FINAL REPORT

In the treatment of primary and secondary liver malignancies, surgical resection is usually considered the only curative therapeutic option. Despite recent and ongoing advancements in surgical techniques, the mortality rates of extended liver resections are still high. The rapid onset of posthepatectomy liver failure (PHLF) is responsible for 75-80% of postoperative deaths. Several causes can be assumed in the initiation of PHLF, however, the insufficient volume of the remaining liver after the operation (future liver remnant (FLR)) proved to be an important, independent factor. Recently, various surgical interventions have been developed to increase the volume and functional capacity of the FLR. These approaches are based on the unique regeneration ability of the liver.

The so called portal vein occlusion (PVO) techniques are leading to the redirection of portal blood away from the liver lobes that are planned to be resected towards the anticipated FLR and resulting in FLR hypertrophy ("programmed" regeneration), while the portal deprived liver lobes undergoing atrophy (atrophy/hypertrophy complex). These surgical interventions make the removal of high tumor load possible without the risk of PHLF. If hepatectomy is urgently needed (e.g. aggressive tumor growth and invasion) classic PVO methods (PVL/PVE) cannot be used due to the slow regeneration of the liver. A novel 2- staged surgical procedure called ALPPS (Associating Liver Partition and Portal vein ligation for Staged hepatectomy) was introduced in 2011 to overcome this issue. In ALPPS, PVL is combined with parenchymal transection (in situ splitting - ISS) during the first step of the operation, which leads to significantly higher regeneration rate and shorter regenerative period compared to the classic PVO techniques.

Although the PVO or ALPPS induced liver regeneration have been examined intensively in the past few years, several important issues regarding the induced regenerative process remains unanswered.

The main goal of the present project was the investigation of programmed liver regeneration, induced by PVO techniques and ALPPS in experimental and clinical scenarios.

I.) PVO AND "PROGRAMMED" LIVER REGENERATION

I/a Assessment of PVO induced morphological alterations:

In our experiment, we surgically ligated portal branches supplying 70, 80 and 90% of the liver mass and asses the alteration in liver morphology and circulation.

The total liver mass remained essentially unchanged in all groups throughout the 7 days of the experiment, which can be attributed to the balance of the atrophy of ligated lobes and the hypertrophy of non-ligated lobes. The regeneration ratio in case of a 90% ligature significantly exceeded the values of both the 80% and the 70% occlusion.

Histologic analysis of the non-ligated lobes showed a marked increase in the mitotic cell count in each of the three experimental groups. The mitotic activity in the PVL 90% group significantly exceeded the mitotic activity found in groups PVL 80% and PVL 70% groups. The hematoxylin-eosin stained slides of the ligated lobes showed prominent necrotic cell degradation, predominantly around the central veins. At the 24 postoperative hour, extended necrotic zones were visible in each groups. Thereafter, with simultaneous appearance of inflammatory cells, the percentage of necrotic areas decreased gradually. By the end of the experiment, complete resorption and microstructural restoration was observable without postnecrotic fibrosis.

PVL provoked an immediate and steep increase of portal pressure in each of the experimental groups. The increase in portal pressure strongly correlated with the volume of liver parenchyma affected by the ligation. Consequently, the spleen weight showed a significant increase in each group. Our results support the anticipated role of portal hypertension concerning the induction and regulation of liver regeneration ("blood-flow theory").

Related article:

Lauber DT, Tihanyi DK, Czigany Z, Kovacs T, Budai A, Drozgyik D, Fulop A, Szijarto A: Liver regeneration after different degrees of portal vein ligation., J SURG RES 203: (2) 451-458, 2016.

I.b. Assessment of functional alterations after PVO

- organic anion (bilirubin, bile salts, etc.) transport

For the basic evaluation of functional changes cell cultures were created. Hepatocytes from both lobes unequivocally maintain polarity and bile duct formation capacity. To assess in vitro function taurocholate and bilirubin transport measurements were performed. Results of the in vitro analysis confirmed a transient, but marked reduction of taurocholate and bilirubin transport in both lobes without significant intracellular accumulation.

To assess in vivo bile excretion, selective biliary drainage of the ligated and non-ligated was performed. The bile output was progressively decreased, and oppositely, significantly increased in the ligated and non-ligated lobes, respectively, with unchanged overall bile output.

To assess in vivo global hepatic functions dynamic liver function tests were performed. The ICG-clearance test exhibited a marked drop between 24–72 h, which rapidly normalized by 168h. These results were echoed by the hepatobiliary scintigraphy (HBS) test. Blood half-life, reflecting global hepatic uptake function significantly increased at 48–72h, and rapidly normalized thereafter. First duodenal appearance, reporting on global hepatic excretory function showed similar changes. Taken together, contrary to the excretion of endogenous organic anions such as bilirubin, the elimination of exogenous ligands suffered a temporary reduction, indicating a transient suppression in global liver function.

Nuclear imaging techniques enable sophisticated evaluation of regional liver function. Therefore, a planar 99mTc-mebrofenin HBS test was performed to assess hepatic function separately in the ligated and non-ligated. Our observations indicate a transient, bilateral

deterioration of regional liver function, followed by the compensatory functional gain of the non-ligated lobes resulting in a massive divergence of liver function.

To further investigate local changes in liver function Cellvizio® endomicroscopy was applied on the non-ligated lobes to evaluate the uptake and excretion of ICG by hepatocytes. Accordingly, the excretory function of non-ligated lobes temporary decline in the first 48 hours. After the 2nd day gradual functional increment can be assessed.

In conclusion, the induced liver regeneration causes significant alterations in global and regional liver function. The observed shift of hepatic function towards the non-ligetad lobes, leading to massive liver functional inhomogeneity underlines, that beyond volumetric analysis, liver functional testing is critically important during PVO procedures.

- Cytochrome P450-Mediated Drug Metabolism

Pentobarbital-induced sleeping time was measured to evaluate in vivo hepatic CYP activity. Sleeping time gradually increased after PVL with a significantly elevated peak at 72 h, and thereafter normalized to control levels.

The "intrinsic" microsomal CYP activities were assessed by high-performance liquid chromatographic analyses. Transcriptional expression of CYP enzymes were analyzed by RT-PCR. We verified a solid, disparate difference between the drug metabolism of the ligated and non-ligated lobes. As a result of an adaptive response of active transcriptional upregulation, drug metabolism in the non-ligated lobes was increased and re-established, meanwhile permanently depressed in the ligated lobes. Hepatic drug metabolism became overwhelmingly redistributed, depending nearly exclusively on the non-ligated lobes. Our findings also proved the isoform-dependence of CYP enzyme activity and transcription alterations following PVL in the rat (somewhat different expression and activity profiles of CYP1A, CYP2B, and CYP3A as compared to CYP2C enzymes).

In conclusion, the alterations in hepatic drug metabolism and underlying CYP enzymes is critically important for a better understanding of post-PVO liver function, as well as for the prevention of drug-related complications. Our study verified a transient suppression of global hepatic function, followed by the adaptive response of non-ligated and the permanent deterioration of the ligated lobes, resulting in solid interlobar differences. Furthermore, changes were found to be isoform dependent. Results may contribute to knowledge regarding liver function as well as personalized medication strategies during induced liver regeneration, although further investigation is required.

Related articles:

Lauber DT, Fulop A, Kovacs T, Szigeti K, Mathe D, Szijarto A: State of the art in vivo imaging techniques for laboratory animals, LABORATORY ANIMALS: THE INTERNATIONAL JOURNAL OF LABORATORY ANIMAL SCIENCE AND WELFARE 51: (5) pp. 465-478., 2017

Kovacs T, Mathe D, Fulop A, Jemnitz K, Batai-Konczos A, Veres Z, Torok G, Veres DS, Horvath I, Szigeti K, Homolya L, Szijarto A: Functional shift with maintained regenerative potential following portal vein ligation, SCIENTIFIC REPORTS 7: 18065, 2017

Kovacs T, Deri M, Fulop A, Palhazy T, Hafra E, Sirok D, Kiss AF, Lotz G, Szijarto A, Monostory K: Isoform-Dependent Changes in Cytochrome P450-Mediated Drug Metabolism after Portal Vein Ligation in the Rat., EUROPEAN SURGICAL RESEARCH 59: (5-6) pp. 301-319., 2018

I/c Assessment of the molecular mechanism of liver regeneration - the role of miRNA:

The recently discovered non-protein-coding RNAs, miRNAs constitute a class of endogenous post-transcriptional regulators of genes that orchestrate proliferation in organogenesis and cancer evolution. Their role, however, in liver regeneration is largely unknown and have not been investigated in case of PVL/ALPPS.

In the present project we plan to evaluate the miRNA induction during the PVL/ALPPS induced regenerative process.

Microarray analysis: Changes of the miRNA pattern in the regenerated lobe/non-regenerated lobe was assessed by Affymetrix® miRNA 4.1 Array. We identified 83 miRNA which were shown significantly different (increased/decreased) expression profile after the postoperative 4th hours between the ALPPS and PVL groups. We have taken this information and we identified 7 miRNA (miR-21-5p, miR-155-5p, miR-200b-3p, miR-350, miR-455-5p, miR1224, miR-1848-5p) which play role in liver regeneration by DAVID 6.8 (Database for Annotation, Visualization and Integrated Discovery, https://david.ncifcrf.gov/) target prediction database.

Related articles:

Publication is in progress.

I/d Clinical evaluation of PVO

On the basis of study I/a-c we have started a human liver tissue based investigations, which will include the checking of the aforementioned Cyp enzyme profiles from human blood and liver specimen gained during portal vein embolization and liver resections.

Since the number of PVO procedures decreased significantly in the last years, the sample collection delayed compared to planned. Evaluation of the data are in progress.

II.) PRECLINICAL AND CLINICAL INVESTIGATION OF ALPPS

II/a small animal models of ALPPS

Our surgical research group created a rat model similar to the one reported by Yao et al. After the ligation of the portal branches leading to the right lateral, left median, left lateral, and caudate lobes, the transection is performed with intraparenchymal U sutures and blunt preparation alongside the transition line (visible border between the perfused and deportalized

liver lobes). Any encountered intraparenchymal portoportal shunts are ligated and cut. In this manner, blood loss can be minimalized and major damage to the hepatic veins can be avoided. Electrocautery is carefully utilized to seal the liver wounds of the median lobe but without damaging too much parenchyma. This model proved to be standard and reproducible.

Related articles:

Budai A, Fulop A, Hahn O, Onody P, Kovacs T, Nemeth T, Dunay M, Szijarto A: Animal Models for Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy (ALPPS): Achievements and Future Perspectives, EUROPEAN SURGICAL RESEARCH 58: (3-4) pp. 140-157., 2017

II/b morphological and functional alterations after ALPPS

- *Liver regeneration*

The weight gain and pace of hypertrophy of the non-ligated lobes was significantly higher after ALPPS than after PVL. The Ki-67 index was also significantly higher in the ALPPS group compared to PVL group.

- *Mitochondrial function*

Isolated mitochondrial function was measured using colorimetric reaction for ATP production (adding succinate or glutamate-malate to the mix induces the respiratory complexes). For oxygen consumption measurement high resolution respirometer was used. According to our results PVL-induced liver regeneration is a well-balanced process, which has a sufficient energy supply provided by the increased mitochondrial function. Regarding ALPPS, although oxygen consumption and ATP production increased temporarily after the operation, mitochondrial activity decreased suddenly at 48h, resulting in significantly reduced mitochondrial function compared with that in the PVL group. Accompanied by intense cell division, these changes may lead to imbalances in energy demand and supply of hepatocytes. The energy imbalance of hepatocytes increases the vulnerability of the liver after the operation.

- *Mitochondrial biogenesis*

Mitochondrial function is maintained through mitochondrial biogenesis. Therefore, the proliferator-activated receptor γ co-activator 1-α (PGC1-α) controlled biogenesis pathway was assessed. PVL-induced liver regeneration is characterized by upregulated, well balanced mitochondrial biogenesis, which results in sufficient energy production for the immense cell division. The PGC1-α concentrations, however, showed different kinetics in the ALPPS group. PGC1-α levels remained significantly lower in the ALPPS group than in the PVL group. In addition, the mitochondrial transection area was also significantly smaller at 48 h in the ALPPS group. These data, together with the functional and biogenetic parameters, suggest that, although the number of mitochondria increases after ALPPS, these organelles are rather immature and lack proper function owing to inadequate biogenesis. According to the authors' hypothesis, the immense inflammation – a critical factor in ALPPS-induced accelerated liver regeneration – might suppress mitochondrial biogenesis.

In sum, ALPPS caused an overwhelming inflammatory response that interfered with the peroxisome proliferator-activated receptor γ co-activator 1-α-coordinated, stress-induced,

mitochondrial biogenesis pathway. This resulted in the accumulation of immature and malfunctioning mitochondria in hepatocytes during the early phase of liver regeneration (bioenergetic destabilization). These findings might explain some of the high morbidity if confirmed in patients.

Since physical exercise has positive effect on mitochondrial function, it could be used (physical prehabilitation) before ALPPS to mitigate energy imbalance of hepatocytes. Therefore, our further aim was to assess the beneficial effects of exercise on mitochondrial function and biogenesis after ALPPS.

Regeneration rate, as well as cell cycle entry characteristics were profoundly influenced by physical prehabilitation. In the ALPPS+Prehabilitation group regeneration rate and Ki-67 index significantly surpassed those of the ALPPS group. No clear explanation can be found for this phenomenon, but a few effects must be counted in:

1. Prehabilitation leads to better ATP production with the same amount of O2-consumption after ALPPS.

2. ALPPS leads to a massive inflammatory response, while prehabilitation increases the expression of anti-inflammatory cytokines.

3. Prehabilitation enhances mitochondrial biogenesis via PGC1-Alpha pathway.

As physical exercise has positive effect on mitochondrial biogenesis and function, it could be used before ALPPS to decrease postoperative morbidity and mortality.

Related articles:

Budai A, Horvath G, Tretter L, Radak Z, Koltai E, Bori Z, Torma F, Lukats A, Rohlich P, Szijarto A, Fulop A: Mitochondrial function after associating liver partition and portal vein ligation for staged hepatectomy in an experimental model, BRITISH JOURNAL OF SURGERY 106: (1) pp. 120-131., 2019

Budai A, Szijarto A, Fulop A et al.: Improved Liver Regeneration with attenuated inflammatory response - The effect of aerobic prehabilitation on mitochondrial function in an Associating Liver Partition and Portal Vein Ligation for Staged Hepatectomy (ALPPS)- rodent model. Under publication.

II/c Assessment of the molecular mechanism of liver regeneration - the role of miRNA:

See above (I/c chapter).

II/d Clinical evaluation of ALPPS

Since the number of ALPPS procedures decreased significantly in the last years, the sample collection delayed compared to planned. Evaluation of the data are in progress.