Strategies to improve outcome after partial liver resection

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Chapter 7

Portal vein ligation is as effective as sequential portal vein and hepatic artery ligation in inducing contralateral liver hypertrophy

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Chapter 7

Abstract

**Purpose.** Dual embolization of hepatic artery and portal vein has been proposed to enhance contralateral liver regeneration prior to resection. The aim of this study was to evaluate the effect of portal vein ligation compared to dual ligation, either simultaneously or with sequential approach, on regeneration, proinflammatory response and liver damage.

**Methods.** Single hepatic artery, portal vein ligation (PVL) of 70% or dual ligation of hepatic artery and portal vein of 70% either simultaneously or sequentially with a 48h interval was performed in a rat model. Liver regeneration, proinflammatory mediators, hepatocellular synthetic function and injury, histopathology and apoptosis were assessed up to 14 days after surgery.

**Results.** Sequential dual ligation resulted in faster increase in hepatocyte proliferation at 24h without additional increase in liver mass compared to PVL after 14 days. Both the dual ligations significantly increased proinflammatory response in plasma and in the regenerating liver compared to PVL alone. 14 days after PVL, the hepatic parenchyma was completely restored compared to fibro-necrosis in sequentially dual ligated and complete necrosis in simultaneously ligated groups. Increased apoptosis in the regenerating liver and prolonged hepatic dysfunction was observed after both dual ligations.

**Conclusions.** PVL is as effective as dual ligation in inducing liver regeneration. No additional benefit of arterial ligation was observed.
Introduction

Severe postoperative complications and mortality after extensive resection are directly related to the size and function of the remnant liver. Especially patients with parenchymal liver disease have an increased risk of postoperative liver dysfunction due to already impaired preoperative function combined with an impaired regenerative capacity leading to slower recovery of liver mass after resection. Portal vein embolization (PVE) was introduced to enable more extensive liver resections by inducing compensatory hypertrophy in the non-embolized future remnant liver and atrophy in the embolized lobe planned for resection. With PVE, the future remnant liver volume can be increased up to 40%, consequently decreasing liver-dysfunction related complications caused by insufficient remnant liver. Dual embolization of hepatic artery and portal vein has been suggested to induce sufficient liver regeneration in patients with potentially impaired liver regeneration. The obvious advantage of dual embolization compared to PVE is the complete occlusion of both portal and arterial blood supply to the tumor bearing liver segments. However, if PVE and transarterial embolization are combined simultaneously, the total occlusion of blood supply potentially causes hepatic infarction and massive hepatocellular necrosis consequently triggering systemic proinflammatory cytokine response. This might as a result lead to acute liver failure impairing hepatic synthetic and metabolic functions. Therefore, a sequential embolization with 48h interval has been suggested to prevent these life-threatening complications. However, no studies are available assessing the potential systemic or local effect directly after sequential embolization and the impact of sequentially ligated liver lobes on adjacent regenerating lobes is unclear. Currently used approaches to evaluate hepatocellular injury such as histopathology of the resected liver earliest after 4-8 weeks after embolization or plasma aminotransferases, give only an estimation of the actual response and give no information of the state of the regenerating lobes.

The purpose of this study was to compare portal vein ligation as surrogate PVE, with simultaneous or sequential dual ligation of hepatic artery and portal vein. The effect of ligation on liver proliferation and hepatocellular damage and function was assessed along with evaluation of local and systemic proinflammatory cytokine response.

Materials and methods

Animals

Male Wistar rats (250-300g) were obtained (Harlan CPB, Zeist, Netherlands). The animals were housed at constant 24 °C with 12 h light- dark cycle and were fed a standard rodent chow (Hope farms, Woerden, The Netherlands) and water ad libitum. Rats were allowed to acclimatize 7 days to laboratory conditions before surgery. During all procedures the animals were treated according to the guidelines of the Dutch legislation and international standards for animal care and handling. The protocol was approved by the Animal Ethics Committee of University of Amsterdam, The Netherlands.
Experimental design and surgical procedures

Surgery was performed under inhalation anesthesia of a mixture of $O_2/N_2O$ (1:1 V/V, 2.1 l/min) and isoflurane (1-2 % Florene, Abbott laboratories Ltd, Queensborough, UK) and pain medication (Temgesic i.v. 0.033 mg/0.1 kg). Ligation of the portal vein and/or the hepatic artery to the median and left lateral liver lobes was performed, occluding perfusion of 70% of total liver mass. Rats were divided into 5 groups (n=6); only mobilization of the liver (SHAM), only arteria hepatica ligation (AHL), only portal vein ligation (PVL), simultaneous ahl and pvl (DUAL0) and subsequent ahl and pvl after 48h (DUAL48). After operation, all animals were allowed to recover in a warm environment with free access to water and food. At 6 h, 72 h and 7 days postoperatively blood was collected via tail vein puncture under general anaesthesia and a maximum of 10 % of total blood volume per rat was collected. Under above mentioned general anaesthesia animals were sacrificed (n=6 per time point) after 24 h and 48 h for evaluation of early response and after 14 days for the end point analysis. Blood was collected by heart puncture, centrifuged (10 min, 3,000 RPM, 4°C) and plasma was stored at −80 °C until analysis. The liver lobes were removed, weighed and thin slices were immersed in 10% formalin for light microscopy (H& E and Sirius red staining) and for immunohistochemistry.

Assessment of liver regeneration

The weight of the non-ligated liver lobes divided by the total liver weight was used as a parameter to evaluate the regenerative capacity of the liver. For assessment of hepatic proliferation, MIB-5, a rat equivalent of Ki-67 antibody was used which detects all active parts of the cell cycle. The MIB-5 index has a strong positive correlation with proliferating antigen expression, bromodeoxyuridine incorporation and thymidine incorporation. Briefly, 4 μm sections were deparaffinized, preheated and boiled (citric acid pH 6.0, 2 bar, 120°C, 20 min) in a pressure cooker. Sections were incubated with a MIB-5 antibody (dilution 1:50, 60 min, DAKOCytomation, Glostrup, Denmark). After incubation with a secondary antibody (dilution 1:1, Poly-HRP, Invitrogen, Carlsbad, US) 3,3-diaminobezidine (Sigma chemical, Munich, Germany) was used to visualise the peroxidase complexes together with haematoxylin counterstaining. The proliferative index was determined in 30 high power fields (HPF) at 40X magnification and expressed as the percentage of positive cells per 1000 hepatocytes.

Hepatocellular damage and hepatic synthetic function

Plasma was analysed for aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin (T-Bil) and for albumin and prothrombin time using in the Department of Clinical Chemistry (AMC) using standard laboratory methods.

Proinflammatory cytokine response

Liver samples were homogenized in buffer (PBS, pH 6.0), centrifuged (10,000 x g, 4 °C, 10 min) and supernatant was used for analysis of IL-1β, IL-6 and TNF-α, the most important acute phase response cytokines produced by hepatic macrophages. Plasma and hepatic concentrations were measured using an enzyme-linked immunosorbent assay (Quantikine Rat TNF-α, Duoset Rat IL-1β and IL-6, RnD Systems Europe Ltd, England) according to
manufacturer's instructions. All samples were measured in duplicate in a 96-well microtitre plate and the concentrations were calculated from a standard curve. The hepatic protein concentration was measured with a BCA Protein Assay kit (Pierce, Rockford, US) and the hepatic cytokine concentrations were expressed in pg/mg protein.

**Hepatic neutrophil activity**

For neutrophil activation, hepatic myeloperoxidase (MPO) activity was measured by as described by Krawisz et al. Briefly, tissue samples were homogenized (PBS, pH 6.0), centrifuged for 10 min at 10,000 rpm at 4 °C. The pellet was homogenized in HTAB-EDTA buffer (0.5% hexadecyltrimethyl ammonium bromide, 10mM EDTA in PBS, pH 6.0, Sigma Chemicals, Munich, Germany) sonicated and centrifuged. The supernatant was incubated (2h, 60°C) and the MPO activity was measured spectrophotometrically after addition of o-dianiside hydrochloride (Sigma Chemical, Munich, Germany). The MPO activity was expressed as units/mg protein and one unit was defined as the amount of enzyme necessary to produce a change in absorbance of 1.0 per minute.

**Apoptosis**

For primary and secondary antibody, a cleaved caspase-3 (dilution 1:200, Cell Signaling Technology, Frankfurt, Germany) and Poly-HRP (dilution 1:1, Invitrogen, Carlsbad, US) respectively were used. The apoptotic index was determined at 40X magnification in 30 HPF and expressed as the amount of positive cells per 1000 hepatocytes.

**Histopathology**

Paraffin-embedded liver samples were routinely stained with haematoxylin-eosin (H&E) and Sirius red (0.1% Fast red in picric acid, Immunotech, The Netherlands). Histology of the ligated and non-ligated lobes was observed by light microscopy. Examination was performed by two independent investigators blinded to the treatment groups. Necrosis was expressed as percentage of necrotic tissue: 0 = no necrosis, 1 = < 25%, 2 = 25-50%, 3 = 50-75%, 4 = >75% necrosis. Inflammatory activity was determined as follows: 1 = focal collections of mononuclear cells, 2 = diffuse infiltrates of mononuclear cells, 3 = focal collections of polymorphonuclear cells in addition to mononuclear cells, 4 = diffuse infiltrates of polymorphonuclear cells.

**Statistical analysis**

Data analysis was performed with GraphPad Prism 3.02 for Windows (GraphPad Software Inc., San Diego, US). The results are presented as mean± SEM. Significant differences between groups were tested using Kruskal-Wallis one-way analysis and Mann-Whitney’s U-test. P values less than 0.05 were considered significant.
Results

Hepatic proliferation, regeneration and apoptosis

Both the regeneration ratio and hepatocyte proliferation index, measured by MIB-5 positive cells in the nonligated lobes, were increased in the PVL and both dual ligation groups at all time points compared to the SHAM and the AHL groups (Fig. 1A, B, respectively). Furthermore, the regeneration ratio in the DUAL0 group was lower compared to the PVL and the DUAL48 groups at all time points (p<0.05). At 24h, the regeneration ratio was significantly increased in the DUAL48 group compared to the PVL group. At 24h, the hepatocyte proliferation index was significantly lower in the PVL group compared to the DUAL0 and DUAL48 groups (p<0.05). The percentage of caspase-3 positive cells was higher in both the dual groups compared to the other groups and in the DUAL0 compared to DUAL48 group at 24 h and 48h after surgery (p<0.05) (Fig.1C).

Figure 1. Regeneration ratio, MIB-5 positive cells and caspase-3 positive cells

There were no changes after 14 days in the liver regeneration ratio (A) between PVL and sequential dual ligation. The proliferation index (B) of MIB-5 positive hepatocytes at 24h was increased in sequential ligation group, however no changes were seen at 48h. Both regeneration ration and MIB-5 index were decreased after simultaneous dual ligation. The apoptotic index (C) was increased in the non-ligated liver lobes of the both dual ligated groups. * = p<0.05 compared to the to SHAM and AHL groups, † = p<0.05 compared to DUAL0 group.
Hepatocellular damage

At all time points, no differences were observed in hepatocellular damage between the SHAM and the AHL groups. The PVL and both the dual groups had significantly increased AST levels at 24h, 48h and 72h compared to SHAM group (p<0.05). In the DUAL0 group, plasma AST was increased already at 6h compared to the all other groups and remained increased until 72h (p<0.05)(Fig.2A). In the DUAL48 group, AST was also elevated already at 6h compared to the SHAM and PVL groups. In the PVL group, AST was increased at 48h and 72h compared to the DUAL48 group (p<0.05). ALT followed the pattern of AST with the exception that there were no differences between both dual groups at 6h postoperatively (Fig. 2B). Plasma bilirubin followed the pattern of AST and ALT in all groups (data not shown).

Figure 2. Plasma levels of hepatocellular damage markers AST (A) and ALT (B)

There was a significant increase at 6h and 24h in AST and ALT after dual ligation groups compared to PVL groups in which peak was seen at 48h. * = p<0.05 compared to the to SHAM and AHL groups, †= p<0.05 compared to PVL group.

Hepatic synthetic function

No differences in plasma albumin concentrations were seen between the SHAM and the AHL groups at all time points. Plasma albumin concentration was increased in the DUAL0 group at 6 h postoperatively compared to all other groups. In both dual groups, the albumin was decreased compared to SHAM at 24h and was decreased compared to SHAM and the PVL groups up to 14 days (p<0.05). In the PVL group, no differences were seen after 72h compared to the SHAM group. PT followed the pattern of albumin in all groups (data not shown).
Figure 3. Hepatocellular synthetic function evaluated by plasma albumin level

A prolonged dysfunction in both dual ligation groups is visible. * = p<0.05 compared to the SHAM and AHL groups, †= p<0.05 compared to DUAL0 group, ‡= p<0.05 compared to PVL group and § = p <0.05 compared to DUAL48 group.

Systemic proinflammatory cytokine response

No differences were seen in plasma cytokines between the SHAM and the AHL groups at all time points. Plasma TNF-α was increased at 6h and 24h in the PVL and both dual groups compared to the SHAM group (p<0.05) (Fig. 4A). At 24h, TNF-α was elevated in the DUAL0 group compared to the other groups (p<0.05). Plasma IL-1β at 6h was elevated in the PVL, the

Figure 4. The systemic proinflammatory cytokine response TNF-α (A), IL-1β (B) at 6h, 24 and 48h and the local hepatic response TNF-α, IL-1β and IL-6 at 48h (C)

* = p<0.05 compared to the SHAM and AHL groups, †= p<0.05 compared to PVL group and § = p <0.05 compared to DUAL48 group.
DUAL0 and the DUAL48 groups compared to the SHAM group (Fig. 4B). At 24h and 48h, the DUAL0 group had increased plasma IL-1β levels compared to the other groups (p<0.05). Plasma IL-6 remained below detection level (75ng/ml) in all groups at all time points (data not shown).

Hepatic TNF-α, IL-1β and IL-6 in the nonligated liver lobes were elevated in the PVL and in both the DUAL groups compared to the SHAM group at 24h (p<0.05) (data not shown). At 48h (Fig. 4C), TNF-α was increased in the AHL, the PVL and both the dual groups compared to SHAM group and in the both the dual groups compared to the PVL group.

Figure 5. Histopathology and necrosis score of dual ligation groups and PVL group

Necrosis score at 24h, 48h and 14 days after simultaneous dual ligation remained almost 100% at all time points. However, sequential ligation with 48h interval led to less and decreasing necrosis. After PVL, no necrosis was seen at 14 days. (A). * = p<0.05 compared to the to SHAM and AHL groups, † = p<0.05 compared to DUAL0 group, ‡ = p<0.05 compared to PVL group.

The histopathological changes after 14 days in ligated lobes; no changes in portal vein ligated (B, magnification 100X, H&E staining), complete necrosis after simultaneous ligation (C, magnification 40X, H&E staining) and combined necrosis and fibrosis after sequential ligation (D, magnification 400X, H&E and E, Sirius red staining for collagen). Black arrow indicates the border of encapsulation and complete parenchymal necrosis, white arrow indicates collagen deposition.
(p<0.05). At 48h, hepatic IL-1β was increased in both dual groups compared to the other groups (p<0.05) and hepatic IL-6 was increased in PVL and both the dual groups compared to the SHAM group (p<0.05).

**Hepatic MPO activity**

Hepatic MPO levels evaluating neutrophil activity were increased at 24h in the PVL, the DUAL0 and the DUAL48 groups compared to the SHAM and the AHL groups (p<0.05). At 48h, the MPO level was significantly increased in the DUAL0 group compared to the PVL and the DUAL48 groups (p<0.05) (data not shown).

**Histopathology**

In the ligated and non-ligated liver lobes of the SHAM and AHL groups, no pathologic changes were seen. In the ligated liver lobes, the PVL group had less necrosis compared to both dual groups at all time points and no changes were seen after 14 days (Fig. 5A, B) (p<0.05). In the DUAL48 group significantly less necrosis was visible at 48h and 14 days compared to DUAL0 (p<0.05). In the DUAL0 group a complete necrosis (Fig. 5C) and in the DUAL48 group an extensive fibrosis with occasional necrosis was seen (Fig. 5D-E).

In the ligated lobes, the inflammation score was increased in the DUAL0 and the DUAL48 groups compared to the PVL group at all time points (Fig. 6A). In the DUAL48 group, the score was less compared to the DUAL0 group after 48h and 14 days (p<0.05) (Fig. 6A). In the nonligated liver lobes, the score was increased in the DUAL0 group compared to the PVL and DUAL48 groups at 24h (Fig. 6B). At 48h and 14 days, the score was significantly less in the PVL group compared to the both dual groups (p<0.05).

**Figure 6. The inflammation score of ligated as well as non-ligated lobes**

The inflammation score remained increased in the ligated (A) lobes after simultaneous ligation. However after both simultaneous and sequential ligation inflammation persisted in the non-ligated, regenerating, liver lobes (B) up to 14 days. At all time points no pathological changes were seen SHAM and AHL groups. †= p<0.05 compared to DUAL0 group, ‡= p<0.05 compared to PVL group.
Discussion

In the present study, PVL and both simultaneous and sequential dual ligation induced liver regeneration via activation of hepatocyte proliferation. The acceleration of hepatocyte proliferation after sequential dual ligation is most likely induced by the increased hepatic tissue TNF-α detected after arterial ligation as TNF-α has been reported a potential primer of hepatocytes and increases postresection liver regeneration \(^{16}\). The primed hepatocytes consequently respond more rapidly to the stimulus from subsequent PVL leading to an increased hepatocyte proliferation as seen 24h after sequential dual ligation. The exact mechanisms of TNF-α release after arterial ligation are uncertain and further research in this field is needed.

However, the acceleration after sequential ligation did not result in additional increase in the regeneration ratio after 14 days indicating that a maximal regenerative response can be reached with PVL only. Apparently during proliferation, the excessive hepatocytes in the cell cycle are eliminated by apoptosis as reflected by the increased amount of apoptotic hepatocytes in the regenerating liver after sequential ligation. However, after simultaneous ligation showing inadequate proliferation, apoptosis is most likely induced by another mechanism. One possible explanation is that inflammatory cytokines as TNF-α activate caspase-3 subsequently triggering hepatocytes into apoptosis. In contrast, after PVL, no increase of apoptosis was detected. This is in accordance with a study by Ikeda et al reporting an unchanged number of apoptotic hepatocytes during hepatic proliferation at 24 and 48 h in the remnant liver after hepatectomy \(^{17}\). In contrast, Kong et al report a beneficial effect on sequential ligation on liver regeneration \(^{11}\). However, the length of follow-up was in the latter study longer making the comparison of results difficult.

After PVL, hepatocellular damage was dramatically less than in both the dual ligation groups. The initially observed necrosis after PVL, had completely resolved while the liver parenchyma was fully restored after 14 days. This restoration can be attributed to the hepatic artery buffer response which increases blood flow and thus oxygen supply via the hepatic artery to the ligated liver lobes after PVL \(^{18}\). This notion is supported by the increased hepatic IL-6 levels found after PVL as IL-6 is a recognized marker of mechanical stress to hepatic sinusoidal cells after changes in blood flow and pressure \(^{19}\).

Simultaneous dual ligation of both arterial and portal blood supply led to massive hepatocellular necrosis in the ligated liver lobes. This effect might be advantageous if complete destruction of a fast growing tumor like HCC is sought before resection. However, massive necrosis also led to a substantial increase in release of aminotransferases and albumin from the injured hepatocytes. This consequently contributes to activation of systemic and local proinflammatory cytokine release as was demonstrated by significantly increased plasma and hepatic TNF-α and IL-1β. Also the activation of neutrophils contributed to the inflammatory response as reflected by increased MPO activity in the liver. This inflammatory response in the regenerating lobes most likely hindered regeneration after simultaneous ligation as hepatic TNF-α triggers hepatocytes to apoptosis and necrosis instead of proliferation \(^{20}\). Also, the persisting inflammation in regenerating lobes as seen in both the dual groups most likely is responsible for the prolonged hepatic synthetic and metabolic dysfunction as demonstrated by increased plasma PT after both dual ligations \(^{21}\).
Interestingly, entirely different local responses were observed in the ligated liver lobes after either sequential or simultaneous dual ligation. The ligated liver lobes were encapsulated by adherent peritoneal tissue already at 24 h after simultaneous dual ligation, as a local protective response to the massive necrosis. The isolation of necrotic ligated lobes presumably protected the adjacent regenerating lobes as the inflammatory response in the latter, was not significantly increased after simultaneous ligation as compared to sequential dual ligation. After sequential ligation, an extensive intraparenchymal fibrosis developed as a restorative response enclosing the necrotic areas by fibrotic scar formation. Furthermore, as sequential ligation induced less hepatocellular damage and a local restorative response, sufficient hepatocyte proliferation could occur in contrast to simultaneous ligation. These events might be useful in a clinical setting, when both tumor destruction and induction of contralateral hypertrophy is desired. Even though the biological response in the PVL model does not differ from the embolization model in experimental studies, it is possible that in the clinical setting this aspect is not the same. Also, another potential limitation when extrapolating these results into clinical situation is the different arterial vs portal blood supply to liver seen in all rodents. As the portal blood supply in rodents is greater than in humans, the negative effect of sequential ligation might be misjudged in our study. Also, underlying parenchymal diseases as are often present in patients undergoing liver surgery might influence the liver regeneration or affect hepatocellular injury. Therefore, the results of our study should be confirmed in a clinical setting with carefully standardized protocol.

In conclusion, this study shows that portal vein ligation is as effective as sequential dual ligation in inducing liver regeneration. No additional benefit of arterial ligation was observed.

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Reference list


The effect of portal vein and/or hepatic artery ligation


