HEPATIC CELL PROLIFERATION PEAKS ONE WEEK AFTER PORTAL VEIN LIGATION AND IS NOT ENHANCED BY INSULIN (EXPERIMENTAL STUDY)

O PICO DA TAXA DE PROLIFERAÇÃO DAS CÉLULAS HEPÁTICAS OCORREU UMA SEMANA APÓS A LAQUEAÇÃO PORTAL E NÃO FOI INFLUENCIADO PELA INSULINA (ESTUDO EXPERIMENTAL)

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ABSTRACT

Introduction: Selective portal vein ligation is currently used to induce hypertrophy of healthy liver segments before partial hepatectomy performed to treat metastatic or primary liver cancer. A major limitation of this procedure is the time interval needed between the portal ligation and hepatic resection, potentially leading to tumour progression. We report the characterization of the liver regeneration kinetics after portal ligation on a rat model, also testing the usefulness of insulin to accelerate liver hypertrophy after the procedure. **Materials and Methods**: Liver function and morphology in male Wistar rats was evaluated 1, 2 or 4 weeks after portal ligation alone or with concomitant continuous intraperitoneal insulin infusion and compared to those of intact controls. **Results**: Selective portal vein ligation induced ipsilateral lobe atrophy and contralateral lobe hypertrophy. In this model, liver lobe hypertrophy reached its peak at 1 week after portal ligation (PVL:0,13cm; PVL+Insulin:0,34cm) and insulin infusion had no significant additional effect on the liver response to portal ligation. **Discussion and conclusion**: Hepatocyte proliferation after selective portal ligation was a fast phenomenon, peaking as early as 1 week after the procedure. Insulin was not useful as adjuvant therapy to further increase hepatocyte proliferation after portal ligation in the rat.

Keywords: *partial hepatectomy, portal vein ligation, insulin, proliferation.*

RESUMO

Introdução: A laqueação seletiva da veia porta é usada para indução de hipertrofia de segmentos hepáticos, previamente à realização de hepatectomia parcial no tratamento de cancro do fígado primário ou metastático. Uma limitação importante deste procedimento é o intervalo de tempo necessário entre a laqueação portal e a ressecção hepática, com o potencial risco de progressão tumoral. Neste artigo reportamos a caracterização da cinética da regeneração celular hepática após laqueação portal no

rato, testando também a insulina como eventual fator acelerador deste processo. **Material e Métodos:** Foram avaliadas a função e morfologia hepática de ratos Wistar machos 1, 2 e 4 semanas após laqueação portal em exclusivo ou associada a infusão contínua de insulina intraperitoneal, e comparados os resultados com os de ratos controlo intactos. **Resultados:** A laqueação portal induziu atrofia do lobo ipsilateral e hipertrofia do lobo contralateral do fígado. A hipertrofia lobar do fígado atingiu o seu valor máximo 1 semana após o procedimento (PVL:0,13cm; PVL+Insulin:0,34cm). A infusão de insulina não teve qualquer efeito adicional na resposta à laqueação seletiva da veia porta. **Discussão e conclusão:** A proliferação dos hepatócitos após laqueação portal é um fenómeno rápido, atingindo o seu máximo tão cedo como 1 semana após o procedimento. A insulina não teve utilidade como tratamento adjuvante no aumento da proliferação hepatocitária após laqueação portal no rato.

Palavras chave: *hepatectomia parcial, laqueação portal, insulina, proliferação hepática.*

INTRODUCTION

The liver has a unique ability to regenerate upon partial hepatectomy which permits surgical removal of more than half of its segments[1]. In metastatic or primary liver cancer, ligation of the portal vein branches that supply the diseased segments is a standard surgical strategy before performing partial hepatectomy[2]. This procedure incites the healthy non-ligated liver to undergo rapid hepatocyte proliferation that will overcome in weeks or months the transient liver deficit caused by the selective portal vein ligation (PVL)[3]. A major limitation of this treatment approach is the time interval needed between the PVL and hepatic resection, which could lead to tumour progression. Thus, improving the current knowledge on liver regeneration kinetics upon PVL, and eventually developing additional or alternative means to promote or accelerate liver cell proliferation, would be crucial to allow a decrease in the time interval between PVL and partial hepatectomy and to enhance the likelihood of patient survival[4].

To investigate the liver regeneration phenomenon after PVL, we have successfully applied this surgical technique to a rat model. In addition, we have investigated whether liver cell proliferation is enhanced by continuous low dose insulin treatment, since this anabolic hormone has been previously shown to promote cell proliferation[5].

Diabetes has been shown as an independent factor to reduce hypertrophy ratio after portal vein embolization[6]; insulin, as also glucagon, have been implicated in liver regeneration[7]. Based on these two facts and being insulin a low-cost molecule, easily acquired and handled in clinical practice, with low risk of use, the authors decided to test it as a potential hepatotrophic agent.

MATERIALS AND METHODS

Adult 8 week old male Wistar rats (n=42), purchased from a local commercial breeder (Charles River, Barcelona, Spain), were maintained in individual cages to allow food intake assessment, under controlled temperature (21–23 °C), humidity (50-60%) and light (12 h light, 12 h dark, lights on at 7.00 hours), with free access to standard rat chow (4RF21, Mucedola, MI, Italy) and tap water. Animals were acclimatized to our local facilities for 7 days before surgery.

Animals were randomized and divided into weight-matched groups to be submitted either to selective portal vein ligation and intra peritoneal insulin perfusion (PVL-insulin, n=18), portal vein ligation alone (PVL, n= 18) or no intervention and keeping them under the same conditions (control, n=6). The insulin dose was established as the highest tolerated dose that would not cause hypoglycaemia in non-diabetic animals.

The rats of the two groups submitted to PVL were further divided in 3 subgroups that were sacrificed 1, 2 and 4 weeks after the surgery, and compared with the untreated animals at the end of the study. Body weight was measured 3 times weekly using an appropriate weighing cell (Monobloc, Metterr, Toledo, USA) recording to the nearest 1g, and the remaining food in the hopper was weighed at the same time using a precision weight balance (Kern, KB 5000-1) recording to the nearest 0.1 g, which allowed for daily food intake to be calculated. Both rat groups with portal ligation (n=36) were sacrificed 1, 2 or 4 weeks after surgery (n= 6 / per group at each time point).

The experimental protocol and all procedures were approved by the local Ethics Board for Animal Research and followed the European Union laws on animal protection (86/609/EC).

Surgical Procedures

After an overnight 12 h fast, rats were given analgesia and sedation with a subcutaneous injection of acepromazine (2 mg/kg), butorfanol (2.5 mg/kg) and diazepam (2 mg/kg). Anaesthesia was initiated with 3 % isoflurane and maintained with 1.5 % isoflurane administered through a rodent's face mask. The animals were kept in spontaneous ventilation during the entire surgical procedure. Prophylactic antibiotic therapy consisting of cefazolin (100 mg/kg) was administered intraperitoneally immediately before laparotomy, and all surgical procedures were performed under aseptic conditions.

After midline incision, the left and median branches of the portal vein were identified and ligated using ligation surgical clips (5 mm Titanium clips, Weck, USA.) In the PVL-insulin group, the continuous intraperitoneal insulin perfusion was delivered through osmotic mini-pumps (ALZET, 2006 model) with an output of 0.15 IU/ h of regular insulin (Actrapid, Novonordisk, Denmark) into the peritoneal cavity just before surgical wound closure. All animals were given 5 ml sterile warmed saline subcutaneously to avoid dehydration, allowed to recover spontaneously from anaesthesia, returned to their home cages and placed on regular solid diet on the first postoperative day.

Ultrasound evaluation

All rats submitted to surgery, in PVL and PVL-insulin groups, underwent liver ultrasound evaluation with a high resolution linear 12MHz probe (Vivid i, linear probe 6-13MHz, GE ®, CA, USA) before surgery and at the end of the study period. The aim of the image protocol was to exclude focal liver lesions, to confirm portal vein patency, to evaluate liver overall morphology, and to accomplish measurements of the longitudinal, transversal and antero-posterior diameters of the whole liver and of the caudate lobe.

Liver function parameters

At the scheduled times, according to groups protocol, twelve hours fasted rats were sacrificed by exsanguination under deep anaesthesia with CO2. The liver was removed, weighed and measured. Epididymal white tissue pads were also removed to assess relative body composition. Blood was collected by cardiac puncture into chilled lithium heparin tubes containing a protease inhibitor (0.02 ml/10 ml; Trasylol, Bayer, Portugal). The tubes were kept on ice and centrifuged at 3,000 rpm for 8 min at 4 °C and plasma was separated and stored at –20 °C until the assayed. Blood glucose levels were analysed by the glucose oxidase method using a glucometer (One Touch Ultra, Lifescan, Johnson and Johnson, Milipitas, CA, USA), while the other liver function biochemical parameters, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase and albumin were quantified by colorimetric methods using an automated chemistry analyser (Imola, Randox, UK).

Histological analysis

Livers were fixed in 10% buffered formalin for 96 hours, followed by processing and paraffin embedding using routine histology protocols to analyse the morphology of the right and the caudate lobes and of non-ligated lobes. Formalin-fixed, paraffin embedded tissue sections (3 µm) were then mounted on adhesive microscope slides Superfrost (Thermo Scientific®).

To evaluate liver glycogen content, Periodic Acid-Schiff (PAS) staining was used. Sections were successively deparaffinized, rehydrated in graded alcohols and then incubated in PAS 1% for 15 minutes. Sections were rinsed in water and incubated for 25 minutes in Schiff reagent followed by 1 minute in sodium metabisulfite 0.5%. Hematoxylin was used as nuclear counterstaining.

Immunohistochemistry (IHQ) was used to evaluate the cell proliferation index, Ki-67. Sections were deparaffinized, rehydrated in graded alcohols to water, and processed using the Kit Novolink Polymer Detection System (Leica). For antigen retrieval, the sections were boiled for 3 minutes in 0.01 M-citrate buffer at pH 6.0 with 0.05% Tween 20. The endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol, followed by incubation in the protein block solution for 5 minutes. The samples were incubated overnight at 4° C in the primary antibody Ki-67 (27R-14; 1:100; Cell Marque), followed by the post-primary block for 30 minutes, and by the polymer for 30 minutes. Diaminobenzidine was used as chromogen, and hematoxylin as nuclear counterstaining.

Morphometric analysis

The number of nuclei, nucleoli, mitotic figures and bi-nucleated cells were counted, in a minimum of 10 high magnification fields (400x). The bile ducts size was evaluated by measuring the major and minor diameters of 10 bile ducts in Hematoxilin Eosin stained sections with a 200 magnification, using the Image J software.

For the morphometric analysis of PAS and Ki-67 immunohistochemistry stained slides, a minimum of 10 photos were taken for each sample at 200x magnification, using a Leica DFC290 camera and Zeiss image acquisition software (West Germany) for Windows, performed by the same observer under the same magnification and illumination conditions. Images were analysed using the ImageJ software (National Institutes of Health, USA) with the colour deconvolution plug-in ("H Dab" for Ki-67 analysis and "H PAS" for the PAS analysis), which allows the separation of the stained area from the initial image and quantification of the percentage of the stained area.

Data analysis

Results are presented as mean ± standard error of the mean (Mean ± SEM), unless otherwise specified. The Kolmogorov–Smirnov test was used to determine the normality of the groups. Comparison between groups was performed using a one-way ANOVA or a Kruskal–Wallis one-way analysis of variance test accordingly to the previously established normality. When using a one-way ANOVA, a post-hoc Tukey test was used, and the post-hoc Dunn test was performed when the Kruskal–Wallis one-way analysis was used. To evaluate the differences between two independent parameters, an unpaired t-test or a Mann-Whitey was used, according to the normality of the data. Data were analysed with GraphPad Prism (version 5.00).

RESULTS

During the experimental period all rats displayed the same growth pattern, with no significant differences with regards to food intake, body weight or relative body composition in adipose tissue, as assessed by the percentage of epididymal adipose tissue (Table 1).

Table 1 – **Food intake, body weight and percentage of white adipose tissue of the animals in the different study groups (mean ± SEM) submitted to PVL plus insulin infusion (PVL-insulin), PVL alone (PVL) and controls. No statistically significant differences were found between the 3 groups of rats.**

	Daily Food Intake (grams)			Body Weight (grams)			Epididymal white adipose tissue (grams)		
	PVL-Insulin	PVL	Control	PVL-Insulin	PVL	Control	PVL-Insulin	PVL	Control
1 WEEK	17 ± 2	13 ± 3	-	321 ± 8	314 ± 8	-	5,4 ± 0,4	3,9 ± 0,9	-
2 WEEKS	17 ± 1	19 ± 1	-	307 ± 8	312 ± 11	-	5,1 ± 0,5	5,2 ± 0,2	-
4 WEEKS	19 ± 1	20 ± 1	19 ± 1	325 ± 15	319 ± 15	309 ± 9	6,4 ± 0,4	6,0 ± 0,4	4,8 ± 0,4

* No significant differences among the three groups at all study times.

Gross anatomy observation (Fig. 1A and B) and ultrasound evaluation (Fig. 1C and D) of the liver of the rats showed that PVL induced both atrophy of the left/medial lobe (white arrows in Fig. 1 A and B), and compensatory hypertrophy of the right and caudate lobes (black arrows in Fig. 1 A and B). Treatment of PVL rats with additional insulin did not result in any significant difference in weight or longitudinal

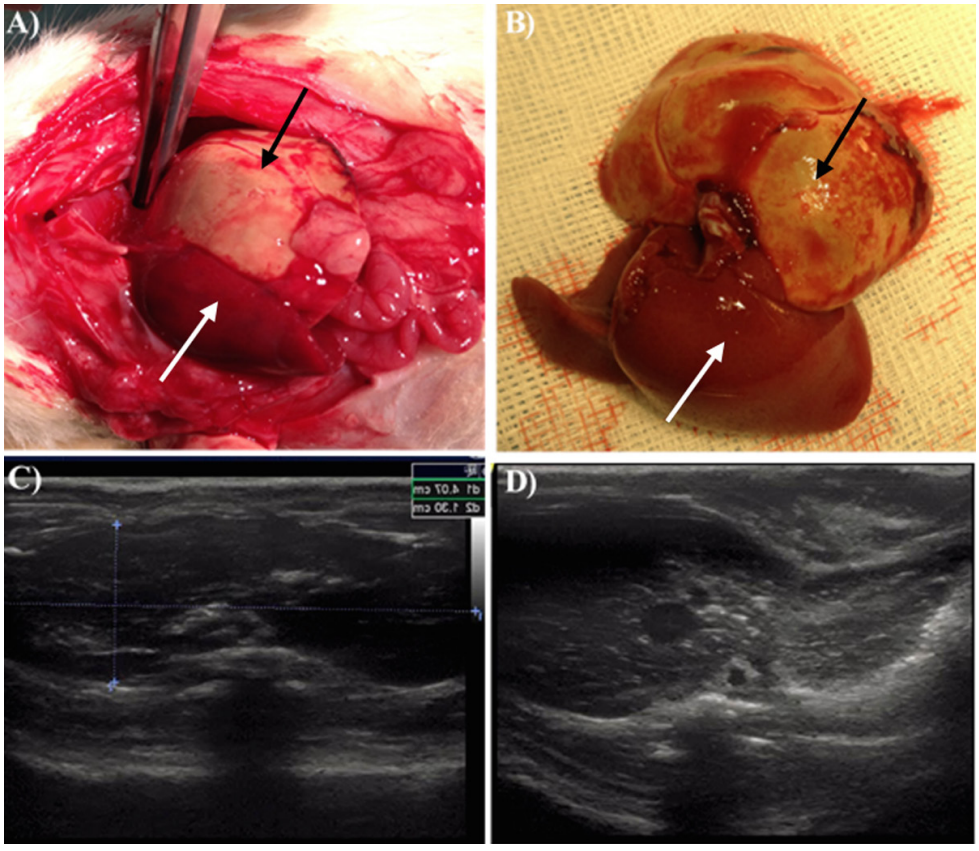

Figure 1 – **(A, B) Photograph displaying a noticeable atrophy of the left/medial lobe (black arrows) and compensatory hypertrophy of the right and caudate lobes (white arrows) of the liver, after portal vein ligation. Ultrasound images of the liver before (C) and after (D) the PVL procedures.**

diameter of the liver (Table 2). Insulin treatment of PVL rats also did not significantly change any biochemical parameter of liver function during the 4 weeks of the study (Table 3).

Light microscopy analysis of the rats' liver showed that PVL caused significant decreased in PAS staining of the glycogen content of hepatocytes; insulin did not trigger any significant change in liver glycogen content of rats submitted to PVL (Fig. 2 A-G). The diameter of liver bile ducts increased in response to PVL and was not altered by insulin treatment (Fig. 3).

The livers of the rats responded to PVL with hepatocyte proliferation, as expressed by the enhanced numbers of mitotic figures and binucleated nuclei observed by light microscopy and confirmed by the increased percentage of liver tissue stained by Ki-67, an immunocytochemical marker of cell proliferation. Treatment of rats submitted to PVL with insulin did not modify any of the histological parameters cited above. No microscopic signs of inflammation, steatosis or fibrosis were observed in the liver of any of the rats (Fig. 4 A-G).

Table 2 – **Liver weight, longitudinal and caudate lobe diameters, evaluated by ultrasound in PVL-insulin, PVL alone groups and controls (mean ± SEM). PVL induced significant increase in the right and caudate lobes and significant atrophy in the left/medial lobe of the liver. No significant differences were observed after insulin infusion.**

	Liver Weight (Grams)			Liver longitudinal diameter (cms)		Caudate lobe longitudinal diameter (cms)	
	PVL-Insulin	PVL	Control	PVL-Insulin	PVL	PVL-Insulin	PVL
BASELINE	-	-	-	2.62 ±0.02	2.73 ±0.03	1.06 ±0.11	1.04 ±0.09
1 WEEK	11.32 ± 0.72	11.32 ± 0.87	-	2.85 ±0.23	2.76 ±0.23	1.41 ±0.54	1.38 ±0.41
2 WEEKS	11.42 ± 1.09	10.35 ± 0.65	-	2.94 ±0.32	2.90 ±0.18	1.81 ±0.9	1.53 ±0.52
4 WEEKS	10.60 ± 0.37	10.23 ± 0.70	11.09 ± 1.31	3.31 ±0.71	3.39 ±0.58	1.85 ±0.71	1.84 ±0.87

* No significant differences among the three groups at all study times.

Table 3 – **Biochemical liver function parameters in PVL-insulin, PVL alone groups and controls, at the different study time points (mean ± SEM). PVL caused statistically significant differences in comparison to intact controls but no significant differences were seen between PVL alone and PVL-insulin.**

	Glucose (mg/dL)			Alanine transaminase (U/L)			Aspartate Aminotransferase (U/L)			Alkaline phosphatase(U/L)			Albumin(U/L)		
	PVL-Insulin	PVL	Control	PVL-Insulin	PVL	Control	PVL-Insulin	PVL	Control	PVL-Insulin	PVL	Control	PVL-Insulin	PVL	Control
1 Week	119.8 ± 11.3	118.5 ± 20.6	-	200 ± 91	110 ± 24	-	369 ± 114	360 ± 71	-	163.3 ± 35.1	152.5 ± 54.3	-	2.9 ± 0.1	2.8 ± 0.1	-
2 Weeks	137.7 ± 14.2	164.2 ± 20.4	-	178 ± 42	119 ± 29	-	848 ± 521	206 ± 34	-	157.2 ± 9.8	185.7 ± 18.6	-	2.9 ± 0.2	3.0 ± 0.1	-
4 Weeks	146.3 ± 7.7	116.5 ± 23.9	226,6 ± 58,1	141 ± 72	75 ± 19	159 ± 104	257 ± 93	181 ± 39	335 ± 117	122.5 ± 13.1	114.0 ± 8.3	-	3.3 ± 0.2	3.0 ± 0.1	3.1 ± 0.2

* No significant differences among the three groups at all study times.

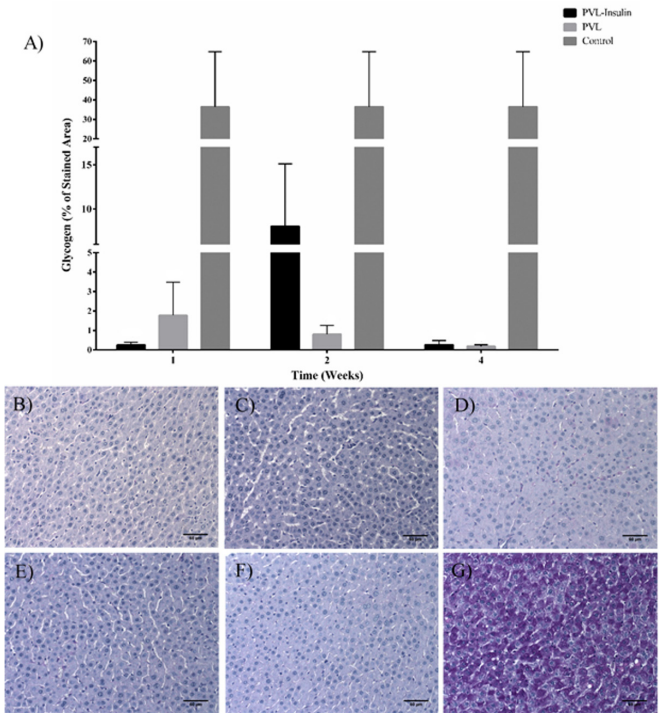

Figure 2 – **(A-G) There were no differences in the liver PAS staining, a surrogate marker for glycogen deposition, between PVL-insulin and PVL rats, at the different study time points, although the PAS staining in the two groups that underwent PVL there was a significantly lower when compared with the staining in liver of control rats (A).**

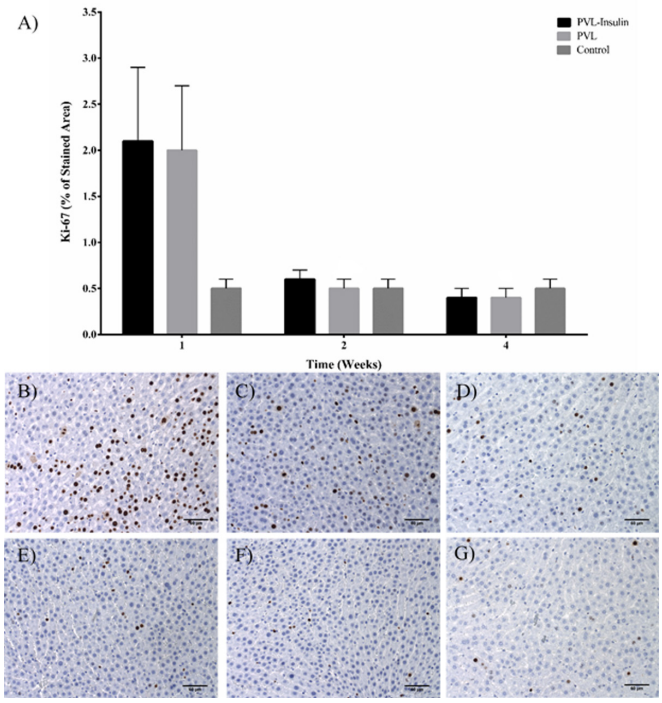

Figure 4 – **(A-G) The percentage area with Ki-67 immunohistochemistry staining in the liver of PVL rats was significantly higher compared to controls. There were not significant differences between the PVL-insulin and PVL at the different study time points. In addition, the percentage area labeled by Ki-67 immunohistochemistry staining and the number of mitotic figures peaked one week after PVL, decreasing significantlythereafter.**

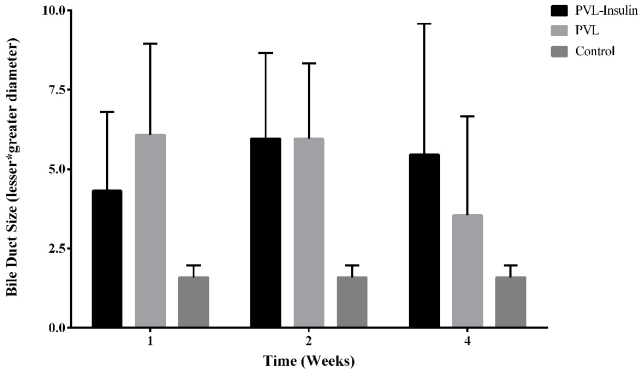

Figure 3 – **Bile duct diameters were significantly increased in PVL rats when compared to control animals. There was no statistically significant difference between bile duct diameters of rats submitted to PVL-insulin and PVL alone.**

DISCUSSION

The main goals of this study were to define the kinetics of the hepatocyte proliferation after selective PVL and to evaluate whether insulin could be used to further promote the compensatory liver hypertrophy that is triggered by PVL.

We found that PVL did not induced any significant changes in food intake, body weight, relative body composition or biochemical liver function parameters, similarly to data regarding PVL reported in human studies[8]. PVL induced significant increase in liver size and weight, compared to control rats. All animals underwent ultrasound examinations, which although not as reliable as computed tomography, allowed the assessment of the overall organ morphology and liver measurements. Liver histological analysis revealed

a significant decrease in glycogen deposits and an increase in cell proliferation markers after PVL, as it is usually observed in the human setting[9].

Taken together, these data reveals that we were able to replicate PVL in the rat and that in this model, liver regeneration after PVL is a fast event peaking as early as one week after surgery. Previous reports have shown that liver tissue regeneration was able to recover the original liver mass within two weeks after partial hepatectomy in rodents[10]. Cell hypertrophy is the first regeneration response, followed by proliferation when hypertrophy is not sufficient to recover the original mass. Hepatocytes have been observed by electronic microscopy to accumulate lipids and glycogen as early as 72h after partial hepatectomy, which contributes to the rapid increase in cell size that returns to baseline with no obvious change in hepatocyte ultrastructure with the exception of the enlarged nuclei[11]. Given that lipid and glycogen accumulation is one of the mechanisms proposed to account for the initial increase in liver volume, it is pertinent to stress out the fact that these features were not observed in our current study. Possible explanations could be that, in our research, the time interval until the first observation was longer and also that the changes in lipid depositions were documented by electronic and not optic microscopy, as performed in the current study[11]. Previous studies in rodents have also shown that liver weight increases up to 8 hours after feeding, with 66% of the volume accountable to water and glycogen[12], which are rapidly depleted after overnight fast. Furthermore, a possible explanation for the decrease in liver glycogen deposits, observed in our study in the rats submitted to PVL surgery, as compared to that observed in intact controls is the depletion of energy deposits used to overcome the surgical stress, since all animals were sacrificed under the same fasting conditions.

Liver regeneration occurs through the proliferation of all existing mature cellular liver populations that synthesize connective tissue proteins and secrete several growth factors. These processes are influenced by humoral factors like interleukin 6, norepinephrine, insulin and bile acids[13, 14]. In order to promote faster liver regeneration, shorten the period of time between PVL and hepatectomy, we have postulated that insulin, because of its anabolic actions, might enhance hepatocyte proliferation in PVL rats. In 1975, Starzl et al. and in 1984 Francavilla et al. discovered that insulin in the portal vein is a hepatotrophic factor that is essential for liver regeneration[15, 16]. The choice of this hormone is related to the knowledge we had from previous studies that diabetes is an independent factor to reduce hypertrophy ratio of unembolized lobe after portal vein embolization[8, 17, 18]. Another very important factor in the selection of this putative hepatotrophic factor, was the ease of acquisition, handling and price.

This hypothesis was not confirmed in our investigation. A possible explanation for the absence of trophic response to the hormone could be that the insulin dose was insufficient to induce the hepatocyte changes observed *in vitro* studies, since the use of higher insulin doses *in vivo* is limited by the risk of inducing hypoglycemia. Another explanation is that exogenous insulin, regardless of the dose used, does not have a hepatotrophic enhancing effect when used in normoglycemic animals. An experimental study, published in 1982, concludes that the exogenous supply of insulin and glucagon by permanent infusion into the portal vein, did not significantly stimulate the rate of regeneration normally controlled by endogenous pancreatic hormones[19].

Six studies published between 1984 and 2014, explore insulin-induced liver regeneration: five experimental studies carried out in post-hepatectomy rats and a clinical study in patients undergoing liver transplantation; globally all conclude that endocrinal hormones, like insulin, are important hepatic mitogens with vital roles in inducing the process of liver regeneration[20]. All of these experiments have in common the performance of hepatectomy in association with exogenous insulin administration alone or in combination.

Hepatocyte proliferation is proportional to the severity of the liver injury/resection[7, 21], therefore,

the stimulus for liver regeneration conditioned by hepatectomy In the present study, to the putative synergy of the effect of the administration of exogenous insulin with liver resection was not present.

This study demonstrated that the peak of liver cell proliferation of the non-ligated liver occurs as early as 7 days after the PVL surgery, with a significant decrease in proliferation markers after this time point. In the clinical setting, the time interval currently used between PVL or portal vein embolization (PVE) and hepatic resection varies between 2 to 9 weeks[17, 22]. This time interval has been set according to previous studies showing that after portal vein occlusion induced ischaemia, the non-ligated or non-embolized liver volume peaked at two weeks after the procedure[23]. However, similarly to our observation in rats, previous studies with pigs have also shown that the hepatocyte replication rates assessed by ultrasonography and nuclear bromodeoxyuridine incorporation peaked 7 days after PVE or PVL[24].

There are many limitations in conducting liver disease research directly in humans, particularly with surgical models, due to the ethical issues.

For several reasons (size, short life span, breeding capabilities, genetic engineering options, and similarity to human hepatic lesions at histological and molecular levels), the mouse and rat has long been regarded as one of the animal models most suitable for this purpose[25]. For that reason, using appropriate animal models, can be advantageous in ascertaining the physiology of pathologic events and in developing new treatment modalities. However, the differences between both models make the conclusions of these animal studies difficult to apply to our patients.

CONCLUSIONS

The investigation herein reported has shown that portal vein ligation is able to induce liver hyperplasia peaking as early as one week after surgery and that this was not improved by the use of adjuvant insulin therapy. The negative result we are reporting here, the non-detected synergy of insulin and PVL, brought to the field of research several issues that, if solved, may have clinical role in reducing the time between PVL and the curative intervention.

If our data may be translated to human liver recovery and function they suggest that the time interval between PVL and partial hepatectomy may be safely shortened without an increased risk of liver failure. The shortening of the interval between PVL and hepatectomy may decrease the risk of tumour progression.

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