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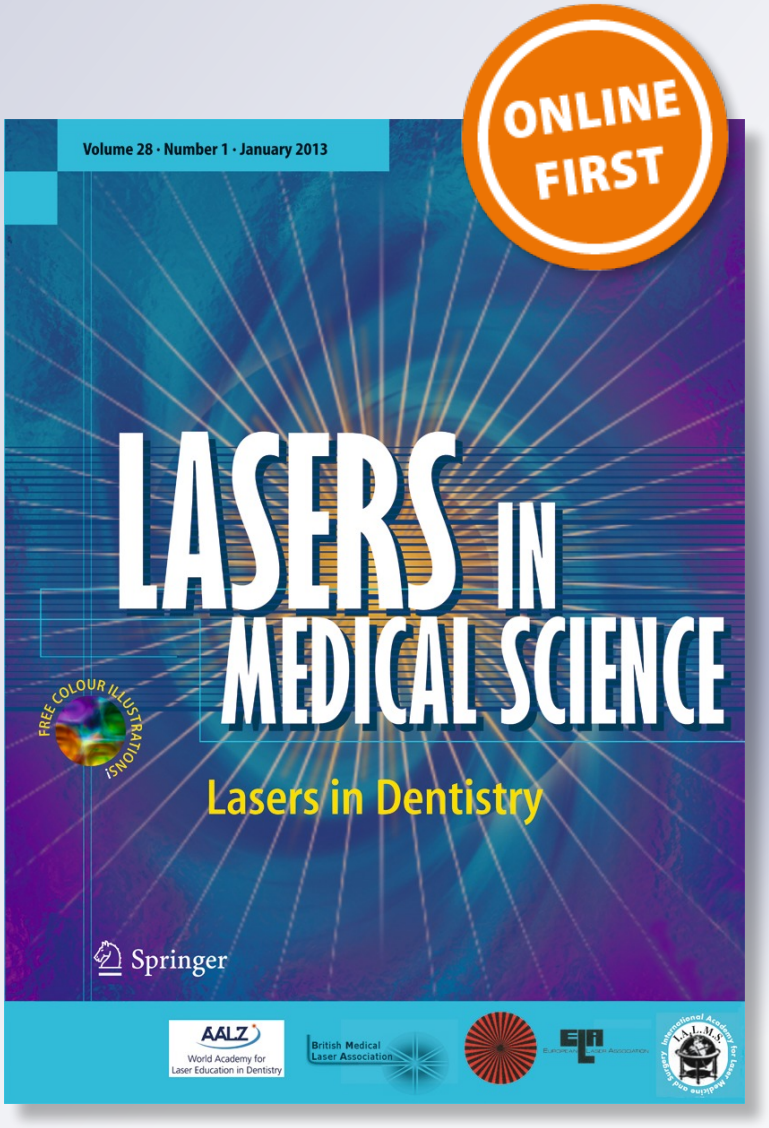

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ORIGINAL ARTICLE

Liver regeneration following partial hepatectomy is improved by enhancing the HGF/Met axis and Akt and Erk pathways after low-power laser irradiation in rats

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Abstract A simple, easy, and safe procedure aiming to improve liver regeneration could be of great clinical benefit in critical situations such as major hepatectomy, trauma, or hemorrhage. Low-power laser irradiation (LPLI) has come into a wide range of use in clinical practice by inducing regeneration in healthy and injured tissues. However, the effect of LPLI on the process of liver regeneration, especially those related to the molecular mechanisms, is not fully understood. Thus, the aim of the present study was to investigate the main molecular mechanisms involved in liver regeneration of partially hepatectomized rats exposed to LPLI. We used Wistar male rats, which had their remaining liver irradiated or not with LPLI (wavelength of 632.8 nm and fluence of 65 mW/cm^2) for 15 min after a 70 % hepatectomy. We subsequently investigated hepatocyte growth factor (HGF), Met, Akt, and Erk 1/2 signaling pathways through protein expression and phosphorylation analyses along with cell proliferation (proliferating cell nuclear antigen (PCNA) and Ki-67) using immunoblotting and histological studies. Our results show that LPLI can improve liver regeneration as shown by increased HGF protein expression and the phosphorylation levels of Met, Akt, and Erk 1/2 accompanied by higher levels of the PCNA and Ki-67 protein in the remnant livers. In summary, our results suggest that LPLI may play a clinical role as a simple, fast, and easy-to-perform strategy in order to enhance the liver regenerative capacity of a small liver remnant after hepatectomy.

Keywords Hepatectomy · Laser · Liver regeneration · HGF · Met · Proliferation · Rats

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Introduction

The capacity for the liver to regenerate has been long recognized and is related to a sequence of molecular and cellular events that result in the induction of DNA synthesis, cell cycle progression, mitosis, and cell division [1, 2]. Several strategies to accelerate these events have been tested with controversial results [3–8]. A simple, easy, and safe procedure aiming to prepare the liver to regenerate could be of great clinical benefit in critical situations such as major hepatectomy, trauma, or hemorrhage.

In the clinical setting, preparing the liver parenchyma for regeneration during surgery is very interesting. Theoretically, it could lead to a better postoperative outcome in cases with a small liver section remaining following liver resection or living-related liver transplantation. In the past,

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several strategies generically known as "pre/post-conditioning" were tested in order to stimulate liver regeneration before massive resection and in situations when the liver remnant may be too small [3, 4, 9].

Different techniques for conditioning the liver remnant can be pharmacological or mechanical. Ischemic preconditioning, intermittent clamping of the hilum, and stutter clamping release are examples of mechanical strategies, but their clinical effectiveness is still under investigation [3–6]. Inhibitors of PPAR-gamma, *n*-acetyl cysteine, and pentoxifiline are examples of pharmacological approaches that have also been used in the past in order to improve liver capacity to respond to ischemic injury. However, these drugs may interfere in important homeostatic systems, such as coagulation, that make their usage very restricted [7, 8]. There is some evidence showing that low-power laser irradiation (LPLI), especially red and near infrared light, may have an important role towards inducing regeneration in healthy and injured tissues [10, 11].

LPLI has come into a wide range of use in clinical practice especially for presenting a very important physiological effect: the induction of cell proliferation [10]. This biostimulatory effect has been shown in many cell type studies in vitro, including keratinocytes [12], fibroblasts from different systems [13–15], human osteoblasts [16], lymphocytes [17], mesenchymal stem cells, cardiac stem cells [18], aortic smooth muscle cells [19], and venous endothelial cells [20]. In particular, LPLI is well known to promote a stimulating effect on cell regeneration accompanied by a consequent improvement in ischemic or mechanical problems in organs such as skeletal muscle, heart, brain, and liver [11, 21–26]. Regarding the main molecular mechanisms involved in the liver regeneration, it is well established that hepatocyte growth factor (HGF) is a mesenchymal-derived pleiotropic cytokine that regulates cell proliferation, anti-apoptosis, motility, morphogenesis, and anti-inflammatory process in hepatic regeneration [27]. These multifaceted events occur via activation of its receptor, a transmembrane tyrosine kinase called Met. Activation of the Met receptor by HGF leads to autophosphorylation on tyrosine residues, followed by the phosphorylation of downstream signaling molecules, including phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathway proteins [27]. However, the effect of LPLI on the process of liver regeneration, specifically related to the molecular mechanisms, is not yet fully understood. The aim of the present study was to investigate the main molecular mechanisms involved in liver regeneration of partially hepatectomized rats (70 % hepatectomy) exposed to LPLI.

Materials and methods

Study subjects

All experimental protocols were approved by the Animal Care and Use Committee at the State University of Campinas (no. 1962-1) and were in accordance with the guidelines for Care and Use of Laboratory Animals. Five-week-old male *Wistar* rats weighing 200–250 g were obtained from the State University of Campinas Central Breeding Center. The animals were maintained under a controlled room temperature (23±2 °C) under a 12/12-h light and dark cycle and were fed with standard laboratory chow and water ad libitum.

All animals were randomly assigned to three groups which consisted of six rats each: group sham-operated controls (sham); group PHx (submitted to partial hepatectomy (70 %)); and group PHx + Laser (submitted to partial hepatectomy (70 %) and laser therapy).

Seventy percent partial hepatectomy (Higgins procedure)

Animals were anesthetized with ketamine 5 % (30 mg/kg) and xylazine 2 % (30 mg/kg) intraperitoneally. Under strict sterile conditions, two thirds partial hepatectomy was performed according to the method of Higgins and Anderson [28]. Briefly, the left lateral and median hepatic lobes, constituting approximately 70 % of the total liver weight, were ligated and resected. In sham-operated controls that were anesthetized as described above, the livers were briefly removed from the peritoneal cavity, but were not tied or excised.

During the surgery, animals were kept warm with a halogen light (45 W, 127 V) and corporeal temperature was monitored by a rectal digital thermometer (YSI Precision 4000A Thermometer) and maintained around 37 °C. Animals were allowed spontaneous ventilation with an oxygen-enriched mixture (40 %) during the entire procedure. To improve the survival rate post-surgery, 20 % glucose was added to the rat's drinking water [29].

Laser treatment

Before closing the surgical wound, the laser group was treated by direct irradiation of the remnant liver with an fluence of 65 mW/cm^2 for a period of 15 min at a distance of 10 cm, with a He–Ne Laser model 3184H V1.0, wavelength of 632.8 nm, and 3.30 mW (Hughes® Aircraft Company Electron Dynamics Division, Culver City, CA, USA). The laser beam was optically expanded to match the entire size of the remaining liver to guarantee uniform exposure. Recordings from the thermocouple placed under the irradiated area showed no elevation of temperature in the abdomen of the rats.

Tissue extraction and immunoblotting

Forty-eight hours after the partial hepatectomy and laser exposure, rats were anesthetized and used 10–15 min later, as soon as anesthesia was assured by the loss of pedal and corneal reflexes. The abdominal cavity was opened and any remaining liver tissue was removed and homogenized immediately in extraction buffer at 4 °C (1 % Triton X-100, 100 mM Tris–HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2.0 mM phenylmethylsulfonyl fluoride, and 0.1 mg of aprotinin/ml) with a Polytron PTA 20 S generator (model PT 10/35; Brinkmann Instruments). Insoluble material was removed by centrifugation for 30 min at 9,000×*g* in a 70 Ti rotor (Beckman, Fullerton, CA, USA) at 4 °C. The protein concentrations of the supernatants were determined by the Bradford dye-binding method [30–32]. In direct immunoblot experiments, protein extracts were separated by SDS-PAGE, transferred to nitrocellulose membranes, and blotted with anti-HGFα, anti-phospho-Met, anti-phospho-Akt, anti-phospho-Erk 1/2, and anti-PCNA. The homogeneity of gel loading was evaluated by blotting the membranes with antibodies against β-actin, Met, Akt, and Erk 1/2 as appropriate.

Immunohistochemistry and histology

To detect Ki-67, microwave postfixation was carried out by a domestic microwave oven which was applied to slides immersed in 0.01 mol/L citrate buffer, pH 6.0, in two 7-min doses separated by a 2-min break. Sections were then incubated at 4 °C overnight with primary monoclonal mouse antihuman Ki-67 clone MIB-1 from Dako (diluted 1:100). The slides were then incubated with avidin–biotin complex LSAB + Kit from DakoCytomation for 30 min followed by the addition of diaminobenzidine tetrahydrochloride as a substrate–chromogen solution. After hematoxylin counterstaining and dehydration, the slides were mounted in Entellan from Merck® [32, 33].

Outcome variables

The outcome variables in this study were changes in the expression levels (arbitrary units) of the proteins (HGF and PCNA), as well as changes in the phosphorylation levels (arbitrary units) of the proteins (Met, Akt, and Erk). These outcomes were obtained by immunoblotting technique. Another outcome variable was obtained by immunohistochemistry method, which consists of the number of cells stained for Ki-67 per field analyzed.

Materials

Antibodies against β-actin (mouse monoclonal, sc-8432), HGFα (rabbit polyclonal sc-7949), phospho-Erk 1/2 (mouse monoclonal, sc-7383), Erk 1/2 (mouse monoclonal, sc-93), and PCNA (mouse monoclonal, sc-25280) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho (Tyr1234/1235)-Met (rabbit monoclonal, #3077), Met (rabbit monoclonal, #8198), phospho (Ser 473)-Akt (rabbit polyclonal, #9271S), and Akt (rabbit polyclonal, #9272) were obtained from Cell Signaling Technology (Beverly, MA, USA). Routine reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless specified otherwise.

Statistical analysis

Data are displayed as mean ± standard error of the mean (SEM). The results of blots are presented as direct comparisons of bands or spots in autoradiographs and quantified by optical densitometry (UN SCAN IT gel®, Silk Scientific Inc., Orem, UT, USA). Multiple comparisons were tested by one-way ANOVA, followed by Tukey's post hoc test, with the significance level set at $p<0.05$ using SPSS software (SPSS for Windows, version 16.0, Chicago, IL, USA).

Results

Laser exposure improves the HGF/Met axis in hepatectomized rats

In this paper, we realized to determine how the HGF expression behaves after laser exposure in hepatectomized rats. As shown in Fig. 1a, the HGF protein expression in the remnant liver of PHx group was increased when compared to the sham group, and the PHx + Laser group exhibited a more pronounced increase when compared with the PHx group.

Since the phosphorylation at tyrosine residues of Met receptor is a hallmark of its activation, we examined the phosphorylation levels of Met. The results showed that tyrosine phosphorylation levels of Met were higher in PHx group compared to the sham group and that the laser exposure induced a more pronounced increase in Met phosphorylation (Fig. 1b). We did not observe differences in Met expression amongst any study groups (Fig. 1b, lower panel).

Laser exposure enhanced Akt and Erk 1/2 signaling along with higher PCNA expression in hepatectomized rats

The downstream pathways of Met mainly related to cell proliferation and growth, such as PI3K/Akt and MAPK pathway, were analyzed. Animals submitted to partial hepatectomy showed higher phosphorylation levels of Akt and Erk 1/2 when compared to the sham group (Fig. 2a, b).We also observed that the PHx + Laser group exhibited a

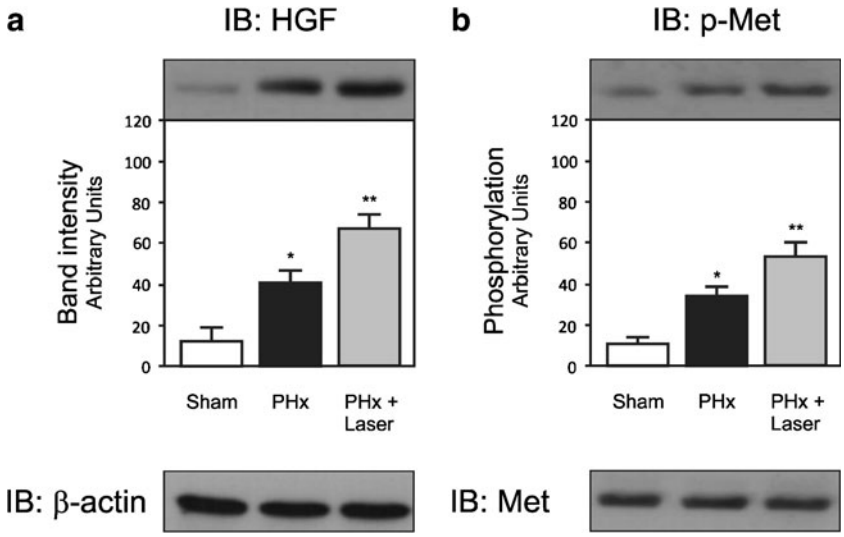

Fig. 1 Representative blottings show HGF content (**a**) and Met activation (**b**) in remaining livers of partial hepatectomized rats exposed or nonexposed to the laser. Total protein expression of Met (**b**). Western blots were quantified after standardization with β-actin. Data were representative of three independent experiments. The values represent the mean ± SEM (n=6). *p<0.05 vs. sham; **p<0.05 vs. PHx. *IB*, immunoblot

significant increase in phosphorylation levels of Akt and Erk 1/2 in comparison with the PHx group (Fig. 2a, b). No changes in Akt and Erk 1/2 protein expression were observed among groups (Fig. 2a, b—lower panels).

In order to strengthen the role of the HFG/Met axis in cell proliferation, we considered PCNA protein expression. The results showed that PCNA expression was increased in the PHx group when compared to sham animals, and that the laser treatment was able to induce an additional increase in PCNA protein expression (Fig. 2c).

Ki-67-positive cells are increased by laser treatment in hepatectomized rats

After 48 h of laser exposure, remaining livers from partially hepatectomized rats were also evaluated by Ki-67 immunohistochemistry to contribute towards data on hepatic regeneration. As expected, we observed a strong increase in the number of positive Ki-67 staining cells from the PHx group in comparison with the sham group (Fig. 3). Indeed, when the partially hepatectomized rats were submitted to laser exposure, the number of positive Ki-67 staining cells was higher than those observed in the PHx group (Fig. 3).

Discussion

Looking for an easy, safe, and intraoperative strategy in order to improve post-hepatectomy liver regeneration, the intraoperative application of LPLI on the remnant liver parenchyma in animal model may be an interesting option. Several studies have demonstrated that LPLI improves hepatic regeneration post-hepatectomy [11, 23–26]. However, the molecular mechanisms behind this effect are not well understood. This paper helps to demonstrate the key

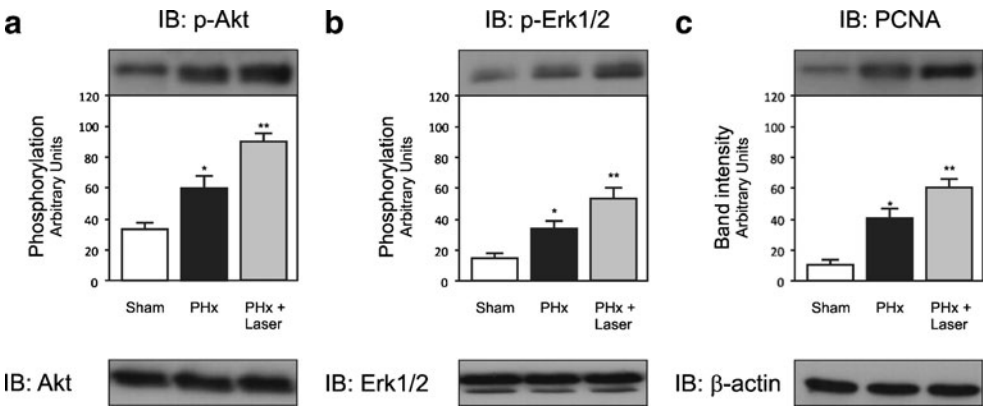

Fig. 2 Representative blottings show Akt (**a**) and Erk 1/2 (**b**) phosphorylation levels, as well as PCNA expression (**c**) in remaining livers of partial hepatectomized rats exposed or nonexposed to the laser. Total protein expression of Akt and Erk 1/2 (**a**, **b**). β-actin was used as a normalization housekeeping protein. Data were representative of three independent experiments. The values represent the mean ± SEM (n=6). *p<0.05 vs. sham; **p<0.05 vs. PHx. *IB*, immunoblot

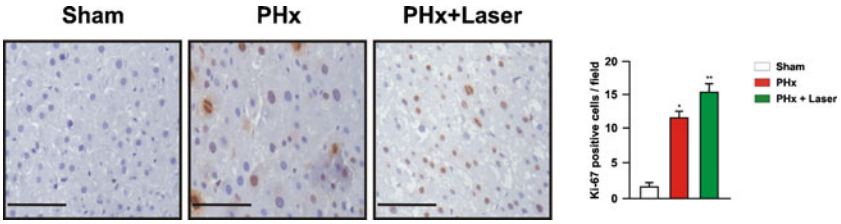

Fig. 3 Representative microphotographs of Ki-67 immunopositive staining hepatocytes on liver and remnant livers of sham, PHx, and PHx + Laser rats. The *graph* indicates the number of Ki-67-positive cells per field; five fields per section. *Scale bars*, 200 μm. The values represent the mean ± SEM (n=6). *p<0.05 vs. sham; **p<0.05 vs. PHx

molecular pathways involved in the LPLI effect on liver regeneration.

In the current study, we used a robust and traditional model of liver regeneration in rodents called the "Higgins procedure," which consists of a two thirds partial hepatectomy [28, 34]. This induces an intense and sustained hyperplasia of the remnant lobes, which reach the original size in about 6–7 days [34]. Furthermore, justifying the approaches chosen in this study, previous research shows that in the model of the partial hepatectomy, the hepatocytes undergo a well-orchestrated cell cycle. DNA synthesis begins 12–16 h after hepatectomy and peaks at 24–48 h. The beginning of mitosis occurs 6–8 h after the surgery, reaching its maximum 48 h later [35]. Based on this information, we studied the remaining livers 48 h after partial hepatectomy surgery and exposure to laser.

With regard to liver regeneration, HGF signaling is a major mechanism that controls cell proliferation and regeneration [35, 36]. When HGF binds to its receptor (Met), this receptor becomes phosphorylated on tyrosine residues, which in turn stimulates the phosphorylation of downstream signaling molecules, including PI3K and MAPK pathway proteins [27]. The PI3K/Akt signaling pathway produces a diversity of the biological effects of HGF, such as proliferation, anti-apoptosis, and migration [37, 38]. Similarly, the Erk 1/2 signaling pathway is also related to mediating proliferation, anti-apoptosis, and differentiation processes [39]. Our data show that after partial hepatectomy, the above-mentioned pathways were most active. It was observed that hepatectomized animals exhibited an increase in the expression of HGF followed by increases in phosphorylation levels of Met and its downstream signaling molecules (Akt and Erk 1/2).

Previous research has focused on the various mechanisms related to the biological effects of LPLI. Studies have demonstrated that the gene or protein expressions of several growth factors, such as transforming growth factor-β, vascular endothelial growth factor, platelet-derived growth factor, insulin-like growth factor I, and others were increased by LPLI [10]. Accompanied with increased expressions of these growth factors, LPLI may induce improved cellular proliferation and differentiation induced by LPLI. Our data have built upon this previous research and demonstrate that HGF expression is increased in rat liver remnants exposed to the laser.

Additionally, LPLI presented mitogenic effects through the activation of specific receptors that are in the "right energetic state" to accept the laser energy, leading to their autophosphorylation and downstream effects [40]. LPLI (632.8 nm) has been reported to induce the phosphorylation of Met receptor and consequently activated downstream signaling molecules [41]. Accordingly, our study demonstrates that the remnant liver of rats exposed to the laser showed an increased phosphorylation of Met accompanied by an augmentation in Akt and Erk 1/2 phosphorylation. In corroboration with our results, studies carried out in myoblasts demonstrated that Akt phosphorylation is augmented when stimulated by LPLI, and when Wortmannin, a specific

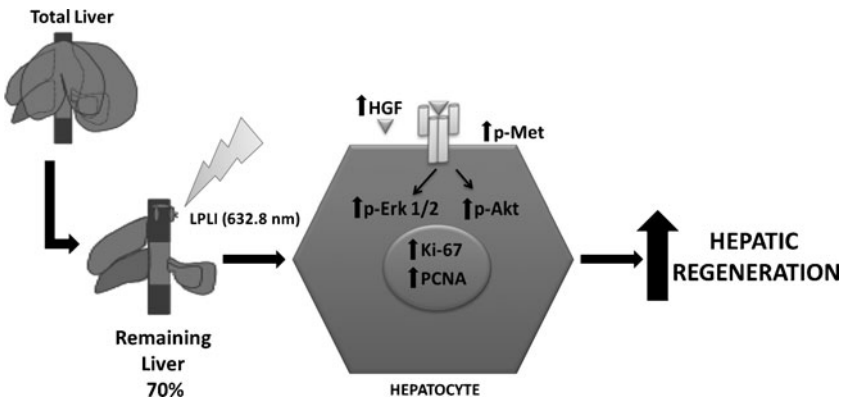

Fig. 4 Schematic representation showing an overview of the LPLI effect on the remaining liver of partially hepatectomized rats. LPLI can improve liver regeneration as evidenced by increasing HGF protein expression and the phosphorylation levels of Met, Akt, and Erk 1/2, along with higher levels of PCNA and Ki-67

inhibitor for the PI3K/Akt pathway was used, the LPLI-induced cell proliferation was attenuated [42]. In addition, another study performed in skeletal muscle cells demonstrates that LPLI also has a role in the MAPK/Erk pathway [41].

In order to confirm and emphasize our results, it is important to consider previously established proliferating markers. PCNA and Ki-67 fulfill this role well, but there are significant differences in expression of these two proteins. PCNA is evident from the M phase to G0 and/or G1, with a much longer half-life. On the other hand, Ki-67 is expressed in mid-G1, through S and G2, and reaches its peak expression in M phase [43]. In our study, in response to the signaling increased on aforementioned pathways (Met → Akt and Erk 1/2), the PCNA expression on the remnant liver of partially hepatectomized rats was increased. Indeed, when the remnant liver was exposed to the laser, PCNA expression presented an additional augmentation. This observation is in accordance with a previous study that also shows the upregulation of PCNA following LPLI stimulation in primary rat satellite cells [44]. In addition, the changes related to improvement in hepatocytes proliferation were consistent with our immunohistochemical study. In this study, we observed a significant increase in the number of hepatocytes stained with Ki-67 in remnant liver exposed to the laser. This approach has reinforced the important effect of the laser in the improvement of liver regeneration.

From a clinical perspective, it is well known that the small liver remnant is an important problem in a liver surgeon's daily practice that may lead to catastrophic postoperative course. It is difficult to predict when the liver remnant is big enough or too small to keep the patient alive in the postoperative period. In this sense, important strategies such as partial portal vein embolization have been proposed in order to avoid this situation, since it improves the volume of the liver remnant [3, 4, 6]. When portal blood flow is redirected to only one half of the liver (the remnant lobe), there is a substantial hyperplasia due to the presence of hepatotropic elements (e.g., insulin) in portal blood [3, 4]. However, this portal embolization must be performed preoperatively and one should wait some weeks (4–5 weeks) for a substantial improvement on the liver remnant. Nowadays, there are not any intraoperative alternative to improve liver regenerative capacity when the surgeon realizes that the liver remnant seems to be too small. This situation often causes a tremendous distress on surgical team. Since it is not possible to improve liver remnant size intraoperatively, an alternative to activate regenerative molecular pathways may be useful in order to avoid postoperative liver failure. To the best of our knowledge, laser has never been used to improve liver remnant regenerative potential. Since low potency laser application is safe and easy to perform and the activation of regenerative molecular pathways does not rely on the depth of laser penetration, thus it is reasonable to propose the utilization of laser during liver resection as a useful therapy in clinical practice.

In conclusion, our results show that LPLI can improve liver regeneration as evidenced by increasing HGF protein expression and the phosphorylation levels of Met, Akt, and Erk 1/2, along with higher levels of PCNA and Ki-67 in the remnant liver of partially hepatectomized rats (Fig. 4). Future research may help to show that LPLI may play a clinical role as a simple, fast, and easy-to-perform strategy in order to enhance liver regenerative capacity of a small liver remnant after hepatectomy.

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Conflict of interest No potential conflicts of interest relevant to this article were reported.

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