bloo samples. Blood gases were maintained at pH 7.3–7.4 P_{CO_2} at 4.0–5.3 kPa (30–40 mmHg) and P_O > 13.3 kPa (> 100 mmHg).

Experimental preparation

The abdomen was opened using a lower left pararect incision, and the infrarenal aorta, aortic bifurcation an both iliac arteries dissected extraperitoneally. T dissection of the left iliac artery provided a no branching arterial conduit from the aorta to the femor arteries, and blood flow to the thigh was measured usi an electromagnetic flow transducer. A polyethyle catheter was placed into the left iliac vein for bloc sampling and pressure monitoring. Both hindlimbs we made ischaemic by applying an atraumatic vascul clamp to the aorta below the renal arteries. N additional preparation to the lumbar arteries w performed. Blood flow to the ischaemic hind limbs w again assessed by an electromagnetic probe, and in pil studies, using aortography. The abdominal incision w closed and the anaesthetized animal was kept at roc temperature during the ischaemic interval of 6 h. Ju before starting reperfusion, an intravenous bolus dose sodium heparin (300 units/kg) was administered, sim lating the clinical situation, when the patient is enteri hospital.

Measurements

Regional blood flow

Dissection of the left iliac artery provides a n branching arterial conduit from the aorta to the femc system and blood flow to the thigh was measured us an electromagnetic blood flow transducer (Flow-Pr SP7515; Statham, Cleveland, Ohio, USA) placed aro the iliac artery and recorded with a two-chan flowmeter (SP2202; Statham, Cleveland, Ohio, USA ml/min.

In pilot studies ($n = 5$), the left hindlimb amputated at the level of the hip joint. The mean(s.e. weight of these limbs was 6.3(0.4) kg whereas mean(s.e.m.) body weight was 47.4(3.0) kg (i.e. l weight was 13% of body weight). In all other pigs, l weight was calculated as 13% of the body weight.

Flow (ml/min per 100 g tissue) in the limb calculated as:

$$\text{Flow} = 100 \times \left[\frac{\text{Flow in the iliac artery}}{\text{Limb weight}} \right]$$

where flow in the iliac artery is measured in ml/min limb weight in grams.

Vascular resistance

Vascular resistance in the iliac artery was calculated as:

$$VR = \frac{(MAP - VP) \times 80}{Flow}$$

where VR is vascular resistance (dynes × s/cm^5), MAP the mean arterial pressure (mmHg) and VP the pressure in the iliac vein (mmHg). Flow is expressed as ml/min and 80 is a constant.

Muscle rigidity

After the animal was anaesthetized and intubated, muscle rigidity was measured by determination of the range of motion of the knee joint in the right limb. Data were recorded by measuring the range of motion in degrees. The measurements were performed before and 6 h after ischaemia, and 90 min after reperfusion.

Enzymes and electrolytes

Blood samples for all parameters were prepared routinely for measurement on the same day (centrifuged for 5 min at 3000 revolutions/min). 'Optimized standard methods' conforming to the 'Deutsche Gesellschaft für Klinische Chemie' were used. Each method was modified for automated analysis with a random access analyser (Hitachi 717; Boehringer Mannheim GmbH, Mannheim, Germany), and potassium, total calcium and creatine kinase were assessed.

Ionized calcium was determined using two different methods: (1) by means of a calcium-selective electrode (Calcium Selective Electrode 93–20; Orion Research, Cambridge, Massachusetts, USA) against a reference electrode, which directly measures calcium ions; and (2) according to the technique described by Siggard-Jersen *et al.*[22]. The latter uses a nomogram in which total calcium, protein and pH must be correlated, and the ionized calcium can be read. Total calcium was measured photometrically with a calcium determination kit (Calcium Test Combination; Boehringer, Mannheim, Germany). Protein was assessed with the biuret method (Merckotest Total Protein; Merck, Darmstadt, Germany), and pH was measured with an electrode (Ingold Electrode 405 S7; Ingold, Steinbach, Germany) and a microprocessor ionalyser (Microprocessor Ionalyzer-901 Device; Orion Research, Cambridge, Massauchesetts, USA).

Oxygen metabolism

Haemoglobin was measured (CO-Oxylite AVL 912; Linz, Austria) at the same time as blood gases (Automatic blood gas system AVL 947; Linz, Austria) from samples drawn from the carotid artery and iliac vein. Oxygen content was calculated as:

$$C_{O_2} = (Hb \times \%sat. \times 1.39) + (P_{O_2} \times 0.0031)$$

where C_{O_2} is oxygen content (ml O_2/100 ml blood), Hb is haemoglobin (mg/dl), % sat. is oxygen saturation (%), P_{O_2} is partial oxygen pressure (mmHg), and 1.39 and 0.0031 are constants.

Oxygen delivery (ml/100 g per min) was calculated as:

$$\text{Oxygen delivery} = Ca_{O_2} \times \text{Flow}$$

where Ca_{O_2} is arterial C_{O_2} (ml/100 ml) and flow is in ml/100 g per min.

Arteriovenous oxygen difference (ml O_2/100 ml blood) was calculated as:

$$avC_{O_2} = Ca_{O_2} - Cv_{O_2}$$ where Cv_{O_2} is venous C_{O_2}

Oxygen consumption ($\dot{V}_{O_2}$) (ml/100 g tissue per min) was calculated as:

$$\dot{V}_{O_2} = avC_{O_2} \times \text{Flow}$$

Measurements and calculations were performed before and 3 and 6 h after ischaemia, and after 5, 30, 60 and 90 min of reperfusion.

Glucose metabolism

Arteriovenous glucose difference (avC_{glu}) is expressed in mg/dl and calculated as:

$$avC_{glu} = \text{Arterial glucose concentration} - \text{venous glucose concentration}$$

Glucose consumption (mg/100 g tissue per min) was calculated as:

$$\text{Glucose consumption} = avC_{glu} \times \text{Flow}$$

where Flow was measured in dl/100 g tissue per min.

Measurements and calculations were performed before and 3 and 6 h after ischaemia and after 5, 30, 60 and 90 min of reperfusion.

Muscle pH and temperature

A precalibrated, 21-gauge stainless-steel electrode (Ingold Elektrode U-402-M3; Ingold, Steinbach, Germany) was inserted into the tibial muscle. The electrode was precalibrated, using pH standard solutions (pH 4 and 7). The electrode was connected to a pH meter (Digital pH Meter; Knick, Berlin, Germany), providing continuous muscle pH readings. An intramuscular needle temperature probe (Myocardial Temperature Sensor; Sims, California, USA) was inserted close to the pH electrode and connected to a temperature recorder. (Tastoterm D 700; Impact, Hanau, Germany), which corrected pH according to the muscle temperature. Care was taken to maintain a constant electrode and needle position. Measurements were performed before and 3 and 6 h after ischaemia, and after 5, 30, 60 and 90 min of reperfusion.

Skeletal muscle water content

Water content in the anterior tibial muscle was determined by weighing the muscle biopsies before and after freeze drying. The weight of the biopsy specimens (approximately 0.6–1.2 g) taken before and 6 h after ischaemia, and after 90 m of reperfusion was ascertained, following which the muscle was dried for 48 h at −50°C in a vacuum lyophilizor (Freeze Dryer Modulyo; Edwards, Crawley, UK). Weighing was performed on a precision balance (Sartorius 2442; Sartorius-Werke GmbH, Göttingen, Germany). Tissue water content was calculated according to the following equation:

$$\text{Water content (\%)} = \frac{\text{(wet tissue weight} - \text{dry tissue weight)}}{\text{wet tissue weight}} \times 100$$

High-energy phosphates

Adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) were analysed from tibialis muscle biopsies with the luciferin–luciferase reaction described by Lundin *et al.*[23]. This highly sensitive luminometric method allows measurement of ATP concentration down to 10^{-11} mol/l. The luciferin–luciferase reagent (ATP monitoring reagent; LKB Wallac, Turku, Finland) emits light of almost constant intensity that is proportional to the ATP concentration and can be measured with a luminometer (type 1250; LKB Wallac, Turku, Finland). Biopsies were taken with a Tru-Cut (Travenol Laboratories, Deerfield, Illinois, USA) needle biopsy before and after 6 h of ischaemia, and after 90 min of reperfusion. They were immediately frozen in liquid nitrogen and homogenized together with 500 µl of perchlorate and 4 mmol ethylenediamine tetraacetic acid. Some 80 mg tris (hydroxymethyl) methylamine was added before centrifugation and a small aliquot of the samples withdrawn for protein assessment. All values were related to the protein content of the biopsy (µmol/g protein). Total adenine nucleotides were determined by adding the concentrations of ATP, ADP and AMP.

Experimental groups

All pigs underwent preparation of the aorta and iliac arteries.

Control group (n = 6)

The animals were observed for 7.5 h (without ischaemia) after preparation of the aorta and iliac arteries.

Normal blood reperfusion after 6 h of acute aortic occlusion (uncontrolled reperfusion)

Eight pigs underwent 6 h of acute infrarenal aortic occlusion followed by reperfusion with normal blood at systemic pressure (accomplished by removing the vascular clamp). Blood samples were collected from the carotid artery, jugular vein and iliac vein after 5, 30, 60 and 90 min of reperfusion. This experimental group mimics acute, prolonged aortic occlusion treated by embolectomy.

Treatment of the ischaemic limb before normal blood reperfusion (controlled limb reperfusion)

In six pigs, the aorta was occluded for 6 h; after 5.5 h during this period, preparations for controlled arterioarterial limb reperfusion were made by cannulating the aorta proximal to the occlusion with a 26-Fr cannula (VC Cath. HKV-36B; Jostra, Hechingen, Germany) which was used as arterial inflow. The aorta distal to the occlusion was cannulated with a 14-Fr cannula (Retroplegia; Research Medical, Salt Lake City, Utah, USA) as outflow for the controlled limb reperfusate (*Figure 1*). Oxygenated blood was drawn from the proximal aorta and modified by the addition of a crystalloid solution in a ratio of 6:1 (blood:crystalloid solution) (*Table 1*). This was passed through a coil and a heat exchanger (at 37°C) (Perfusionsspirale; HP-Medica, Augsburg, Germany), and infused into the aorta distal to the occlusion with a Stöckert roller pump. The conditions of the reperfusion (i.e. intra-aortic pressure 40 mmHg; flow 400–500 ml/min, temperature 37°C) were controlled during the first 30 min of

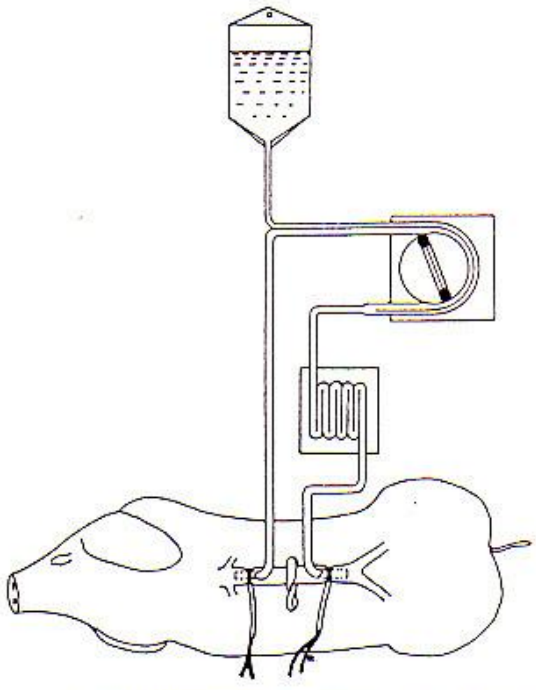

Figure 1 Experimental model of infrarenal aortic occlusion and cannulation for controlled limb reperfusion

Table 1 Composition of the controlled limb reperfusate

Principle	Method	Concentration delivered
Provide oxygen	Blood	Haemoglobin 7.8–8.9 g/dl
Avoid oedema	Hyperosmolarity	340–350 mosmol/l
Provide substrate	Glucose	400–500 mg/dl
	Glutamate	13 mmol/l
	Aspartate	13 mmol/l
Reverse acidosis	Tromethamol	pH 7.5–7.6
Limit Ca^{2+} influx	Citrate–phosphate–dextrose	Total Ca^{2+} 1.7 mmol/l
Prevent free radical formation	Lipoic acid (Thioctacid)	12.5 mg/l (60 μmol/l)

reperfusion. Blood samples were collected at 5 and 30 min during controlled reperfusion from the jugular vein, iliac vein and the controlled reperfusate at the same time. The aorta was decannulated and repaired with 4/0 polypropylene interrupted suture. Normal blood reperfusion at systemic pressure was restored to both limbs and blood samples collected at 60 and 90 min of reperfusion from the jugular vein, iliac vein and carotid artery.

Statistical analysis

Statistical analysis was made using the Epistat computer package and the Bias computer package provided by the Johann Wolfgang Goethe-University, Frankfurt am Main, Germany, in conjunction with Dr H. Ackermann, a biomathematician (Johann Wolfgang Goethe-University). Comparisons between groups were made with the one-way analysis of variance (ANOVA) and nominal data by Fisher's exact test. Differences were considered significant at $P < 0.05$. Data are expressed as mean(s.e.m.).

Results

Severity of ischaemia after 6 h of infrarenal aortic occlusion

In the first five experiments, angiography of the aorta, iliac and femoral arteries was performed and radiography showed no staining of the iliac or femoral arteries; collateral formation was minimal. In all other experiments, blood flow (as measured with an electromagnetic flow probe) was zero and high-energy phosphates (ATP, creatine phosphate and total adenine nucleotides) decreased to 50% of the control values (*Tables 2* and *3*). Muscle temperature decreased from 36.3(0.2) to 30.3(0.4)°C ($P < 0.000001$), muscle pH from 7.4(0.1) to 5.9(0.1) ($P < 0.000001$) and muscle rigidity from 122(1) to 90(2)° ($P < 0.000001$). There was a marked increase in creatine kinase (2068(529) *versus* 513(80) units/l; $P < 0.01$) as well as potassium (6.7(0.2) *versus* 4.4(0.2) mmol/l; $P < 0.00001$) (*Table 4*) in the systemic circulation after 6 h of ischaemia.

Table 2 Water content and tissue levels of ATP, ADP and AMP during 7.5-h observation without ischaemia (control group, $n = 6$) and after 6-h infrarenal aortic occlusion followed by normal blood reperfusion (group 1, $n = 8$) or controlled limb reperfusion (group 2, $n = 6$)

Time (h)	Control	Group 1	Group 2
Water content (%)			
0	76.1(0.5)	76.6(0.4)[†]	76.8(0.2)
6	76.7(0.4)	77.4(0.7)	77.2(0.6)
7.5	76.9(0.4)	80.6(0.7)[*]	77.6(0.4)[‡]
Muscle ATP (μmol/g protein)			
0	34.1(1.1)	36.1(0.9)	34.4(1.4)
6.0	30.0(1.2)	16.3(1.7)	12.9(0.9)
7.5	25.6(0.9)[§]	18.3(1.9)[¶]	20.5(1.2)
Muscle ADP (μmol/g protein)			
0	6.9(1.3)	5.7(0.7)	4.8(1.0)
6.0	7.9(1.2)	4.9(1.2)	3.5(0.8)
7.5	8.5(1.7)	3.9(0.9)	6.8(0.3)
Muscle AMP (μmol/g protein)			
0	5.1(1.0)	3.9(0.7)	3.2(0.2)
6.0	4.9(0.7)	4.1(1.1)	3.3(1.6)
7.5	4.1(0.6)	4.5(0.9)	4.0(1.0)

Values are mean(s.e.m.) [*]$P < 0.0009$ *versus* baseline. [†]$P < 0.04$ *versus* normal blood reperfusion. [‡]$P < 0.002$ *versus* 90-min reperfusion. [§]$P < 0.0001$ *versus* baseline. [¶]$P < 0.002$ *versus* control

Table 3 Tissue levels of creatine phosphate and total adenine nucleotides (μmol/g protein) during 7.5-h observation without ischaemia (control group, $n = 6$), and after 6-h infrarenal aortic occlusion followed by normal blood reperfusion (group 1, $n = 8$) or controlled reperfusion (group 2, $n = 6$)

Time (h)	Control	Group 1	Group 2
Muscle creatine phosphate			
0	34.3(2.7)	28.9(0.7)	38.6(2.3)
6	29.8(2.4)	16.8(2.8)	17.8(2.8)
7.5	23.3(2.4)[*]	21.3(0.9)	25.8(3.1)
Muscle total adenine nucleotides			
0	46.0(2.2)	45.8(1.5)	40.3(1.9)
6.0	42.8(1.8)	25.3(2.5)	19.7(2.5)
7.5	38.2(2.5)[†]	26.3(2.6)	31.3(2.0)

Values are mean(s.e.m.) [*]$P < 0.02$ *versus* baseline. [†]$P < 0.04$ *versus* baseline

Ischaemia and reperfusion

Oxygen metabolism

Oxygen delivery (*Table 5*) was comparable in both groups undergoing reperfusion before ischaemia. However, after ischaemia and 5 min of normal blood reperfusion, there was a severe decrease in oxygen delivery (95.9(9.1) *versus* 36.0(6.2) ml/100 g tissue per min; $P < 0.0001$) and it remained low throughout the

period of uncontrolled reperfusion. In contrast, oxygen delivery was only slightly reduced during the first 30 min of controlled limb reperfusion because of the low flow. Thereafter, however, the increase in oxygen delivery was significant and at the end of the 90-min reperfusion period the values did not differ from the control data (77.0(14.4) *versus* 100.9(5.7) ml O_2/100 g tissue per min) (*Table 5*).

Furthermore, there was a significant reduction in oxygen consumption (*Figure 2*) during the entire period

Table 4 Levels of creatine kinase and potassium in the iliac vein during 7.5-h observation without ischaemia (control group, $n = 6$), and after 6-h infrarenal aortic occlusion followed by normal blood reperfusion (group 1, $n = 8$) or controlled limb reperfusion (group 2, $n = 6$)

Time (h)	Control	Group 1	Group 2
Creatine kinase (units/l)			
0	527(89)	513(60)	468(73)
3	804(151)	930(202)	702(64)
6	968(192)	2068(529)[1]	1717(602)
6.083	968(192)	5119(1048)	1514(762)
6.5	983(192)	7515(1562)	1549(695)
7.0	1031(205)	10456(2163)	1915(370)
7.5	1112(237)[*]	12743(2563)[2]	2618(702)[3]
Potassium (mmol/l)			
0	3.7(0.2)	4.4(0.2)	3.9(0.1)
3	4.0(0.1)	5.8(0.2)	4.7(0.3)
6	4.3(0.2)	6.7(0.2)[**]	5.5(0.4)
6.083	4.3(0.2)	8.3(0.1)	5.1(0.3)
6.5	4.4(0.3)	7.5(0.2)	5.4(0.3)
7.0	4.5(0.3)	8.3(0.4)	5.2(0.5)
7.5	4.7(0.4)[#]	7.9(0.3)[††]	5.1(0.3)[‡‡]

Values are mean(s.e.m.). [*] $P < 0.04$ *versus* baseline. [1] $P < 0.01$ *versus* baseline. [2] $P < 0.0003$ *versus* control. [3] $P < 0.02$ *versus* normal blood reperfusion. [#] $P < 0.03$ *versus* baseline. [**] $P < 0.00001$ *versus* baseline. [††] $P < 0.00001$ *versus* control. [‡‡] $P < 0.0002$ *versus* normal blood reperfusion

Table 5 Oxygen delivery during 7.5-h observation without ischaemia (control group, $n = 6$) and after 6-h infrarenal aortic occlusion followed by normal blood reperfusion (group 1, $n = 8$) or controlled limb reperfusion (group 2, $n = 6$)

Time (h)	Control	Group 1	Group 2
0	88.6(9.1)		
6		95.9(9.1)	100.9(5.7)
6.083	82.1(11.1)	36.0(6.2)[*#]	24.3(2.1)
6.5	80.4(11.6)	34.4(4.7)[1]	23.1(2.1)
7.0	75.0(9.5)	41.1(4.0)[3]	70.9(7.6)[1]
7.5	76.2(8.9)	40.1(4.1)[#]	77.0(14.4)

Values are mean(s.e.m.) [*] $P < 0.002$ *versus* control. [1] $P < 0.04$ *versus* normal blood reperfusion. [2] $P < 0.001$ *versus* control. [3] $P < 0.03$ *versus* control. [#] $P < 0.0001$ *versus* baseline

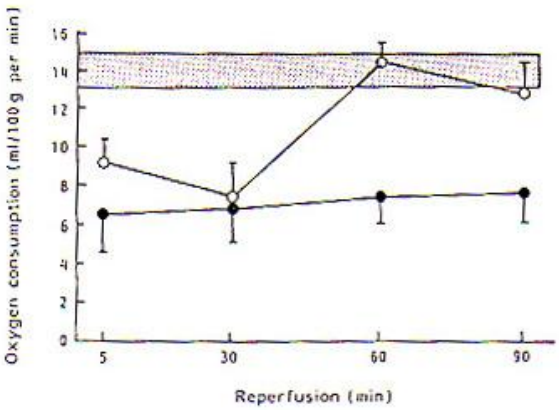

Figure 2 Mean(s.e.m.) Oxygen consumption of skeletal muscle after 6 h of acute infrarenal aortic occlusion followed by normal blood reperfusion (●) or controlled limb reperfusion (○). Control values are shown in the stippled area

of uncontrolled reperfusion (14.3(2.5) *versus* 6.2(1.6) ml/100 g tissue per min; $P < 0.02$). This marker of reperfusion injury could be completely avoided by a period of controlled limb reperfusion after ischaemia and oxygen consumption at the end of reperfusion did not differ from that of the control animals (14.9(3.3 *versus* 13.2(1.6) ml/100 g tissue per min) (*Figure 2*).

Glucose metabolism

In all groups there was a progressive decrease in arteri glucose concentration during the observation period 7.5 h (*Table 6*). The reduction in arterial gluco concentration did not differ between the contro (without ischaemia) and the group with 6 h of ischaem followed by normal blood reperfusion. However, t hyperglycaemic-controlled limb reperfusate increas arterial glucose content significantly during the fi 30 min of reperfusion and hyperglycaemia persist during reperfusion. Furthermore, only in the group wi controlled limb reperfusion was there a highly signi cant glucose uptake during the first 30 min of reperf sion (7.5(2.1) *versus* 238.7(48.9) mg/dl; $P < 0.000$ This increase was present only during the 30 min controlled reperfusion whereas in the subsequent 60 m of normal blood reperfusion, the arteriovenous gluc difference did not differ from the baseline (*Table* even though the arterial glucose levels were s increased (> 150 mg/dl).

Whereas glucose consumption (*Figure 3*) decrea severely after uncontrolled reperfusion (0.19(0. *versus* 0.51(0.01) mg/100 g tissue per dl), treatment ischaemic skeletal muscle by controlled reperfus resulted in a marked increase in glucose consumpt during the first 30 min and controlled values could achieved thereafter (0.58(0.19) *versus* 0.46(0.11) 100 g tissue per min) (*Figure 3*).

ıble 6 Arterial glucose concentration and arteriovenous glucose fference during 7.5-h observation without ischaemia (control group, n =), and after 6-h infrarenal aortic occlusion followed by normal blood perfusion (group 1, n = 8) or controlled limb reperfusion (group 2, = 6)

ime (h)	Control	Group 1	Group 2
Glucose (arterial) (mg/dl)			
)	128(13)	113(15)	93(21)
3	79(11)	75(7)	46(16)
5	52(15)	50(8)	56(11)
5 083	52(15)	46(8)	482(42)
6.5	56(15)	44(9)	429(43)
7.0	56(16)	42(9)	173(7)
7.5	55(18)	36(8)	193(4)
Art	enous glucose difference (mg/dl)		
0	4.5(1.8)	7.4(1.6)	7.5(2.1)
3	6.8(1.7)	22.2(3.1)	12.7(3.0)
6	2.0(0.5)	16.5(2.6)	12.3(3.5)
6.	2.0(0.5)	12.1(3.7)	238.7(48.9)*†
6.5	3.2(0.9)	14.6(7.5)	165.7(48.3)†
7.0	3.5(1.3)	11.3(2.2)	6.4(1.8)
7.5	4.3(0.8)	6.4(1.7)	9.0(2.1)

Values are mean(s.e.m.) *$P<0.002$ *versus* normal blood reperfusion. †$P<0.003$ *versus* normal blood reperfusion. ‡$P<0.0002$ *versus* baseline

Water content of skeletal muscle

Water content did not differ during the 7.5-h observation period in the control group (*Table* 2). Some 6 h of infrarenal aortic occlusion did not produce any increase in water content (77.4(0.7) *versus* 76.6(0.4)%). However, 90 min of normal blood reperfusion produced severe oedema in skeletal muscle (76.6(0.4) *versus* 80.6(0.7); $P=0.0009$). In contrast, normal values for water content were achieved with controlled limb reperfusion (76.8(0.3) *versus* 77.6(0.4)%) and were significantly lower than in those animals undergoing normal blood reperfusion (77.6(0.4) *versus* 80.6(0.7)%; $P<0.04$) (*Table* 2).

High-energy phosphates

Some 7.5 h anaesthesia in the control animals resulted in a marked decrease in tissue ATP (25.6(0.9) *versus* 34.1(1.1) μmol/g protein; $P<0.0001$), creatine phosphate (23.3(2.4) *versus* 34.3(2.7) μmol/g protein; $P<0.02$) and total adenine nucleotides (38.2(2.5) *versus* 46.0(2.2) μmol/g protein; $P<0.04$). The degradation products of ATP (i.e. ADP and AMP) were not affected in the control group (*Table* 2).

The mode of reperfusion did not influence tissue contents of ATP, ADP, AMP and creatine phosphate. However, baseline values for ATP, ADP and AMP in the controlled limb reperfusion group were always lower when compared with those in the normal blood reperfusion group (*Table* 2) so that the total adenine nucleotides, expressed as a percentage of baseline values, were markedly increased in the controlled limb reperfusion group (78% *versus* 57%) (*Figure* 4).

Muscle damage as assessed by creatine kinase and potassium release

In the control animals, there was a steady increase in creatine kinase (1112(237) *versus* 527(89) units/l; $P<0.04$) and potassium (4.7(0.4) *versus* 3.7(0.2) mmol/l; $P<0.03$) in iliac venous blood during the observation period (*Table* 4). There was pronounced and marked muscle damage after reperfusion with normal blood after 6 h of infrarenal aortic occlusion: creatine kinase levels rose from 513(80) units/l to

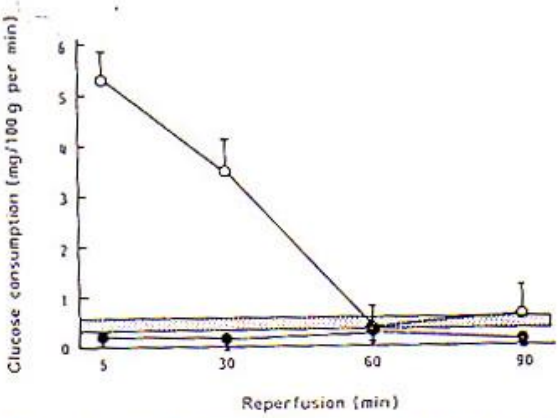

Figure 3 Mean(s.e.m.) Glucose consumption of skeletal muscle after 6 h of acute infrarenal aortic occlusion followed by normal blood reperfusion (●) or controlled limb reperfusion (○). Control values are shown in the stippled area

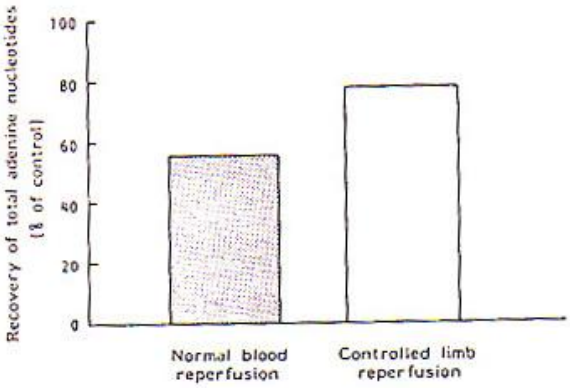

Figure 4 Recovery of total adenine nucleotides, expressed as a percentage of the control, after 6 h of infrarenal aortic occlusion following reperfusion with normal blood at systemic pressure or controlled limb reperfusion

12 743(2563) units/l ($P < 0.0003$) and potassium increased from 4.4(0.2) to 7.9(0.3) mmol/l ($P < 0.00001$). This increase was almost avoided by controlled limb reperfusion. There was only a slight increase in creatine kinase (2618(702) *versus* 1112(237) units/l; not significant) and this was significantly lower compared with the value after normal blood reperfusion (2618(702) *versus* 12 743(2563) units/l; $P < 0.02$). Furthermore, potassium release did not differ from that of the controls (5.1(0.3) *versus* 4.7(0.4) mmol/l; not significant) and again was lower when compared with the uncontrolled reperfusion group (5.1(0.3) *versus* 7.9(0.3) mmol/l; $P < 0.0002$).

Flow and vascular resistance

Normal blood reperfusion was followed by a marked low-reflow phenomenon. Flow in the iliac artery decreased from 453(29) to 191(27) ml/min ($P < 0.00001$) and flow as expressed per 100 g limb weight decreased from 6.4(0.6) to 2.6(0.3) ml/100 g per min ($P < 0.0001$). The low-reflow phenomenon was avoided by controlled reperfusion and even resulted in a slight hyperaemic response (458(20) *versus* 343(58) ml/min or, as expressed for limb weight 5.4(1.1) *versus* 4.6(0.5) ml/100 g per min).

The low-reflow phenomenon after normal blood reperfusion was accompanied by an increase in vascular resistance which occurred already after 5 min of uncontrolled reperfusion from 22.8(1.7) to 37.9(4.3) dynes × s/cm^5 ($P < 0.006$). Controlled limb reperfusion did not result in any increase in vascular resistance, in fact, there was even a slight decrease (14.7(3.4) *versus* 19.5(1.9) dynes × s/cm^5) (*Table 7*).

Calcium

In the control group, there was no significant change in the ionized calcium content of the systemic circulation during the observation period. However, systemic total calcium decreased from 2.6(0.1) to 2.2(0.1) mmol/l; $P < 0.003$ (*Table 8*). A similar reduction in the regional total calcium content (femoral artery and vein) was also observed (2.2(0.1) *versus* 2.5(0.1) mmol/l; $P < 0.01$) (*Table 9*). Normal blood reperfusion after 6 h of ischaemia resulted in a similar fall of the systemic and regional total calcium content. Furthermore, ischaemia and normal blood reperfusion also resulted in a significant decrease in the systemic ionized calcium (1.10(0.03) *versus* 1.33(0.03) mmol/l; $P < 0.02$). In the controlled reperfusion group, a similar drop in ionized calcium was seen as well as a significant decrease in total calcium in the systemic circulation and the femoral artery and vein. The most significant reduction was observed in total calcium content of the femoral artery and vein during hypocalcaemic controlled limb reperfusion (*Table 9*).

Table 7 Flow and vascular resistance during 7.5-h observation without ischaemia (control group, $n = 6$) and after 6 h infrarenal aortic occlusion followed by normal blood reperfusion (group 1, $n = 8$) or controlled limb reperfusion (group 2, $n = 6$)

Time (h)	Control	Group 1	Group 2
Flow in the iliac artery (ml/min)			
0	448(73)		
6.0		453(29)	458(20)
6.083	357(75)	154(19)	162(14)
6.5	353(69)	159(21)	162(14)
7.0	337(45)	198(26)	333(37)
7.5	337(38)	191(27)	343(58)
Flow in the limb (ml/100 g per min)			
0	6.4(0.4)		
6.0		6.4(0.6)	4.6(0.5)
6.083	5.1(0.7)	2.1(0.3)	2.5(0.3)
6.5	5.1(0.7)	2.2(0.3)	2.4(0.2
7.0	5.0(0.7)	2.7(0.3)	5.2(0.8
7.5	5.0(0.6)	2.6(0.3)	5.4(1.1
Vascular resistance (dynes × s/cm^5)			
0	23.0(2.4)		
6.0		22.8(1.7)	19.5(1.9
6.083	22.5(3.3)	37.9(4.3)	17.5(2.6
6.5	23.2(3.2)	40.7(8.6)	17.6(1.1
7.0	22.1(2.6)	29.6(2.3)	15.9(2.2
7.5	21.0(2.2)	29.9(3.9)	14.7(3.4

Values are mean(s.e.m.)

Table 8 Systemic concentration of ionized calcium and total calciur during 7.5-h observation without ischaemia (control group, $n = 6$) an after 6-h infrarenal aortic occlusion followed by normal blood reperfusic (group 1, $n = 8$) or controlled limb reperfusion (group 2, $n = 6$)

Time (h)	Control	Group 1	Group 2
Ionized calcium (mmol/l)			
0	1.38(0.01)	1.33(0.03)	1.28(0.0
6	1.29(0.03)	1.16(0.03)	1.08(0.0
7.5	1.31(0.02)	1.10(0.03)*	0.90(0.0
Total calcium (mmol/l)			
0	2.6(0.1)	2.7(0.1)	2.7(0.1)
3	2.5(0.1)	2.6(0.1)	2.5(0.1)
6	2.3(0.1)	2.3(0.1)	2.3(0.1)
6.083	2.3(0.1)	2.3(0.1)	2.1(0.1)
6.5	2.2(0.1)	2.2(0.1)	1.9(0.1)
7	2.2(0.4)	2.3(0.1)	2.2(0.1)
7.5	2.2(0.1)†	2.3(0.1)	2.2(0.1)

Values are mean(s.e.m.) *$P < 0.02$ *versus* baseline. †$P < 0.003$ ver baseline

Muscle pH

Normal blood reperfusion for 90 min after 6 h of aor occlusion did not reverse the severe tissue acido (5.9(0.1) *versus* 7.3(0.1); $P < 0.000006$). In contra 30 min of controlled limb reperfusion increased tiss

able 9 Total calcium concentration in the femoral artery and vein during 5-h observation without ischaemia (control group, $n = 6$), and after 6-h infrarenal aortic occlusion followed by normal blood reperfusion (group 1, $n = 8$) or controlled limb reperfusion (group 2, $n = 6$)

Time (h)	Control	Group 1	Group 2
Total calcium (femoral artery) (mmol/l)			
0	2.5(0.1)	2.7(0.1)	2.7(0.1)
3	2.5(0.1)	2.5(0.1)	2.5(0.1)
6	2.2(0.1)	2.3(0.1)	2.3(0.1)
6.083	2.2(0.1)	2.2(0.1)	1.7(0.1)
6.5	2.2(0.1)	2.1(0.1)	1.9(0.1)
7.0	2.1(0.1)	2.1(0.1)	2.2(0.1)
7.5	2.2(0.1)*	2.2(0.1)	2.2(0.1)
Total calcium (femoral vein) (mmol/l)			
0	2.5(0.1)	2.7(0.1)	2.6(0.1)
3	2.5(0.1)	2.6(0.1)	2.5(0.1)
6	2.3(0.1)	2.3(0.1)	2.2(0.1)
6	2.3(0.1)	2.2(0.1)	2.0(0.1)
6.5	2.1(0.1)	2.1(0.1)	2.0(0.1)
7.0	2.3(0.1)	2.1(0.1)	2.1(0.1)
7.5	2.2(0.1)	2.1(0.1)†	2.2(0.1)

Values are mean(s.e.m.) *$P < 0.01$ *versus* baseline, †$P < 0.002$ *versus* baseline

pH significantly compared with that of normal blood reperfusion (6.6(0.2) *versus* 5.9(0.1); $P < 0.002$) (*Figure 5*).

Muscle rigidity

Muscle damage, estimated by muscle rigidity, was least if controlled reperfusion was used (106(4) *versus* 122(1)°). In contrast, severe muscle stiffness occurred following normal blood reperfusion after the period of haemia (60(11) *versus* 122(1)°; $P < 0.00008$) (*Figure*

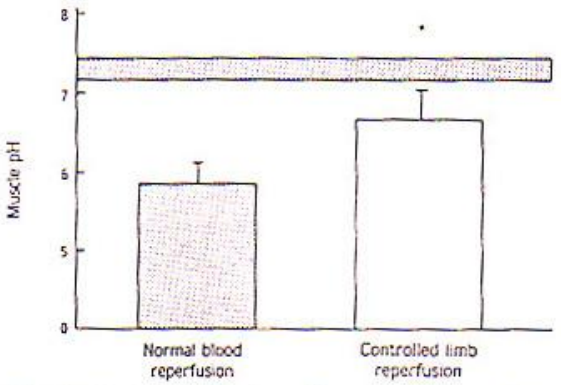

Figure 5 Muscle pH after 6 h of acute infrarenal aortic occlusion followed by normal blood reperfusion or controlled limb reperfusion. Control values are shown in the stippled area. *$P < 0.002$

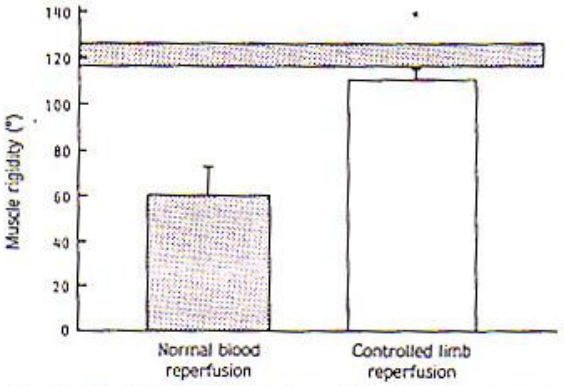

Figure 6 Muscle rigidity after 6 h of acute infrarenal aortic occlusion and normal blood reperfusion or controlled limb reperfusion. Control values are shown in the stippled area. *$P < 0.00008$

Discussion

Studies of ischaemia and reperfusion injury in skeletal muscle have been hampered by the difficulty of adequately reproducing acute aortic occlusion in an experimental model. The *in vivo* pig model was therefore chosen to simulate this clinical situation. The model enabled study of the local and systemic biochemical effects of prolonged ischaemia and reperfusion of skeletal muscle.

This study of severe, prolonged ischaemia of both lower extremities, produced by infrarenal aortic clamping, shows that a significant improvement in the metabolism, structure and function of limbs can be achieved after revascularization, provided that the ischaemic tissue is treated carefully during the initial reperfusion period. Delivery of a hypocalcaemic, hyperosmotic, substrate-enriched limb reperfusate at 40 mmHg for 30 min after 6-h ischaemia resulted in higher postischaemic flow rates, less oedema, higher oxygen and glucose consumption, less creatine kinase and potassium release, and improved high-energy phosphates than normal blood at systemic pressure.

The critical importance of the reperfusion strategy *per se* in determining the fate of ischaemic skeletal muscle cells is emphasized by the production of 'reperfusion injury' characterized by reduced oxygen (50% of control) and glucose consumption (37% of control) (*Figures 2* and *3*) marked oedema (81% water content), a persistent decrease in total adenine nucleotides (57% of control), explosive creatine kinase (2484% of control) and potassium release (179% of control), low-reflow rates (only 41% of the control flow rate) and a marked increase in vascular resistance (131% of control), as well as persistent tissue acidosis (muscle pH 5.9) and increased muscle stiffness in the normal blood reperfusion group. These data emphasize the importance of developing a treatment modality during

the initial reperfusion period that avoids further injury to cells already jeopardized by ischaemia.

It is apparent that the severe damage produced by normal blood reperfusion after 6-h infrarenal aortic occlusion (combination of ischaemic and reperfusion injury) can be reduced when the conditions of reperfusion and the composition of the reperfusate are controlled for 30 min. The initial 30-min controlled limb reperfusion phase restored oxygen and glucose consumption to control levels (*Tables 5* and *6*), avoided tissue oedema (*Table 2*), restored tissue total adenine nucleotides to 78% of control, and significantly limited creatine kinase and potassium release. Furthermore, controlled reperfusion in the limb increased flow above control values (117% of control) and decreased vascular resistance (75% of control) (*Table 7*).

The importance of specific attention to glucose during reperfusion is emphasized by studies[25] showing that hypoxia stimulates glucose uptake in skeletal muscle. Glucose-enriched perfusion solutions increase carbohydrate metabolism, which in turn improves the ischaemic tolerance of skeletal muscle[26]. Further insight into the need to support anaerobic and aerobic metabolism simultaneously can be found in reports[27] suggesting that there is a fundamental compartmentalization of glycolytic and oxidative metabolism even during oxygenation. Recent data suggest that glycolysis supports sarcolemmic function (i.e. sodium–potassium pump activity)[28], curtails postischaemic enzyme release[29], and limits the sensitivity of injured cells to phospholipase activation[30], which may lead to massive calcium overload. Oxidative metabolism and further repair can occur only if sarcolemmic function is preserved and sufficient metabolizable substrate (e.g. glucose) is provided[31]. Normal blood reperfusion resulted in a rapid decline in the arterial glucose concentration and a decrease in glucose consumption (*Table 6*). In sharp contrast the controlled limb reperfusate with marked hyperglycaemia (> 400 mg/dl) during the first 30 min resulted in a 700–1100% increase in glucose consumption compared with that of the controls. This improved anaerobic metabolism cannot be explained by hyperglycaemia alone, because an increase in glucose content above control levels during the subsequent normal blood reperfusion (> 150 mg/dl) resulted in a normal, but not increased, glucose consumption (*Table 6*). Therefore, either marked hyperglycaemia alone (> 400 mg/dl) or the combination of marked hyperglycaemia and other factors of controlled limb reperfusion are responsible for the improved glucose metabolism.

The authors' data show that normal blood reperfusion resulted in severe postischaemic oedema (81%) and increased tissue turgor and muscle rigidity (restriction of joint motion to 50% of control, *Figure 6*). Previous studies[9,32,33] have suggested that these alterations may be caused by calcium influx. Conceivably, some of the muscle contracture that follows normocalcaemic reperfusion is related to calcium loading of previously ischaemic tissue that has insufficient energy production to maintain normal calcium homoeostasis. This pathological mechanism is supported by the present results showing that ionized calcium in the systemic circulation as well as total calcium both show a marked decrease after normal blood reperfusion (*Tables 8* and *9*). This severe reduction in systemic calcium which occurs initially during ischaemia and continues to decrease during reperfusion might be a cause of myocardial depression in patients with severe limb ischaemia.

Postischaemic oedema and increased tissue turgor may be responsible for the 'low-reflow phenomenon' after normal blood reperfusion and thus restrict subsequent oxygen delivery to ischaemic and reperfusion-damaged skeletal muscle. The authors suspect that the marked oedema formation was caused by persistent impairment of the capacity of the skeletal muscle cell to regulate cell volume[34] and was compounded by the sudden reperfusion produced by removal of the vascular clamp at systemic pressure. This is further supported by a recent study by Forrest *et al.*[35] showing that no improvement was seen with vasodilators, suggesting that local vasoconstriction is not the primary mechanism for the low-reflow phenomenon. The role of leucocytes in preventing the low-reflow phenomenon in skeletal muscle is still controversial[14,19,20,36].

The authors' observations on the metabolic response of skeletal muscle to severe prolonged ischaemia followed by reperfusion showed that there is an immediate (within 5 min of reperfusion) decrease in oxygen consumption (*Figure 2*), which remains depressed throughout the observed reperfusion period of 90 min. This decrease in oxygen consumption has been described in previous studies using cardiac muscle[37] and explained by the fact that reperfused muscle has a limited capacity to take up oxygen because of substrate depletion and mitochondrial damage[38,39]. The present authors' controlled limb reperfusion strategy: (1) avoided the development of tissue oedema; (2) reduced vascular resistance; and (3) may have restored some of the cellular regulatory mechanisms. This might explain the retained capacity for oxygen consumption.

Oxygen consumption of skeletal muscle appears to be critically dependent on extracellular fluid pH[40]. Decreased hydrogen ion concentration (i.e. alkalosis) of blood perfusing an isolated canine hind limb was consistently associated with an increase in oxygen uptake by the limb with a change in pH of 0.1 unit leading to a 10% alteration in oxygen consumption[4]. This relationship appears linear throughout the entire physiological pH range (7.0–7.6)[40]. As the present authors' therapeutic approach to ischaemic skeletal muscle using controlled limb reperfusion is based on cellular repair by aerobic and anaerobic metabolism high oxygen consumption is necessary to generate energy. The study by Harken[40] underscores the importance of having an alkalotic initial reperfusate.

Control of reperfusate calcium is critical becau

ytosolic calcium activity gradually increases during chaemia when cellular metabolism is inhibited. This in urn may activate cytosolic or membrane calcium ermeability[41] and may set the stage for massive alcium influx during reperfusion. Furthermore, there is vidence that cellular calcium overload and free radical eneration each contribute to ischaemia–reperfusion njury and that free radical generation and subsequent eperfusion injury is a function of calcium concentration[42].

The authors' proposed approach of controlled limb reperfusion to reduce reperfusion injury in skeletal muscle incorporated *all* the principles of modification of the conditions of reperfusion and the composition of the reperfusate evolved from previous studies[7,9–13,31,37]. T modified *conditions of reperfusion* included: (1) gentle reperfusion pressure (i.e. 40 mmHg) to limit p ischaemic oedema; (2) normothermia (i.e. 37°C) to c mize the rate of cellular repair; and (3) prolonged duration of controlled limb reperfusion (i.e. 30 min) to allow recovery of cellular regulatory mechanisms before regular blood flow is re-established. The *reperfusate composition* was modified to allow incorporation of: (1) hyperosmolarity to minimize postischaemic oedema and allow cell volume regulation to occur more gradually when flow with normal blood is restored; (2) oxygen free radical scavengers (i.e. lipoic acid) to limit the cytotoxic effects of oxygen free radicals; (3) adding citrate–phosphate–dextrose to normal blood, to reduce calcium influx by lowering the calcium concentration of the reperfusate; (4) increased glucose concentration to enhance osmotic effects and perhaps initiate anaerobic energy production at the start of reperfusion; (5) replenishment of amino acid precursors of Krebs' cycle intermediates (i.e. glutamate, aspartate) needed to ensure more effective oxidative metabolism and energy duction for cell repair; and (6) reversal of tissue acidosis with a buffer (i.e. tromethamol, to provide an mal intracellular milieu for effective resumption of metabolic function.

Control of the conditions of reperfusion and composition of the reperfusate was achieved in this study by cannulating the aorta proximal to the clamp with a 26-Fr cannula and the aorta distal to the clamp with a 14-Fr cannula. The controlled limb reperfusate was delivered directly into the distal aorta. The conditions of reperfusion were controlled by a roller pump (flow and pressure) and a heat exchanger (temperature) and the composition of the reperfusate was achieved by mixing the oxygenated blood with a crystalloid solution (six parts blood and one crystalloid solution).

The authors conclude that the fate of temporarily ischaemic skeletal muscle is determined largely by how the tissue is treated during the initial reperfusion phase, before normal blood flow is re-established. The present data show that muscle salvage is possible after 6-h infrarenal aortic occlusion if controlled limb reperfusion is used as the initial treatment for the injured tissue, and controlled limb reperfusion can be performed easily in the operating room. Future studies will determine how further modifications of the initial reperfusion may increase skeletal muscle salvage.

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