Selective portal clamping to minimize hepatic ischaemia–reperfusion damage and avoid accelerated outgrowth of experimental colorectal liver metastases

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Background: Temporary vascular clamping during local ablation for colorectal liver metastases increases destruction volumes. However, it also causes ischaemia–reperfusion (IR) injury to the liver parenchyma and accelerates the outgrowth of microscopic tumour deposits. The aim of this study was to investigate the effects of selective portal clamping on hepatocellular damage and tumour growth.

Methods: Mice carrying pre-established hepatic colorectal micrometastases underwent either simultaneous clamping of both the portal vein and the hepatic artery or selective clamping of the portal vein to the median and left liver lobes for 45 min. Sham-operated mice served as controls. Hepatic injury and tumour growth were assessed over time.

Results: Standard inflow occlusion resulted in a rise in liver enzymes, a local inflammatory response and hepatocellular necrosis. The outgrowth of pre-established micrometastases was accelerated three- to fourfold in clamped compared with non-clamped liver lobes (27.4 versus 7.8 per cent, \( P < 0.010 \)). Conversely, selective portal clamping induced minimal liver injury, tissue inflammation or hepatocellular necrosis, and completely stopped the accelerated outgrowth of micrometastases.

Conclusion: Selective portal clamping does not induce liver tissue damage or accelerate micrometastasis outgrowth and may therefore be the preferable clamping method during local ablative treatment of hepatic metastases.

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Introduction

Colorectal cancer is one of the most prevalent malignancies in the western world and is the third leading cause of cancer-related deaths. Mortality is strongly associated with the development of liver metastases, which eventually occurs in 50–70 per cent of patients with colorectal cancer. Once liver metastases have developed, the natural course of the disease is associated with poor survival rates. Partial liver resection remains the only hope for cure, offering 5-year survival rates of 30–40 per cent; however, only 15–20 per cent of patients are eligible for curative resection. For non-resectable liver metastases, local ablative techniques such as radiofrequency ablation and laser-induced thermotherapy may provide local control and increase life expectancy. In these patients, the tumour is destroyed by heat from energy-transmitting sources, resulting in coagulative necrosis. Unfortunately, even after an apparently complete destruction, most patients will have tumour recurrence in the liver within 2 years. Several mechanisms may underlie tumour recurrence. First, new regional intrahepatic recurrences may develop from previously undetected micrometastases, as reported in 60–90 per cent of patients. Second, local recurrences in and around the primary lesion occur in 10–68 per cent of cases and are the result of incomplete heat-destruction of tumour tissue, or from the outgrowth of previously undetected microsatellite lesions.
Insufficient heat diffusion at the tumour periphery may cause unsuccessful treatment, especially in tumours greater than 4 cm16,17. Finally, local recurrences may develop from viable tumour cells that survive around blood vessels thanks to the cooling effect of the bloodstream, or the heat sink effect18,19. To overcome these obstacles, vascular inflow occlusion, or the Pringle manoeuvre, is advisable to reduce dissipation of the generated heat, providing increased destruction volumes and greater tumour-free margins13,20–23.

As 75 per cent of hepatic blood flow is carried by the portal vein, the heat sink effect is determined mainly by portal flow. Indeed, selective clamping of the portal vein results in increases in lesion size similar to simultaneous clamping of the hepatic artery and the portal vein20,24–27.

The main drawback of interrupting the hepatic blood supply is ischaemic injury to the liver parenchyma. On restoration of the blood flow, reperfusion injury will aggravate the ischaemic damage, which may contribute to postoperative liver dysfunction. The adverse effects of ischaemia–reperfusion (IR) on the liver parenchyma have been well documented28–30. In addition, the authors have recently shown that the outgrowth of pre-established micrometastases was strongly stimulated following temporary vascular clamping31. The authors have hypothesized that selective clamping of the portal vein may not only prevent ischaemic damage to the liver parenchyma, but may also protect against accelerated outgrowth of residual tumour cell deposits. The aim of this study was to assess the effects of selective clamping of the portal vein on hepatic tissue damage and micrometastasis outgrowth in a highly standardized murine model.

**Methods**

**Animals**

All experiments were carried out in accordance with the guidelines of the Animal Welfare Committee of the University Medical Center Utrecht, The Netherlands. Male BALB/c mice (10–12 weeks) were bought from Charles River (Sulzfeld, Germany) and were housed under standard laboratory conditions.

**Murine model of hepatic ischaemia–reperfusion**

Animals were operated under inhalation anaesthesia with a 1.5–2 per cent isoflurane/oxygen mixture using a mask. Buprenorphine (3 μg/mouse) was given intramuscularly before surgery for intra- and postoperative analgesia. Surgical procedures were performed under aseptic conditions and surgical foil was placed over the laparotomy wound to avoid dehydration. Heparin was not administered. Body temperature was maintained at 36.5–37.5°C by placing the animals on a heated table and covering them with aluminium foil. After all procedures, a small amount of saline was left in the abdominal cavity, and the peritoneum and skin were separately closed with 5-0 Vicryl. A model of partial IR to the left plus median lobes was used, corresponding to approximately 70 per cent of the liver mass. After laparotomy, the liver hilus was exposed and the portal vein was carefully freed from the hepatic artery by microsurgical dissection. By randomization, either both the portal vein and the hepatic artery were clamped simultaneously for 45 min or the portal vein to the left and median liver lobes was clamped selectively (n = 8 in each group). Sham-operated mice underwent laparotomy with exposure of the liver and dissection of the vascular structures, but without interruption of hepatic blood flow.

**Blood pressure measurements**

Blood pressure was measured in a separate set of mice by placing a 26 gauge catheter in the carotid artery in sham-operated mice and in mice subjected to 45 min of ischaemia to the left and median lobes followed by 40 min of reperfusion (n = 3 in each group). Mean arterial blood pressure was continuously measured for at least 120 min from the onset of anaesthesia.

**Liver enzymes**

The degree of early liver tissue damage was assessed in separate groups by plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels. Animals were allowed to recover after surgery and were reanaesthetized for blood withdrawal following 6 h of reperfusion (n = 8 in each group). Heparin plasma samples (500 μl) were obtained by cardiac puncture and centrifuged at 14 000 r.p.m. for 10 min. Plasma levels of ALT and AST were analysed using commercially available diagnostic kits (Instruchemie BV, Delfzijl, The Netherlands).

**Cell culture and induction of liver micrometastases**

The murine colon carcinoma cell line C26 was cultured in Dulbecco’s modified Eagle’s medium supplemented with 5 per cent heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a 5 per cent carbon dioxide environment. Confluent cultures were harvested by brief trypsinization (0.05 trypsin
in 0.02 per cent ethylenediamine tetra-acetic acid) and, after centrifugation, single-cell suspensions were prepared in phosphate buffered saline to a final concentration of $5 \times 10^6$ cells/100 µl. Cell viability was determined by trypan blue staining, and was always at least 98 per cent. Colorectal liver metastases were induced in the mice: through a left lateral flank incision, $5 \times 10^4$ C26 colorectal carcinoma cells were injected into the splenic parenchyma. Single tumour cells reach the liver through the portal vein where a subset grows out to form intrahepatic micrometastases. After 10 min, the spleen was removed to prevent intrasplenic tumour growth. Micrometastases were allowed to develop throughout the liver for 5 days. At that time, animals were subjected to clamping of the portal vein plus hepatic artery, selective clamping of the portal vein or sham treatment. Morphological assessment of tumour growth was performed on non-clamped and clamped lobes harvested 5 days later.

**Tumour analysis**

Intrahepatic tumour load was scored as the hepatic replacement area (HRA), the percentage of hepatic tissue replaced by tumour cells. For each liver lobe, at least 100 fields were selected on two non-sequential sections stained with haematoxylin and eosin using an interactive video overlay system including an automated microscope (Leica-Q-Prodit, Leica Microsystems, Rijswijk, The Netherlands) at a magnification of 40×. Using a four-point grid overlay, the ratio of tumour cells to normal hepatocytes plus necrotic cells was determined for each field. Tumour load (HRA) was expressed as the mean area ratio of all fields. The two observers were blinded to treatment. Finally, HRA ratios between clamped and non-clamped lobes were calculated for each animal to express the proportional increase in HRA in the clamped (left plus median) lobes versus the non-clamped (right) lobes.

**Quantification of hepatocellular necrosis**

The percentage of hepatocellular necrosis was scored simultaneously with tumour HRA analyses on two non-sequential haematoxylin and eosin-stained sections. The ratio of necrotic cells to healthy hepatocytes plus tumour cells was determined for each field. The percentage of hepatocellular necrosis was expressed as the mean area ratio of all fields.

**Statistical analysis**

Statistical differences between groups were analysed by the Mann–Whitney U test for non-parametric data. Values are expressed as mean(s.e.m.); $P < 0.050$ was considered statistically significant.

**Results**

The model of partial hepatic IR induced by occluding the vascular inflow of the left lateral liver lobe has been described previously. However, in mice, selective clamping of the portal vein at the level of the left lateral liver lobe is technically complicated. Therefore, a model of partial IR to the left plus median lobes was used, corresponding to approximately 70 per cent of the liver mass. Selective occlusion of the portal flow (Fig. 1a) was confirmed by injection of methylene blue into the portal system, showing a lack of staining of the left and median lobes (not shown).

![Fig. 1](image_url) Standardized murine model of left and median lobar ischaemia–reperfusion (IR) of the liver. a Schematic image of selective portal clamping technique. The portal vein is carefully freed from the hepatic artery by microsurgical dissection and is selectively occluded to the left (L) and median (M) liver lobes by a microvascular clamp for 45 min, leaving the oxygen-rich blood supply via the hepatic artery intact. b Mean arterial blood pressure (MAP) during IR of the left and median lobes in comparison to sham-operated mice.
Haemodynamic stability in models of IR is crucial, as hypotension and systemic hypo-oxygenation may induce temporary tissue ischaemia. In addition, hypothermia affects haemodynamic stability and reduces IR-induced injury. Therefore, special attention was paid to anaesthesia, haemodynamic stability and body temperature. After clamping both the portal vein and hepatic artery to the left plus median lobes, blood pressure remained stable for at least 120 min before and during IR (Fig. 1b) without overt changes in blood pH, partial pressure of oxygen or partial pressure of carbon dioxide (not shown). Vascular inflow obstruction to 70 per cent of the liver mass did not adversely affect haemodynamic stability in this model.

Standard clamping of the median and left liver lobes induced severe early hepatocellular damage, as shown by elevated plasma ALT and AST levels after 6 h of reperfusion (Figs 2a and b). Areas of severe early hepatocellular damage were seen 6 h after standard clamping in all clamped liver lobes, characterized by haemorrhage, eosinophilic hepatocytes, signs of nuclear pyknosis and loss of cell–cell contact (Fig. 2c). Five days

![Graphs showing ALT and AST levels](image)

**Fig. 2** Hepatocellular injury after sham operation, ischaemia–reperfusion (IR) and portal clamping. **a** Plasma alanine aminotransferase (ALT) and **b** aspartate aminotransferase (AST) at 6 h of reperfusion. **c** Microscopic haematoxylin and eosin-stained sections of the non-clamped and clamped liver lobes, harvested 6 h after clamping. After IR, liver cell damage in the clamped lobes is characterized by haemorrhage (arrows), eosinophilic hepatocytes, signs of nuclear pyknosis and loss of cell–cell contact. Portal clamping induced minor haemorrhage in the clamped lobes (arrow) without signs of hepatocellular injury (original magnification × 20). **d** The percentage of liver tissue necrosis 5 days after clamping. Values are presented as mean(s.e.m.). *P < 0.010 (Mann–Whitney U test)
Selective portal clamping and colorectal liver metastases

The outgrowth of pre-established micrometastases 5 days after sham operation, hepatic (ischaemia–reperfusion IR) and portal clamping. a Tumour growth, expressed as the hepatic replacement area (HRA). b Proportional increase in HRA in the clamped (left plus median) lobes versus the non-clamped (right) lobe, expressed as HRA ratio. Values are presented as mean(s.e.m.). *P < 0.010 (Mann–Whitney U test). c Microscopic haematoxylin and eosin-stained sections of the non-clamped and clamped liver lobes, harvested 5 days after clamping. IR-induced acceleration (t, tumour cells) is observed around necrotic areas (n) (original magnification × 10)

after standard clamping, necrotic areas were observed in all animals, covering 14 per cent of the clamped liver tissue (Fig. 2d). Thus, 45 min of 70 per cent hepatic ischaemia caused considerable liver tissue damage without any mortality, allowing long-term evaluation of hepatocellular damage and tumour growth.

Ten days after intrasplenic tumour cell injection, tumour load in the right lobe was similar to that in the left plus median liver lobes in sham-operated mice (Fig. 3a, 6.9(2.1) versus 5.3(1.5) per cent, P = 0.571). Consequently, the ratio between the HRA values was approximately 1 (Fig. 3b). Based on these results, it can be concluded that in this model the right liver lobe may serve as an internal control for tumour growth after selective clamping of the left and median lobes.

Standard clamping of both the left and median lobes caused a significant increase in tumour load in occluded liver lobes (Fig. 3a). Tumour growth was stimulated three- to fourfold compared with the non-occluded lobes (Fig. 3b). Similar to the authors’ previous findings on selective left lobar pedicle clamping, accelerated tumour growth was located in perinecrotic tissue areas (Fig. 3c), where tumour cells preferentially grew into zones of inflammation surrounding these necrotic areas.

It was reasoned that portal clamping alone would reduce ischaemic damage, as the oxygen-rich blood supply through the hepatic artery would be maintained. Selective portal clamping prevented early hepatocellular damage by more than 90 per cent, indicated by plasma ALT and AST levels 6 h post-clamping (Fig. 2a,b). Despite focal spots of minimal haemorrhage, no signs of severe liver cell damage were observed after portal clamping compared with standard clamping (Fig. 2c). In addition, late hepatocellular damage, or liver tissue necrosis, as occurred after standard clamping, did not ensue following selective portal clamping (Fig. 2d). Next, it was determined how selective portal clamping affected the outgrowth of pre-established micrometastases. As demonstrated in Fig. 2a,b, selective clamping of the portal vein, in contrast to standard clamping, did not accelerate the outgrowth of micrometastases. Moreover, microscopic examination showed no signs of liver tissue necrosis or inflammation (Fig. 2c).
Discussion

IR due to standard inflow occlusion of the left plus median liver lobes for 45 min resulted in a three- to fourfold increase in outgrowth of pre-established micrometastases. This is in concordance with the authors’ previous findings on selective left lobar clamping. When the portal vein to the left and median lobes was selectively clamped, leaving the oxygen-rich blood supply via the hepatic artery intact, liver tissue damage was minimal and outgrowth of micrometastases remained unaffected. Based on these results, it appears that ischaemic tissue damage due to clamping of the arterial blood supply in addition to the portal vein is crucial for stimulation of tumour growth following IR in the liver.

Several mechanisms may underlie the stimulating effect of IR on tumour outgrowth. First, although relatively short, the oxygen deprivation during standard clamping may directly activate several hypoxia-dependent proliferation-stimulating signalling pathways in the tumour cells. During portal clamping, the oxygen supply to the liver tissue is maintained, which prevents tissue hypoxia and, by inference, hypoxia-stimulated tumour growth. Second, ischaemic damage following standard clamping is characterized by widespread liver cell death, the influx of inflammatory cells and microcirculatory disturbances. These local responses may create an ideal milieu for tumour cell proliferation or invasion. Portal clamping did not result in severe liver tissue damage, as liver enzymes were only marginally increased and only minor histopathological changes were observed. Selective portal clamping may avoid stimulation of tumour growth by failing to induce liver tissue damage.

The acceleration of tumour growth following vascular clamping could theoretically decrease the life expectancy of patients undergoing local tumour ablation under standard hepatic inflow occlusion, but clinical data to support this are lacking. As local ablative treatments become more accepted for resectable liver metastases, an increasing number of patients may be expected to undergo hepatic clamping. During local ablation, particularly of large colorectal liver metastases (bigger than 4 cm) or lesions close to large vessels, it is essential to occlude intrahepatic blood flow to obtain a safe margin around the ablated tumour. Moreover, to avoid unacceptably high local recurrence rates, inflow occlusion has been shown to be mandatory in a recently published meta-analysis on this topic. Here an alternative occlusion technique is presented that minimizes IR injury, successfully reduces the heat sink effect and eliminates the risk of accelerated tumour outgrowth after standard inflow occlusion.

Portal clamping has several advantages over standard inflow occlusion, but its application in surgical practice may depend on the specific clinical circumstances. Selective clamping of the portal vein may be the clamping method of choice during open local ablation and during ablation of multiple liver tumours, particularly when the expected ablation time is relatively long. In those patients, occlusion time during surgery is not restricted as oxygen supply to the liver is maintained. Moreover, the hepatoprotective effect of portal clamping may contribute to a decreased postoperative morbidity. Portal clamping may be particularly useful in high-risk patients in whom partial resection was abandoned because of poor medical condition. Nonetheless, in patients who have undergone previous liver surgery, portal clamping is technically challenging because of adhesion formation in the perihilar region, and may increase operating times.

Intermittent inflow occlusion may also be a safe alternative during local ablation, as complete absence of accelerated outgrowth of micrometastases following intermittent compared with continuous clamping has been reported. For single tumours and after previous operations, intermittent clamping may be easier than portal clamping, as it avoids dissection of the hepatoduodenal ligament. When large hepatic arteries run close to or within the tumour, the hepatic artery may be clamped in addition to the portal vein for short periods to avoid local recurrence that may develop from viable tumour cells around these arteries. For multiple tumours or complicated cases, the disadvantage of intermittent clamping is that it needs stringent time management and additional manipulation during ablation. In contrast, using continuous portal clamping, the operation is not delayed by successive periods of reperfusion between clamping periods.

Selective arterial embolization has also been reported to increase lesion size and may be easier than selective portal clamping during the laparoscopic and percutaneous approach. However, several authors have shown that arterial clamping alone is not sufficient to produce larger coagulation diameters.

In combination with image-guided probe positioning and monitoring and adjuvant chemotherapy, selective portal clamping may improve the oncological outcome of patients with colorectal cancer.

Selective portal clamping induces minimal post-ischaemic liver tissue injury and does not promote outgrowth of resident micrometastases. Prospective studies in patients are required to assess whether selective portal clamping can help reduce hepatic tumour recurrence and improve the prognosis of patients undergoing local ablation of colorectal liver metastases.

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References


