

New Hope for an Old Cure: A Pilot Animal Study on Selective Venesection in Attenuating the Systemic Effects of Ischaemic-Reperfusion Injury

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Abstract

Introduction: Reperfusion of acutely ischaemic tissue may, paradoxically, lead to systemic complications. This phenomenon is believed to be initiated by humoral factors that have accumulated in the ischaemic tissue. The ancient art of venesection may reduce the load of these mediators at the point of reperfusion. The aim of this study is to test if selective venesection, by removing the initial venous return from the ischaemic tissue, can attenuate the systemic effects of the ischaemic-reperfusion injury using a porcine model of acute limb ischaemia. **Materials and Methods:** The right femoral arteries of anaesthetised female pigs were clamped. Twelve pigs were divided into 2 groups (n = 6 per group). In the treatment group, 5% of blood volume was venesected from the ipsilateral femoral vein upon reperfusion; the other arm served as control. The animals were sacrificed after 4 days for histological examination. A pathologist, blinded to the experimental groups, graded the degree of microscopic injury. **Results:** For the control group, the kidneys showed glomeruli and tubular damage. The livers demonstrated architectural distortion with cellular oedema. There was pulmonary oedema as well as extensive capillary congestion and neutrophil infiltration. Such findings were absent or reduced in the venesected animals. Consequently, the injury scores for the kidney, lung, liver and heart were significantly less for the venesected animals. **Conclusion:** Selective venesection reduces the remote organ injuries of the ischaemic-reperfusion phenomenon.

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Key words: Acute ischaemia, Humoral factors, MODS, Pulmonary oedema

Introduction

Ischaemia-Reperfusion (I/R) injury describes a paradoxical phenomenon where reperfusion of tissues following a prolonged period of acute onset ischaemia causes further injury both to the tissues involved as well as distant organs uninvolved in the initial ischaemic event. The remote effects of I/R injury are most frequently observed in the lungs.^{1,2} Such distant effects are associated with the development of the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS).^{2,3} Respiratory failure is followed by hepatic, renal and gastrointestinal dysfunction, as well as central nervous system involvement and myocardial failure.⁴ Consequently, revascularisation of an acutely ischaemic limb is associated with mortality rates of 10% to 20% and morbidity rates of 20% to 30%.⁵⁻⁸

The remote effects of I/R injury are believed to be mediated by humoral factors.⁹ These factors include pro-inflammatory cytokines,^{10,11} activated polymorphonuclear (PMN) leukocytes^{12,13} and oxidants.¹⁴ Up-regulation of adhesion molecules leads to increased leukocyte sequestration in multiple vascular beds.¹⁵⁻¹⁷ Such activated leukocytes, along with the xanthine/xanthine oxidase system and mitochondrial electron transport chain leakage, are documented sources of reactive oxygen species.^{18,19} However, the use of anti-cytokine therapy, adhesion molecule-directed antibodies and antioxidants, while showing some promise in the laboratory, has been disappointing in clinical practice.^{14,20,21}

While the source of the various mediators implicated for these remote effects remains hypothetical, what is certain is that the remote effects only manifest after reperfusion.²² This suggests that the factors responsible had either originated

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from the ischaemic tissue² or were activated by mediators or metabolites that have accumulated in the ischaemic tissue. Steinau²³ had probably recognised this link and hence recommended that limbs subjected to prolonged ischaemia be amputated so as to save the body. In addition, SIRS has been shown to manifest within an hour of reperfusion,² which suggests that the initial venous return from the ischaemic tissue carries a significant, if not the highest, load of such humoral factors.

Venesection has been practiced in almost all societies for at least two and a half millennia.²⁴ It was used to remove excess stimulation or irritation from the body,²⁵ as the Ancients subscribed to the Hippocratic theory of humours.²⁶ In modern day medicine, therapeutic venesection has evolved and is now an established treatment for ailments such as haemochromatosis and polycythaemia rubra vera. To our knowledge, there has been no study reported in the literature testing this technique in the prevention of remote effects of I/R injury.

We believe that the systemic manifestations of I/R injury are initiated by the various metabolites and mediators that have accumulated in the ischaemic organ. Such factors can either cause the damage themselves, or play a secondary role in activating other mediators capable of initiating tissue damage. Upon reperfusion, these are then flushed into the systemic circulation. We also believe that the immediate venous return upon reperfusion will carry the largest volume load of such metabolites and mediators.

We therefore hypothesise that if part of the initial venous return is removed from the ischaemic tissue upon reperfusion, we can ameliorate, if not prevent, the remote manifestations of the I/R injury. In this study, we test this hypothesis in a porcine model of acute hind limb I/R injury against histopathological endpoints of pulmonary, hepatic, renal and myocardial injury.

Materials and Methods

Animals and Anaesthesia

In the handling and care of all animals, the principles for animal research as stipulated by WHO Chronicle (1985) and as adopted by the Laboratory Animal Centre, National University of Singapore, were followed. In total, 14 female Landrace-Large White Cross pigs with a mean weight of 30.6 kg (range, 27.4 to 37.3) were used.

The pigs were sedated using a mix of Ketapex at 10mg/kg (ketamine; APEX Laboratories Pte Ltd, Australia) and Atrosite at 0.05mg/kg (atropine sulphate; Troy Laboratories Pte Ltd, Australia). These were injected intramuscularly. Anaesthesia was then induced with intravenous Thiopental at 10mg/kg (thiopentone 0.5g injection; Biochemie GmbH, Vienna, Austria) through an intravenous cannula (24G x

3/4"; Terumo, Japan) placed into the left ear vein. The animal was then intubated with an endotracheal tube (Mallinckrodt Medical, Ireland). Anaesthesia was maintained with inhalational isoflurane (Isoflo; Abbott Laboratories, Chicago, USA) at 2% to 3% with oxygen. Analgesia was achieved with intravenous buprenorphine at 0.1mg/kg (Temgesic; Schering-Plough, England). Ventilatory settings were set to a tidal volume of 15 mL/kg at a rate of 12 to 15/min. A continuous infusion of 0.9% sodium chloride (F8B1323, Baxter Healthcare Ltd., Philippines) at 3 mL/kg/hr was administered from the point of induction for the entire length of the experiment. Heart rate and oxygen saturation were recorded at 15 minute intervals using a pulse oximeter (model 8500AV; Nonin Medical Inc, Minneapolis, USA). Reversal was achieved by withdrawing the anaesthetic gas and allowing the animal to recover spontaneously.

Experimental Protocol

Experiments were performed in 2 groups: following surgically-induced ischaemia of the right hind limb, animals in the control group (n = 6) were not venesected, whilst those in the treatment group (n = 6) were subjected to selective venesection of the ischaemic limb upon reperfusion. Two pigs were allocated as sham controls. Within each group, pigs were further divided into 3 subgroups (n = 2) with an ischaemic clamp-time of 4 hours, 5 hours and 6 hours, respectively (Fig. 1). This protocol was designed to determine whether the duration of ischaemia affects the magnitude of remote organ injury, and whether the proposed treatment might be beneficial. Following reversal from anaesthesia, the animals were cared for in individual cages. On the fourth postoperative day, the animals were sedated with ketamine and atropine, following which euthanasia was performed with an overdose of intravenous pentobarbitone (Euthanasia Fort solution; APEX Laboratories Pte Ltd, Australia). Tissue

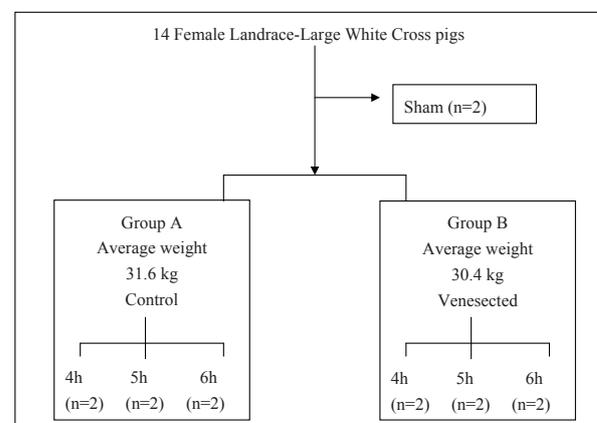


Fig. 1. Schematic illustration of the experimental protocol.

samples were then harvested from the heart, the lung, the kidney and the liver, to assess for histological evidence of injury in these organs.

Surgical Procedure

Following induction of anaesthesia, a right groin incision was made. The right common femoral artery was isolated just below the inguinal ligament and a DeBakey arterial clamp (Codman, Johnson & Johnson Medical; Singapore) was applied to induce acute limb ischaemia. This is to simulate the clinical situation of acute limb ischaemia secondary to an embolus. We attempt to verify complete limb ischaemia using the BLF21 Laser Doppler Flowmeter (Transonic System Inc.; New York, USA) with comparison to the contralateral limb. The timer was started and the wound loosely packed with gauze moistened with normal saline. The clamp was applied for 4, 5 or 6 hours depending on the ischaemic clamp-time group allocation.

At the end of the pre-determined ischaemic clamp-time period, arterial clamps were released for animals in the control groups, their wounds were closed and anaesthesia was reversed.

For animals in the treatment groups, at the end of the ischaemic period and prior to arterial clamp release, the adjacent femoral vein was isolated and occluded using vessel loops wound around the vein in a figure-of-8 fashion. The vein was then cannulated distal to the point of vein occlusion (i.e. closer to the foot) using a 21 G Venofix butterfly cannula (B Braun, Malaysia) so as to allow the aspiration of venous blood from the ischaemic limb. The arterial clamp was then released to allow arterial inflow before commencement of venous aspiration. In this manner, following the re-perfusion of the limb, we prevented the venous return from the limb from entering the systemic circulation. A pre-determined calculated amount of the venous return from the right hind limb, based on the body weight of the respective animals, was aspirated via the butterfly needle into a syringe, and hence prevented from returning into the systemic circulation. Five per cent of total blood volume (calculated at 7% of the animal's body weight) was aspirated per animal. To avoid hypovolemia as a result of the venesection, normal saline was infused at a ratio of 3:1 to the volume of blood drawn. Following venesection, the vessel loop was removed and the cannula withdrawn. Haemostasis of the puncture point was achieved with light digital pressure.

The groin wounds were closed with interrupted Prolene 3/0 (W538, Johnson & Johnson Medical; Singapore) and covered with an occlusive dressing.

Histopathological Assessment

To determine the presence and extent of remote

microscopic injury, the animal's liver, kidney, lung and heart were harvested and fixed in 10% neutral buffered formalin. They were then dehydrated in an ascending series of alcohol, cleared with toluene and embedded in paraffin wax. Tissue blocks were taken from areas which were grossly abnormal, with a cuff of adjacent normal looking tissue for comparison. Sections of 5µm thickness were cut in standard fashion, stained with haematoxylin and eosin, and examined for microscopic injuries under 200x magnification. The number of blocks harvested as well as the number of sections made was at the discretion of the pathologist.

All slides were read by a single pathologist who was blinded to the allocation arm of the animal as well as the ischaemic clamp-time period to which the animal was assigned. She was asked to grade the microscopic injuries seen in the lung, the liver, the kidney and the heart using a semi-quantitative scoring system, with zero indicating no discernable injury and 4 indicating the presence of severe injury. These scores were allocated based on her assessment of the following histological features present in each organ: cellular oedema, interstitial oedema, neutrophil infiltration, capillary congestion and structural distortion. These are all well-documented morphological alterations seen in remote organ injuries of the I/R phenomenon.^{2,14,21}

Statistical Analysis

Statistical analysis was carried out using SPSS software Version 11.2. Descriptive statistics such as median scores and range were presented. The histological scores for the 3 time groups within each arm were compared first. If there was no significant difference between them, then the injury scores for the 3 time groups in the control arm will be pooled together for comparison with the pooled scores of the venesected arm. Comparison of median histological scores between pooled venesected and control animals was performed using the Mann-Whitney U test. All reported '*P*' are 2-sided, and *P* values of <0.05 were considered statistically significant.

Results

For pigs that were assigned to the treatment arm, a mean of 106mL of blood was venesected (range, 96 to 130mL). All pigs survived until planned euthanasia.

When we examined the relationship between the injury scores and the duration of ischaemia within each experimental arm, we were not able to detect any significant differences between the 3 ischaemic clamp-time periods of 4 hours, 5 hours and 6 hours in all 4 organ systems for either the venesected groups or the control groups (Fig. 2). However, when we looked at the pooled injury scores of the 3 time groups and compared it with the pooled scores

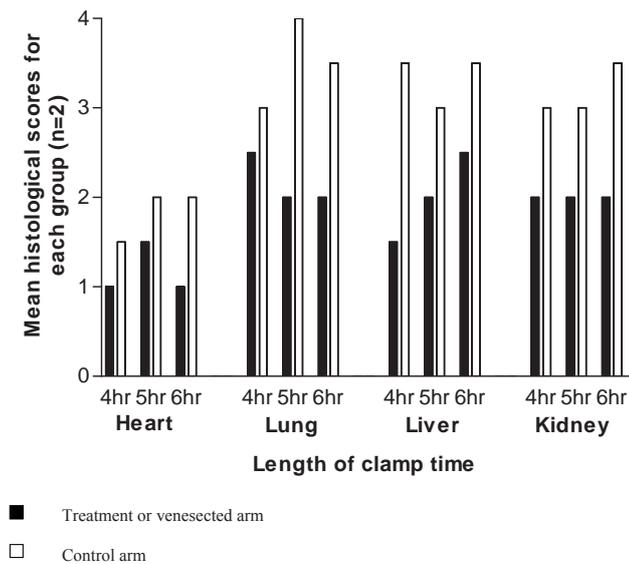


Fig. 2. Figure shows the mean histological injury score of the heart, the lung, the liver and the kidney respectively of 2 animals in each group of venesectioned (black bars) and control (white bars) with 4, 5 and 6 hours of ischaemic clamp-time.

Table 1: The Effect of Selective Venesection on Microscopic Remote Organ Injury

Organ	Median score (range)		Mann-Whitney U test <i>P</i> value for difference
	Venesectioned (n = 6)	Control (n = 6)	
Heart	1 (1-2)	2 (1-2)	0.027
Lung	2 (2-3)	4 (3-4)	0.006
Liver	2 (1-2)	3 (3-4)	0.007
Kidney	2 (2)	3 (3-4)	0.002

from the other arm, we were able to detect statistically significant differences between the 2 investigation arms with the treatment arm or venesectioned arm having lower injury scores (Table 1). This difference is the largest between the median scores for the kidney ($P = 0.002$), followed by the lung ($P = 0.006$) and the liver ($P = 0.007$). The difference is less marked in the heart ($P = 0.027$).

Figure 3 shows the slides of the kidney for the 5h venesectioned animal (A) and the 5h control animal (B), respectively. The morphology seen in the venesectioned animal closely resembles that of the sham control animal. However, for the control animal, gross architectural distortion with obvious glomeruli and tubular damage can be seen. There is also a large amount of cellular debris signifying extensive necrosis. Figure 4 shows the slides of the lung for the 5h venesectioned animal (A) and the 5h control animal (B). In the venesectioned animal, pulmonary oedema is seen, probably due to the residual load of mediators in the venous

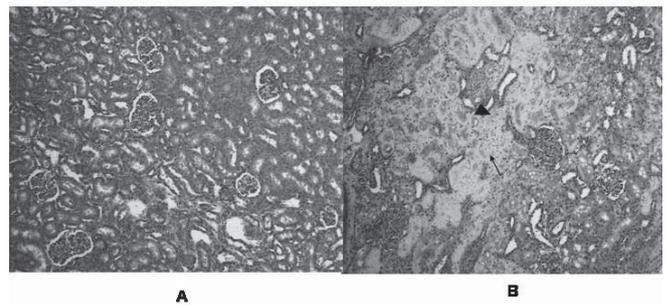


Fig. 3. Kidney: (A) 5h ischaemic clamp-time; venesectioned. (B) 5h ischaemic clamp-time; control. Arrow shows cellular debris with interstitial oedema. Arrowhead shows glomerular damage. (Haematoxylin and eosin, magnification 200X)

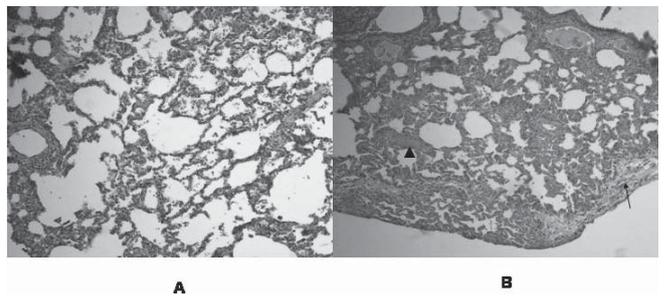


Fig. 4. Lung: (A) 5h ischaemic clamp-time; venesectioned. (B) 5h ischaemic clamp-time; control. Arrow shows pulmonary oedema. Arrowhead shows pulmonary congestion. (Haematoxylin and eosin, magnification 200X)

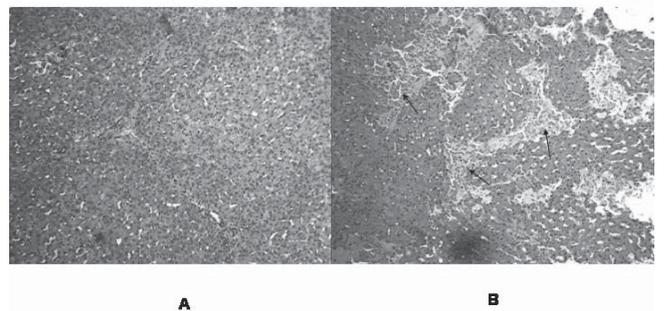


Fig. 5. Liver: (A) 5h ischaemic clamp-time; venesectioned. (B) 5h ischaemic clamp-time; control. Arrows show blood within the bile canaliculi. Haematoxylin and eosin, magnification 200X)

return which is sufficient to cause an observable end organ effect. This substantiates the observation that the lung is the most vulnerable organ in I/R injury.¹⁴ For the control animal, there is evidence of a greater magnitude of injury. Besides pulmonary oedema, there is also extensive capillary congestion and neutrophil infiltration. Figure 5 shows the slides of the liver for the venesectioned animal (A) and the control animal (B), respectively. In the control animal, there is obvious distortion of the usual liver architecture with hepatocyte oedema and widening of the sinusoids. There is also leakage of blood into the bile canaliculi. Such changes are attenuated in the venesectioned animal. Thromboembolism is evident in the slides of the myocardium of animals in

the control groups but is absent in those of the venesectioned groups.

Discussion

The findings from our experiment have strongly suggested 2 aspects of the systemic manifestations of I/R injury. Firstly, it is indeed factors that have accumulated in the ischaemic limb that initiate the cascade of events leading to injury in the distant organs. Thus by removing some of these factors via venesection, remote organ injuries were ameliorated in the treated animals. Secondly, the initial venous return upon reperfusion carries the largest volume of such factors, since we were able to reduce the remote changes through a one-off procedure performed only at the point of reperfusion. This conclusion could also be inferred from another experiment in which administration of superoxide dismutase, a free radical scavenger, after the onset of reperfusion, had proved ineffective. This led the investigators to conclude that the generation of free radicals is likely to be a transient early event.²⁷ In a study which had looked at the profile of pro-inflammatory cytokines following visceral ischaemia, the authors found an early appearance of tumour necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, IL-10, p55 and p75 in the blood 1 to 4 hours after reperfusion.²⁸ Subsequently, concentrations of TNF- α , IL-6, IL-8, IL-10 decreased towards baseline within 24 hours. Furthermore, the clinical effects of remote organ injury usually manifest early.²⁹ Consequently we decided to sacrifice the animals on the fourth postoperative day, by which time, we were fairly confident that, in the event of any remote organ injuries, they would be evident histologically. We did not dissect out the ischaemic limb to look for histological evidence of local reperfusion injury as the specific aim of this study was to look at the remote effects only.

Our findings support the humoral theory of I/R injury,³⁰ the basis being that the effectors of distant organ injury are blood-borne. However, while many of these mediators have been identified, it is still debatable whether they play a primary or a secondary role.³¹ It is clear, though, that the formation of these primary mediators must be closely related to prolonged ischaemia. During periods of prolonged hypoxia, cellular energy stores become depleted, resulting in the failure of cellular homeostasis as characterised by the loss of ionic gradients across the cell membranes.¹⁴ Toxic metabolites accumulate, consequent to anaerobic metabolism. If ischaemic tissues are to be salvaged, there is little doubt that blood flow and tissue oxygenation must be restored. Re-establishing blood flow to ischaemic tissue restores the energy supply and removes the toxic metabolites from the limb. However this comes at the price of introducing such mediators into the systemic circulation. Reperfusion with oxygen-rich blood carries an additional

penalty from the formation of highly reactive oxygen metabolites. Such reactive species can subsequently lead to the generation of lipid mediators of inflammation and the up-regulation of endothelial cell adhesion molecules.³² The net effect of these oxidant-dependent changes is an acute and often intense systemic inflammatory response, which can result in leukocyte-mediated tissue injury. Finally, the re-vascularisation of severely ischaemic skeletal muscles is accompanied by the subsequent release of potassium, hydrogen ions and myoglobin. These may result in impaired myocardial, pulmonary and renal function.³³ Not surprisingly, MODS is a documented consequence of skeletal muscle reperfusion³ and is the leading cause of death in critically ill patients.^{1,34}

Oxidants, activated leukocytes and pro-inflammatory mediators or cytokines have been implicated as the main drivers of remote organ injuries.¹⁴ Plasma xanthine oxidase activity has been shown to increase following aortic occlusion-reperfusion,³⁵ and given its capacity to generate superoxide (O₂⁻) and H₂O₂, it is believed to have a significant contribution towards reperfusion-induced distant organ injury. However, several clinical trials³⁶⁻³⁸ to investigate the efficacy of free radical antagonists have not found any difference in outcome. Activated neutrophils can produce free radicals too.¹⁸ In addition, they can also produce potent proteases capable of disrupting endothelial barrier integrity.¹³ Anti-neutrophil clinical trials have yet to reap dividends.³⁹⁻⁴¹ Pro-inflammatory mediators are able to activate and/or attract circulating neutrophils,¹⁴ amongst other actions. In particular, TNF α and IL-1 are generally agreed to have a central role in initiating and sustaining the proinflammatory cytokine cascade.⁴² Several clinical trials have examined anti-TNF and anti-IL-1 therapies, but the overall results of these anti-cytokine trials have been discouraging.²¹

Recognising that the factors responsible are humoral in nature, haemofiltration has been used.⁴³ While it remains investigative, it is disadvantaged by being cumbersome to apply and costly as well. In addition, there are also known complications of dialysis such as electrolyte imbalance and haemolysis. Selective venesection can achieve the same objective of removing these humoral factors before they enter the systemic circulation, and in an easier and faster manner. The main concern is whether venesection can cause hypovolemia, which in the extreme situation can lead to shock. The total body ischaemia that ensues could confound the outcome. Physiological studies have shown that blood loss is generally well tolerated up to an absolute or relative reduction of 15% to 25% of circulating blood volume.⁴⁴ Beyond this, physiological derangements due to generalised cellular hypoxia with end-organ injury, such as acute renal failure, can occur. Advanced Trauma

Life Support (ATLS) principles state that haemorrhage of up to 15% of blood volume has minimal physiological disturbance in a healthy individual, and thus no replacement is required.⁴⁵ Without the benefit of historical experimental evidence, we decided to be conservative and venesect just 5% of the total blood volume. It is possible that venesection of larger volumes of blood could be tolerated, leading to a greater improvement in injury scores of organs damaged by the I/R phenomenon.

We were not able to demonstrate a correlation between ischaemic clamp-time period and injury scores, as had been demonstrated by other studies.²⁸ A plausible explanation is that the formation of pro-inflammatory mediators is an early event during tissue ischaemia, and the total amount of such mediators accumulating in the ischaemic limb may have reached a plateau by the time the arterial clamp is released in the animals with the shortest clamp time, such that no difference is seen with an even longer duration of ischaemia. It is also possible that, beyond a threshold concentration of these mediators required to produce end organ damage, any further increase in the concentration will not be reflected by a greater degree of the injury caused.

One shortfall of our study is the small sample size. However, as it was only a pilot study, from an ethical point of view, we felt that we should not sacrifice too many animals before we have some preliminary data. The study was also based on the assumption that the common femoral artery of the pig is the main, if not the major, arterial inflow of the lower limb. And by applying the arterial clamp, we hoped to simulate the clinical scenario of acute limb ischaemia secondary to an embolus. The ideal test of absolute ischaemia is to demonstrate cessation of muscle perfusion. However, we have no access to a more invasive form of monitoring other than to measure skin perfusion as a surrogate indicator of muscle perfusion although we were aware of the limitations of the Laser Doppler Flowmeter.

In conclusion, the data from this study provide evidence that the mediators that lead to remote organ injury in an I/R limb model are humoral in nature, and that the primary mediators that initiated the inflammatory cascade are formed and have accumulated within the ischaemic tissue. Upon reperfusion, the immediate venous return would carry the highest volume load of such mediators. Selective venesection of 5% of total blood volume, by removing the immediate venous return and the mediators that it carried, could reduce or even avert remote organ injury. Further studies will certainly include biochemical assays of the venous effluent in an attempt to uncover the identities of these mediators, as well as to profile the systemic levels of pro-inflammatory cytokines and markers of oxidative injury.

Whilst the development of the “magic bullet” for reperfusion-related injuries could take years to realise, this surgical technique may be an attractive alternative in the meantime. It can potentially be applied to any organ system that has been subjected to prolonged ischaemia and in which the venous outflow can be easily isolated. The beauty of this procedure lies in its simplicity and the fact that it can be performed quickly without the need for very costly equipment and additional procedures. It would be interesting to test this technique on an aortic-occlusive model. The limitations of this pilot study notwithstanding, the results were indicative. This merely means that more work needs to be done and we can proceed to apply for a study with a larger sample size to validate this pilot study. Nevertheless, this study does suggest that a novel use of an ‘old cure’ can perhaps bring new hope in reducing or even averting the devastating remote organ injuries of the I/R phenomenon.

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