Associating liver partition and portal vein ligation for staged hepatectomy: establishment of an animal model with insufficient liver remnant

Alexandra Dili^{1,2} · Valérie Lebrun¹ · Claude Bertrand² · Isabelle A. Leclercq¹

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Abstract

Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) allows extended hepatectomy in patients with an extremely small future liver remnant (FLR). Current rodent models of ALPPS do not include resection resulting in insufficient-for-survival FLR, or they do incorporate liver mass reduction prior to ALPPS. Differences in FLR volume and surgical procedures could bias our understanding of physiological and hemodynamic mechanisms. We aimed to establish a rat ALPPS model with minimal FLR without prior parenchymal resection. In rodents, the left median lobe (LML) represents 10% of total liver. Partial hepatectomy (PHx) sparing LML and pericaval parenchyma represents our reference 87% resection. The first step in the procedure is either portal vein ligation (PVL) corresponding to ligation of all but the LML portal branches, or PVL with transection between the left and right median lobe segments (PVLt), and is defined as ALPPS stage-1. Second, ligated lobes were removed: PVL-PHx represents a conventional 2-stage hepatectomy, while PVLt followed by PHx is a strict reproduction of human ALPPS. In Group A, liver hypertrophy was analyzed after PVL ($n = 38$), PVLt ($n = 47$), T ($n = 10$), and sham ($n = 10$); In group B, mortality and FLR hypertrophy was assessed after PHx ($n = 42$), Sham-PHx ($n = 6$), PVL-PHx ($n = 37$), and PVLt-PHx ($n = 45$). In group A, PVLt induced rapid FLR hypertrophy compared to PVL ($p < 0.05$). Hepatocyte proliferation was higher in PVLt remnants ($p < 0.05$). In group B, PHx had a 5-day mortality rate of 84%. Sham operation prior to PHx did not improve survival ($p = 0.23$). In both groups, major fatalities occurred within 48 h after resection. PVL or PVLt prior to PHx reduced mortality to 33.3% ($p = 0.007$) or 25% ($p = 0.0002$) respectively, with no difference between the 2 two-stage procedures ($p = 0.6$). 7-day FLR hypertrophy was higher after the PVLt-PHx compared to PVL-PHx and PHx ($p = 0.024$). Our model reproduces human ALPPS with FLR that is insufficient for survival without liver resection prior to the stage-1 procedure. It offers an appropriate model for analyzing the mechanisms driving survival rescue and increased hypertrophy.

Introduction

Surgical resection is the only treatment with curative intent for patients suffering from a primary or secondary liver tumor. The extend of liver resection depends on tumor location, and/or burden. Case series show that, when

operated upon, patients with colorectal liver metastases, even multiple and initially unresectable, can achieve higher survival rates compared to chemotherapy alone [1]. Tumor burden but also tumor biology may have an important impact on survival [2, 3]. Recent guidelines propose to attempt curative resection [4, 5, 6], and this is also recommended for multiple metastases in spite of the lack of evidence from randomized controlled trials [7]. In order to propose resection, the surgeon must respect oncological limits and “liver-related” limits determined by the size and function of future liver remnant (FLR). Resection must leave in place at least 20–25% of the initial liver volume when the parenchyma is healthy, or 40% in case of diseased liver with reduced functional capacity [8]. If resection is performed without respecting hepatic-related limits, patients risk postoperative liver insufficiency due to

✉ Isabelle A. Leclercq
isabelle.leclercq@uclouvain.be

¹ Laboratory of Hepato-Gastroenterology, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain, Brussels, Belgium

² Department of Surgery, Centre Hospitalier Universitaire UCLouvain, Brussels, Belgium

“small for size syndrome (SFSS)”. SFSS is a clinical entity that combines postoperative hyperbilirubinemia, ascites and coagulopathy and is related to high mortality rates [9, 10].

To increase, when needed, the size of the future liver remnant, the gold standard is embolization or ligation of portal branches (PVL/E) nourishing the diseased, to-be-excised, liver in order to stimulate hypertrophy of the FLR by compensatory growth [11]. Portal vein occlusion achieves hypertrophy of the future liver remnant up to 52.8% of its initial size within 4–6 weeks, in some reports. However, up to 30% of the patients who gain insufficient liver mass recovery or experience a progression of their oncogenic disease during this period will be ineligible for liver resection [12].

Associated liver partition and portal vein ligation for staged hepatectomy (ALPPS) is a technique that combines portal vein ligation and parenchymal in situ split to obtain rapid and enhanced hypertrophy of the future liver remnant [13]. Although the use of the technique is controversial [12], the cautious selection of patients and indications seem paramount to reduce the high morbidity and mortality rates initially reported [14, 15, 16]. ALPPS is proposed for patients with an extremely small FLR [17], or even as a rescue technique for patients whose liver failed to hypertrophy after portal vein occlusion [18]. ALPPS patients obtain accelerated and enhanced FLR hypertrophy [19] without developing liver insufficiency [13, 20], despite the extent of parenchymal resection.

Since the first report published in 2012 [13], experimental attempts have been made to study the mechanism of enhanced FLR hypertrophy in ALPPS. To this end several ALPPS rodent models were developed. However, these models do not include liver resection with insufficient-for-survival FLR [21–26], or they do incorporate liver mass reduction prior to ALPPS technique [27, 28, 29]. Differences in volume of FLR and in surgical events could introduce bias in our understanding of pathophysiological and hemodynamic mechanisms. This study aimed therefore to establish a rat model of ALPPS with extended hepatectomy ($\pm 90\%$) without prior parenchymal resection mimicking the human procedure.

Methods

Animals

All animal experiments were conducted in accordance with European regulations and FELASA guidelines for human care for laboratory animals established by the Université catholique de Louvain (UCLouvain, Belgium), and the study protocol was approved by the university ethics committee.

Data were reported according to the ARRIVE guidelines. To avoid gender-related effects only male Wistar rats of ~350 g (UCLouvain Medical school, Brussels, Belgium) were used. Animals were housed in a temperature- and humidity-controlled environment in a 12-h light/12-h dark cycle. They had free access to food and water at all times before and after the surgical procedure. In order to take into account circadian variations in liver regeneration, all procedures were performed between 7 and 12 am. In a preliminary experiment, we measured the volume of the liver and of each individual lobe in 10 control rats.

Surgical procedures and experimental design

During the operative procedures, animals were allowed to breathe spontaneously in a glass cylinder filled with a mixture of oxygen (2 l/min) and isoflurane (2.5%) (IsoFlo, Zoetis BelgiumSA, Louvain-la-Neuve, Belgium). Animals had a median laparotomy after subcutaneous injection of 5cc of warm physiological sterile solution to increase the circulatory blood volume. Liver ligaments were gently freed and left lateral lobe (LLL) and median lobe (ML) were lifted with cotton-tipped sticks out of the abdomen and wrapped in gauzes to keep them humidified.

Conversely to the human liver, the rat liver is already partitioned, and the only lobe that receives two distinct glissonian pedicles is the median one: the right segment of the median lobe (RML) receives a pedicle directly from the hilum, whereas the left segment (LML) shares a common pedicle with the left lateral lobe (LLL). This bifurcation usually takes place into the LLL parenchyma, rendering the dissection of the portal vein branch from the arterial and biliary elements difficult. Indeed, into the parenchyma, glissonien elements are fragile, adherent, and surrounded by connective tissue. Based on microsurgical techniques already described [30, 31, 32], microscope-assisted dissection ($\times 20$ magnification) and pedicular hydrodissection permitted to individualize precisely the portal vein branch irrigating the LLL, which is posterior to the arterial and biliary elements. Arterial and biliary elements of the LLL were lifted with a Prolene 7/0 (Ethicon) to avoid damage. Cautious dissection allowed preservation of the whole glissonien pedicle destined to LML. LML represents 10% of the total rat liver mass [33, 31] and can be partitioned from the RML. Therefore, in all procedures (ALPPS, portal vein ligation or extended hepatectomy, see below), LML is the future liver remnant. Another technically difficult part of the operation is the dissection of the right lobe and of the pericaval parenchyma (evaluated at 2–5% of total liver weight [33, 31]) without damaging the inferior vena cava. Even with gentle fine microdissection, liver pericaval parenchyma could not completely be removed.

Fig. 2 LML is the ideal future liver remnant (FLR) as it represents approximately 10% of the total liver parenchyma and can be partitioned from its right counterpart. Analysis of relative weight of rat liver lobes compared to total liver weight in 10 control rats (**a + b**). Schematic representation of a partial hepatectomy leaving as FLR the left segment of the median and the pericaval tissue (87% of total liver weight) (**c**). LLL left lateral lobe, LC caudate lobe, LR right lobe, RML right medial lobe, LML left medial lobe, PC pericaval tissue

Assessment of liver growth and cell proliferation

Five markers of liver regeneration were evaluated: (a) The restitution of liver mass was determined as the weight of LML to body weight ratio (FLR/BW); (b) The Kinetic Growth Ratio (KGR) was assessed as the gain of LML weight/BW/day; (c) Immunohistological staining (IHC) and morphometrical quantification of nuclear expression of the proliferation marker Ki67; (d) DNA synthesis determined by immuno-detection of BrdU incorporated in newly synthesized DNA, and (e) mitotic figures of hepatocytes were counted on 8–10 high power fields ($\times 20$ magnification) per sample. To confirm the mitotic cell type, we performed double immunofluorescence staining. HNF4 α (R&D systems; PP-H1415-00; 1/350) for hepatocyte, pHH3 (Cell Signalling; 9701-S; 1/200) for mitotic cells, Hoechst for nuclear staining and Lyve-1 (R&D Systems; AF2125-SP; 1/100) with TSA (Thermo Fisher; Alexa Fluor™) for liver sinusoidal cell.

Liver injury assessment

Serum aspartate (ASAT) and alanine (ALAT) aminotransferases were measured using an automatic procedure in a serum multiple biochemical analyzer (FUJI DRI-CHEM NX500, Fujifilm Corporation, Tokyo).

Statistical analysis

GraphPad Prism software (San Diego, CA, USA) was used for graphs and statistics. In graphs, we report individual data (dots), the mean $\pm$ standard error (SEM). Survival curves were analyzed using the LogRang test (Mantel-Cox). Unpaired two-tailed *t*-test was used for simple comparison or one-way and two-way ANOVA followed by Bonferroni's post-hoc correction for multiple comparison. Statistical significance was assumed for *p* values < 0.05 (**p* < 0.05 ; ***p* < 0.01 ; ****p* < 0.001).

Results

The left segment of median lobe, an ideal FLR for our study

Ten rats were harvested as control group to analyze the relative weight of each lobe or segment compared to the total liver weight (Fig. 2a, b). In accordance with other authors [31, 33] the left segment of the median lobe represents 10.24% (range: 7.29–13.1%), while the pericaval parenchyma represents 2.7% (range: 1.1–5.8%) of the total liver (Fig. 2c). Thus, a partial hepatectomy leaving the LML + pericaval parenchyma as FLR corresponds approximately to a 87% hepatectomy.

Fig. 3 Accelerated liver regeneration after ALPPS step I procedure. **a** Weight of future liver remnant (LML)/ body weight evolution (two-way ANOVA: surgery: $p < 0.005$, timing: $p < 0.0001$; interaction: ns). **b** Kinetic Growth Ratio (KGR) calculated as the gain of LML weight/BW/day as per Fig. 1a protocol. **c** Hepatocyte proliferation assessed by quantification of Ki67 positive hepatocyte nuclei after PVLT: day 1 ($n = 10$), day 2 ($n = 4$), day 3 ($n = 5$); PVL: day 1 ($n = 7$), day 2 ($n = 4$), day 3 ($n = 5$); or Sham: ($n = 3$), (two-way ANOVA: surgery:

$p < 0.0001$, timing: $p < 0.0001$, interaction: $p < 0.001$). **d** Quantification of BrdU immunostaining (% of positive hepatocyte nuclei) after PVLT: day 1 ($n = 10$), day 2 ($n = 4$), day 3 ($n = 5$); PVL: day 1 ($n = 10$), day 2 ($n = 4$), day 3 ($n = 2$), Sham: ($n = 4$), (two-way ANOVA: timing: ns, surgery: $p < 0.001$, interaction: ns). **e** Mitotic count (per $\times 20$ microscopic field) in day 3 PVLT ($n = 6$); PVL ($n = 5$) and sham ($n = 3$) (One-way Anova: ns). * $P < 0.05$ and ** $p < 0.01$ using Student *t*-test. ns not significant

Compared to PVL, PVLT (ALPPS step I) boosts the hypertrophy of the FLR

There was an important release of ASAT and ALAT at day 1 after PVL and PVLT with no significant difference between the groups (data not shown). Both PVL and PVLT induced a hypertrophy of the FLR. The hypertrophy was however greater after PVLT than after PVL at day 2 and 3 ($p < 0.05$) but not at day 7 post-surgery (Fig. 3a). The kinetic growth ratio assessing the daily rate of growth confirmed that the recovery occurred during the first 3 days in PVLT while growth is more constant over the 7 days period after PVL (Fig. 3b). There was no hypertrophy of the LML in animals undergoing transection alone or in sham-operated animals (data not shown). The proportion of cycling hepatocytes, assessed by Ki67 (Figs. 3c, 4c) and the proportion of hepatocytes engaged into the DNA synthesis phase of the cell cycle (BrdU+) (Figs. 3d, 4b) were significantly higher in PVLT than in PVL at day 3. The number of mitotic figures at the 3 day time point was however similar between the 2 groups (Figs. 3f, 4a). Immunohistochemistry and double immunofluorescent staining confirmed that hepatocytes were the main cell type undergoing DNA synthesis and cell division at this time point (Figs. 4 and 5). Thus, PVL associated with

parenchymal in situ split accelerates FLR hypertrophy compared to PVL without transection.

ALPPS reduces mortality due to SFSS

Extended 87% partial hepatectomy (PHx) is associated with a high 84.21% mortality (16 out of 19) at day 7 (Fig. 6). Sham operation prior to 87% partial hepatectomy (Sham-PHx) did not improve survival compared to the upfront extended hepatectomy (83.33%, 5 out of 6, $p = 0.23$). In both groups, major fatalities occurred within the first 48 h after extended resection (PHx: 68.4% and Sham-PHx: 66.6%). Thus, the extended PHx provokes a SFSS.

When PHx is preceded by PVL (PVL-PHx) or PVLT (PVLT-PHx or ALPPS) the seven-day mortality caused by the hepatectomy significantly dropped, compared with PHx, to 33.3% (4 out of 12, $p = 0.007$) or 25% (4 out of 16, $p = 0.0002$), respectively, with no significant difference between the 2 two-stage procedures ($p = 0.6$) (Fig. 6a). Our surgical strategy that models ALPPS dramatically reduced the mortality due to SFSS that occurs with a minimal FLR.

5 days after hepatectomy, FLR hypertrophy was significantly higher after the PVLT-PHx (or ALPPS) procedure compared to PVL-PHx ($p = 0.024$) and to SFSS (PHx) survivors ($p = 0.024$; Fig. 6b).

Fig. 4 Histological illustrations of hepatocyte proliferation PVL and PVLT at day 3 after the operation. Representative pictures from **a** H&E-stained liver sections readily showing hepatocyte mitosis (black arrows point to the mitotic figures). **b** Identification of BrdU incorporation into newly synthesised DNA. Lower panel are a higher magnification and show BrdU incorporation preferentially in hepatocytes, although few non parenchymal cells (broken-line arrow) are also in the synthesis phase of the cell cycle. **c** Ki67, a marker of cycling cells, IHC. Bar size: 100 μ m

At day 1 after 87% partial hepatectomy, hepatocyte proliferation was already engaged as shown by the increased number of Ki67 positive hepatocyte nuclei (Fig. 6c). PVL triggered hepatocyte proliferation as at day 1 post PVL the proportion of Ki67 positive nuclei rose to the same extent as after PHx ($p = 0.5$). Importantly, PVLT alone, on the first day after the initial step, triggered an even higher regenerative response ($p = 0.0085$ compared to PHx; Fig. 6c). In addition, at day 5 post hepatectomy (step II procedure), the number of hepatocytes still engaged in DNA synthesis (BrdU + hepatocytes in Fig. 7) or in mitosis was significantly higher in PVLT-PHx compared to PVL-PHx ($p = 0.0027$; Fig. 6d). This supports that in addition to initial higher liver hypertrophy obtained after step I procedure, there is a higher rate of hepatocyte proliferation following hepatectomy concurring to increased liver mass recovery after ALPPS (Fig. 6b).

Discussion

ALPPS is a surgical technique implemented for patients needing hepatectomy with very small FLR. It achieves a rapid boost in FLR hypertrophy compared to the classical two-stage hepatectomy with portal vein ligation/occlusion. This accelerated and enhanced hypertrophy could diminish the drop out of planned resections after portal vein ligation or embolization due to progression of the underlying primary or secondary tumor or insufficient liver regeneration [34].

Since 2014, 9 publications describe rodent models of ALPPS. Dahr et al. initially proposed a model in which the right median lobe (corresponding to 26–28 % of the original liver mass (+3% of pericaval liver tissue) is the future liver remnant [21]. This model was subsequently used by several groups [26, 22, 24, 27]. In another model described by Perez et al. [23], the left median lobe, caudate lobe and

Fig. 5 Immunofluorescent staining of **a** HNF4 α , a hepatocyte specific transcription factor, in red, phosphohistone H3 (PHH3), a marker of mitotic cells, in green and nucleus in blue (DAPI) and **b** Lyve-1, a endothelial cell marker, in red, PHH3, in green and nucleus in blue (DAPI) in PVLV liver 3 days after first procedure confirming that hepatocytes (DAPI-HNF4 α + cells) but not LSEC (Lyve-1 + cells) undergo cell proliferation. Bar size 10 μ m

pericaval tissue amounting to 39% of the total liver volume represent the FLR. It is well documented that the rate of liver regeneration is related to the extent of hepatic resection [35] as well as to the extent of occlusion of the portal bed after PVL [36, 35, 37, 38]. In the context of extended hepatectomy Zhang et al [37] even highlighted that a 5% difference in the volume resected significantly impacts on mortality and regeneration rate. In all ALPPS models mentioned above, a large future liver remnant is left in place such as the resection corresponds to a 70% partial hepatectomy, an historical model with excellent survival and in which 7 days suffice to achieve full regeneration [39]. In this regards, the above models do not mimic the minimal, insufficient for survival FLR that would impose an ALPPS procedure in humans.

Other investigators resect part of the liver during the first step of the procedure: Wei and co-workers [27] resected the caudate lobe while Schlegel et al. [28], and Shi et al. [40], remove the left lateral lobe in mouse and rat, respectively, at the time of PVLV. This corresponds to an 8–30% upfront partial hepatectomy respectively or a wedge hepatectomy during human ALPPS procedure. Previous literature abundantly supports that such an hepatectomy cannot be seen as trivial as it is sufficient to prime the liver and engage a regenerative response [41, 42], thereby introducing a confounding factor when analyzing the ALPPS-dependent effects.

In the model we describe here a minimal, incompatible with survival FLR is left in place as proved by the tremendous mortality rate after PHx, unless ALPPS is performed. In addition, no liver tissue is resected during the first step of the procedure. This offers thus a clean model to analyze and understand the mechanisms driving the increase in hypertrophy of the FLR and the rescue of survival. We showed that the first step ALPPS procedure, namely PVL and parenchymal transection, rapidly engaged hepatocytes into cell cycle in an accelerated fashion and to a greater extent than conventional PVL. This accelerated entrance into regeneration allows for significantly increased mass and function recovery and animal survival after hepatectomy.

Metrics in FLR mass recovery were evaluated by mass measurements during harvesting. In order to integrate inter-individual differences in animals' body weight, we reported FLR mass as liver mass-to-body weight ratio, a valuable and largely used method to assess liver mass and liver mass recovery. Repeated CT or MRI volumetry, in which each animal serves as its own control, would probably be more adequate to appreciate FLR recovery but this would be at the cost of a higher stress to the animal and perhaps interference with the normal regenerative process. While 90% partial hepatectomy in rats usually associates with high mortality, some groups report little lethality, especially when the caudate lobe is left in place [31]. The capacity of regeneration of the individual remnant lobes is significantly

Fig. 6 ALPPS prevents mortality caused by extended hepatectomy. **a** Kaplan–Meier survival curve after PHx ($n = 19$), PVL-PHx (or ALPPS) ($n = 16$), PVL-PHx ($n = 12$) and Sham-PHx, $n = 6$). PVL-PHx but also two-stage hepatectomy (PVL-PHx) procedure significantly improved survival ($p = 0.0001$ using Log-rank (Mantel-Cox) test). However, Sham operation prior to 87% partial hepatectomy had no favorable impact on survival compared to an upfront extended resection ($p = 0.23$ using Log-rank (Mantel-Cox) test). The high mortality rate of PHx proved that our animal model represents SFSS. **b** Future Liver remnant (FLR) mass recovery after PVL-PHx procedure compared to extensive 87% hepatectomy and to PVL-PHx 5 days after surgery (ALPPS $n = 12$, PHx $n = 3$ survivors, PVL-PHx $n = 8$). One-way ANOVA proved a significant improvement in FLR mass recovery in ALPPS compared to PVL-PHx and to PHx ($p =$

0.024, respectively). **c** Ki67 HIC at day 1 after operation (87% hepatectomy, PVL = ALPPS step I procedure and PVL = PVL-PHx step I procedure) (Sham ($n = 3$), PVL-PHx ($n = 7$), PVL-PHx ($n = 10$), PHx ($n = 4$)). ANOVA one-way analysis showed a significant difference in proliferation of PVL-PHx and PVL-PHx compared to Sham ($p = 0.0055$ and $p < 0.0001$ respectively). Hepatocyte proliferation was significantly more important in the PVL-PHx group compared to PHx ($p = 0.0085$), whereas no significant difference was observed between PVL-PHx and PHx operations ($p = 0.59$). **d** Mitotic count (per $\times 20$ microscopic field) in day 5 post hepatectomy PVL-PHx ($n = 10$); PVL-PHx ($n = 7$), PHx ($n = 3$), and sham ($n = 3$) (One-way ANOVA and Bonferroni post hoc: $p = 0.0027$ in PVL-PHx compared to PVL-PHx)

different [43]. Differences in the nature of the FLR and in surgical dissection imposed by various anatomical localizations, genetic strains of the animals or age at the time of procedure may contribute to variable survival. The experiments reported in this study were performed by a senior surgeon with large experience in rodent liver surgery (>500). In addition, mortality curve in the cohort reported in the paper is similar to that of a separate cohort repeated months later.

In conclusion, we here describe the first rat model with minimal FLR, leading to high mortality due to SFSS unless ALPPS is applied. The degree of liver growth and kinetic growth ratio confirms that in situ split combined to PVL

boosts liver hypertrophy more than PVL alone. In addition, a second benefice might be expected as hepatectomy induces a higher rate of hepatocyte proliferation that concurs to increased liver mass recovery after ALPPS. This clean model reproduces the objectives intended in human conventional ALPPS and should be of great value for studying the physiological mechanisms leading to accelerated regeneration and rescue from SFSS.

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Fig. 7 BrdU immunostaining in Sham, PVL-PHx, PVLT-PHx (ALPPS), PHx 5 days post hepatectomy at low magnification

(upper panels) and higher magnification (lower panels). Bar size: 100 μ m

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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