Functional recovery after liver resection
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chapter 10

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Portal vein ligation is as effective as sequential portal vein and hepatic artery ligation in inducing contralateral liver hypertrophy in a rat model.
Introduction

Severe postoperative complications and mortality after extensive resection are directly related to the size and function of the remnant liver (1,2). In particular, patients with parenchymal liver disease have an increased risk of postoperative liver dysfunction as a result of already impaired preoperative function combined with an impaired regenerative capacity leading to slower recovery of liver mass after resection (1-3). Portal vein (PV) embolization 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 (4-5). With PV embolization, the future remnant liver volume can be increased as much as 40% consequently decreasing liver dysfunction related complications caused by insufficient remnant liver (6-8). 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 PV embolization is the complete occlusion of both portal and arterial blood supply to the tumor-bearing liver segments (9,10). However, if PV embolization 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 (9). Therefore, a sequential embolization with 48h-interval has been suggested to prevent these life-threatening complications (9-11). However, we are aware of no available studies that have assessed the potential systemic or local effects 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 histopathologic examination of the resected liver 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 (9-12).

The purpose of this study was to compare PV ligation as surrogate PV embolization, with simultaneous or sequential dual ligation of hepatic artery and PV. 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 vol/vol; 2 L/min) and isoflurane (1-2% Fluorene, Abbott laboratories Ltd, Queensborough, UK) and pain medication (Temgesic i.v. 0.033 mg/0.1 kg). Ligation of the PV and/or the hepatic artery to the median and left lateral liver lobes was performed with occlusion of perfusion of 70% of total liver mass (13). Rats were divided into five groups (n=6), which were treated with only mobilization of the liver (sham group), hepatic artery ligation (HAL) only, PV ligation only, simultaneous HAL and PV ligation (dual-0 group) and subsequent HAL and PV ligation after 48h (dual-48 group). After operation, all animals were allowed to recover in a warm environment with free access to water and food. At 6 hours, 72 hours and 7 days after the operation, blood was collected via tail vein puncture under general anaesthesia and a maximum of 10% of total blood volume per rat was collected. Under the aforementioned general anaesthesia animals were killed (n=6 per time point) after 24 hours and 48 hours for evaluation of early response and after 14 days for the endpoint analysis. Blood was collected by heart puncture, centrifuged (10 minutes, 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 nonligated 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 (14). Briefly, 4-μm sections were deparaffinized, preheated and boiled (citric acid pH 6.0, 2 bar, 120°C, 20 minutes) in a pressure cooker. Sections were incubated with a MIB-5 antibody (dilution 1:50, 60 minutes; DAKO Cytomation, Glostrup, Denmark). After incubation with a secondary antibody (dilution 1:1, 30 minutes; 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 at 40X magnification and expressed as the percentage of positive cells per 1,000 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 according to standard laboratory methods.

Proinflammatory cytokine response

Liver samples were homogenized in buffer (phosphate-buffered saline solution, pH 6.0), centrifuged (10,000g; 4°C; 10 minutes) and supernatant was used for analysis of interleukin(IL)-1β, IL-6 and tumor necrosis factor (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-1b and IL-6, RnD Systems Europe Ltd, UK) 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 picograms per milligram protein.

**Hepatic neutrophil activity**

For neutrophil activation, hepatic myeloperoxidase activity was measured by as described by Krawisz et al. (15). Briefly, tissue samples were homogenized (PBS, pH 6.0), centrifuged for 10 minutes 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, 50°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 40 magnification in 30 high-power fields and expressed as the amount of positive cells per 1,000 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 PV ligation and both dual ligation groups at all time points compared to the sham and the HAL groups (Fig. 1A, B, respectively). Furthermore, the regeneration ratio in the dual-0 group was lower compared to the PV ligation and the dual-48 groups at all time points (p<0.05). At 24 hours, the regeneration ratio was significantly increased in the dual-48 group compared to the PV ligation group. At 24 hours, the hepatocyte proliferation index was significantly lower in the PV ligation group compared to the dual-0 and dual-48 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 dual-0 compared to dual-48 group at 24 hours and 48 hours after surgery (p<0.05) (Fig.1C).

![Graph A](image)

**Figure 1.** 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 HAL groups. The PV ligation and both the dual groups had significantly increased AST levels at 24 hours, 48 hours and 72 hours compared to SHAM group (p<0.05). In the dual-0 group, plasma AST was increased already at 6 hours compared to the all other groups and remained increased until 72h (p<0.05)(Fig.2A). In the dual-48 group, AST was also elevated already at 6 hours compared to the sham and PV ligation groups. In the PV ligation group, AST was increased at 48 hours and 72 hours compared to the dual-48 group (p<0.05). ALT followed the pattern of AST with the exception that there were no differences between both dual groups at 6 hours postoperatively (Fig. 2B).

Plasma bilirubin followed the pattern of AST and ALT in all groups (data not shown).

![Figure 2](image)

**Figure 2.** Plasma levels of hepatocellular damage markers AST (A) and ALT (B). These 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 concentration were seen between the sham and the HAL groups at all time points. Plasma albumin concentration was increased in the dual-0 group at 6 hours postoperatively compared to all other groups. Further in the both dual groups, at 24 hours the albumin was decreased compared to the SHAM and was up to 14 days decreased compared to the sham and the PV ligation groups (p<0.05). In the PV
Figure 3. Hepatocellular synthetic function evaluated by plasma albumin level, showed a prolonged dysfunction in both dual ligation groups. * = 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 and § = p<0.05 compared to DUAL48 group.

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 to SHAM and AHL groups, † = p<0.05 compared to PVL group and § = p<0.05 compared to DUAL48 group.

No differences were seen in plasma cytokines between the sham and the HAL groups at all time points. Plasma TNF-α was increased at 6 hours and 24 hours in the PV ligation and both dual groups compared to the sham group (p<0.05) (Fig. 4A). At 24 hours, TNF-α was elevated in the dual-0 group compared to the other groups (p<0.05). Plasma IL-1β at 6 hours was elevated in the PV ligation, the dual-0 and the dual-48 groups compared
to the sham group (Fig.4B). At 24 hours and 48 hours, the dual-0 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 PV ligation and in both the dual groups compared to the sham group at 24 hours (p<0.05) (data not shown). At 48 hours (Fig.4C), TNF-α was increased in the HAL, the PV ligation and both the dual groups compared to sham group and in the both the dual groups compared to the PV ligation group (p<0.05). At 48 hours, hepatic IL-1β was increased in the both

Figure 5. Figure 5A 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.

5BCDE 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.
dual groups compared to the other groups (p<0.05) and hepatic IL-6 was increased in PV ligation and the both dual groups compared to the sham group (p<0.05).

Hepatic MPO activity

Hepatic MPO levels evaluating neutrophil activity were increased at 24 hours in the PV ligation, the dual-0 and the dual-48 groups compared to the sham and the HAL groups (p<0.05). At 48 hours, the MPO level was significantly increased in the dual-0 group compared to the PV ligation and the dual-48 groups (p<0.05) (data not shown).

Histopathology

In the ligated and non-ligated liver lobes of the sham and HAL groups, no pathologic changes were seen. In the ligated liver lobes, the PV ligation 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 dual-48 group significantly less necrosis was visible at 48 hours and 14 days compared to dual-0 (p<0.05). In the dual-0 group a complete necrosis (Fig.5C) and in the dual-48 group an extensive fibrosis with occasional necrosis was seen (Fig.5D-E).

In the ligated lobes, the inflammation score was increased in the dual-0 and the dual-48 groups compared to the PV ligation group at all time points (Fig.6A). In the dual-48 group, the score was less compared to the dual-0 group after 48 hours and 14 days (p<0.05) (Fig.6A). In the nonligated liver lobes, the score was increased in the dual-0 group compared to the PV ligation and dual-48 groups at 24 hours (Fig. 6B). At 48 hours and 14 days, the score was significantly less in the PV ligation group compared to the both dual groups (p<0.05).

![Figure 6](image_url)

**Figure 6.** 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, PV ligation and 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 PV ligation, leading to an increased hepatocyte proliferation as seen 24 hours 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 PV ligation 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 that shows inadequate proliferation, apoptosis is most likely induced by another mechanism. One possible explanation is that inflammatory cytokines such as TNF-α activate caspase 3, subsequently triggering hepatocytes into apoptosis. In contrast, after PV ligation, no increase of apoptosis was detected. This is in accordance with a study by Ikeda et al that reported an unchanged number of apoptotic hepatocytes during hepatic proliferation at 24 hours and 48 hours in the remnant liver after heptectomy (17). In contrast, Kong et al report a beneficial effect on sequential ligation on liver regeneration (11). However, the length of follow-up was longer in the latter study, making the comparison of results difficult.

After PV ligation, hepatocellular damage was dramatically less than in the dual ligation groups. The initially observed necrosis after PV ligation had completely resolved and 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 therefore oxygen supply via the hepatic artery to the ligated liver lobes after PV ligation (18). This notion is supported by the increased hepatic IL-6 levels found after PV ligation 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 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 hepatocellular carcinoma 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 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 seen in both the dual-ligation groups is 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 hours 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 (22). In addition, 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, in which tumor destruction and induction of contralateral hypertrophy is desired.

Even though the biologic response after PV ligation model does not differ from embolization model in experimental studies, it is possible that in clinical setting this aspect is not the same. Also, another potential limitation when extrapolating these results into clinical situation is the different arterial and portal blood supply to liver seen in all rodents. Because 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 often present in patients undergoing liver surgery might influence the liver regeneration or affect the 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.

Reference List


