Title：The maturity of ALPPS-derived liver regeneration in a rat model

Abstract: Despite the rapid proliferation of future liver remnant induced by associating liver partition and portal vein ligation for staged hepatectomy (ALPPS), the high mortality and morbidity remained alarming. A plausible reason was the functional proliferation lagged behind increase in volume. In this study, a rat model was established to evaluate the maturity of ALPPS-derived hepatocytes. Through the identification of hepatic characteristics, detection of liver function, and analysis of functional gene expression, we revealed the immaturity of ALPPS-derived proliferation in early regenerative response, which indicated volumetric assessment overestimated the functional proliferation. And clinically, the stage II of ALPPS should be performed prudently in patients with marginally adequate FLR, as the ALPPS-derived proliferation in volume lags behind the functional regeneration.

Background: With the improvement of technology and accumulation of experience, the mortality of ALPPSS, decreased to less than 10%, remains too high.[13] A rational possibility is that volumetric assessment overestimating the functional. Thus, we establish this rat model to minic the ALPPS procedure in clinic and thereby to evaluate the proliferation functionallly. The traditional PHx and sham models were regarded as positive and negative control group. Each groups contained 6 rats.

Objectives: The functional proliferation is the primary objective and the volumetric proliferation iss the second objective.

Ethical statement: The protocol of this study was reviewed and approved by the animal ethics committee of the Zhejiang University, Hangzhou, China. All experiments were performed in accordance with relevant approved guidelines and regulations.

Study design:

The ALPPS, PHx, and sham groups were defined as experimental, positive and negative control groups in this study. ALPPS group: ligation of the portal vein belonging to left lateral, right, caudate lobes, and transection of parenchyma of middle lobe. PHx group: removal of left lateral, right and caudate lobes. Sham group: Open and close the abdominal cavity. .

Flowchart: Preliminary study------Establishment of ALPPS, PHx and sham groups-----comparison of the proliferation in different groups------The characteristics of induced hepatocytes----Detection of hepatocyte function------Cluster analysis of different expression of functional genes

Experimental procedures: All rats were fasted 8 hours before operation. Under the general anesthesia with 8% of chloral hydrate (5.0 ml/kg) by intra-abdominal injection, the abdominal transverse incision was adopted. For the ALPPS procedure, dissection of the left lateral lobe followed by ligation of the portal vein supplying the corresponding lobe with 5-0 silk were performed. While artery and biliary duct branches were maintained. Then, the same procedure was conducted in the portal branches of the right and caudate lobes, respectively. Along with the ischemic demarcation line of the middle lobe, parenchyma was partitioned by 5-0 silks. And five days after stage I, the stage II was performed, in which the deportalization lobes were removed. For PHx model, the left lateral, right and caudate lobes were removed after corresponding hepatic pedicle were ligated with 3-0 silks. And for sham group, Opening followed by closing the abdominal cavity was performed.

Experimental animals: In the present study, male Sprague-Dawley rats, weighing 180 to 230 g from experimental animal center of Zhejiang province, Hangzhou, China, were used.

Housing and husbandry: All the rats were housed in a restricted access room with controlled temperature (23 ℃) and a light/dark (12 hours: 12 hours) cycle, and freely access to food and water before and after treatment.

Sample size: Each group in different time points contained six rats. Half of them were used for evaluating the efficiency of proliferation, and the other three rats were used for primary hepatocyte isolation and subsequent detection of hepatic function. Each study contained three reduplications.

Allocating animals to experimental groups: Randomly allocation, and all the feeding condition are the same.

Experimental outcomes: In our study, ALPPS, partial hepatecotmy (PHx) and sham rat models were established initially. In the setting of accelerated proliferation in volume at second and fifth day after ALPPS, the characteristics of newborn hepatocytes were identified. Afterwards, the detection of liver function followed by cluster analysis of functional gene expression were performed to evaluate the maturity.

Statistical methods: Data are expressed as mean with standard deviation or number with percentage. Correspondingly, Student T test or Chi-square test was used to analyze the difference. Significance was considered when a two-tailed P value was less than 0.05. Statistical analysis was performed using SPSS, version 22.0 for Windows (IBM Corporation, Armonk, NY).

Baseline data: In the present study, male Sprague-Dawley rats, weighing 180 to 230 g from experimental animal center of Zhejiang province, Hangzhou, China, were used.

Numbers analysed: Initially, a preliminary study was simply performed to screen the feasible models (n=5, each group). Then in formal study, each group in different time points contained six rats. Half of them were used for evaluating the efficiency of proliferation, and the other three rats were used for primary hepatocyte isolation and subsequent detection of hepatic function.

Outcomes and estimation：Compared with PHx and sham groups, the proliferation of FLR was significantly high in ALPPS group ($p=0.023$ and 0.001 at second day, $p=0.034$ and $p<0.001$ at fifth day after stage I). However, the characteristics of ALPPS-induced hepatocytes indicated limited function in early proliferative stage. Additionally, the detection of liver function and functional genes expression confirmed the immaturity of renascent hepatocytes derived in early stage of ALPPS-derived liver regeneration.

Adverse events：Initially, the feasibility and proliferative capacity of different models were compared in the preliminary experiment. The sham group was adopted as baseline control. For ALPPS, the stage II, removal of deportalization lobes was performed in the fifth day after stage I (n=5). The mortality of extended PHx group (removal of left lateral, left middle, right and caudate lobes, n=5), which presented

the same extension of stage II of ALPPS, was 80%. On the contrary, no rat in ALPPS group (removal of left lateral, left middle, right and caudate lobes) was dead in this study. This indicated ALPPS could decrease the mortality (0% vs 80%, p=0.053) by inducing rapid hepatic proliferation.

Interpretation/scientific implications： Our study revealed the immaturity of ALPPS-derived proliferation in early regenerative response, which indicated volumetric assessment overestimated the functional proliferation.

Generalisability/translation：This could be a convincing evidence that the stage II of ALPPS should be performed prudently in patients with marginally adequate FLR, as the ALPPS-derived proliferation in volume lags behind the functional regeneration.

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National Centre
for the Replacement
Refinement & Reduction
of Animals in Research

The ARRIVE Guidelines

Animal Research: Reporting of *In Vivo* Experiments

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The ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines were developed as part of an NC3Rs initiative to improve the design, analysis and reporting of research using animals – maximising information published and minimising unnecessary studies. The guidelines were published in the online journal *PLOS Biology* in June 2010 and are currently endorsed by scientific journals, major funding bodies and learned societies.

The guidelines are intended to:

- Improve reporting of research using animals.
- Guide authors as to the essential information to include in a manuscript, and not be absolutely prescriptive.
- Be flexible to accommodate reporting a wide range of research areas and experimental protocols.
- Promote reproducible, transparent, accurate, comprehensive, concise, logically ordered, well written manuscripts.
- Improve the communication of the research findings to the broader scientific community.

The guidelines are NOT intended to:

- Promote uniformity, stifle creativity, or encourage authors to adhere rigidly to all items in the checklist. Some of the items may not apply to all studies, and some items can be presented as tables/figure legends or flow diagrams (e.g. the numbers of animals treated, assessed and analysed).
- Be a guide for study design and conduct. However, some items on the checklist, such as randomisation, blinding and using comparator groups, may be useful when planning experiments as their use will reduce the risk of bias and increase the robustness of the research.

Who are the guidelines aimed at?

- Novice and experienced authors
- Journal editors
- Peer reviewers
- Funding bodies

What kind of research areas do the guidelines apply to?

- The guidelines will be most appropriate for comparative studies, where two or more groups of experimental animals are being compared; often one or more of the groups may be considered as a control. They apply also to studies comparing different drug doses, or, for example, where a single animal is used as its own control (within-subject experiment).
- Most of the recommendations also apply to studies that do not have a control group.
- The guidelines are suitable for any area of bioscience research where animals are used.

How might these guidelines be used?

The guidelines provide a checklist for those preparing or reviewing a manuscript intended for publication.

References

1. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG (2010) Improving Bioscience Research Reporting: The ARRIVE Guidelines for Reporting Animal Research. *PLOS Biol* 8(6): e1000412. doi:10.1371/journal.pbio.1000412
2. Schulz KF, Altman DG, Moher D, the CONSORT Group (2010) CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *BMJ* 340:c332.

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Further Information

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	ITEM	RECOMMENDATION
Title	1	Provide as accurate and concise a description of the content of the article as possible.
Abstract	2	Provide an accurate summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study.
INTRODUCTION		
Background	3	a. Include sufficient scientific background (including relevant references to previous work) to understand the motivation and context for the study, and explain the experimental approach and rationale. b. Explain how and why the animal species and model being used can address the scientific objectives and, where appropriate, the study's relevance to human biology.
Objectives	4	Clearly describe the primary and any secondary objectives of the study, or specific hypotheses being tested.
METHODS		
Ethical statement	5	Indicate the nature of the ethical review permissions, relevant licences (e.g. Animal [Scientific Procedures] Act 1986), and national or institutional guidelines for the care and use of animals, that cover the research.
Study design	6	For each experiment, give brief details of the study design including: a. The number of experimental and control groups. b. Any steps taken to minimise the effects of subjective bias when allocating animals to treatment (e.g. randomisation procedure) and when assessing results (e.g. if done, describe who was blinded and when). c. The experimental unit (e.g. a single animal, group or cage of animals). A time-line diagram or flow chart can be useful to illustrate how complex study designs were carried out.
Experimental procedures	7	For each experiment and each experimental group, including controls, provide precise details of all procedures carried out. For example: a. How (e.g. drug formulation and dose, site and route of administration, anaesthesia and analgesia used [including monitoring], surgical procedure, method of euthanasia). Provide details of any specialist equipment used, including supplier(s). b. When (e.g. time of day). c. Where (e.g. home cage, laboratory, water maze). d. Why (e.g. rationale for choice of specific anaesthetic, route of administration, drug dose used).
Experimental animals	8	a. Provide details of the animals used, including species, strain, sex, developmental stage (e.g. mean or median age plus age range) and weight (e.g. mean or median weight plus weight range). b. Provide further relevant information such as the source of animals, international strain nomenclature, genetic modification status (e.g. knock-out or transgenic), genotype, health/immune status, drug or test naïve, previous procedures, etc.
Housing and husbandry	9	Provide details of: a. Housing (type of facility e.g. specific pathogen free [SPF]; type of cage or housing; bedding material; number of cage companions; tank shape and material etc. for fish). b. Husbandry conditions (e.g. breeding programme, light/dark cycle, temperature, quality of water etc for fish, type of food, access to food and water, environmental enrichment). c. Welfare-related assessments and interventions that were carried out prior to, during, or after the experiment.
Sample size	10	a. Specify the total number of animals used in each experiment, and the number of animals in each experimental group. b. Explain how the number of animals was arrived at. Provide details of any sample size calculation used. c. Indicate the number of independent replications of each experiment, if relevant.
Allocating animals to experimental groups	11	a. Give full details of how animals were allocated to experimental groups, including randomisation or matching if done. b. Describe the order in which the animals in the different experimental groups were treated and assessed.
Experimental outcomes	12	Clearly define the primary and secondary experimental outcomes assessed (e.g. cell death, molecular markers, behavioural changes).
Statistical methods	13	a. Provide details of the statistical methods used for each analysis. b. Specify the unit of analysis for each dataset (e.g. single animal, group of animals, single neuron). c. Describe any methods used to assess whether the data met the assumptions of the statistical approach.
RESULTS		
Baseline data	14	For each experimental group, report relevant characteristics and health status of animals (e.g. weight, microbiological status, and drug or test naïve) prior to treatment or testing (this information can often be tabulated).
Numbers analysed	15	a. Report the number of animals in each group included in each analysis. Report absolute numbers (e.g. 10/20, not 50%[2]). b. If any animals or data were not included in the analysis, explain why.
Outcomes and estimation	16	Report the results for each analysis carried out, with a measure of precision (e.g. standard error or confidence interval).
Adverse events	17	a. Give details of all important adverse events in each experimental group. b. Describe any modifications to the experimental protocols made to reduce adverse events.
DISCUSSION		
Interpretation/ scientific implications	18	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including any potential sources of bias, any limitations of the animal model, and the imprecision associated with the results[2]. c. Describe any implications of your experimental methods or findings for the replacement, refinement or reduction (the 3Rs) of the use of animals in research.
Generalisability/ translation	19	Comment on whether, and how, the findings of this study are likely to translate to other species or systems, including any relevance to human biology.
Funding	20	List all funding sources (including grant number) and the role of the funder(s) in the study.

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The ARRIVE Guidelines: Animal Research: Reporting of *In Vivo* Experiments. Originally published in *PLOS Biology*, June 2010[1]