THE ROLE OF PHYSICAL AND PHARMACOLOGICAL PREHABILITATION IN ACCELERATED LIVER REGENERATION

PhD thesis

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1. Introduction

Over the decades the limitations of liver surgery have immensely expanded. By improving intensive care and anesthetic methods, deepening the knowledge in hepatic anatomy and refining surgical technique, new doors have opened in the operating room and the boundaries of accomplishing safe liver resections are continuously widening. Now, we are living the era of liver surgery when the main and most important restraint of curative resection is the insufficient volume and function of future liver remnant (FLR) following operation. To overcome even this restrictive factor and further broaden the indication of major liver resections, augmentation techniques have been invented to push patients from unresectable to resectable status by inducing liver regeneration. Currently portal vein occlusion (PVO) techniques are standardly used in hepato-pancreato-biliary surgery to preoperatively increase FLR size, however still more than a third of patients must be excluded from performing major hepatectomy due to the insufficient growth rate of the FLR after PVO, which also requires a time interval, whilst tumor progression could compromise the second stage of the procedure. To overcome these major issues a novel surgical procedure, associating liver partition and portal vein ligation for staged hepatectomy (ALPPS), has been introduced, which could induce a more rapid and robust regeneration, offering a therapeutic approach for patients initially considered unresectable. However, unfortunately high postoperative morbidity and mortality were reported initially following ALPPS. In order to put ALPPS in favor of patients with hepatic malignancies, novel approaches

must be found to mitigate the adverse postoperative outcome. Prehabilitation is a new multimodal concept in oncological surgery increasing patients' bearing capacity and decreasing perioperative stress.

One crucial pillar of prehabilitation is physical prehabilitation (PP), which improves the functional status of the patient by various types of exercises. However, well-designed, mechanistic studies on the effect of preoperative exercise therapy still lack after major liver surgery, while there is no data to our knowledge regarding the effect of PP after ALPPS, which are hampering the comprehensive application of such a highly useful, easily utilizable, non-pharmacological therapeutic strategy.

Adding to this, the assessment of the signaling pathways regulating accelerated liver regeneration after ALPPS could help to identify pharmacological targets and create the ground for "pharmacological prehabilitation". By pharmacologically influencing these target molecules, beneficial effects similar to the ones of PP could be achieved in case of frail, weakened patients, who could not tolerate extra physical burden.

According to our previous studies, the improvement of mitochondrial dysfunction could contribute to better postoperative outcomes following ALPPS. The inhibition of Cyclophilin D (CypD) has recently been introduced as a mitochondrial therapy in liver diseases, however there is no literature data regarding the effect of CypD inhibition after two-stage hepatectomy as PVL or ALPPS.

Bile acids (BA) own a key role in the early phase of liver regeneration. BAs bearing mitotic properties are functioning as signaling molecules after binding and activating nuclear BA receptor farnesoid X receptor

(FXR), which triggers hepatocyte proliferation. Although the role of FXR pathway in liver regeneration following PVL has already been described, the relevance of the hepatic and intestinal pathways has not yet been distinguished and there is no literature data regarding FXR signaling after ALPPS.

2. Objectives

In our translational studies we aimed to identify potential preoperative therapeutic approaches to mitigate the adverse postoperative outcomes of ALPPS by investigating the effects of "physical prehabilitation" and identifying molecular targets to create the ground of the "pharmacological prehabilitation".

Aims of the I. experiment
I./1 the effect of physical prehabilitation on postoperative volumetric liver regeneration,
I./2 the effect of physical prehabilitation on postoperative functional liver regeneration,
I./3 the effect of physical prehabilitation on postoperative vulnerability in endotoxemia model,
I./4 the effect of physical prehabilitation on body composition,
I./5 the connection between changed body composition and improved postoperative outcome.

Aims of the II. experiment
II./1 the effect of CypD depletion on mitochondrial function,

II./2 the effect of CypD depletion on mitochondrial biogenesis,
II./3 the effect of CypD depletion on liver regeneration.

Aims of the III. experiment
III./1 the liver regeneration after ALPPS juxtaposed to PVL,
III./2 the hemodynamical changes after ALPPS juxtaposed to PVL,
III./3 the systemic and portal bile acid concentration after ALPPS juxtaposed to PVL,
III./4 the expression of bile acid transporters and production enzymes after ALPPS juxtaposed to PVL,
III./5 the hepatic and ileal FXR signaling pathway after ALPPS juxtaposed to PVL.

3. Methods

I. experiment
Male Wistar rats (n = 106) were divided to physical prehabilitation (PP) and sedentary (S) groups. Both groups underwent ALPPS procedure. Changes in liver weight, Ki67 index and liver volume by magnetic resonance imaging (MRI) was evaluated. Liver function was assessed by standard laboratory parameters (plasma aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and total bilirubin (tBil)). Dynamic liver function test ^{99m}Tc-mebrofenin nano single photon emission computed tomography (SPECT) hepatobiliary scintigraphy (HBS) was also performed. Characteristic parameters were computed from kinetic curves such as time of maximum (T_{max}) and tracer half-life

($T_{1/2}$). The uptake of the FLR can be characterised by the midpoint of the ascending section of the curve, which fits well with a linear function. The difference in isotope concentration in the bound lobe and in blood was determined at two time points. This value is then normalized by the applied activity. The washout was determined with same method at the descendent section of the kinetic curve after the inflexion point of the isotope activity curve. In a lipopolysaccharide (LPS) induced endotoxemia model postoperative vulnerability was investigated by determining mortality and sepsis-related laboratory parameters (C-reactive protein level, platelet count, neutrophil percentage (%) and lymphocyte %). In vivo fat and muscle volumes were determined by MRI volumetry.

II. experiment

Male wild type (WT) BL6/jk (n=30) and CypD KO (n=30) mice (originating from the C57Bl6/J strain, mated with C57Bl6/F mice and afterwards backcrossed with C57Bl6/J mice for at least eight generations to ensure homologous genetic background) underwent ALPPS procedure. Animals were terminated pre-operatively and 24, 48, 72 or 168 h after the operation. Mitochondrial functional studies (ATP production and oxygen consumption) and proteomic analysis of mitochondrial biogenesis proteins (peroxisome proliferator-activated receptor γ co-activator 1-α (PGC1-α), Nuclear Respiratory Factor (NRF) 1, oxidative phosphorylation system (OXPHOS), caspase-3) were performed. Regeneration rate and cell proliferation with ki67 index and mitotic rate were assessed.

III. experiment

Male Wistar rats underwent portal vein ligation (PVL) (n = 30) or ALPPS (n = 30). Animals were sacrificed pre-operatively and at 24, 48, 72, or 168 hours after intervention. Regeneration rate, Ki67 index, hemodynamic changes (liver microcirculation and portal pressure), and systemic and portal BA levels were assessed. Transcriptome analysis of molecular regulators involved BA transport, and BA production (sodium taurocholate co-transporting polypeptide (Ntcp), organic anion transporting polypeptide (Oatp)1a4, multidrug resistance protein (Mrp)3, Mrp2, bile salt exporting pump (Bsep), cytochrome P450 isoform 7A1 (Cyp7a1)) were performed. Transcriptome analysis of molecular regulators of the FXR signaling pathway (hepatic Fxr, forkhead box (Fox)m1b, hepatic Shp, ileal Fxr, ileal Shp, fibroblast growth factor (Fgf)15, fibroblast growth factor receptor (Fgfr)4) was also conducted.

4. Results

I. experiment

ALPPS induced more expressed liver growth of the FLR in the PP group compared to the S group, resulting in a significant difference between the groups from 48 h until the end of the experiment. Supporting the increase observed in liver mass, Ki67 index was also higher in the PP group. MRI liver volumetry also showed increased volume of FLR in the PP group than in the S group.

The level of ALT and AST were significantly lower in the PP group at 24 h compared to the S group. Similarly, tBil

level was significantly lower in the PP group compared to the S group at 24 h.
Measured with ^{99m}Tc-mebrofenin HBS, T_{max}, which characterizes the organic anion uptake capacity of the liver and ^{99}mTc-mebrofenin uptake did not show difference between the S and PP group. However, $T_{1/2}$ was significantly lower at 48 h in the PP group compared to the S group, indicating a more effective hepatic excretion after ALPPS in the PP group. Supporting this, ^{99}mTc-mebrofenin washout was significantly higher in the PP group compared to the S group at 48 h, too.
In the vulnerability a notable difference could be observed between the two groups, as the survival rate after LPS injection was 91.67 percent in the PP group, while only 41.67 percent in the S group. Laboratory results also showed better stress-tolerance in the PP group, as the CRP level was higher, platelet count lower, neutrophil % higher and lymphocyte % lower in the S animals compared to the PP group, indicating markedly expressed inflammation, thrombocytopenia, neutrophilia and lymphocytopenia in the sedentary animals.
Confirming that exercise was properly implemented, the percentage of both visceral and subcutaneous fat was reduced in the PP group compared to the S group on the preoperative MRI. On univariable linear regression analysis, the subcutaneous fat percentage correlated with FLRV at 48 h, while only a tendentious correlation could be observed at 120 h.

II. experiment
Endogenous and stimulated ATP production of remained significantly preserved in the CypD KO group compared

to the WT group. Basal and stimulated oxygen was notably higher in the CypD KO group compared to the WT group. PGC1-α was significantly elevated in the CypD KO group compared to the WT group. Despite of the changes in PGC1-α, NRF 1 level did not change significantly neither in the WT, nor in the CypD KO group.

Level of OXPHOS complex I in the CypD KO group was higher compared to the WT group preoperatively and at 24 h, in case of complex II the same disparity could be observed at 48 h, at the most vulnerable time points following ALPPS.

The level of uncleaved proenzyme form of caspase-3 did not change in the WT group, while a significant increase could be observed from 48 h until the end of the experiment in the CypD KO group. The level of the activated cleaved form of caspase-3 was elevated at 24 h in the WT group and remained increased by the end of the experiment, which is in line with previous results on apoptotic activity following ALPPS. In contrast, active caspase-3 level in the CypD KO group increased significantly only from 72 h to168 h, in the remodeling period of ALPPS.

Both in the WT and CypD KO groups, liver mass gradually increased. However, while liver growth achieved only about 100% growth in the WT group, the CypD KO group displayed a 150% growth, resulting significantly higher liver mass in the CypD KO group at 168 h compared to the WT group. No difference could be observed nor between the mitotic rates either the ki67 index of the groups.

III. experiment

Increase in liver mass of the FLR was higher in the ALPPS group compared to the PVL group at all time points. The Ki67 index in the FLR was higher in the ALPPS group compared to the PVL group.

Contrived occlusion of blood supply to portions of the liver causes a divergence of blood flow between the ligated segments and the FLR. In line with this, both PVL and ALPPS resulted in an increase in microcirculatory flow in the right median lobe while flow in the ligated lobe had decreased compared to the pre-ligation flow. The transection part of ALPPS exerted no additional effect on flow in the FLR but further decreased flow in the ligated lobe. Both interventions were associated with an increase in portal pressure immediately and at 24 h post-intervention, after which the pressure gradually normalized by the end of the experiment. The increase in portal pressure was greater in the ALPPS group compared to the PVL group during the first 24 h.

Systemic and portal BA concentration were higher in the ALPPS group compared to the PVL group.

No difference in the transcript level of Ntcp, Oatp1a4, Mrp2, Bsep, and Cyp7a1 could be observed between the ALPPS and PVL groups. The transcript levels of the basolateral exporter Mrp3 were significantly higher in the ALPPS group compared to PVL.

Hepatic Fxr levels decreased after PVL in the hypertrophy response phase (72 h to 168 h). ALPPS also caused a decline in Fxr levels, but earlier than 24 h, and remained lower relative to preoperative levels up to 168 h after the intervention. No intergroup differences were observed at any of the time points. Forkhead box (Fox)m1b, the

downstream target of Fxr, exhibited a similar trend to Fxr in the PVL-exposed animals in that its transcript levels were decreased compared to baseline. In the ALPPS group Foxm1b levels were also lower relative to preoperative levels at all time points. Shp did not seem to interfere in BA signaling following PVL and ALPPS given the absence of dysregulation.

Ileal Fxr showed no change after PVL but exhibited a spike at 24 h after ALPPS, while remaining at similar levels to baseline at all other time points in both groups. This resulted in a notable difference between ALPPS and PVL at 24 h. Concomitantly, Shp had remained unchanged in the PVL group, while its upregulation was observed in the ileum of ALPPS-subjected animals at 24 h post-intervention, which had receded to baseline levels at the subsequent time point. Ileal Fgf15coincidentally did not change in the PVL group. A single time point spike was observed after ALPPS at 24 h. Upregulation of Fgfr4 was not observed after PVL. ALPPS-exposed hepatocytes had upregulated Fgfr4 at 24 h, 48 h, and 168 h.

5. Conclusions

Conclusions of the I. experiment:

I./1 Our study revealed the beneficial impact of PP on volumetric liver regeneration, as it could further augment the inherently robust regeneration after ALPPS.

I./2 Along with volumetric growth of the liver, PP also improved the functional regeneration after ALPPS.

I/3 Moreover, our study raised evidence that PP improved the vulnerability following ALPPS, and hence significantly reduced the postoperative mortality.

I/4 The validity of our model was also confirmed, as PP notably improved the body fat composition of the animals.
I/5 Our study also revealed the correlation between reduced body fat composition and enhanced volumetric liver regeneration after ALPPS, strongly confirming the beneficial effect of PP.
Based on the findings of the I. experiment, the disadvantageous aspects of ALPPS could be mitigated with physical prehabilitation, therefore we propose the further clinical implementation of preoperative aerobic physical exercise protocols to improve patients' safety after surgery.

Conclusions of the II. experiment:
II./1 CypD depletion significantly enhanced mitochondrial function, as both ATP production and oxygen consumption improved after ALPPS.
II./2 Along with enhanced function, CypD depletion also enhanced mitochondrial biogenesis following ALPPS.
II./3 CypD depletion improved liver regrowth after ALPPS, possibly via the alteration of apoptosis.

The results of the II. experiment draw attention to the relevance of mitochondrial therapy after ALPPS. Therefore, we propose the further investigation of CypD inhibition as a potential target of "pharmacological prehabilitation" to enhance the post-operative outcomes following ALPPS.

Conclusions of the III. experiment:

III./1 Our model was adequately utilized, as liver growth induced by ALPPS was significantly greater compared to PVL.

III./2 Hemodynamical changes were more expressed after ALPPS compared to PVL, as microcirculation notably decreased in the ligated lobes while portal pressure parallelly increased in the FLR.

III./3 Both systemic and portal BA concentrations were considerably higher following ALPPS than PVL.

III./4 Nor the expression of BA production enzymes, nor BA transporters changed significantly with the exception of the Mrp3 basolateral transporter.

III./5 In the final analysis we exposed that BA-activated mitotic signals could be characterized by the activation of the intestinal Fxr pathway rather than hepatic Fxr signaling after ALPPS.

In conclusion, the findings of the III. experiment point to a different pattern of BA-induced liver regeneration following ALPPS. Based on these results, we propose that intestinal Fxr should be investigated as a potential therapeutic target of “pharmacological prehabilitation” to enhance post-operative outcomes following ALPPS, which can be achieved through mitogenic BA mimetics that have been cleared by regulatory agencies and are being clinically implemented for other indications.

6. Bibliography of the candidate's publications

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