The Effect of Lower Limb Ischaemia-reperfusion on Intestinal Permeability and the Systemic Inflammatory Response

W. K. Edrees†1, L. L. Lau1, I. S. Young3, M. G. Smye3, K. R. Gardiner2, B. Lee1, R. J. Hannon1 and C. V. Soong1

1Vascular Surgery Unit, Belfast City Hospital, Lisburn Road, Belfast BT9 7AB, Northern Ireland, Departments of 2Surgery and 3Medicine, The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BJ, Northern Ireland

Objectives: a relationship has been demonstrated between increased intestinal permeability, endotoxaemia and the development of the systemic inflammatory response syndrome (SIRS) after aortic surgery. The aim of this study was to evaluate whether isolated lower limb ischaemia-reperfusion (I/R) injury affects intestinal mucosal barrier function and cytokine release.

Patients and Methods: four groups of patients were investigated, group I, patients with critical limb ischaemia (CLI) undergoing infra-inguinal bypass surgery (n = 18); group II, patients with intermittent claudication (IC) undergoing infra-inguinal bypass surgery (n = 14); group III, patients with CLI unsuitable for arterial reconstruction, undergoing major amputation (n = 12); and group IV, patients undergoing carotid endarterectomy for symptomatic carotid stenosis (n = 13). Intestinal permeability, endotoxaemia and urinary soluble tumour necrosis factor receptors were assessed (p55TNF-R).

Results: an increase in intestinal permeability was observed on the 3rd postoperative day only in CLI group. This was found to correlate with arterial clamp time. Patients who had a femoro-distal bypass had significantly higher intestinal permeability compared to those who had femoro-popliteal bypass. Endotoxaemia was not detected in any of the groups. Postoperative urinary p55TNF-R concentrations were significantly higher in CLI group compared to the other groups. These did not correlate with the increased intestinal permeability.

Conclusions: our results support the hypothesis that revascularisation of critically ischaemia limbs leads to intestinal mucosal barrier dysfunction and cytokine release. They also suggest that the magnitude of the inflammatory response following I/R injury is related to the degree of initial ischaemia.

Key Words: Ischaemia; Reperfusion injury; Permeability; Sepsis syndrome.

Introduction

The involvement of the gastrointestinal tract in ischaemia-reperfusion (I/R) injury is of interest not only because it functions as a target organ but also as a potential effector of the multiple organ dysfunction associated with reperfusion injury.1,2 When impaired, the gut mucosa allows translocation of bacteria and their toxins into the extraintestinal tissues and produces free radicals and cytokines that potentiate the development of multiple organ dysfunction.3–6 Increased intestinal permeability with portal endotoxaemia and cytokine release has been observed after repair of elective and ruptured abdominal aortic aneurysms (AAA).7–9 I/R injury has been suggested as an aetiological factor, with increased intestinal permeability occurring because of hypovolaemia and aortic clamping. However other workers have found mucosal barrier dysfunction only after transperitoneal repair of AAA and not with the extraperitoneal approach, suggesting that intestinal manipulation and mesenteric traction are more important factors.10 Increased intestinal permeability has also been observed in patients with intermittent claudication after exercise, which may be reversed after arterial revascularisation. Repeated attacks of low-grade I/R injury during exercise in these patients have been suggested as the cause of the loss of intestinal mucosal integrity.11 In experimental models, isolated lower extremity I/R injury has been found to be associated with structural changes in the mucosa associated with increased intestinal permeability, release of IL-6, endotoxaemia, and higher mortality.12,13 The role of the mucosal barrier in the development of a systemic inflammatory response after isolated
Ischaemia-reperfusion and Intestinal Permeability

lower limb ischaemia-reperfusion injury has not so far been examined in a clinical setting. The objective of this study was, therefore, to evaluate the effect of isolated lower limb I/R injury on intestinal mucosal barrier function and the development of endotoxemia and cytokine release in patients with varying degrees of limb ischaemia.

Materials and Methods

Four groups of patients admitted for elective surgery in the Vascular Surgery Unit, Belfast City Hospital were studied prospectively after informed written consent:

- Group I: patients undergoing infra-inguinal bypass surgery for critical limb ischaemia (CLI).
- Group II: patients undergoing infra-inguinal bypass surgery for disabling intermittent claudication (IC).
- Group III: patients with critical limb ischaemia, unsuitable for reconstructive surgery, undergoing above or below knee amputation.
- Group IV: patients undergoing carotid endarterectomy (CEA) for symptomatic carotid stenosis (control).

For the purpose of this study CLI was defined as chronic ischaemia with persistent rest pain requiring analgesia for more than 2 weeks, ulceration, gangrene of the foot, or an ankle systolic pressure below 50 mmHg. Disabling claudication was defined as short distance IC interfering with the patient’s lifestyle. Patients in group III were considered unsuitable for reconstructive surgery by a consultant vascular surgeon on the basis of angiographic findings or clinically after failure of previous bypass surgery. The study was approved by the Research Ethics Committee of The Queen’s University of Belfast.

Patients were excluded from the study if they had a history of vasculitis, arterial lesions involving the abdominal aorta or the iliac arteries, large ischaemic ulcers >2 cm, infected ulcers, uncontrolled diabetes mellitus (blood glucose >12 mmol/L on admission), chronic renal failure (serum creatinine >200 μmol/L on admission), inflammatory bowel disease, other chronic inflammatory disorders or Methicillin Resistant Staphylococcus Aureus (MRSA) infection. Patients undergoing CEA for carotid stenosis were also excluded if they had IC or CLI.

Intestinal permeability

Intestinal permeability was assessed preoperatively (PO), at day 1 (D1), day 3 (D3) and day 7 (D7) by measuring the urinary excretion of orally administered lactulose and mannitol. Patients were fasted for 6h before the test and a pre-test urine sample was obtained. A test solution consisting of 10 g of lactulose and 5 g of mannitol mixed in 100 ml of water was administered orally. All urine was then collected for 6h and refrigerated. At the completion of the collection, the urine was divided into aliquots and frozen at −20 °C until assayed.

The concentrations of lactulose and mannitol were measured enzymatically by reduced nicotinamide adenine dinucleotide (phosphate)-linked enzymatic assay, using a Cobras Faro centrifugal analyzer (Roche Diagnostics, Welwyn Garden City, U.K.).14,15 Interference from glucose in the lactulose assay was overcome by incubating the sample with glucose oxidase and catalase for 3 h at 37 °C before lactulose analysis.16 Inter and intra-assay coefficients of variation were <5%.

Blood samples

Five ml of systemic blood was collected into endotoxin-free (Falcon 111548, Labtech International Ltd, Sussex, U.K.) heparinised (20 iu/ml of blood) tubes from a peripheral vein in the antecubital fossa. In the CLI & IC groups, samples were collected immediately before arterial clamping (PC), at maximum ischaemia (IS), immediately and 1 h after reperfusion and then daily at 8 am for 7 days. For the amputation group, samples were collected preoperatively (PO), 1 h after amputation and then daily as above. For the CEA group, samples were collected PO, 1 h postoperatively and then daily for 3 days. These samples were centrifuged immediately at 500 g for 10 min at 4 °C. The plasma was then aliquoted into sterile endotoxin free tubes (Nunc 363401, Intermed, Rosklide, Denmark) and stored at −70 °C until assayed. Strict aseptic techniques were maintained throughout the handling of the samples.

Endotoxin measurements were made using the chromogenic Limulus Amoebocyte Lysate (LAL) technique (Coatest Endotoxin, Quadratech, Epsom, U.K.).17 Antiendotoxin antibodies were measured using the EndoCAb assay, which measures the concentrations of antibodies (IgG and IgM) to the core region of the lipopolysaccharide molecule.18

Urine samples

Five ml of urine was collected at PO, D1, D3 and D7. Urinary concentrations of p55TNF-R were measured by an ELISA assay as described by Liabakk.19
Statistical analysis

Results are expressed as median and interquartile ranges (IQR) or mean ± standard error of mean as appropriate depending on distribution. Kruskal–Wallis test, Mann–Whitney U-test, Wilcoxon Signed Rank and Chi Square tests were used as appropriate. For correlation, the Spearman Rank Correlation Coefficient was used. The area under the curve was calculated using the Trapezium Rule. Significance was taken at the 5% level.

Results

Demographic details

Sixty-three patients were initially recruited into the study. However, two patients withdrew their consent and did not complete the study, three patients underwent intraoperative radiological intervention and were excluded and one patient developed major stroke after carotid endarterectomy (CEA) and was also excluded. Fifty-seven patients were studied to completion (group I: n = 18, group II: n = 14, group III: n = 12, group IV: n = 13).

Apart from a significantly younger age in the IC group compared to the CLI group, all other preoperative parameters and co-morbid conditions were similar between the groups. The operating time was significantly shorter and the blood loss was significantly higher in the amputation group (Table 1).

Nine patients in the CLI group and two patients in the IC group had femoro-distal bypass, while nine patients in the CLI group and 12 patients in the IC group had femoro-popliteal bypass surgery (p = ns). In the amputation group, nine patients had above knee amputation and three patients had above knee amputation.

Intestinal permeability

There was no significant difference between the four groups in the preoperative L/M ratio. A significant increase in the L/M ratio was observed in the CLI group at D3 (0.04(0.02–0.07)) compared to PO level (0.01(0.01–0.03), p < 0.05. This was also found to be significantly higher than that of IC group at D3 (0.02(0.01–0.04), p < 0.05). There was no significant change in L/M ratio in IC, amputee and CEA groups at any of the time points (Fig. 1).

A significant correlation (r = 0.58, p = 0.046) between the increased L/M ratio at D3 and clamp time in the CLI group but no correlation was found with the other parameters.

Within the CLI group, patients who had femorodistal bypass had significantly higher L/M ratio at D3 (0.07(0.05–0.9)) compared to those who had femoro-popliteal bypass (0.03(0.01–0.3), p < 0.05). This was not related to operating time nor clamp time.

Endotoxin and EndoCAb antibodies

No significant endotoxaemia was detected (Fig. 2), nor was there any change in the EndoCAb antibody concentrations intra or postoperatively in any of the groups (Table 2).

![Fig. 1. Lactulose/mannitol ratio. *p < 0.05 vs PO (Wilcoxon Signed Ranks test), †p < 0.05 vs group II (Mann–Whitney U-test).](image1)

![Fig. 2. Relative frequency of detection of endotoxin in groups I, II, III and IV. (Preop) preoperative, (IS) maximum ischaemia, (Postop) postoperative (Chi square test).](image2)

Table 1. Patients’ demographic and operative details (median, IQR).

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>73.7(14)</td>
<td>67(16.5)*</td>
<td>71(10)</td>
<td>67(9)</td>
</tr>
<tr>
<td>Sex</td>
<td>7M, 11F</td>
<td>12M, 2F</td>
<td>9M, 3F</td>
<td>10M, 3F</td>
</tr>
<tr>
<td>Operating time (min)</td>
<td>200(67.5)</td>
<td>172(101.3)</td>
<td>50(20)‡</td>
<td>140(35)</td>
</tr>
<tr>
<td>Clamp time (min)</td>
<td>66(31)</td>
<td>65(30.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood loss (ml)</td>
<td>305(143.7)</td>
<td>250(158)</td>
<td>500(200)†‡</td>
<td>200(190)‡</td>
</tr>
</tbody>
</table>

*p < 0.05 vs group I.
‡p < 0.05 vs group I, II and IV (Mann–Whitney U-test).
Table 2. EndoCAb/Tetanus antibodies ratio (mean ± SEM).

<table>
<thead>
<tr>
<th>Group</th>
<th>PO</th>
<th>IS</th>
<th>1 h</th>
<th>D1</th>
<th>D3</th>
<th>D5</th>
<th>D7</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>618(91)</td>
<td>724(128)</td>
<td>685(123)</td>
<td>932(295)</td>
<td>687(110)</td>
<td>998(402)</td>
<td>723(110)</td>
</tr>
<tr>
<td>II</td>
<td>374(82)</td>
<td>513(122)</td>
<td>436(82)</td>
<td>534(147)</td>
<td>454(99)</td>
<td>411(90)</td>
<td>559(140)</td>
</tr>
<tr>
<td>III</td>
<td>804(180)</td>
<td>822(199)</td>
<td>1090(234)</td>
<td>955(134)</td>
<td>992(289)</td>
<td>731(153)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>1259(196)</td>
<td>1320(219)</td>
<td>1229(208)</td>
<td>1219(225)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Urinary p55 soluble TNF-receptors. *p < 0.05 vs PO (Wilcoxon Signed Ranks test), †p < 0.05 vs groups II, III and VI (Mann–Whitney U-test).

**Urinary p55 soluble TNF-receptors**

A persistent elevation of urinary p55TNF-R at D1 and D3 postoperatively was observed in the CLI and IC groups (Fig. 3). However, this was demonstrated to be significantly greater in the CLI group when area under the curve was considered. There was no significant correlation between the increased urinary p55TNF-R concentrations in CLI group and the operative parameters or L/M ratio.

**Discussion**

Increased intestinal permeability has been well documented in patients with intestinal inflammation associated with increased morbidity and mortality, after major trauma, thermal injury, obstructive jaundice, acute pancreatitis, following major vascular surgery including cardiopulmonary bypass and abdominal aortic aneurysm repair.\(^{21,27}\) Increased intestinal permeability associated with the development of MODS has also been observed in critically ill intensive care unit patients.\(^{28}\)

The finding of increased intestinal permeability in patients with CLI undergoing bypass surgery only supports the hypothesis that isolated lower limb I/R injury leads to intestinal mucosal barrier dysfunction and provides evidence of remote organ injury following I/R injury.\(^{11–13}\) The results suggest that the magnitude of injury following reperfusion is related to the degree of initial ischaemia since the increased intestinal permeability was observed only in patients with CLI.

The pathogenesis of this increase in intestinal permeability is unclear. Roumen et al.\(^ {8}\) reported increased intestinal permeability after elective and emergency repair of aortic aneurysms. They found no difference in increased permeability between patients who underwent elective surgery and those who presented with shock due to rupture of their aneurysms and suggested that reperfusion rather than ischaemia is the cause of increased intestinal permeability. However, other workers observed that the increased intestinal permeability following transperitoneal repair of aortic aneurysms may be avoided by the extraperitoneal approach suggesting that intestinal manipulation and mesenteric traction were important in causing the increased intestinal permeability.\(^ {10}\)

The significant positive correlation between L/M ratio and the clamp time suggests that I/R injury of the lower limb may be important in causing the disruption in intestinal mucosal integrity. This remote organ injury concurs with the findings of others who have observed a significant correlation between clamp time and impairment of pulmonary, renal, hepatic and haematopoietic functions.\(^ {29}\)

Within the CLI group, L/M ratio was also significantly higher in those who had femoro-distal bypass compared to those who had femoro-popliteal bypass even though no significant difference was found between these two subgroups in any of the operative parameters. This may be explained by the clinical observation that patients who needed distal bypass surgery had more severe ischaemia than those with more proximal disease.

These findings may suggest that the increased intestinal permeability in the CLI group may be related to the difference in the degree of initial ischaemia. On the other hand, it is possible that femoro-distal bypass is more traumatic than more proximal bypass.
The relationship between endotoxaemia, increased intestinal permeability, SIRS and MODS is still controversial. Endotoxaemia and increased intestinal permeability were reported after major vascular surgery including abdominal aortic aneurysm repair. However, endotoxaemia was detected before and not at the very moment of the documented increase in intestinal permeability, with no temporal correlation between them, indicating that endotoxaemia and increased intestinal permeability may be independent phenomena. On the other hand, endotoxaemia could have contributed to the rise in the already abnormal intestinal permeability. A single dose of endotoxin may increase intestinal permeability in healthy volunteers. Other workers have detected systemic endotoxaemia in the immediate postoperative period following major surgery without relation to subsequent complications and they further questioned the relationship between endotoxaemia and the development of MODS.

The absence of relationship between systemic endotoxaemia and increased intestinal permeability in this study agrees with the findings of other workers who could not detect systemic endotoxaemia even after aortic surgery inspite of increased intestinal permeability. They also found no correlation between the increased intestinal permeability and endotoxaemia or between endotoxaemia and the development of MODS. This may be explained by the fact that the increased intestinal permeability was transient and mild, and did not lead to endotoxaemia, or that systemic endotoxaemia was intermittent and was not detected because of the infrequent sampling times. In this study, the increased intestinal permeability was not accompanied by systemic endotoxaemia. However, other workers have found a significant correlation between intestinal mucosal dysfunction and portal endotoxaemia following aortic surgery even though the portal endotoxaemia was only detected intraoperatively and did not seem to be related to the systemic endotoxaemia. In addition, the increase in intestinal permeability was observed 24 h later.

Endotoxin works via the release of proinflammatory mediators such as TNF-α. Unfortunately, the detection of TNF-α, like endotoxin, in plasma following I/R injury has been inconsistent due to its short half life, burst-like release, limited frequency of sampling and assaying techniques. These problems in detecting TNF-α may be avoided if soluble p55TNF-R is measured. This is a more stable compound and urinary p55TNF-R has been shown to be an accurate measure of the systemic inflammatory state. In addition, measuring urinary p55TNF-R has the advantage of avoiding plasma inhibitors and haemodilution which can interfere with plasma p55TNF-R measurements. In the present study, the significantly greater p55TNF-R response in CLI group implies a greater inflammatory response in this group of patients. Unlike intestinal permeability no difference was observed between those undergoing femoro-distal bypass and those who had femoro-popliteal bypass suggesting that the inflammatory response may be influenced by the degree of initial ischaemia.

In conclusion, these results suggest that intestinal mucosal barrier dysfunction and cytokine response following the revascularisation of chronic limb ischaemia may be related to the degree of initial ischaemia.

Acknowledgements

This work was generously supported by the vascular research funds from Belfast City Hospital and by generous awards from the Royal Victoria Hospital and Down Lisburn Trust, Belfast.

References


Accepted 16 December 2002