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# Hepatic regeneration in a rat model is impaired by chemotherapy agents used in metastatic colorectal cancer

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## Abstract

*Purpose*: Administering Oxaliplatin prior to resection of colorectal liver metastases often induces a Sinusoidal Obstruction Syndrome (SOS), which can affect postoperative patient outcome. Bevacizumab (Anti-VEGF-A) can decrease the severity of SOS and the associated risk of postoperative liver failure. We investigated the impact of both Oxaliplatin (Oxali) and Bevacizumab on liver regeneration in a rat model.

*Material and methods*: Male Wistar rats underwent a 70% partial hepatectomy (PH) 3 days after a 2 ml intraperitoneal injection of either saline (controls, n = 17), or Oxaliplatin 10, 20 or 50 mg/kg, 5-Fluorouracil 100 mg/kg (5-FU) and Bevacizumab 5 or 10 mg/kg in various combinations (total 98 rats, 11 groups, n = 5–18/group). Liver regeneration was assessed by remnant liver weight recovery and cell proliferation by immunodetection of BrDU incorporation (days 1, 2, 3, 7). Hepatic mRNA expression levels of VEGF-A and of its 2 receptors (Flt-1 and KDR) were quantified by PCR technique.

*Results*: Liver regeneration was impaired for 3 days post PH by Oxali 20 alone and Oxali 10 + 5-FU, without any rescue effect by neither Bevacizumab 5 nor 10 mg/kg. Unlike in humans, there were no sinusoidal changes. VEGF-A mRNA expression and receptor 2 (KDR) expressions decreased 24 h post PH in a similar fashion in controls, Oxali 20 and Oxali 10 + 5-FU groups. All groups had recovered over 60% of their liver weight by day 7.

*Conclusion*: Oxaliplatin causes early hepatocyte proliferation impairment post PH, unaffected by Bevacizumab and unexplained by changes in VEGF-A signalling in a Wistar rat model.



*Keywords:* Chemotherapy; Liver regeneration; Bevacizumab

## Introduction

Patients suffering from colorectal adenocarcinoma have or will develop liver metastases in 60% of the cases.[1] Liver resection remains the standard treatment for patients with resectable colorectal liver metastases (CLRM) and is the only single-modality therapy associated with cure. A five-year-survival rate after liver resection of CLRM as high as 58% has been reported.[2–4] Only a minority (15–30%) of patients suffering from CLRM have metastases that are resectable at the time of diagnosis.[5,6] For others, a chemotherapy neoadjuvant treatment for tumour downstaging or downsizing is necessary prior to resection.[7] A response rate of 54–56% is obtained after treatment with 5-fluorouracil (5-FU) combined either to Oxaliplatin, a platinum derivative, or to Irinotecan, a topoisomerase I inhibitor.[8–10] However, these chemotherapeutic agents may induce toxic side-effects on the non-tumoral liver parenchyma, potentially leading to liver dysfunction or defective

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hepatic regeneration post resection. Oxaliplatin is known to cause sinusoidal damage in the form of a Sinusoidal Obstruction Syndrome (SOS),[11] whilst Irinotecan can induce steatohepatitis, associated with 15% perioperative mortality following liver resection, mainly linked to liver failure.[12] In Oxaliplatin-induced SOS, hepatic sinusoids are dilated associated to extravasation into centrolobular hepatic zones, leading to portal hypertension in the most severe form, called Nodular Regenerative Hyperplasia (NRH).[13,14] Several authors have indeed reported an increased morbidity with liver failure after major hepatectomy, an increased need for transfusion and a longer hospital stay after liver resection in patients treated preoperatively with Oxaliplatin.[15–17] However, recent newly developed molecular targeted therapies such as Bevacizumab, a monoclonal antibody to vascular endothelial growth factor (VEGF), and Cetuximab, an anti-epidermal growth factor receptor (EGFR) appear promising. These drugs, usually administered in combination with cytotoxic agents, are reported to induce a cytostatic response rate reaching 70%.[18] In addition to its antitumoral effects, Bevacizumab has been shown to decrease both the severity of SOS[19–21] and the associated risk of postoperative liver failure.[22] Experimental studies have to date, however, yielded conflicting results regarding the impact of anti-VEGF therapies on liver regeneration.[23,24] In this study we investigated whether Oxaliplatin alone or combined to 5-FU had a direct impact on liver regeneration in a Wistar rat model, and whether Bevacizumab modified this effect.

## Material and methods

### *Animals*

In total, 98 male Wistar 6–7-week-old rats weighing 175–250 g (Centre d'élevage JANVIER, Le Genest-Saint Isle, France) were housed in our animal facility and were kept in a 12-h-light cycle, temperature and humidity controlled environment where they had ad libitum 24-h access to water and food. Animals were handled following the guidelines for humane care for laboratory animals established by the Université catholique de Louvain (UCL), in accordance with European regulations.

### *Chemotherapy*

Three days prior to partial hepatectomy (day-3), following tail-blood collection, 98 rats were given an intraperitoneal (IP) injection of either chemotherapy diluted into 2 ml of saline or saline alone (controls, 2 ml) under light diethyl ether anaesthesia. There were 11 groups of rats (including 1 control group, n = 17), receiving Oxaliplatin (Oxali) 10 mg/kg alone (Oxali 10, n = 17), 20 mg/kg (Oxali 20, n = 18), or 50 mg/kg alone (n = 5), or 5-FU 100 mg/kg alone (5-FU 100, n = 6), Oxali 10 + 5-FU 100 (n = 7), Oxali 20 + 5-FU 100 (n = 5), Bevacizumab 5 mg/kg (Beva 5, n = 5) or 10 mg/kg (Beva 10, n = 6), Oxali 10 + 5-FU 100 + Beva 5 (n = 6), Oxali 10 + 5-FU 100 + Beva 10 (n = 6) (Fig. 1A).

### *Partial hepatectomy*

After tail-blood collection, a partial hepatectomy (PH) 70% was performed (day 0) according to the method described by Higgins and Anderson.[25,26] A midline ventral abdominal incision was performed on anaesthetised animals, and, after mobilization of the liver, a ligature was tied around the pedicle (including vessels and bile ducts) of the anterior lobe (including left lateral and median lobes). This lobe was then removed and the abdomen was closed.

Animals were sacrificed 1, 2, 3 or 7 days after PH in the initial experiment. In following experiments, the analyses were performed at 24 h post PH time point. At time of sacrifice, blood was withdrawn by puncture of the inferior vena cava and the remnant posterior lobe (including right lateral and caudate lobes) was excised, weighed and sampled. Liver wedges were frozen in liquid nitrogen or fixed in paraformaldehyde for further analyses. BrdU was administrated IP at the dose of 50 mg/kg 2 h prior to sacrifice.

### *Clinical measurement and biological and specimen collection*

Animals were weighed and blood samples from either the tail or inferior vena cava were taken at every step of the experimental procedures. Blood samples were centrifuged and stored until assayed. Aspartate aminotransferase (AST, mmol/l) and alanine aminotransferase (ALT, mmol/l) were measured in all blood samples.

### *Histology, immunochemistry, BrdU immunostaining*

Paraffin liver 3 μm thick sections were stained with haematoxilin and eosin, using standard histological procedures. Morphological analysis included routine liver examination, mitosis count and assessment of sinusoidal dilation. In addition, sections were immunostained with 5-bromo-2-deoxyuridine (BrdU). BrdU positive cells were counted in at least five randomly selected high-power fields per slide.

### *Liver regeneration*

Restitution of hepatic liver mass was determined as the percentage of regenerated liver mass calculated as follows:

Liver mass recovery (%) = 100 × (Weight of the posterior lobe at the time of final resection/estimated total liver weight), the estimated total liver weight being extrapolated from hepatectomized anterior lobes representing 70% of the liver mass. DNA synthesis was determined by

A

| Chemotherapy (n=98) | | PH | Harvest | | | |
|---|---|---|---|---|---|---|
| | Day -3 | Day 0 | Day +1 | Day +2 | Day +3 | Day +7 |
| Vehicle (n= 17) | 17 | 17 | 5 | 4 | 4 | 4 |
| Oxali 10 ( n=17) | 17 | 17 | 5 | 4 | 4 | 4 |
| Oxali 20 ( n=18) | 18 | 18 | 5 | 5 | 4 | 4 |
| Oxali 50 ( n=5) | 5 | - | - | - | - | - |
| 5-FU 100 (n=6) | 6 | 3 | 3 | - | - | - |
| Oxali 10 + 5-FU (n=7) | 7 | 7 | 7 | - | - | - |
| Oxali 20 + 5-FU (n=5) | 5 | 5 | 5 | - | - | - |
| Beva 5 (n=5) | 5 | 5 | 5 | - | - | - |
| Beva 10 ( n=6) | 6 | 6 | 6 | - | - | - |
| Beva 5 + Oxali 10 + 5-FU ( n=6) | 6 | 6 | 6 | - | - | - |
| Beva 10 + 5 + Oxali 10 + 5-FU (n=6) | 6 | 6 | 4 | - | - | - |

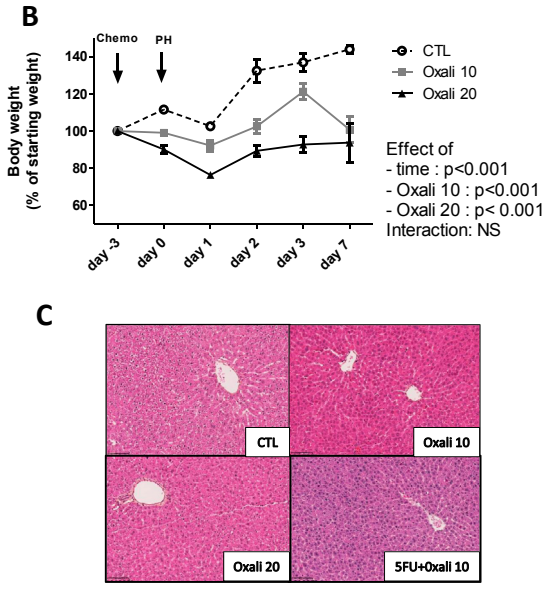


Figure 1. **Study design** (A): Chemotherapy (a single dose) was administered on day-3, 70% partial hepatectomy (PH) performed on day 0 and sacrifice was on days 1, 2, 3 or 7. Chemotherapy consisted of Oxaliplatin (Oxali) at the dose of 10, 20 or 50 mg/kg, 5-FU 100 mg/kg alone (5-FU 100), Oxali 10 + 5-FU 100, Oxali 20 + 5-FU 100, Bevacizumab (Beva) 5 mg/kg, or 10 mg/kg, Oxali 10 + 5-FU 100 + Beva 5, Oxali 10 + 5-FU 100 + Beva 10. Note that all rats (n = 5) receiving Oxali 50 mg/kg died within 2 days following the injection. (B) Body weight evolution (as percentage of BW at the start of the experiment) in rats treated with Oxali 10 or 20 mg/kg or vehicle. Data were analysed by bivariate Rank test. (C) Histological analysis of liver section at the time of PH (3 days post chemotherapy) demonstrating a normal histological appearance in all groups (whether treated with Oxali alone, a combination of Oxali 10 mg/kg and 5-FU or not) and the absence of histological lesion or sinusoidal lesion or dilation in chemotherapy-treated livers. Original magnification ×20.

immunodetection of BrdU incorporation into newly synthetized DNA.

### *Growth factor mRNA expression*

Total RNA was prepared from frozen liver using TRIpure isolation reagent (Roche). Hepatic mRNA expression levels of VEGF-A and of its 2 receptor-type tyrosine kinases (Flt-1 and KDR) were quantitated by real-time PCR. Primer pairs for transcripts of interest were designed using the Primer Expresst design software (Applied Biosystems). The ABI Prism 5700 PCR platform and SYBR-green[s] mastermix were used for detection of the amplification product. The relative amount of mRNA was calculated by reference to a calibration curve and normalized to the level of expression of RPL19 mRNA, an invariant control.

### *Statistics*

Data are presented as means ± SD. Given the non-normal distribution of data even after transformation in 2 out of 3 data sets and heteroskedastic residues of ANOVA, we used a bivariate Rank test. It is known that this test is convenient, only slightly conservative for small sample size.[27] Relative changes in gene expression were analysed using paired t-test, with the anterior (resected) lobe serving as the pre-hepatectomy value and the post lobe as post-hepatectomy value for each individual animal (Fig. 5A); Kruskal–Wallis test was used for between group comparisons (Fig. 5B). A p value of $<0.05$ was considered statistically significant.

## Results

All rats treated with Oxaliplatin at the dose of 50 mg/kg (n = 5) died within 2 days post-injection (Fig. 1A). We were able to pursue experiments with those groups of rats receiving either Oxali 10 or Oxali 20. Partial Hepatectomy was performed 3 days after chemotherapy treatment and liver regeneration evaluated on day 1, 2, 3 and 7 post PH (n = 4–5 per subgroup and time point). Body weight loss was used as an indicator of sufficient dose of chemotherapy administration. Compared to controls, Oxaliplatin injections (Oxali 10 and Oxali 20) caused significant body weight loss ($p < 0.001$). Oxaliplatin did not aggravate BW loss caused by PH on D1, but on D7, there was a significant difference in body weight between rats pre-treated with Oxali 20 and controls ($p < 0.001$) (Fig. 1B). Oxaliplatin did not cause significant alterations in ALT and AST levels (not shown) or sinusoidal lesions as assessed by histological analysis of resected anterior lobes (Fig. 1C). As expected, partial hepatectomy induced an increase in volume of the remnant lobe in controls (Fig. 2A) and liver mass recovery reached 63% of the initial liver mass after 7 days (Fig. 2B). In Oxaliplatin pre-treated rats, the mass

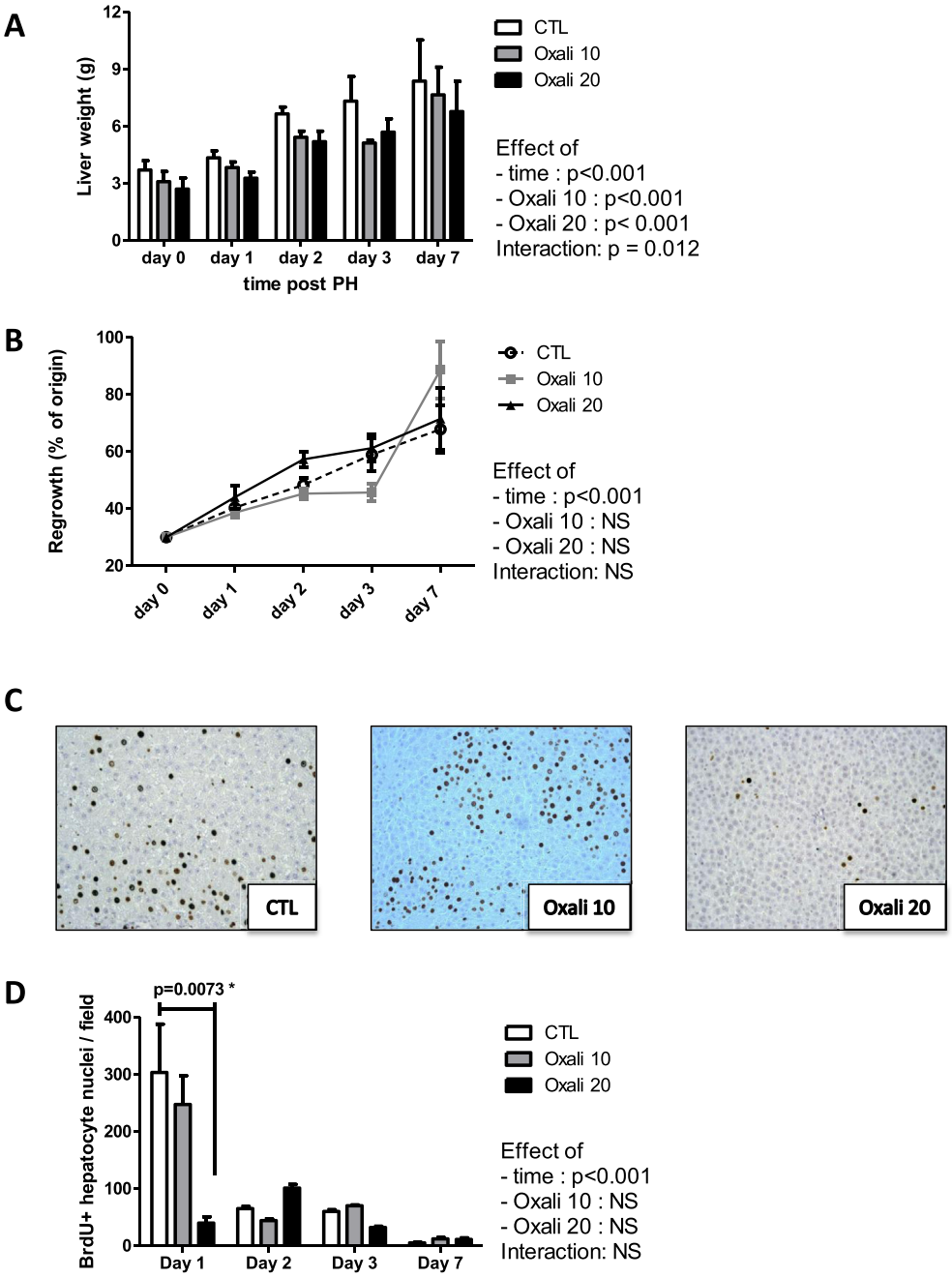


Figure 2. **Defective liver regeneration in early phases post PH in rats pre-treated with chemotherapy**. (A) Weight of the remnant posterior livers in rats pre-treated with Oxaliplatin 10 or 20 mg/kg (Oxali 10 or 20) or vehicle (control group; CTL) at the time of hepatectomy (Day 0; estimated from the weight of resected livers considered as 70% of the total liver mass) and on days 1, 2, 3 and 7 post hepatectomy (measured at the time of sacrifice). In Oxaliplatin-treated rats the weight of the remnant liver was smaller than in controls at time of PH, 24 h, 48 h and 72 h post PH, while increasing with time. (B) Regrowth of the remnant liver calculated as: 100 × (Weight of the posterior lobe at the time of final resection/estimated total liver weight), the total liver weight being extrapolated from hepatectomized lobes representing 70% of the liver mass. (C) Representative pictures of BrdU immunostaining of the regenerating lobe on day 1 post PH (Magnification ×20) and (D) Quantification of the number of BrdU + hepatocyte nuclei per field. Data were analysed by bivariate Rank test. * denotes significance using Rank test on Day 1 data only.

of the remnant liver was significantly smaller than in controls (Fig. 2A). The weight of the regenerating liver remained smaller in Oxali-treated rats. Oxaliplatin pre-treatment did not significantly alter liver mass recovery (Fig. 2B). The proliferative index was calculated as the mean number of BrdU positive hepatocyte nuclei per field (Fig. 2C). In controls, the proliferative index was over 300 BrdU positive-hepatocytes nuclei per field 24 h post PH and decreased over time (Fig. 2D). The proliferative index was similar in the Oxali 10 group. By contrast, BrdU positive hepatocytes were significantly fewer 24 h post PH in the Oxali 20 rats ($p = 0.0073$). Seven days post PH, the number

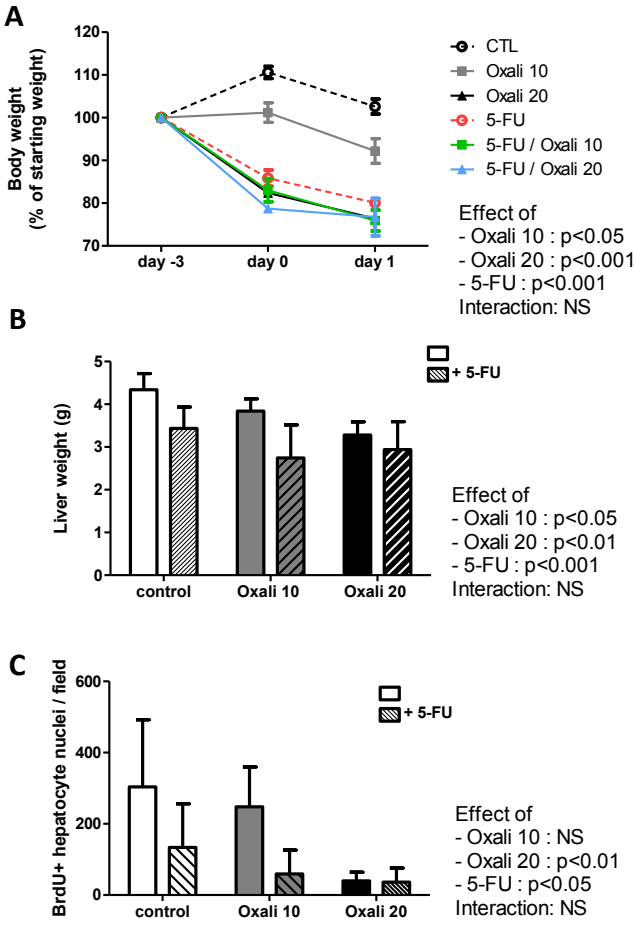


Figure 3. **5-fluorouracyl (5-FU) impaired liver regeneration when added to Oxali 10 but did not further aggravate defective liver regeneration caused by Oxali 20**. (A) Body weight evolution (as percentage of BW at the start of the experiment) in rats treated with Oxali 10 or 20 mg/kg or vehicle (CTL; control group) with or without 5-FU. 5-FU alone and combined to Oxali 10 caused body weight loss but did not aggravate body weight loss caused by Oxali 20 or PH-induced body weight loss. (B) Weight of posterior lobe and (C) proliferative index measured as the number of BrdU + hepatocyte nuclei per field 24 h post PH. In rats treated with 5-FU alone or combined with chemotherapy. Data were analysed by bivariate Rank test.

of BrdU positive hepatocytes was similarly low in all groups (Fig. 2D).

We then proceeded to test the effect of 5-FU 100 in combined therapy with Oxaliplatin and analysed liver regeneration 24 h post PH. 5-FU alone or in addition to Oxali 10 caused a significant body weight (BW) loss ($p < 0.001$), but did not aggravate BW loss caused by Oxali 20 (Fig. 3A). However, 5-FU alone or combined therapy did not aggravate BW loss caused by PH (Fig. 3A). Combined chemotherapy and partial hepatectomy did not significantly alter AST and ALT levels (not shown).

The weight of the remaining liver lobes 24 h post PH was significantly lower in rats pre-treated with 5-FU whether alone or associated to Oxali 10. Additional administration of 5-FU to Oxali 20 did not further decrease the low liver weight observed in the Oxali 20 group (Fig. 3B). The addition of 5-FU to controls or Oxali 10 treated rats decreased the proportion of proliferating BrdU positive hepatocytes ($p < 0.05$) while it did not further alter the already low proliferation when added to Oxali 20 (Fig. 3C).

We therefore examined the mRNA expression of VEGF-A and its 2 receptors (Flt-1 and KDR) in the regenerating livers. Liver regeneration in controls was associated with a decrease of VEGF-A expression and KDR, 24 h post PH (Fig. 4A). Chemotherapy, including Oxali 10, Oxali 20 and 5-FU 100 + Oxali 10 did not alter VEGF-A or VEGF receptors expression (Fig. 4B). In the regenerating lobes of rats pre-treated with Oxali 20 and 5-FU + Oxali 10 (associated with decreased hepatocyte proliferation) we found a down regulation of VEGF-A and KDR expression, similar to that of controls (Fig. 4A).

We then tested the effect of Bevacizumab on liver regeneration: rats pre-treated with combined 5-FU 100 and Oxaliplatin 10 mg/kg received an IP injection of Bevacizumab (5 or 10 mg/kg) at the time of chemotherapy (D-3). The addition of Bevacizumab had no effect on AST and ALT levels (not shown). The weight of the regenerating lobe was similar in control rats and in rats treated with Bevacizumab alone. The addition of Bevacizumab whether at the dose of 5 or 10 mg/kg to Oxali 10 + 5-FU did not increase the weight of the liver remnants 24 h post PH (Fig. 5A). The mean number of BrdU positive proliferative hepatocytes post PH was similar in livers treated with Beva 5 or 10 alone compared to the control group. The addition of Bevacizumab to 5-FU 100 and Oxali 10 was not associated with a significant increase in BrdU incorporation (Fig. 5B).

## Discussion

Pre-operative chemotherapy is increasingly used in patients with colorectal liver metastases,[18,28] and in view of mixed reports on newly introduced regimens including Oxaliplatin, we investigated its impact on hepatic regeneration post 70% PH in a Wistar rat model. As expected and in accordance with literature reports,[29] most of the various chemotherapy regimens investigated (Oxali 20, 5-FU alone, Oxali 10 + 5-FU, Oxali 20 + 5-FU) induced a body weight loss, and a significant unexplained reduction in liver weight (Figs. 1B and 2A). However, there were no differences in serum AST and ALT levels between controls and chemotherapy treated rats, as also reported for combined Oxali and 5-FU in both a mouse and a Wistar rat model.[29,30] Contrary to observations in human livers,[11] we did not find morphological alterations or lesions of the hepatic sinusoids or significant morphological damage in the liver of rats treated with chemotherapy. The absence of sinusoidal lesions in rats treated with Oxaliplatin is in accordance with other reports in both mice and rats.[29,31]

The main point of this study was to determine if exposure to chemotherapy had an impact on the ability of the

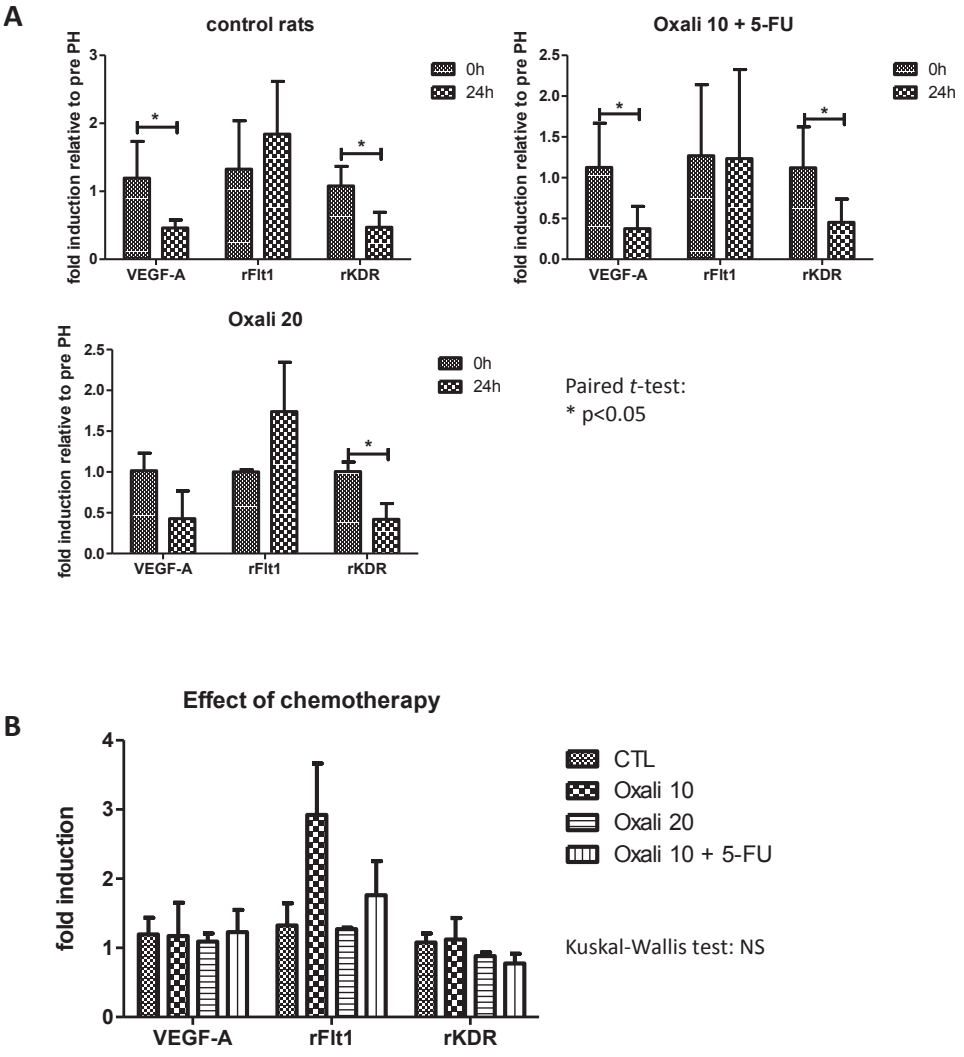


Figure 4. **The expression of VEGF-A and rKDR was down regulated after PH and not influenced by chemotherapy**. (A) Relative expression of VEGF-A, rFlt1 and rKDR prior (anterior resected lobes) and 24 h post PH in control rats and rats pre-treated with Oxali 10 + 5-FU or Oxali 20 + 5-FU.$p < 0.05$ using paired *t*-test. (B) Expression of VEGF-A, rFlt1 and rKDR in the anterior lobes of control rats or rats treated with Oxali with or without 5-FU. No statistical difference using Kruskal–Wallis test.

liver to regenerate. This was assessed by measuring the weight of the remaining liver after PH and by immunostaining for BrDU. In the control group, liver cells proliferation assessed by BrdU incorporation was maximum 24 h post PH and the liver recovered 63% of its initial mass 7 days post PH.

The addition of 5-FU 100 to Oxali 10 led to a significant reduction of positive BrdU hepatocytes, although 5-FU alone did not significantly decrease hepatocyte proliferation rate (Fig. 3C). This 5-FU effect on hepatocytes could not be observed with Oxali 20 as the proliferation rate was already significantly lower than controls on Day 1. Similar results were found in the study of Manekeller et al.[30] where, in a Wistar rat model, liver regeneration was impaired after PH performed 24 h following IP injection of similar doses of Oxaliplatin (85 mg/m$^2$) + 5-FU (1000 mg/m$^2$). Importantly, we observed that hepatocyte proliferation at later time points remained low until Day 3, confirming that chemotherapy impedes rather than delays regeneration, fully recovered on Day 7, as reported by Manekeler et al.[30] Despite defective hepatocyte proliferation, regrowth of the remnant liver appeared similar in controls and chemotherapy treated rats. In summary, we observed in all rats pre-treated with chemotherapy an early impairment of hepatocyte proliferation (24 h), which was not associated with morphological damage of the liver sinusoids nor with delayed recovery of liver mass.

To treat colorectal liver metastases in human, recent protocols combine anti-VEGF treatment (Bevacizumab) with 5-FU and Oxaliplatin with a global benefit on carcinogenic control and postoperative recovery. Indeed, blocking VEGF-A dependent signalling is thought to not only decrease the severity of SOS but also prevent liver failure by improving liver mass recovery.[19–22] However, several

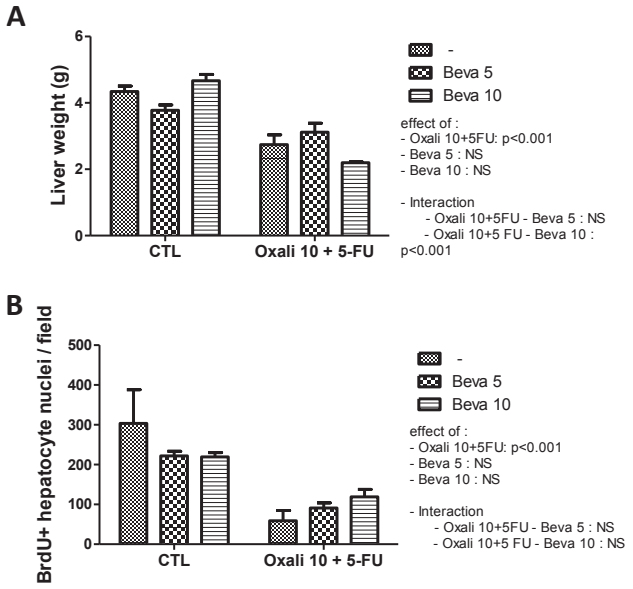


Figure 5. **Bevacizumab did not rescue defective liver regeneration in rats pretreated with Oxali 10 and 5-FU**. (A) Weight of the posterior lobes and (B) BrdU + hepatocyte nuclei 24 h post PH. Data were analysed by bivariate Rank test.

reports suggest that anti-VEGF treatment alters liver regeneration. Bockhorn et al.[23] reported that VEGF blockade almost completely suppressed hepatocyte proliferation measured by Ki67 immunostaining 24 h post PH in rats. Similarly, Taniguchi et al.[32] reported an inhibition of proliferative activity (Ki 67 immunostaining) of hepatocytes as well as of endothelial cells 48 h and 96 h after 70% PH in rats pre-treated with anti-VEGF. Our findings contradict these results. Indeed, we found that Bevacizumab did not significantly alter early hepatocyte proliferation post PH in control rats. Kasuya et al.[24] also reported the absence of effect of Bevacizumab (4 mg/kg, administered IP 6 times from D1 to D10 after PH), on liver regeneration in mice. In chemotherapy pre-treated rats that exhibited a decreased proliferative response post PH, we observed that Bevacizumab tended to enhance hepatocyte proliferation early post PH in a dose-dependent manner.

VEGF plays a major role in angiogenesis that is necessary for healing of injured tissue as it is in liver regeneration after partial hepatectomy.

In our control rats, VEGF-A mRNA expression and its receptor 2 (KDR) were decreased 24 h post PH. This is in contradiction with reports of increases in VEGF expression (48 h–72 h post PH) and in its receptors Flt-1 and KDR expression (72–168 h post PH).[32,33] We also observed that both control and chemotherapy treated rats had decreased expressions of VEGF-A and receptor 2 (RKDR) 24 h post PH. This suggests that the studied chemotherapy agents, though leading to decreased hepatocyte proliferation, do not have additional specific effects on hepatic VEGF-A and VEGF receptors' expression. The observed decreased hepatocyte proliferation cannot be explained by a direct chemotherapeutic toxic effect on either VGF-A or its receptors. Bevacizumab treatment had no significant effect on liver cells proliferation post PH in rats treated with chemotherapy. The latter observation supports the hypothesis that anti-VEGF therapy has no direct effect on liver regeneration, except when SOS is present, as reported in human studies.[19,20,22] In rats receiving Oxaliplatin, the impairment of liver regeneration in the early phase is not due to SOS. In the absence of SOS, Bevacizumab has no effect on liver regeneration.

## Conclusions

Oxaliplatin, alone or combined to 5-FU, impaired early hepatocyte proliferation post PH, which was unrelated to sinusoidal alteration and did not compromise further liver regeneration in a Wistar rat model. Bevacizumab alone does not alter (nor improve) liver regeneration compared to controls. Even when combined to 5-FU 100 and Oxaliplatin 10, Bevacizumab did not improve the significantly altered liver regeneration process linked to chemotherapy. The mechanism of hepatocyte proliferation impairment due to chemotherapy cannot be explained by a change of VEGF-A or of its receptors' expression. Future studies would be necessary to investigate both liver weight reduction on D0 and hepatocyte proliferation impairment linked to Oxaliplatin and 5-FU.

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## Conflict of interest statement

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence their work.

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