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ORIGINAL ARTICLE

Hypoxia protects the liver from Small For Size Syndrome: A lesson learned from the associated liver partition and portal vein ligation for staged hepatectomy (ALPPS) procedure in rats

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Portal hyperperfusion and "dearterialization" of the liver remnant are the main pathogenic mechanisms for Small For Size syndrome (SFSS). Associating liver partition and portal vein ligation for staged hepatectomy (ALPPS) induces rapid remnant hypertrophy. We hypothesized a similar increase in portal pressure/flow into the future liver remnant in ALPPS and SFSS-setting hepatectomies. In a rodent model, ALPPS was compared to SFSS-setting hepatectomy. We assessed mortality, remnant hypertrophy, hepatocyte proliferation, portal and hepatic artery flow, hypoxia-induced response, and liver sinusoidal morphology. SFSS-hepatectomy rats were subjected to local (hepatic artery ligation) or systemic (Dimethyloxalylglycine) hypoxia. ALLPS prevented mortality in SFSS-setting hepatectomies. Portal hyperperfusion per liver mass was similar in ALLPS and SFSS. Compared to SFSS, efficient arterial perfusion of the remnant was significantly lower in ALPPS causing pronounced hypoxia confirmed by pimonidazole immunostaining, activation of hypoxia sensors and upregulation of neo-angiogenic genes. Liver sinusoids, larger in ALPPS, collapsed in SFSS. Induction of hypoxia in SFSS reduced mortality. Hypoxia had no impact on hepatocyte proliferation but contributed to the integrity of sinusoidal morphology. ALPPS hemodynamically differ from SFSS by a much lower arterial flow in ALPPS's FLR. We show that the ensuing hypoxic response is essential for the function of the regenerating liver by preserving sinusoidal morphology.

KEYWORDS
animal models, basic (laboratory) research/science, disease pathogenesis, liver disease, liver transplantation/hepatology, translational research/science

Abbreviations: ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; BW, body weight; DAB3, 3′-diaminobenzidine; DAMPs, Damage Associated Molecular Patterns; DMOG, dimethyloxalylglycine; DMOG-PHx, intraperitoneal injection of DMOG 12 hours before 87% partial hepatectomy; ELISA, enzyme-linked immunosorbent assay; FLR, Future Liver Remnant; HABR, hepatic arterial buffer response; HAF, total hepatic artery flow; HGF, Hepatic Growth Factor; HIF, Hypoxia Inducible Factor; HMGB1, High Mobility Group Box 1; IF, Immunofluorescence; iHAF, indexed hepatic artery flow; IHC, immunohistochemistry; IL1beta and 6, Interleukin 1b and 6; IP, intraperitoneal; iPVF, indexed portal vein flow; LLL, left lateral lobe; LML, left segment of median lobe; LSEC, Liver Sinusoidal Endothelial Cells; LT, liver transplantation; Lyve-1, Lymphatic Vessel Endothelial Hyaluronan Receptor 1; ML, median lobe; PC, pericaval tissue; PHLF, Post-Hepatectomy Liver Failure; PHx, 87% partial hepatectomy; PHx-LHA, hepatic artery ligation during 87% partial hepatectomy; PVF, total portal vein flow; PVL, portal vein ligation; PVLT, portal vein ligation and transection, ALPPS step I procedure; PVLT-PHx, portal vein ligation and transection followed by 87% partial hepatectomy at day 2; qPCR, quantitative Polymerase Chain Reaction; RML, right segment of the median lobe; SFSS, small for size syndrome; Sham-PHx, sham operation followed by 87% partial hepatectomy at day 2; VEGF, vascular endothelial growth factor.

1 | INTRODUCTION

Post-hepatectomy liver failure (PHLF) and small for size syndrome (SFSS) are clinical entities characterized by persistent hyperbilirubinemia, coagulopathy, intractable ascites, and delayed recovery of synthetic function leading to liver insufficiency.[1] They develop after major hepatectomy leaving a very small future liver remnant (FLR) or after liver transplantation (LT) with a small graft. PHLF is responsible for a 90-day mortality rate up to 75% after extended liver resection[2-4] and the size of the graft has been recognized as the only independent predictor factor of SFSS after LT.[2]

Redirection of the whole portal flow into a small FLR causes excessive sinusoidal shear stress[5] and a compensatory constriction of the common hepatic artery (the hepatic arterial buffer response-[HABR]).[6] The ensuing "dearterialization" and hypoxia of the remnant liver are the proposed primary factors for life-threatening postoperative liver failure.[7-10] Hence, reduction of the portal inflow into the remnant liver is a suggested strategy to prevent SFSS. Somatostatin treatment, creation of portosystemic shunts, ligation of splenic artery or splenectomy[11-15] have been employed to this end.

Conversely, other reports claim the beneficial effect of increasing the portal flow, and the inflow of hepatotrophic factors,[16] on liver regeneration.[9,17,18] Based on this observation, portal vein occlusion of the branches nourishing the diseased, to be excised, liver, either by radiological embolization or by surgical ligation, has been proposed for patients in need of major hepatectomy.[19] Portal vein occlusion is now the gold standard procedure before extended liver resections. However, hypertrophy of the FLR requires time (4-6 weeks). Recently, a new technique best known under the acronym ALPPS for "associated liver partition and portal vein ligation for staged hepatectomy" has been proposed to boost liver regeneration.[20] This variant of the standard two-stage hepatectomy combines portal vein ligation (PVL) and parenchymal transection between the diseased and healthy liver in the first step procedure (ALPPS step I) to obtain rapid and enhanced FLR hypertrophy.

Portal ligation in the ALPPS step I procedure redirects the entire portal blood flow through a small liver remnant sinusoidal network, while parenchymal transection excludes all intrahepatic collaterals and possibilities of neo-vascularization. Studies have shown that the extent of the parenchymal split, and thus the number of intrahepatic collaterals abrogated, correlates with the mass recovery of the FLR.[21,22] The contribution of the parenchymal transection to accelerated and enhanced hypertrophy is further underlined by the observation that in-situ split has been used as a rescue technique for patients whose liver fails to hypertrophy after portal vein occlusion.[23,24] Clinical studies document that ALPPS procedure causes an immediate and steep rise of the portal pressure and flow into the liver remnant accompanied by a compensatory arterial constriction (HABR).[25,26] In experimental models, in ALPPS step I, but not in portal vein occlusion alone, the HABR was associated with hypoxia.[26]

While enhanced liver regeneration has been linked to a severe portal hemodynamic stress in a critically small FLR in ALPPS, a similar hemodynamic condition is considered to be at the origin of liver failure in SFSS-setting hepatectomy. We hypothesized that the hypoxic response in ALPPS step I procedure has a protective impact on liver function. We therefore compared liver hemodynamic in a SFSS-setting hepatectomy and in a model of ALPPS leaving a too small for survival FLR recently published by our group.[27] We showed that hypoxia and hypoxia-induced signaling associated with accelerated regeneration and better survival in ALPPS. We further experimentally demonstrated that induction of hypoxia improves survival in SFSS conditions, confirming the protective role of hypoxia.

2 | METHODS

2.1 | Animals

All animal experiments were conducted in accordance with the European regulations and guidelines for human care for laboratory animals. The study protocol was approved by the university ethics committee. Wistar rats (UCLouvain Medical school, Brussels, Belgium) 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. Because hormonal status[28] and circadian cycle influence regeneration, all procedures were performed in male rats and between 7 and 12 AM. Because it fluctuates with food intake, the portal flow was measured on 12-hours fasten animals.

2.2 | Surgical procedures

We recently described an animal model of ALPPS in rats with a minimal, insufficient for survival FLR.[27]

The left segment of the median lobe (LML—10% of the total rat liver mass[27,29]) can be partitioned from the right segment of the median lobe (RML). Therefore, LML is the future liver remnant in all procedures. As the pericaval parenchyma (evaluated at 2%-5% of total liver[27,29]) cannot be resected without damaging the inferior vena cava, the resection amounts to 87% of the initial liver weight.

During operation, 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). All animals had a median laparotomy after subcutaneous injection of 5 cc of warm physiological sterile solution to increase the circulatory blood volume.

Sham operation: liver ligaments were gently freed, left lateral and median lobes were lifted with cotton-tipped sticks out of the abdomen and wrapped in gauzes to keep them humidified. The hilum was gently manipulated.

ALPPS model: In step-one procedure we ligated all portal veins but the one perfusing the LML (90% PVL) and transected between the left and right segments of the median lobe (PVLT). As inferior vena cava is intrahepatic in rodents, 2 mm of liver parenchyma was left around the vena cava to avoid vascular trauma. Two days after PVLT, we resected the deportalized lobes leaving in place the LML and pericaval tissue (ie, 13% of the initial volume) (PVLT-PHx or ALPPS).

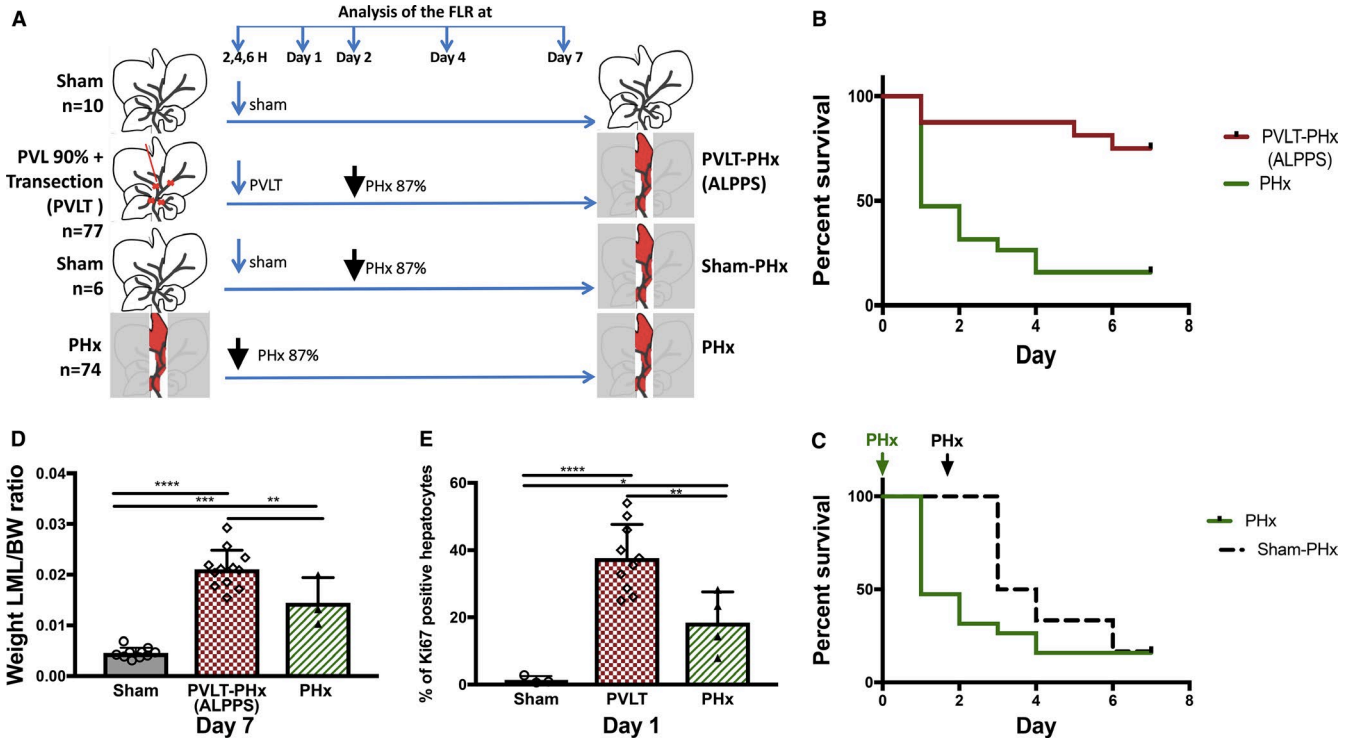

FIGURE 1 Eighty-seven percent partial hepatectomy causes a SFSS and ALPPS procedure prevents extended hepatectomy mortality. Schematic representation of the study design (A). For PHx, 87% of the liver mass was resected by removing all liver segments except the LML and pericaval parenchyma at day 0. A total of 74 animals were included with n = 10 randomized at 2 hours timepoint, n = 10 at 4 hours, n = 12 at 6 hours, n = 11 at day 1, n = 7 at day 2, n = 5 at day 4, and n = 19 at day 7. Animals in the Sham-PHx group underwent laparotomy and mobilization of hepatic ligaments at day 0 and 87% extended hepatectomy at day 2 (n = 6). PVLT-PHx (ALPPS): during the first procedure at day, we ligated all portal vein branches but the one irrigating the LML and transected the parenchyma between RML and LML; we resected all deportalized segments on day 2. A total of 77 animals were investigated, n = 6 at 2 hours, n = 15 at 4 hours, n = 11 at 6 hours, n = 11 at day 1, n = 7 at day 2, n = 11 at day 4, and n = 16 at day 7 after the initial intervention. Sham-operated animals were no hepatectomy controls (n = 10). Kaplan-Meier survival curves (B) after PHx (n = 19), PVLT-PHx (or ALPPS) (n = 16) and (C) PHx (n = 19) and Sham-PHx (n = 6). Arrows in (C) indicate the timing of hepatectomy. Extended PHx causes high mortality, ALPPS significantly improved survival (P = .0002 using Log-rank [Mantel-Cox] test) and Sham operation prior to 87% partial hepatectomy had no favorable impact on survival compared to an upfront extended resection (P = .23 using Log-rank [Mantel-Cox] test). D, FLR mass recovery expressed as liver remnant weight in relation to body weight after PVLT-PHx compared to extensive 87% hepatectomy and Sham 7 days after surgery (ALPPS [n = 12], PHx [n = 3 survivors], Sham [n = 10]). One-way ANOVA proved a significant improvement in FLR mass recovery in ALPPS and in PHx compared to Sham (P = .0001 and P = .0002, respectively). Postoperative FLR hypertrophy was more important in PVLT-PHx group compared to PHx (P = .007). E, Ki67 HIC at day 1 after operation (87% hepatectomy [n = 4], PVLT = ALPPS step I procedure [n = 10] and Sham [n = 3]). ANOVA one-way analysis showed a significant difference in proliferation of PVLT-PHx and PHx compared to Sham (P < .0001 and P = .05, respectively). Hepatocyte proliferation was significantly more important in the PVLT-PHx group compared to PHx (P = .006). ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; FLR, future liver remnant; HIC, immunohistochemistry; LML, left segment of the median lobe; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection; RML, right segment of the median lobe; SFSS, small for size syndrome. *P < .05, **P < .01, ***P < .001, ****P < .0001

Extended PH: We resected all liver lobes but the LML segment (10%) and pericaval tissue (3%), achieving an one-step extended 87% partial hepatectomy (PHx). To evaluate the effect of laparotomy on survival, Sham operation 2 days prior to 87% PHx was also performed (Sham-PHx).

We clinically observed each animal during the first 6 hours post-surgery and twice daily thereafter and recorded mortality. At selected time points (2, 4, 6 hours and days 1, 2, 3, and 7, as indicated), portal and systemic (cardiac puncture) blood was obtained and liver recovered. Figure 1A depicts our experimental protocol. In the figure legends, we report the number of animals and samples on which each analysis was performed.

Induction of hypoxia: To evaluate the impact of hypoxia on animal survival and FLR mass recovery, we ligated the hepatic artery (LHA) at the level of the hepatic pedicle (PHx-LHA) at the time of extended partial hepatectomy. In a separate group of animals, we injected Dimethyloxalylglycine (DMOG) (200 mg/kg BW) intraperitoneally 12 hours before extended partial hepatectomy (DMOG-PHx).[26]

2.3 | Assessment of liver proliferation

We evaluated two markers of liver regeneration: (A) The restitution of liver mass was determined as the weight of LML to body weight ratio (FLR/BW); and (B) the expression of ki67 in hepatocyte nulcei by

immunohistological staining (IHC) and morphometrical quantification (see Supplementary Materials for technical details).

2.4 | Blood flow analysis

After an overnight fasting, animals underwent a laparotomy and microscope-assisted dissection (×20 magnification) with hydrodissection of the liver hilum to individualize precisely portal vein and common hepatic artery. A Sham, a PVLT (ALPPS step I procedure) or a PHx was performed. Total portal flow (PVF) and hepatic artery flow (HAF) were measured for 6 minutes once a steady point was reached with MA2PSB (2 mm) and MA0.5PSB (0.5 mm) flow probes (Transonic Systems Inc., Ithaca, NY), respectively. PowerLab hardware and software were used for analysis. As the portal vein perfuses the whole liver in Sham, but only 10% of the liver in PHx and PVLT, we calculated the "indexed" portal vein flow into the FLR (iPVF) as followed (FLR = LML = 10% of the total liver mass): Sham: iPVF = total PVF × 0.1; PVLT: iPVF = total PVF (as all the segmental branches of the PV were ligated except the one vascularizing the LML [FLR]). PHx: iPVF = total PVF. The indexed hepatic artery flow (iHAF) in the FLR was calculated similarly: Sham: iHAF = total HAF × 0.1; PHx: iHAF = total HAF; PVLT: iHAF= total HAF x 0,1 (as in PVLT, we performed 90% PV ligation whereas the arterial perfusion was preserved for the entire liver).

2.5 | Pimonidazole staining for quantification of tissue hypoxia

In hypoxic cells, pimonidazole undergoes chemical reduction, binds to SH-containing molecules, and the resulting complexes accumulate in tissues.[26] Pimonidazole (60 mg/gr BW) was injected in the tail vein 1 hour prior to surgery and detected tissue-bound pimonidazole by IHC (see Supplementary Materials).

2.6 | Gene and protein analyses

Gene expression of angiogenic proteins[30] was measured by qPCR on RNA extracted from whole LML tissue (primer sets in Supporting Information, Figure S1). We used enzyme-linked immunosorbent assay (ELISA) to measure serum concentration of IL1-beta, IL-6, VEGF, HGF (RLB00, R6000B, RRV00 and MHG00, respectively, R&D systems, UK), and High Mobility Group Box 1 (HMGB1) (ST51011, Gentaur, IBL International GmbH). Hypoxia Inducible Factor (HIF) 1α and 2α were quantified in liver nuclear and cytosolic exctracts[31] using ELISA (LS-F11633; LSBio, Seattle).

2.7 | Assessment of the liver endothelial bed

Immunofluorescent (IF) staining of Lyve-1, a molecular marker of lymphatic and sinusoidal endothelial cells[32] was performed (see supplementary materials). We analyzed five images (20× magnification) taken ad random per liver. The smallest diameter of the transversally cut sinusoids was manually measured by two investigators on anonymized images using Fiji software (LOCI, University of Wisconcin, Madison, WI).

2.8 | Statistical analysis

GraphPad Prism software (San Diego, CA) was used for graphs and statistics. In graphs, we report individual data (dots), the mean ± 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 $P < .05$ (*$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$).

3 | RESULTS

3.1 | ALPPS reduces mortality due to SFSS

Extended 87% partial hepatectomy (PHx) is associated with a high 84.2% mortality (16 out of 19 rats) at day 7 with the majority of death (66%) occurring in the first 48 hours reflecting a SFSS (Figure 1B). When PVLT (PVLT-PHx or ALPPS) preceded PHx the 7-day mortality was significantly lower (25%, $P = .0002$) (Figure 1B). Sham operation prior to 87% partial hepatectomy (Sham-PHx) did not modify survival compared to the upfront extended hepatectomy (83.3%, 5 out of 6, $P = .23$) (Figure 1C). ALPPS, but not Sham operation, dramatically reduced the mortality that occurs after a SFSS-setting hepatectomy.

3.2 | Hepatocyte does regenerate in SFFS but ALPPS enhances FLR mass recovery

After 7 days, FLR hypertrophy was significantly higher in PVLT-PHx (or ALPPS) compared to SFSS (PHx) survivors ($P = .007$). FLR mass recovery was also significantly enhanced in the PHx group compared to Sham ($P = .0002$) (Figure 1D). At day 1 after surgery, hepatocytes were already engaged into the cell cycle as shown by the increased number of Ki67 positive hepatocyte nuclei in both PVLT and PHx groups compared to Sham ($P < .0001$ and $P = .05$, respectively). Thus, hepatocytes proliferate after extended PHx. PVLT alone, on the first day after the initial step, triggered a higher regenerative response compared to PHx ($P = .006$) (Figure 1E).

3.3 | FLR are exposed to severe hypoxia after ALPPS step I

We measured the flow in the portal vein and hepatic artery for 6 minutes directly after completion of the surgical procedure. Total portal vein flow (PVF) was equally reduced after PVLT and PHx compared to Sham ($P < .0001$) (Figure 2A). Because 87% of the liver parenchyma was excluded from the portal circulation in PVLT and PHx, the portal flow per mass of perfused liver parenchyma (indexed PVF or iPVF) increased by a factor of 4 to 5 compared to the flow into LML in Sham animals ($P = .0001$), with no difference between PVLT and PHx ($P = .2$) (Figure 2B). The total hepatic artery flow (HAF) decreased in both

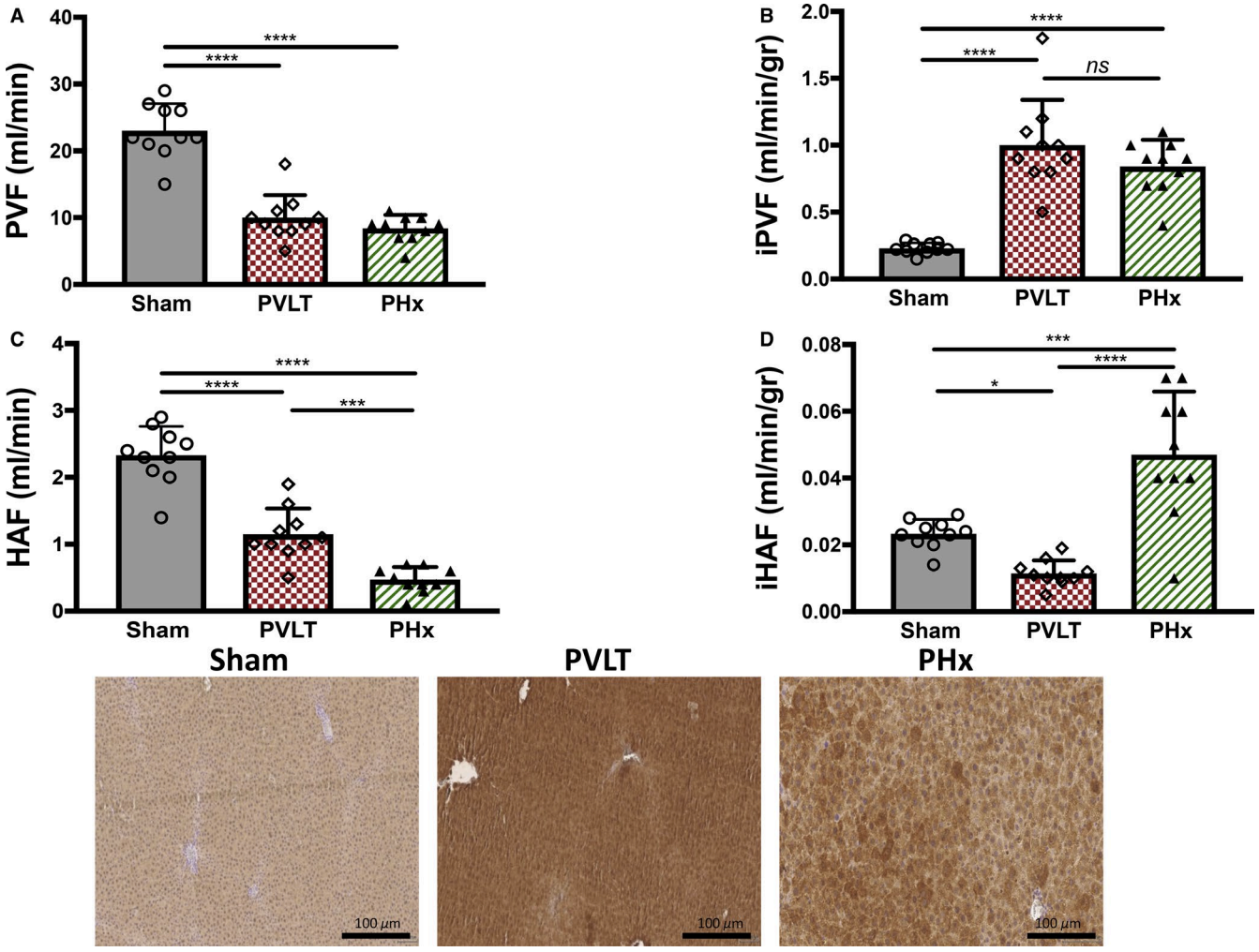

FIGURE 2 Hemodynamic variations after ALPPS step I (PVLT) and PHx. A, Total Portal Flow (PVF) (mL/min) and B, portal flow per liver mass perfused (iPVF) (mL/min/g) in Sham (n = 10), PVLT (n = 10), and PHx (n = 10) procedure. ANOVA one-way analysis showed a significant reduction in the total portal flow in both procedures compared to Sham ($P < .0001$) and a significant increase in the indexed portal flow in both PVLT and PHx compared to Sham ($P < .0001$), with no difference in the inflow between the two procedures ($P = .2$). C, Total HAF and D, hepatic artery flow per liver mass perfused (iHAF) in Sham (n = 10), PVLT (n = 10) and PHx (n = 10). ANOVA one-way analysis showed a significant reduction of the total hepatic artery inflow in both procedures compared to Sham ($P < .0001$). Compared to Sham, there was a tremendous increase in the indexed arterial flow in PHx ($P < .0002$) but a decrease in PVLT ($P = .05$). Thus, FLRs in ALPPS step I receive significantly less arterial blood compared to PHx ($P < .0001$). E, Representative histological pictures of Pimonidazole IHC on FLR on the 1st postoperative day in Sham (n = 2), PVLT (n = 4), and PHx (n = 4). Bar size = 100 μm. ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; FLR, future liver remnant; HAF, hepatic artery flow; iHAF, indexed hepatic artery flow; iPVF, indexed portal vein flow; *ns*, not significant; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection. $^{*}P < .05$, $^{***}P < .001$, $^{****}P < .0001$ [Color figure can be viewed at wileyonlinelibrary.com]

PVLT and PHx compared to Sham ($P < .0001$) (Figure 2C) and was significantly lower after PHx compared to PVLT ($P = .0004$) (Figure 2C). While arterial blood is distributed to the entire liver in PVLT, it perfused only the remnant in PHx (ie, the 13% of the original liver mass). Thus, compared to Sham, the indexed arterial flow into the FLR (iHAF) was increased after PHx ($P < .0002$) while it tended to decrease after PVLT ($P = .05$) (Figure 2D). The iHAF in PVLT was significantly lower compared to PHx ($P < .0001$). This supports that the FLR receives less arterial, well-oxygenated blood in ALPPS than in SFSS.

Pimonidazole staining, an indicator of hypoxia, was significantly increased in PVLT and PHx compared to Sham on the first postoperative day ($P = .0002$ and $P = .03$, respectively), supporting hypoxia in the FLR. At 24 hours, staining was significantly more intense in FLR tissue after PVLT than after PHx ($P = .01$) (Figure 2E, Supporting Information, Figure S2 for quantification). The difference persisted, but to a lower extent, on day 2 (data not shown).

3.4 | Hypoxia in ALPPS step I induces an angiogenic response

Hypoxia is known to activate and induce nuclear translocation of HIF-1α and 2α transcription factors, driving thereby an angiogenic

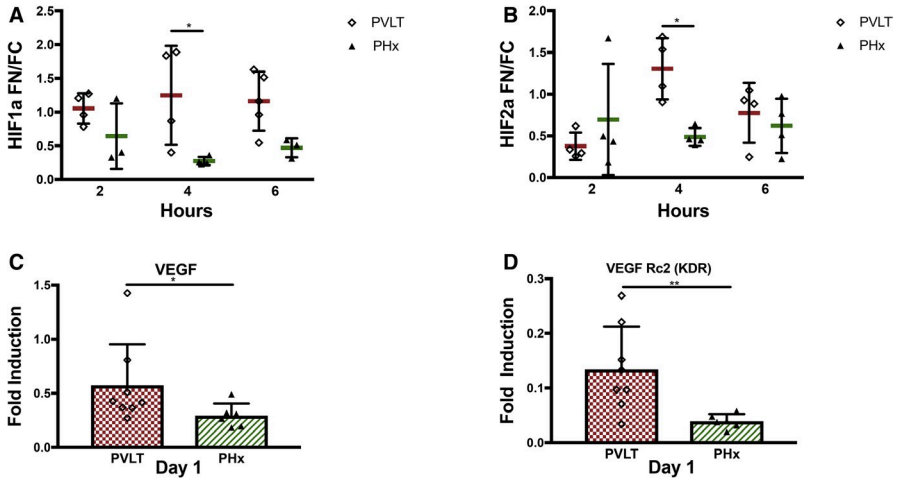

FIGURE 3 Angiogenic response to hypoxia. Ratio of nuclear to cytoplasmic HIF1α (A) and HIF2α (B) as measured by ELISA 2, 4, and 6 hours post-procedure in PVLT (n = 4, n = 4 and n = 5, respectively) and PHx (n = 4, n = 4, and n = 4, respectively). Data were analyzed by two-way ANOVA. VEGF (C) and VEGF Rc2 (D) gene expression measured by qPCR one day after the operation in PVLT (n = 8) and PHx (n = 6). Data are expressed as fold induction compared to Sham (controls) and analyzed using the Mann-Whitney test. Individual data mean and SEM per group are presented. ANOVA, analysis of variance; ELISA, enzyme-linked immunosorbent assay; HIF, hypoxia inducible factor; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection; qPCR, quantitative polymerase chain reaction; Rc2, Receptor 2; SEM, standard error of the mean; VEGF, vascular endothelial growth factor. $^{*}P < .05$, $^{**}P < .01$ [Color figure can be viewed at wileyonlinelibrary.com]

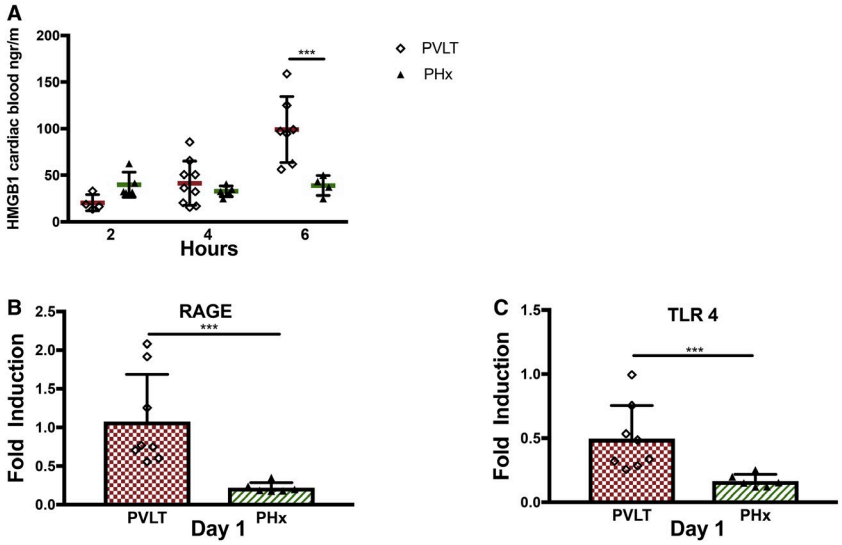

FIGURE 4 DAMPS response to hypoxia. HMGB1 concentrations measured by ELISA in serum prepared from cardiac blood (A) in PVLT (2 hours, n = 3; 4 hours, n = 9;6 hours, n = 7) and PHx (2 hours, n = 5; 4 hours, n = 5; 6 hours, n = 4). Data are analyzed by two-way ANOVA. RAGE (B) and TLR4 (C) gene expression measured by qPCR one day after the operation in PVLT (n = 8) and PHx (n = 6). Data are expressed as fold induction compared to Sham (controls) and analyzed using Mann-Whitney test. Individual data mean and SEM per group are presented. ANOVA, analysis of variance; DAMPS, damage-associated molecular patterns; ELISA, enzyme-linked immunosorbent assay; HMGB1, High Mobility Group Box 1; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection; qPCR, quantitative polymerase chain reaction; RAGE, receptor for advanced glycation end products; SEM, standard error of the mean; TLR, toll like receptor. $^{***}P < .001$ [Color figure can be viewed at wileyonlinelibrary.com]

response.[30] The ratio of nuclear to cytosolic HIF-1α was higher in PVLT than in PHx 4 hours after intervention ($P = .01$) (Figure 3A). Similarly, at the same timepoint, HIF-2α nuclear to cytoplasmic ratio was also higher in PVLT compared to PHx ($P = .01$) (Figure 3B). On the first postoperative day, VEGF gene expression was significantly increased in PVLT compared to PHx ($P = .02$), so was the expression of VEGF Receptor 2 (KDR) ($P = .007$) (Figure 3C,D). Serum levels of VEGF, IL1-β, IL-6, and HGF measured by ELISA were similar in PVLT and PHx (data not shown). Damage-associated molecular patterns (DAMPs) are released by damaged or hypoxic cells. HMGB1, one of such DAMPs, was significantly increased in systemic blood at 6 hours after PVLT compared to PHx ($P = .0004$) (Figure 4A). In parallel, the expression of the receptor for advanced glycation end products (RAGE) and Toll like receptor 4 (TLR4), two receptors

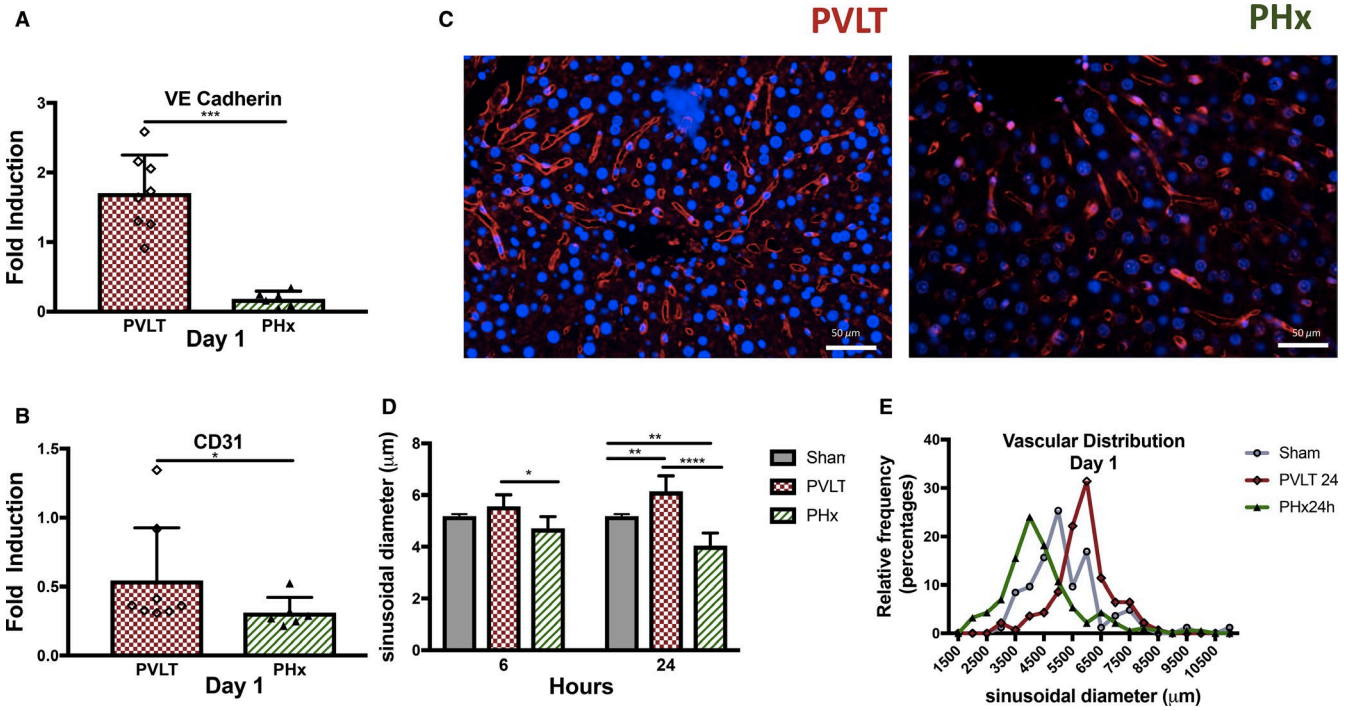

FIGURE 5 In ALPPS FLRs the sinusoidal bed is preserved. VE-Cadherin (A) and CD31 gene expression (B) one day after the operation in PVLT (n = 8) and PHx (n = 6) expressed as fold induction compared to Sham (controls) and analyzed using the Mann-Witney test. Lyve-1 fluorescent staining of sections at 6 hours, and 1 day after PHx and PVLT: (C) Representative pictures at day 1 showing collapsed sinusoids in PHx while they remain patent in PVLT. Lyve-1 in red and nuclei in blue. Bar size = 50 µm. (D) Sinusoidal diameter in Sham (n = 4), PVLT (6 hours, n = 7; day 1, n = 9) and PHx (6 hours, n = 5; day 1, n = 5) (mean + SEM, data analyzed by ANOVA) and (E) distribution of sinusoid diameter at day 1 post-surgery in Sham (n = 4), PVLT (n = 9), and PHx (n = 5). At 6 hours after surgery, sinusoidal diameter was already significantly larger in PVLT compared to PHx ($P = .01$) and this difference widened at day 1 ($P < .0001$). Of note, 1 day after surgery, sinusoids in PHx are collapsed compared to Sham ($P = .002$). ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; FLR, future liver remnant; PHx, 87% partial hepatectomy; PVLT, portal vein ligation and transection; SEM, standard error of the mean . *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$

on which HMGB1 binds to activate neoangiogenesis,[33-35] was significantly higher in FLR after PVLT than after PHx ($P = .0007$) (Figure 4B,C). All angiogenic factors tested showed higher gene expression in the ALPPS step I group compared to PHx (Supporting Information, Figure S3). The expression of VE-Cadherin, a glycoprotein indispensable for proper vascular development, and of CD31, which reflects the endothelial cells pool, were significantly upregulated in PVLT compared to PHx ($P = .0007$ and $P = .04$, respectively) (Figure 5A,B).

3.5 | ALPPS step I rescues liver sinusoidal morphology

As visualized on Lyve-1 immunostaining (Figure 5C), the hepatic sinusoids appeared large in PVLT and small and sometimes collapsed in PHx FLRs. Morphometric quantification confirmed the difference. Compared to Sham, the mean diameter of the sinusoids was significantly larger in PVLT after 6 hours ($P = .01$) and even more so at day 1 ($P < .0001$) while sinusoids were significantly smaller after extended hepatectomy (Figure 5D). The curve of distribution of sinusoid size confirmed this finding (Figure 5E).

3.6 | Hypoxia improves survival in SFSS without affecting FLR mass recovery

Thus, our data support the hypothesis that ischemic signals in the FLR in ALPPS protect from SFSS. To test this, we induced hypoxia upon extended partial hepatectomy either by ligation of hepatic artery during PHx (PHx-LHA) or by administration of DMOG, an inducer of hypoxic signaling,[26] prior to PHx (DMOG-PHx) (Figure 6A). We confirmed increased pimonidazole staining with the two procedures compared to extended PHx alone (Figure 6B, Supporting Information, Figure S4A for representative histological pictures). As shown in Figure 6C, hepatic artery ligation and DMOG treatment significantly improved survival compared to extended PHx alone (66.7% and 83.3%, respectively compared to 15.8%, $P < .0001$), such as survival is comparable to that obtained with ALPPS. Neither local nor systemic hypoxia affected the final FLR mass recovery (Figure 6D). Hepatocyte proliferation attested by Ki67 IHC was induced in all three groups compared to Sham ($P < .05$) but was higher in hepatic artery ligation model (PHx-LHA) compared to PHx alone ($P = .01$) (Figure 6E).

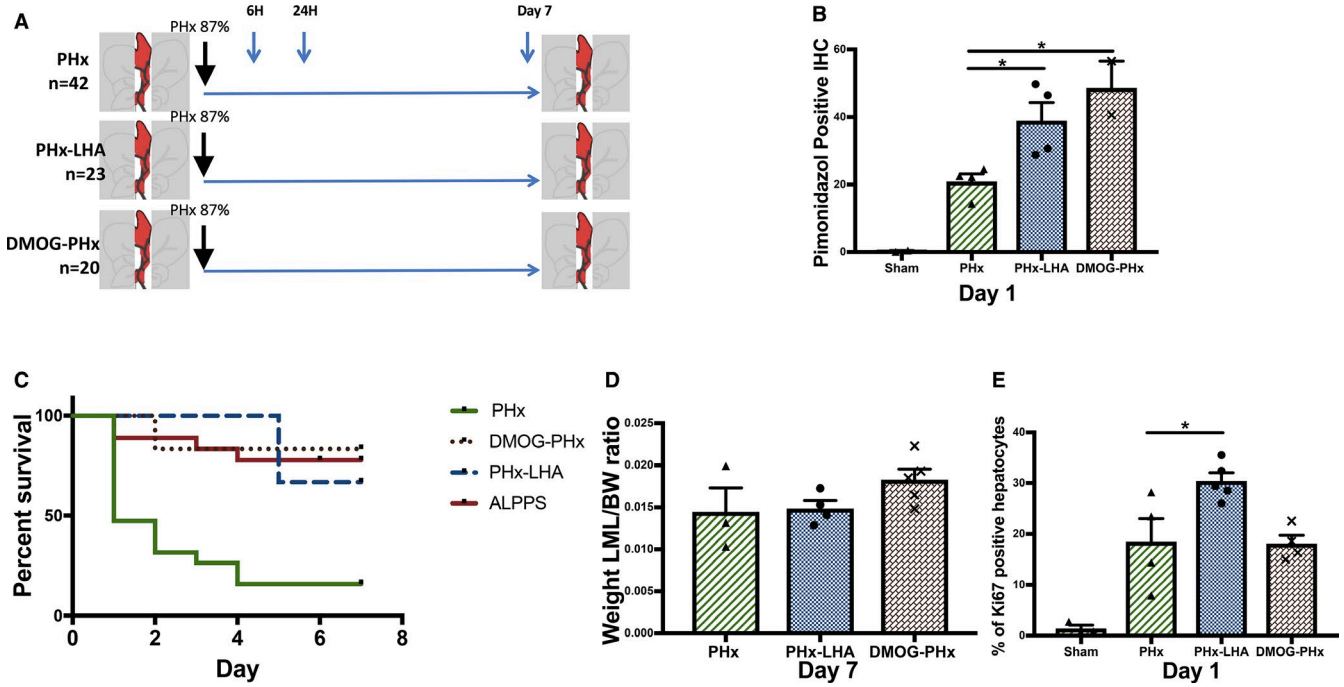

FIGURE 6 Hypoxia in SFSS rescues survival. (A) Schematic representation of the study designed: 42 animals underwent 87% extended hepatectomy (PHx; n = 12 at 6 h, n = 11 at 1 day, and n = 19 at 7 days); 23 animal had a ligature of the hepatic artery at the time of extended PHx (PHx-LHA; n = 12 at 6 hours; n = 5 at day 1 and n = 6 at day 7); 20 animals received a DMOG injection (I.P.) 12 hours prior to extended PHx (DMOG-PHx; n = 9 at 6 hours; n = 5 at day 1, and n = 6 at day 7). (B) Morphometrical quantification of Pimonidazole IHC on FLR on the first postoperative day in Sham (n = 2), PHx (n = 4), PHx-LHA (n = 4), and DMOG-PHx (n = 2). ANOVA one-way analysis showed hypoxia in FLR tissues in PHx-LHA and DMOG-PHx (P = .04 and P = .01, respectively). (C) 7-day Kaplan Meier survival curve after ALPPS (n = 16), PHx (n = 19), PHx-LHA (n = 6), and DMOG-PHx (n = 6). Hepatic artery ligation or DMOG injection prior pour PHx had a favorable impact on survival compared to PHx (P < .0001 using Log-rank [Mantel-Cox] test). Hypoxic model PHx showed similar survival to ALPPS. (D) Mass of the liver remnant relative to body weight at day 7 after initial hepatectomy, and (E) morphometrical quantification of the percentage of Ki67+ hepatocyte nuclei in Sham (n = 3) and at day 1 after hepatectomy in PHx (n = 4), PHx-LHA (n = 5), and DMOG-PHx (n = 4). ANOVA one-way analysis showed a significant difference in proliferation index in PHx-LHA compared to PHx (P = .01). All models of partial extended hepatectomy showed significant proliferation index compared to Sham (P = .0036 for PHx, P < .0001 for PHx-LHA and P = .004 for DMOG-PHx). Individual data, mean and SEM are presented. Data are analyzed by ANOVA. ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; DMOG, dimethyloxalylglycine; DMOG-PHx, intraperitoneal injection of DMOG 12 hours before 87% partial hepatectomy; FLR, future liver remnant; IHC, immunohistochemistry; PHx, 87% partial hepatectomy; PHx-LHA, hepatic artery ligation during 87% partial hepatectomy; SEM, standard error of the mean. *P < .05 [Color figure can be viewed at wileyonlinelibrary.com]

3.7 | Hypoxia improves sinusoidal morphology

There was a significant activation in HIF-1α (Figure 7A), but not in HIF-2α (Figure 7B), 6 hours after surgery in the PHx-LHA group compared to PHx (P = .04). Expression of angiogenesis markers Angiopoietin 1 (Figure 7C), Angiopoietin 2 (Figure 7D), and Platelet-derived growth factor b (Figure 7E) as well as VE-Cadherin gene expression (Figure 8A) was also enhanced in the PHx-LHA group compared to PHx. Compared to PHx, hepatic artery ligation did not enhance HMGB1 systemic release. Systemic DMOG administration associated with high circulating HMGB1 compared to PHx (P = .01) at early time points (Supporting Information, Figure S4B). Induction of hypoxia in SFSS, whether through hepatic artery ligation or DMOG treatment, did not alter expression of HMGB1 receptors RAGE and TLR4 (Supporting Information, Figure 4C,D).

Lyve-1 IF evidenced that local hypoxia as well as DMOG treatment preserved sinusoidal diameter compared to collapsed sinusoids in PHx (PHx-LHA vs. PHx, P < .0001, DMOG-PHx vs. PHx, P = .0001) (Figure 8B). Thus, with the imposition of hypoxia, the diameter of sinusoids and the distribution of the sinusoids' size upon extended PHx (PHx-LHA or DMOG-PHx) are similar than after ALPPS (Figure 8C,D).

4 | DISCUSSION

The present study provides evidence that hypoxia, experimentally induced by the ligation of the hepatic artery or systemic administration of DMOG, rescues survival after extended hepatectomy. This sharply contrasts with the current theory on the origin of post-hepatectomy liver failure.[5,36] Our understanding of SFSS is incomplete but has been related to a small for flow syndrome, ie, a damage caused by an excessive portal blood flow into a small remnant.[5] Works over the years indeed support a major contribution of excessive shear stress and endothelial damage to liver failure.[5,37] When the full portal flow enters a much-reduced liver parenchyma, the pressure building up in the portal vein not only damages the sinusoidal bed but effectively vasoconstricts hepatic

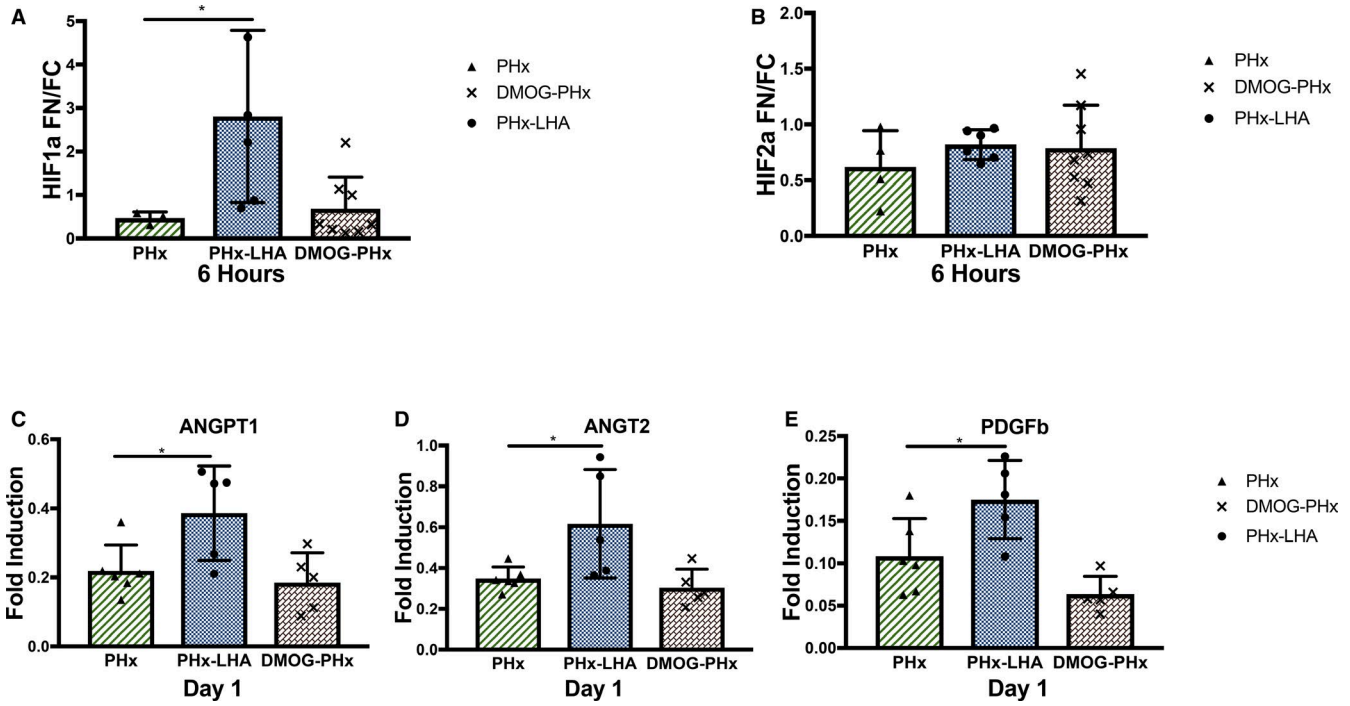

FIGURE 7 Hepatic artery ligature induced a hypoxic response the liver remnant. Ratio of nuclear to cytoplasmic HIF1α (A) and HIF2α (B), as measured by ELISA 6 hours post procedure in PHx (n = 3), PHx-LHA (n = 6), DMOG-PHx (n = 8). Angiopoietin 1 (C) and 2 (D) and PDGFb (E) gene expression measured by qPCR 1 day after the operation in PHx (n = 6), PHx-LHA (n = 5), DMOG-PHx (n = 5). Individual data, mean, and SEM are presented. Data were analyzed using ANOVA. ANOVA, analysis of variance; DMOG, dimethyloxalylglycine; DMOG-PHx, intraperitoneal injection of DMOG 12 hours before 87% partial hepatectomy; ELISA, enzyme-linked immunosorbent assay; HIF, hypoxia inducible factor; PDGFb, platelet-derived growth factor b; PHx, 87% partial hepatectomy; PHx-LHA, hepatic artery ligation during 87% partial hepatectomy; qPCR, quantitative polymerase chain reaction; SEM, standard error of the mean. *$P < .05$ [Color figure can be viewed at wileyonlinelibrary.com]

arterioles. Such "de-arterialization" of the FLR is considered to be the cause of SFSS.[8] On the other hand, clinical and experimental studies support that increased portal flow/pressure triggers liver regeneration.[38] Shear stress is needed for regeneration[39] but increased flow itself is insufficient to stimulate liver growth as demonstrated by the inefficacy of arterio-portal shunt to enhance liver regeneration.[40]

In our experimental model, preconditioning of the FLR by portal vein ligation and in-situ split (ALPPS step I) prevented mortality and boosted liver hypertrophy[27] although the procedure caused the same sudden and steep rise of efficient portal inflow into the FLR as in SFSS.

The total portal flow significantly dropped after PVLT compared to Sham. This was however not associated with a compensatory rise of the total hepatic artery flow, as it would be expected according to the HABR concept. Data pertaining to liver hemodynamics in ALPPS in humans are scarce and conflicting. In their study, Schadde et al[26] found no difference in total portal venous flow during PVLT compared to baseline while Tomassini et al[25] recorded a significant reduction. In the sectorial right and left (to the FLR) arterial branches, the former but not the latter observed an HABR. The divergence between these two studies in similar surgical context highlights that the adaptation of liver hemodynamics during ALPPS step I is complex. The HABR is likely triggered at the level of hepatic microvasculature according to the "adenosine washout" theory, and involves multiple interrelated mechanisms for a dynamic regulation of hepatic inflow as to maintain, acutely and chronically, a constant hepatic blood flow-to-liver mass ratio.[6]

In our study, we measured the portal and arterial flow at the main vascular trunks, while the efficient portal or arterial inflow into the FLR was calculated (indexed portal/arterial flow). The indexed portal venous flow was significantly and similarly increased up to four times over controls both in ALPPS step I and in SFSS. Bucur et al recently showed that a portal venous flow per unit of liver mass exceeding four times the normal amount is incompatible with survival.[41] In our hands, the overflow was associated with mortality in the SFSS group but with survival and liver mass recovery in the ALPPS group. The hemodynamic differences came from the hepatic artery. The indexed arterial flow is much lower after ALPPS than SFSS. Thus, perfusion of the FLR with oxygenated blood is lower in ALPPS. We further demonstrate that FLRs in ALPPS are more hypoxic than after SFSS-setting hepatectomy. This suggests that although portal hyperperfusion plays a pivotal role in triggering regeneration, hypoxia is key to preventing liver insufficiency.

In accordance with clinical[42] and experimental studies,[43,44] we show here that liver insufficiency after hepatic resection in SFSS-setting is not due to the failure of hepatocytes to regenerate. Ninomya et al, however, suggested that liver failure is related to an asynchronous proliferation between hepatocytes and liver sinusoidal endothelial cells (LSEC), keeping clusters of newly generated hepatocytes away

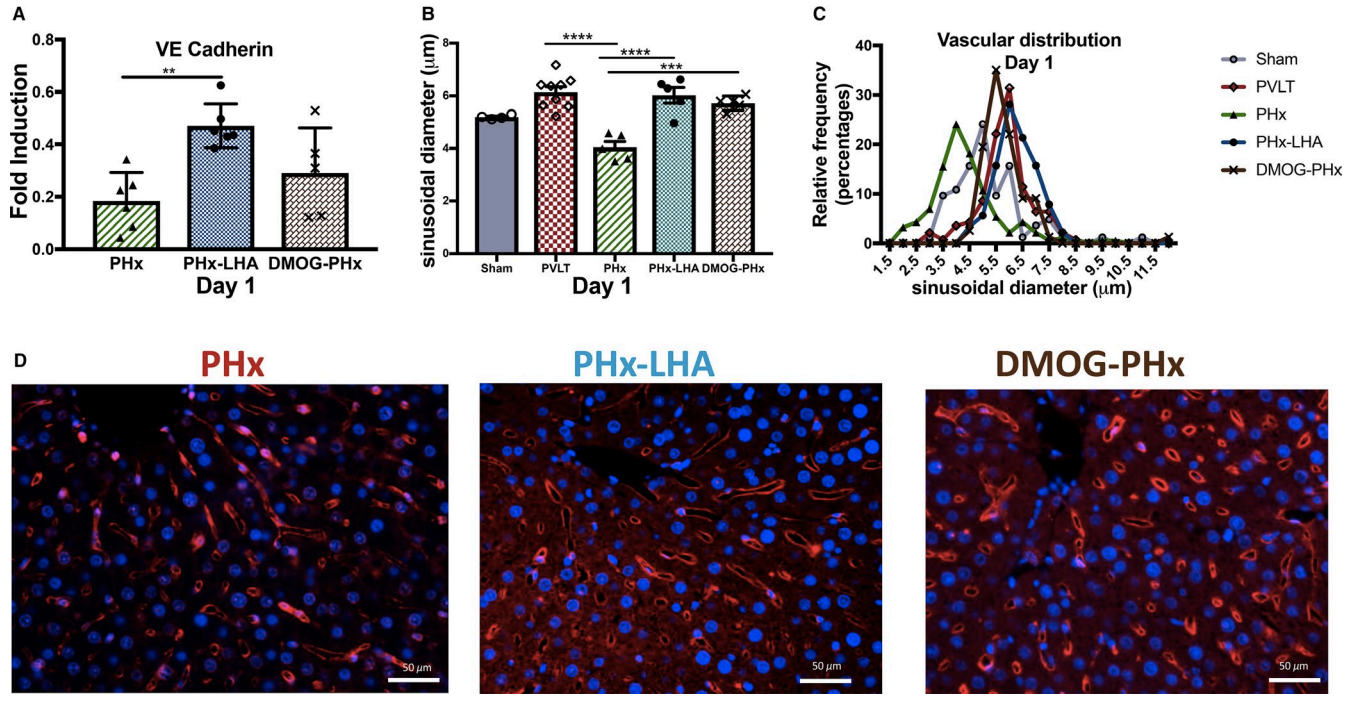

FIGURE 8 Induction of hypoxia maintains sinusoid patency in post-extended PHx remnants. (A) VE-Cadherin gene expression, (B) sinusoidal diameter, (C) distribution of sinusoid diameter as measured on Lyve-1 IF pictures in Sham (n = 4), and at day 1 in PVLT (n = 9), PHx (n = 6), PHx-LHA (n = 6), and DMOG-PHx (n = 5). (D) Representative picture of Lyve-1 IF in PHx, PHx-LHA, and DEMOG-PHx. Lyve-1 in red and nuclei in blue. Bar size = 50 μm. ANOVA one way showed significant increase in sinusoidal diameter in PHx-LHA and DMOG-PHx compared to PHx ($P < .0001$ and $P = .0001$, respectively), so as there is no difference in vascular morphology of these hypoxic models compared to ALPPS step I. ALPPS, associating liver partition and portal vein ligation for staged hepatectomy; ANOVA, analysis of variance; DMOG, dimethyloxalylglycine; DMOG-PHx, intraperitoneal injection of DMOG 12 hours before 87% partial hepatectomy; PHx, 87% partial hepatectomy; PHx-LHA, hepatic artery ligation during 87% partial hepatectomy; PVLT, portal vein ligation and transection. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

from sinusoidal structures. The authors showed that deceleration of hepatocyte division (using MEK/ERK pathway blockers) rescued animals' survival after 90% partial hepatectomy.[44] This could explain recent publications that claim that volume and postoperative FLR hypertrophy are not always predictive of function.[45] In our model, SFSS survivors had a significantly increased hepatocyte proliferation resulting in an enlargement of the liver remnant. Hypoxia did not affect liver mass recovery. Thus, the beneficial effect of hypoxia on survival in SFSS-setting hepatectomy is not due to enhanced parenchymal cell proliferation.

LSEC proliferation is needed to support the function in the regenerating liver.[46] Hypoxia might account for preservation of the sinusoidal bed, hepatic architecture, and thus function during regeneration. We indeed demonstrate that hypoxia in ALPPS step I was associated with early activation of hypoxia sensors HIF-1α and HIF-2α, known to play an important role in neovascularization, and with upregulation of VEGF and VEGF Rc2, the HIF-driven mediators of neo-angiogenesis. Kron et al[47] recently reported that hypoxia induces LSEC proliferation after 68% partial hepatectomy in mice. The authors suggested that HIF-2α was the hypoxic signal inducing LSEC proliferation. In our model, even if we observed both HIF-1α and HIF-2α upregulation in ALPPS compared to SFSS-setting hepatectomy, HIF-2α was not upregulated when hypoxic signals were induced by DMOG or hepatic artery ligation. Thus, the favorable impact of hypoxia on survival and sinusoidal preservation cannot be solely ascribed to HIF-2α activation. A surge of hypoxia in extended hepatectomy (such as obtained by hepatic artery ligation) triggers an early neo-angiogenic response as supported by upregulation of pro-angiogenic genes and VE cadherin. Activation of angiogenic signals, both in ALPPS and upon hepatic artery ligation, associates with enlarged sinusoidal diameter patency. DMOG administration had exactly the same effect. It is thus tempting to speculate that increased hypoxic signals in FLR stimulate early neo-angiogenesis (thus overcoming the asynchronism in hepatocyte-LSEC proliferation), rebuild an efficient sinusoidal bed and preserve the hepatic lobular architecture, thus maintaining the function of proliferating hepatocytes.

The release of danger signals by hypoxic hepatocytes might be an additional mechanism favoring the regeneration of the hepatic sinusoids. Damage associated molecular patterns such as HMGB1 are intranuclear proteins that translocates in the cytoplasm before being released in the extracellular space by necrotic cells or cells exposed to life-threatening stress, including hypoxia.[35,48] Besides activation of immune response, HMGB1 has been recognized as a putative proangiogenic factor capable to stimulate endothelial cell proliferation, attract circulating endothelial progenitors and improve neo-angiogenesis.[33,34,49,50] Here, we found enhanced circulating HMGB1

at early time points in ALPPS and DMOG-treated extended PHx, but not after hepatic artery ligation. Several groups studied ALPPS step I procedure and suggested that rapid liver regeneration was the response to the trauma caused by parenchymal transection.[51-53] In our experience, the rise in HMGB1 was mostly attributed to hypoxia due to portal vein ligation and abrogation of intrahepatic collaterals. Indeed, in our study, HMGB1 release was not ascribed to surgical trauma alone, as liver transection alone or kidney necrosis did not increase circulating HMGB1 (data not shown). This observation is shared by others.[48] Whether in a too small for survival liver remnant, HMGB1 release helps hepatic cells to turn hypoxic signals to their advantage and triggers tissue repair via neo-angiogenesis remains to be explored.

To our knowledge, this is the first study that compares FLR inflow hemodynamics in ALPPS and in SFSS-setting hepatectomy. We bring experimental evidence that sensing of hypoxia may be the catalyst of early neo-angiogenesis favoring the functional organization of the proliferating hepatocytes. Further studies are required to dissect the mechanisms that have a protective impact on SFSS-setting hepatectomy and test whether they apply in conditions of transplantation of a small graft. If verified, induction of hypoxia or pharmacological mimicry of hypoxia-driven response could be proposed during transplantation of a small graft at risk of SFSS.

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AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AD, CB, IL. Performed the experiments: AD, VL, BP. Analyzed the data: AD, VL, BP, CB, IL. Contributed to writing: AD, CB, IL. Revised the manuscript: AD, CB, IL.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

All data are enclosed in the manuscript, there are no data available elsewhere.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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