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RELEVANCE OF VEGFA IN RAT LIVERS SUBJECTED TO PARTIAL HEPATECTOMY UNDER ISCHEMIA-REPERFUSION

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SUPPLEMENTAL MATERIAL AND METHODS

MATERIAL AND METHODS

Experimental animals

Male homozygous obese (Ob) (400–450 g) and heterozygous lean (Ln) Zucker rats (350–400 g) for the genetic obesity experimental model, and male Sprague Dawley rats (SD) (200–220 g) for the experimental model of steatosis induced by choline-deficient diet (CDD) were purchased from Charles River (Paris, France). Animals were allowed to acclimatize to the animal room conditions for two weeks prior to surgical procedures. Animals were housed with one or two cagemates of the same strain in a standard, shoebox-style polycarbonate cage (24x46x20 cm) with standard stainless-steel lids, hardwood chip bedding and environmental enrichment. The environmental conditions in the animal room were: lighting, 300 to1000 lumens/m2 at cage level and lights on from 0700 to1900; temperature, 20 to 28 ºC); and relative humidity, 30% to 60%. Zucker rats were given water and standard laboratory food (rat chow) ad libitum. Liver steatosis was induced in SD rats by feeding a CDD for 10 days [1, 2]. The diet was purchased from Dyets Inc. (Bethlehem, PA), and its analytical composition (a modified #518753 choline deficient diet, g/kg) was: Alcohol-extracted peanut meal 90, Soy protein isolate (low in choline) 80, L-Cysteine 2, Cellulose fibre 10, Cornstarch 100, Dextrin 100, Sucrose 413, Choline bitartrate 0, Vitamin-free casein 10, Salt mix 35, Primex (hydrogenated vegetable oil) 100, Vitamin mix 10 and Corn oil 50. Control rats (SD) were kept on a standard chow diet containing adequate levels of choline (14.48 g/kg of choline at the expense of sucrose). After 10 days, no differences in rat weight, ranging between 350 and 380 g, were noted in SD and CDD-SD rats.

Ob Zucker and CDD-SD rats showed severe macrovesicular and microvesicular fatty infiltration in hepatocytes (60–70% steatosis) [1-4]. All procedures were started at 0900 and were performed under isofluorane anesthesia. Blood pressure, heart rate, and body temperature were monitored. All procedures were approved by the Laboratory Animal Care and Use Committee of Barcelona University and by the Generalitat de Catalunya. European Union regulations (Directive 86/609 EEC) for animal experiments were respected.

Sample collections

In view of the results of previous studies based on the peaks in the parameters of hepatic injury and liver regeneration [5-8], hepatic injury (transaminases and damage score) and liver regeneration (percentage of Ki67 and cyclins) were determined after 24 h of liver surgery in groups from protocols 1-4. To reinforce

the deleterious effects of VEGFA in obese animals, hepatic damage (plasma AST, ALT, GLDH, ALP and bilirubin levels; and damage score) and liver regeneration (percentage of Ki67 and cyclin E levels) were also determined 72 h after liver surgery in Ob Zucker animals from groups of protocol 1. For survival studies, a sub-group of 20 Ln and 20 Ob Zucker rats were subjected to interventions described for groups corresponding to protocols 1and 3 and the survival was monitored for 14 days [9]. Animals that died in the follow-up period underwent autopsy. The different treatments were administered immediately before surgery and the doses were selected on the basis of previous studies [2, 9-15] and preliminary studies from our group. The interventions and measurements for protocols 1-4 are summarized in Supplementary Figure 1.

Biochemical determinations

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) in plasma and malondialdehyde (MDA) and myeloperoxidase (MPO) in liver were measured as described elsewhere [9, 16, 17]. Alkaline phosphatase (ALP), bilirubin, Von Willebrand factor (vWF), hyaluronic acid (HA) and sFlt1 in plasma were measured using commercial kits (ALP: Abcam, UK; bilirubin: Sigma-Aldrich, USA; vWF and sFlt1: Elabscience Biotechnology, USA; HA: R&D Systems, USA).

Western blotting

Liver and peripheral adipose tissues were homogenized and Western blotting was performed as described elsewhere [10, 18-23], using the antibodies against: VEGFA, p-VEGFR2, Id1, Wnt2, cyclin E, cyclin A, p-AKT (Santa Cruz Biotechnology, USA), PI3K (Cell Signaling Technology, USA), cyclin D1 and β-actin (Sigma-Aldrich, USA). To visualize the bound and the free forms of VEGFA, the same protocol was carried out but with a non-reducing 10% gel [24]. Immunoreactive protein bands were quantified densitometrically with Quantity One software.

RT-PCR

Total RNA was isolated from frozen liver and peripheral adipose tissue using TRIzol Reagent (Invitrogen, USA) and was reverse transcribed using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA). Real-time PCR was performed with PowerUp SYBR Green Master Mix using primers for: *Vegfa* 5'-CAAACCTCACCAAAGCCAGC-3' (forward) and 5'-TTCTCCGCTCTGAACAAGGC-3' (reverse), *Flt1* 5'-GGTGTCTATAGGTGCCGAGC-3' (forward) and 5'-GGGTGATCAGCTCCAGGTTT-3' (reverse), s*Flt1* 5'-GAAGACTCGGGCACCTATGC-3'

(forward) and 5'-GCAGTGCTCACCTCTAACGA-3' (reverse), and *Gapdh* 5'-AGTGCCAGCCTCGTCTCATA-3' (forward) and 5'-TAACCAGGCGTCCGATACG-3' (reverse) as control gene. It is well known that sFlt1 is an alternatively spliced, secreted isoform of the cell-surface receptor membrane-bound Flt1. Therefore, sFlt1 lacks the transmembrane and tyrosine kinase domains of Flt1 [24, 25]. In addition, Flt1 can also derive from the endoproteolytic cleavage of Flt1 in endothelial cells [26].

Histology, Red Oil staining, and Immunohistochemistry

Liver and peripheral adipose tissue sections were fixed by immersion into a solution of 10% formaldehyde and paraffin-embedded. To appraise the severity of hepatic injury, we graded hematoxylin and eosin–stained sections with a point counting method on an ordinal scale: (0) minimal or no evidence of injury; (1) mild injury (cytoplasmic vacuolation and focal nuclear pyknosis); (2) moderate to severe injury (extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and a loss of intercellular borders); (3) severe necrosis (disintegration of hepatic cords, haemorrhaging, and neutrophil infiltration); and (4) very severe necrosis (showing the latter manifestations to extreme degree) [27]. Liver steatosis was evaluated via red oil staining [9]. Liver samples were immunostained with a rabbit monoclonal antibody against Ki67 (DAKO, USA). For immunohistochemistry analysis of VEGFA, liver sections were immunostained with a mouse monoclonal antibody against VEGFA (Santa Cruz Biotechnology, USA). For immunohistochemistry analysis of Flt1 and sFlt1, adipose tissue was immunostained with a rabbit monoclonal antibody against sFlt1 and Flt1 (Abcam, UK). Samples were developed with diaminobenzidine, and counterstained with haematoxylin [9]. At least 30 high-power fields per slide were counted. The slides were blinded to the examiners who had extensive experience in evaluating liver pathology. At least four sections were examined per liver and adipose tissue sample.

Statistics

Statistical significance of differing variables was determined via non-parametric Kruskal-Wallis test. Mann-Whitney U test was applied to groups showing significant differences, and adjusted p-values were calculated using False Discovery Rate (FDR) method ($p.adj<0.05$ considered significant). Survival estimates, obtained by Kaplan-Meier method, were then compared using both Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests, since Gehan-Breslow-Wilcoxon test emphasizes early differences [28], with statistical significance set at $p<0.05$.

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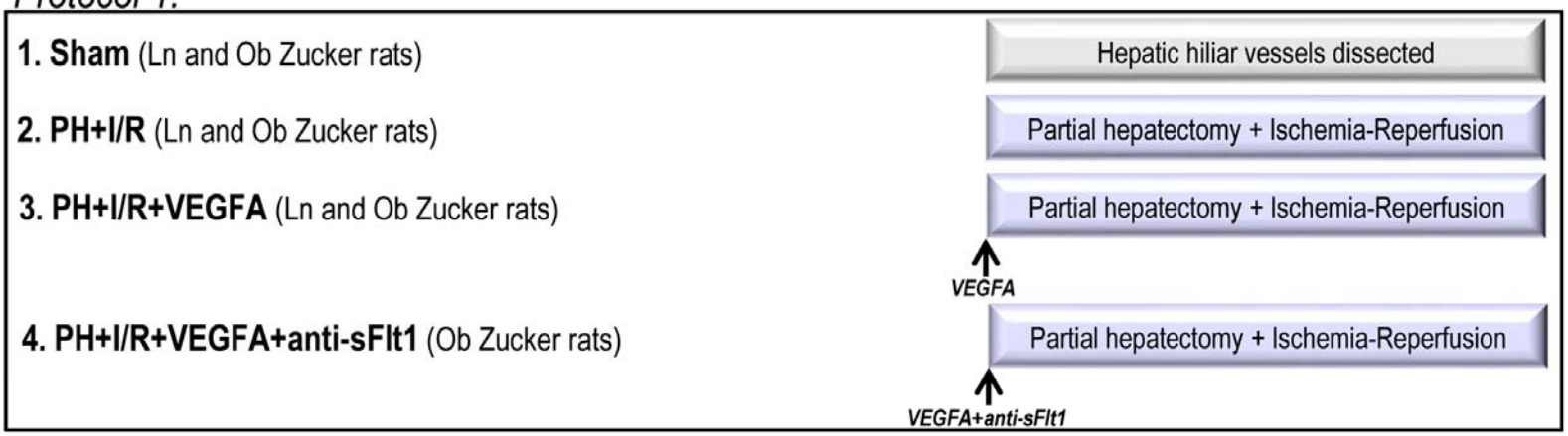
SUPPLEMENTARY FIGURE LEGENDS

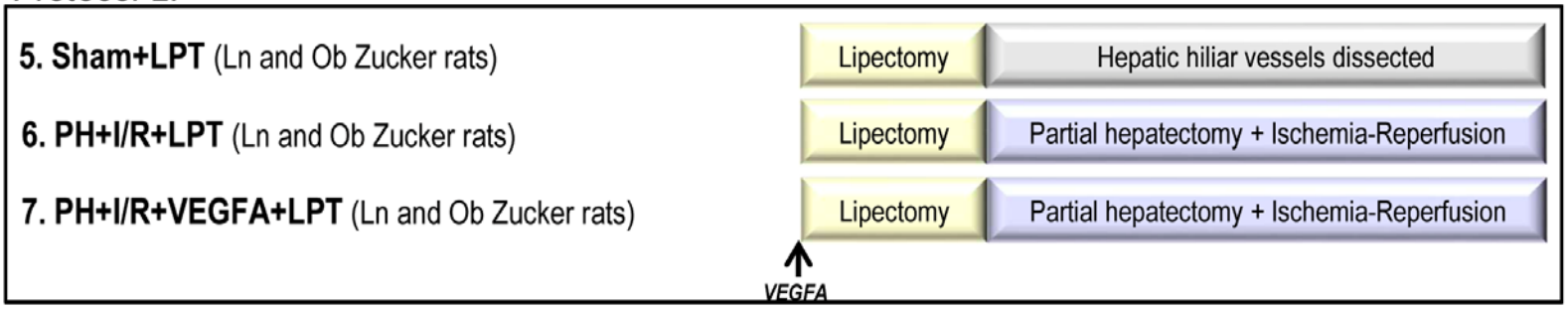
Supplementary Figure 1. Flow chart of the interventions and measurements corresponding to protocols 1-4. Protocol 1) VEGFA impact and availability in Ln and Ob Zucker rats undergoing PH+I/R; Protocol 2) Role of adipose tissue in the circulating sFlt1 levels in Ln and Ob Zucker rats undergoing PH+I/R; Protocol 3) Underlying mechanisms of VEGFA in Ln and Ob Zucker rats undergoing PH+I/R; Protocol 4) VEGFA impact and availability in SD and CDD-SD rats undergoing PH+I/R.

Experimental desing

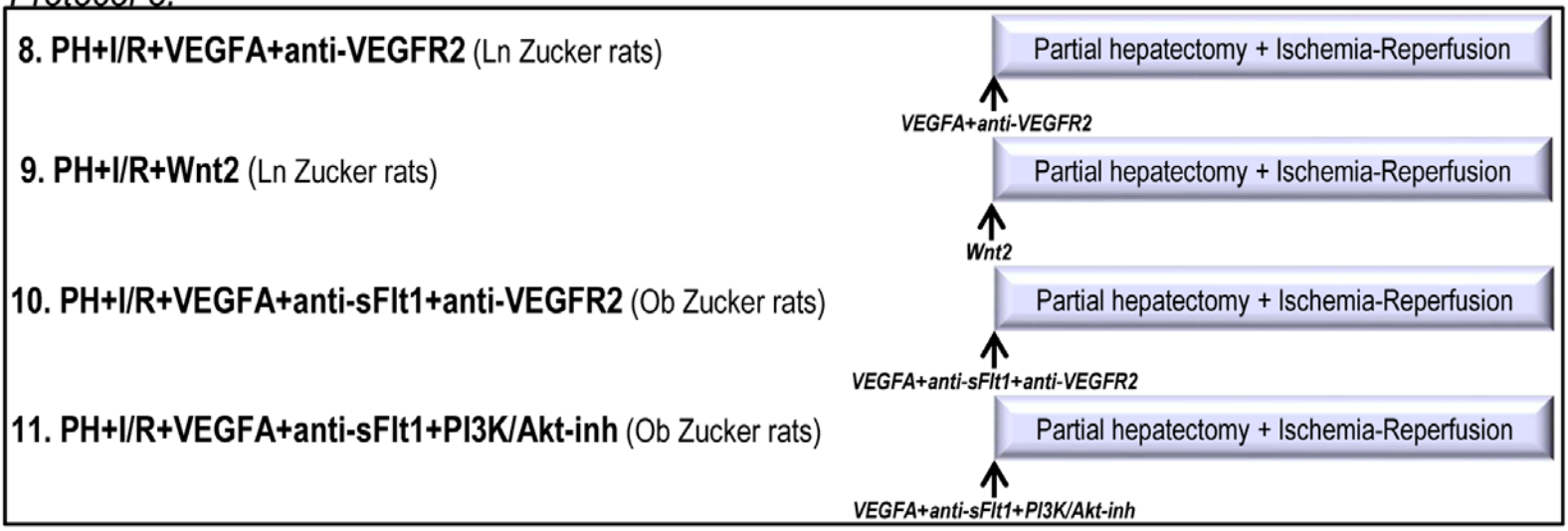
Protocol 1.



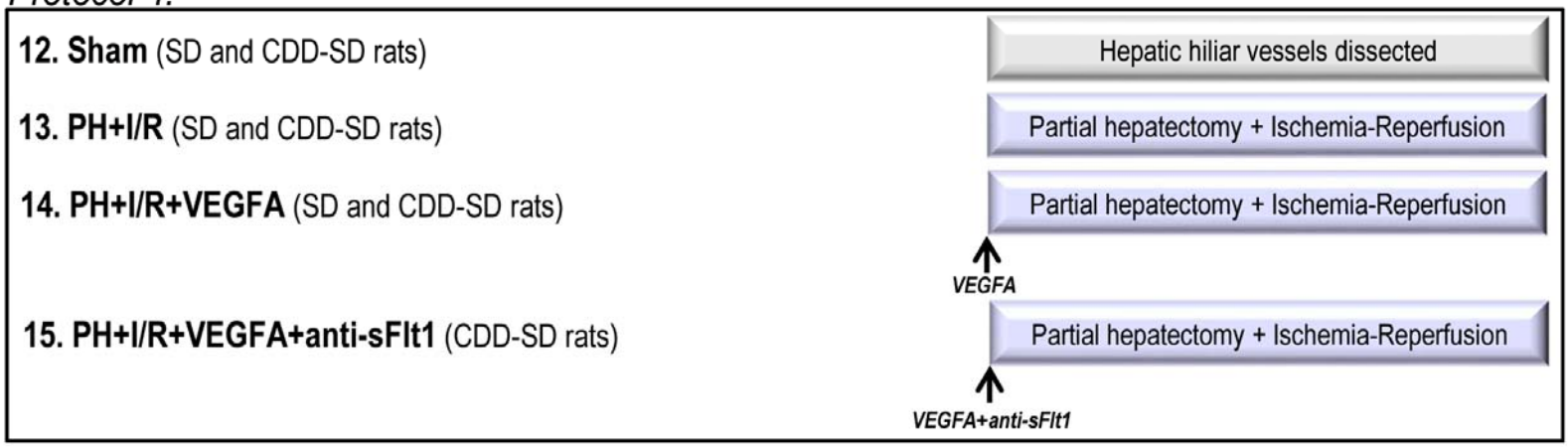
Protocol 2.



Protocol 3.



Protocol 4.



Test at 24 h and 72 h after liver surgery. Plasmatic VEGFA, transaminases, GLDH, ALP, Bilirubin, vWF, HA, sFlt1. Hepatic VEGFA, damage score, MDA, MPO, Ki-67 positive hepatocytes, cyclines, sFlt1, Flt1, VEGFR2, Id1, Wnt2, PI3K, Akt. sFlt1, Flt1, VEGFA in adipose tissue. Survival was monitored for 14 days.