

Partial hepatectomy enhances growing of rat colorectal cancer cells CC531

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Research

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Abstract

Background

Nowadays, partial hepatectomy (PHx) is the gold standard for the treatment of colorectal cancer liver metastases. After removing a substantial amount of hepatic tissue, however, growth factors are released to induce liver regeneration. These cytokines may promote the proliferation of liver micrometastases or circulating tumour cells still present in the patient. The aim of this study was to assess the effect of PHx on the growth of liver metastases after intrasplenic cell inoculation.

Methods

Liver tumours were induced in 18 WAG/RijHsd male rats, by seeding 250,000 syngeneic colorectal cancer cells (CC-531) into the spleen. Twenty-eight days after tumour induction, the animals were sacrificed and the liver was removed and sliced to assess the relative tumour surface area (RTSA%).

Results

The RTSA% was significantly higher in animals which had undergone PHx than the controls (not hepatectomised) ($46.98 \pm 8.76\%$ vs. $18.73 \pm 5.65\%$; $p < 0.05$). While no differences were observed in the right lateral lobe, in either hepatectomised or non-hepatectomised animals, there were significant differences in liver surface occupied by metastases in the paramedian and caudate lobes in both groups.

Conclusion

In this experimental model, the process of growth of colorectal cancer cells in the liver clearly accelerates following PHx.

Background

Colorectal cancer (CRC) is the major cause of cancer-related morbidity and mortality in developed regions. In the European Union (EU), it is the second leading cause of death, while in developing countries it is the fifth (1, 2). In 2014, over 153,000 people died as a result of CRC in the EU, this representing 11% of cancer-associated deaths and 3.1% of all deaths (2, 3).

Metastases are the main cause of CRC-associated morbidity and mortality. The liver is the most common site of metastases derived from CRC (CRCLM), due to the fact that the blood from colon and proximal parts of the rectum drains through the portal system to the liver (4–7). More than 50% of patients with CRC develop CRCLM in their lifetime (2, 6), and without treatment only approximately 20 to 30% of them survive to 1 year after the diagnosis of metastatic disease (8).

Nowadays, partial hepatectomy (PHx) is the only option offering potential cure for patients with CRCLM (9, 10). Regardless of advances in this field, less than 20% of patients are eligible for PHx (11). If performed with curative intent, PHx can achieve a 5-year survival rate of 23 to 51% and median survival up to 60 months (11, 12). Conversely, in untreated CRCLM patients, median survival is 3–20 months, with a 1-year survival rate of less than 30% and 5-year survival rate of less than 5% (8).

Liver regeneration

After CRCLM resection, like after other damage to liver tissue, proliferation of hepatocytes allows liver regeneration (13). Mature hepatocytes are long-lived cells and do not normally undergo cell division processes but, after tissue damage (e.g., PHx, toxic damage or infection), they recover their proliferative capacity to compensate for damaged or lost liver tissue (14). Liver regeneration consists of three stages: the transition of the quiescent/dormant hepatocyte into the cell cycle, called initiation, proliferation and termination (15).

After hepatic resection, many molecules which have stimulatory and inhibitory effects, such as TNF- α , IL-6, immunomodulating mediators and cytokines, are released and induce hepatocytes to leave the dormant state and enter the cell cycle in the first stage (16). The objective of this priming stage is to prepare hepatocytes to respond to growth factors (GFs) in the proliferative stage. Several genes associated with the transcription process, inflammatory response, extracellular matrix (ECM) modifications or cell cycle are involved (17).

In the next step, the proliferation stage, DNA synthesis and hepatocyte mitosis occur by the action of some GFs (hepatocyte growth factor [HGF], epidermal growth factor [EGF], heparin-binding EGF-like growth factor [HB-EGF], tumour growth factor alfa [TGF- α], and amphiregulin, among others) (16, 18). Recently, it also has been shown that changes in the activity or total amount of urokinase-type plasminogen activator (UPA), its receptor (UPAR) and plasminogen activator inhibitor type-1 (PAI-1) are strongly involved in hepatic regeneration, playing a role in the ECM modification process (16, 19).

Termination is not a well-understood stage. After PHx, once the liver reaches its pre-hepatectomy size, the proliferative process is seen to stop (20, 21). This may happen because of proliferation-inhibiting factors, like the TGF- β superfamily, as suggested by some authors (22)

Residual metastatic disease

Metastasis recurrence following tumour resection is a common phenomenon, most frequently resulting from the outgrowth of a residual disease, also called residual metastatic disease (23, 24).

Some studies suggest that these tumour microimplants or some circulating tumour cells may be in a kind of “dormant state” which is suddenly abandoned to begin rapid growth (25, 26). The dormant state might consist in a balance between proliferation and apoptosis and be regulated by a vast range of molecules, including GFs (such as VEGF and EGF), immunological factors, matrix proteins (thrombospondin), oncogenes, and tumour suppressor genes (19, 20).

Surgical procedures, such as PHx, lead to major changes in levels of GF and cytokines, which induce changes in the microtumour implant environment. In addition, the disruption and rearrangement of the extracellular tumour matrix may promote detachment and migration of tumour cells within the remaining hepatic mass (RHM) or its incorporation into the systemic circulation, which facilitates intra- or extrahepatic tumour recurrence. In fact, we have already demonstrated that serum obtained from hepatectomized rats enhances the growth of a rhabdomyosarcoma cell line in vitro (27). Using the same cell line, we have also established that PHx performed after intrasplenic cell inoculation increases the number and size of liver tumour implants. We wanted to explore whether it was something specific to our rhabdomyosarcoma, which is known to be a very aggressive tumour, or common to other tumours. For this reason, we adapted our model for a syngeneic CRC cell line, in order to analyse the potential of hepatectomized rat serum (HRS) to enhance CC531 growth in vitro and to check the effect of 40% PHx on the growth of CC531 cancer cell implants in the liver.

Material And Methods

All procedures involving animals were performed in strict accordance with the recommendations of current national legislation on experiments involving animals or biological agents. All protocols were approved by the Ethics Committee on Animal Experimentation (CEEA) (ref. number: M20/2015/054) and Ethics Committee for Research involving Biological Agents and Genetically Modified Organisms (CEIAB) (ref. number: M30/2018/022) of the University of the Basque Country (UPV/EHU).

Animals

The experiment was carried out in 24 three-month-old male WAG/RijHsd rats, weighing 270–300 g. Animals were kept in a temperature- and humidity-controlled room with a 12-h light/dark cycle and free access to standard laboratory diet and water. Animals were randomly allocated to one of three groups: (1) hepatectomized rats, to obtain serum for in vitro experiments (n = 6), (2) tumour-inoculated rats (control group, n = 9), or (3) tumour-inoculated rats which then underwent PHx (n = 9).

To induce liver cancer implants, CC531 cells were injected into the spleen. Once the animal was properly anaesthetised with isoflurane 1.5%, a midline incision was performed to expose both the liver and the spleen. The left lateral lobe (LLL) vascular pedicle was blocked with a Yasargil vascular microclamp, to prevent cancer cells entering this lobe (as such cells would be lost in animals undergoing 40% PHx). Then, the spleen was exposed and 0.5 ml of 500,000 cells/ml cell suspension was slowly inoculated into the organ. Once the injection had been completed, both the liver and the spleen were returned to the abdominal cavity and the laparotomy was closed with a running suture. Five minutes later, the laparotomy was reopened to excise the spleen and remove the microclamps. At this point, in the hepatectomized group, the LLL was ligated and excised. The abdominal wall was then closed with interrupted stiches, and meloxicam (2 mg/kg) was administered. The animals were kept under observation until full recovery. On days 15 and 20, an ultrasound examination of the liver was performed looking for any tumour foci. These examinations were carried out using a MyLab 60 Xvision system

(Esaote, Genoa, Liguria, Italy), equipped with a multifrequency linear probe operated at 18 MHz and the focus was set at a depth of between 0.5 and 1.5 cm. On day 28, under isoflurane anaesthesia, the liver was removed and fixed in 10% formaldehyde.

The animals in the first group underwent 40% PHx, using the same anaesthetic regimen. Forty hours later, they were anesthetized, had blood collected from the abdominal cava vein, and were then sacrificed. After centrifugation for 10 minutes at 3000 r.p.m., the serum was obtained and stored at -20° C until use.

Relative tumour surface area

Each liver lobe was identified, excised and sliced into 1 mm slices. Then, all slices from each lobe were photographed separately and labelled with a blinded code. Using Image J Software®, absolute tumour surface area (ATSA) and absolute liver surface area (ALSA) were measured in square pixels (p^2), as shown in Fig. 1. Then, relative tumour surface area (RTSA%) was calculated [RTSA%= (ATSA/ALSA)x100]. Once the measurements had been completed, the blind was broken and the data sorted by individual animal and experimental groups.

In vitro assessment of hepatectomized rat serum

To assess the potential of HRS to enhance cell proliferation, a series of in vitro cell proliferation studies were carried out.

CC531 cells were incubated at 37°C and 5% CO₂ in RPMI-based culture medium supplemented with 10% (v/v) foetal calf serum (FCS), antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) and antimycotics (0.25 µg/ml amphotericin B). Once the culture reached semi-confluence, the cells were harvested and seeded on 24-multiwell culture plates at 30,000 cells/ml. The control wells were supplemented with 10% FCS, while those of experimental groups were supplemented with either 5, 10 or 20% normal rat serum (NRS) (obtained from non-hepatectomized rats) or 1, 2.5, 5, 10, or 20% HRS. Cell proliferation was assessed using NucleoCounter® NC-100™ (Chemometec, Allerod, Denmark) and xCELLigence® RTCA SP (ACEA Biosciences Inc. San Diego, Ca, USA).

Statistical Analysis

Statistical analyses were performed using PRISM 6© (GraphPad Software, USA). The normality of all our data was checked with the Kolmogorov-Smirnov test, and results were summarized using means and standard deviations. Comparisons between two groups were carried out using two-tailed t-tests with a confidence level of 95% and between more than two groups with analysis of variance followed by Tukey's test.

Results

In this experiment, both in vitro and in vivo studies showed that liver resection is a strong stimulus for tumour cell proliferation.

Effect of hepatectomized rat serum on in vitro cell proliferation

In order to select appropriate control conditions, first of all, we analysed the effect of adding serum obtained from normal rats to our cultures, comparing their growth rate with that of cultures enriched with conventional 10% FCS. Though no differences were observed during the first 48 hours (Fig. 2), looking at cell counts on the third day, all the cultures enriched with NRS showed lower cell concentrations than cultures with FCS, though the differences were not statistically significant. Notably, however, of the three different concentrations of NRS, the lowest (5% NRS) appeared to be the strongest stimulus.

Then, we compared the effect of HRS to that of NRS and FCS, and observed that none of the cultures receiving HRS showed stronger growth rates than those with FCS. And again, the best result was seen with the lowest concentration of HRS (5%) (Fig. 3). With this concentration no differences were detected between cultures stimulated with HRS and those stimulated with NRS (Table 1). In contrast, with higher concentrations, the decrease in cell count was larger in cultures receiving HRS (Table 1); that is, higher concentrations of HRS were more inhibitory than their equivalent of NRS.

Table 1
Cell count after 72 hours of exposure to different concentrations of rat serum. Cell count is expressed as media and standard deviation.

Serum concentration (v/v)	Normal rat serum (cell/ml)	Hepatectomized rat serum (cell/ml)	p value
20%	$2.84 \times 10^5 \pm 1.12 \times 10^5$	$1.27 \times 10^5 \pm 0.32 \times 10^5$	< 0.0001
10%	$2.95 \times 10^5 \pm 0.93 \times 10^5$	$2.25 \times 10^5 \pm 0.40 \times 10^5$	< 0.0001
5%	$3.22 \times 10^5 \pm 0.82 \times 10^5$	$3.20 \times 10^5 \pm 0.70 \times 10^5$	n.s.
2.5%	$4.32 \times 10^5 \pm 0.75 \times 10^5$	$5.18 \times 10^5 \pm 0.38 \times 10^5$	< 0.0001
1%	$3.98 \times 10^5 \pm 0.72 \times 10^5$	$4.34 \times 10^5 \pm 0.33 \times 10^5$	0.0018

Given these findings, we performed another set of experiments with even lower concentrations of rat serum. We were able to observe that with lower concentrations the difference in effect in favour of HRS was greater (Fig. 4). In fact, when serum concentration was reduced to 2.5%, HRS-enriched cultures had higher cell counts by 48 h than those enriched with NRS. The difference was statistically significant both on day 2 and 3 ($p < 0.05$).

In vivo model assessment

All of the animals which were given an intrasplenic injection of CRC cells developed liver implants, regardless of whether they underwent PHx. Tumour foci were randomly distributed through the whole

organ, without a specific pattern, though the concentration of tumour implants was lower in the paramedian lobe. We were unable to obtain any useful data by ultrasound examination of the animals with our device, it not being possible to accurately assess either the size or the number of tumour foci.

Effect of 40% hepatectomy on tumour development

After completion of the experimental procedure and surgical removal and analysis of each liver, we found that hepatectomy stimulated tumour growth as assessed by all the measures considered (Table 2).

Considering the percentage of the whole liver surface area occupied by tumour tissue in the histological sections, we observed a 2.5-fold difference: 18.7% in control group vs 47% in hepatectomized group ($p < 0.05$; Fig. 5).

Table 2
Relative tumour surface area occupied by CRCLM in total liver mass (TL-RTSA%), caudate lobe (CL-RTSA%), paramedian lobe (PL-RTSA%) and right lateral lobe (RLL-RTSA%).

	TL-RTSA%	CL-RTSA%	PL-RTSA%	RLL-RTSA%
Control Group	18.73 ± 5.6%	36.02 ± 12.80%	5.9 ± 1.4%	32.69 ± 10.19%
PHx Group	46.98 ± 8.8%	70.91 ± 10.35%	40.33 ± 12.1%	45.38 ± 11.24%
p value	0.015	0.04	0.03	0.42

Analysing the liver lobes separately, two different patterns were observed. In the paramedian and caudate lobes, the RTSA% was significantly higher (40.3% vs 5.9%; 70.9 vs. 36.0%). In contrast, in the right lateral lobe, though the percentage was also slightly higher (45.4% vs. 32.7%), the difference was not statistically significant ($p = 0.42$; Fig. 5).

Discussion

Experimental tumour induction model

The aim of this work was to evaluate, in a rat model, how liver resection (like that which may be performed in a metastasectomy) might modify metastatic growth in the remaining liver tissue. Laboratory animals are still needed for this type of research, because it is not ethically or legally possible to undertake the necessary experiments in humans (28).

It could be argued that the ideal animal model to study this phenomenon would be based on naturally-appearing primary CRC which subsequently develops CRCLM. Then, PHx could be performed to remove metastases, and the recurrence of liver metastases could be studied. On the other hand, such a model based on natural or genetic predisposition to develop primary CRC and then CRCLM has several drawbacks. Major problems that discourage use of such a model include: low rates of development of primary tumours or metastases, the longer time needed for metastatic disease to become evident, the

limited number of models (syngeneic and xenograft), metastatic disease not being confined to a single location and the asynchronous development of metastatic disease, as well as greater associated costs of animal housing (29–31).

Chemical induction has also been accepted as an appropriate tool for tumour induction. Various compounds, including dimethylhydrazine and its metabolites methylnitrosoguanidine, N-Nitroso-N-methylurea and azoxymethane, have been shown to induce tumour and/or metastases growth in laboratory animals (32–36). These compounds have alkylating activity, which cause breakage of DNA chains, abnormal pairing of bases, and inhibition of cell division, finally, resulting in cell death. Despite these products being well known, they have several disadvantages when used to induce tumours. For example, long induction time, low rates of tumour development and undefined site of tumour development, meaning that larger numbers of animals would be required for each experimental group. Furthermore, as these chemical products are carcinogenic in humans, the risk of researcher exposure should be taken into account.

Heterotopic implantation of cancer cells is also a validated model to induce tumour development in laboratory animals, particularly in rats and mice. Cells may be implanted directly into the liver parenchyma, via subcapsular injection, and this approach achieves a high tumour development rate: up to 60–70% of the injected animals (37–39). But this model is not appropriate for our purposes because it only allows the development of single well-defined and localized tumour implants, and also it lacks the process of tumour cell dissemination through the vascular tree and subsequent extravasation to produce metastatic disease in the liver (40). Another heterotopic-implantation method to induce CRCLM development is intrasplenic injection. The success rate with this model is, however, very heterogeneous, ranging between 20–100% (41, 42).

For our experiments, we chose intrasplenic injection because it mimics, as closely as possible, the natural haematogenous dissemination pathway through the portal vein of CRC cells from primary colorectal tumours; as well as all the other natural events involved in the spreading of metastases, such as extravasation, implantation, ECM remodelling and CRCLM growth and development.

The immune barrier is another factor to be considered when performing tumour induction by cell injection. To achieve a successful model, allo- or xeno-rejection has to be avoided. This problem can be overcome by the use of cell lines that grow in syngeneic hosts, also called isografts (43) or using immunocompromised host, animals which have an immune system that is suppressed or depleted and does not respond when cells or tissues from other species are implanted (43, 44).

As the immune system plays an important role in cancer, we decided to avoid interfering with it. For this reason, we chose a CRC cell line (CC531) which is syngeneic to WAG/RijHsd rats. When CC531 cells are injected into the spleen of this strain of rats, rejection seldom occurs (45) and a high rate of success is achieved. In fact, in our study, we saw no cases of rejection at all, and a 100% success rate in CRCLM development. The fact that tumour foci were observed in every liver lobe is in consonance with the natural pattern of metastasis development, which reinforces our idea of it being a good experimental

model for studies on liver metastases. Nonetheless, it is also true that this model lacks the genetic variability which is observed in human tumours (46). Moreover, Robertson et al. sustain that as rat liver architecture and homeostasis are quite different from those in humans, caution should be exercised with any extrapolation of results from this model to a clinical setting (44).

Effects of 40% PHx on CRC growth

Though many experiments support the idea that GFs stimulate cancer cells, in our experiment, high concentrations of HRS clearly inhibited cell growth percentage (Fig. 3). Doubling HRS concentration (10%) reduced cell counts after 3 days by 30% (Table 1). The inhibitory effect was even stronger with 20% HRS, the cell count falling to less than 50% of that observed with 5% HRS ($3.22 \times 10^5 \pm 8.24 \times 10^4$ vs. $1.27 \times 10^5 \pm 3.17 \times 10^4$; $p < 0.01$).

In vitro studies have taught us some interesting lessons. Though the name “growth factors” suggest they stimulate cell growth, their real effect is highly dependent on their concentration. We have seen a “window” in which they markedly increase cell proliferation rate, but when levels are higher than this window, they start to act as inhibitors (47–49). Our results are in consonance with those published by other authors.

A second important idea emerges from our experiment. When comparing low concentrations of serum from either normal or hepatectomized rats, we have seen that there is something in the latter (possibly growth factors) that makes it a stronger stimulus to cancer cell growth in vitro (50–52). This could explain the high rate of recurrence after surgery for colorectal liver metastases.

In fact, the experiments we have carried out in vivo have shown that 40% PHx strongly increases CRCLM development. Authors including Panis et al., García-Alonso et al., Krause et al. and Harun et al. have obtained similar results. Specifically, in an intraportal induction model of CRCLM (DHDK12 CRC cell line and syngeneic BDIX rats), Panis et al. demonstrated that three times more rats developed metastases among those that had undergone 70% PHx than those that had not undergone hepatectomy (62% vs. 20%, $p < 0.05$) (53). García-Alonso et al., working with S4MH (a rhabdomyosarcoma cell line, syngeneic for WAG rats) and performing 40% PHx, reported stimulatory effects on liver metastasis development, compared to that observed in controls (non-hepatectomised) (54). Regarding the behaviour of each liver lobe, our results are quite similar to this latter study. The percentage of the liver parenchyma occupied by tumour implants was significantly higher in the paramedian and caudate lobes in hepatectomised animals (33% vs. 24% and 47% vs. 17%, respectively) and also higher in the right lateral lobe, though the difference did not reach significance (20% vs. 17%). These findings are in accordance with our results described above. Krause et al., with CC531 cells and WAG rats, also observed a significantly higher RTSA% in the PHx group than the non-hepatectomized group (2.8 vs. 2.5, respectively) ($p < 0.05$) (55).

In 2007, Harun et al. (56) compared the effects of both early and late liver regeneration stages in intrasplenic-induced CRCLM after 40% and 70% PHx, performed at the same time as CRCLM induction or 6 days later. They observed that metastatic development was only affected by 70% PHx and also that it

was mainly factors involved in late stages of hepatic regeneration that interfered in the progression of metastasis. This group also demonstrated that tumour growth and progression were caused by an upregulation of c-Met in the peripheral area of CRCLM (57).

In summary, the performance of our experimental model and our preliminary results are in accordance with data published by other authors, demonstrating that liver resection to remove the macroscopic metastases, though effective in improving short- and medium-term survival, carries the risk of promoting the proliferation of any tumour cells remaining in the patient. Our in vitro results suggest that the mechanism involved could well be related to GFs. If this were to be proven beyond doubt, targeting these GFs could be a reasonable approach to prevent tumour recurrence following surgical treatment for CRCLM.

Conclusions

In our model, hepatic resection performed on tumour-bearing livers promotes both the growth of cancer cells both in the remnant liver parenchyma and also in colorectal cancer cells in vitro. This finding supports the hypothesis that GFs induced by partial liver resection are responsible for cancer recurrence following surgical oncological treatments. These biological mechanisms should be further explored.

LIST OF ABBREVIATIONS

ALSA absolute liver surface area

ATSA absolute tumour surface area

CRC colorectal cancer

CRCLM colorectal cancer liver metastases

ECM Extracellular matrix

FCS foetal calf serum

GF growth factors

HB-EGF heparin-binding EGF-like growth factor

HGF hepatocyte growth factor

HRS hepatectomized rat serum

LLL left lateral lobe

NRS normal rat serum

PAI-1 plasminogen activator inhibitor type-1

PHx partial hepatectomy

RTSA% relative tumour surface area

TGF- α tumour growth factor alfa

UPA urokinase-type plasminogen activator

UPAr UPA receptor

DECLARATIONS

Declarations

Ethics approval and consent to participate

This project has been approved by the Ethics Committee on Animal Experimentation (CEEA) (ref. number: M20/2015/054) and Ethics Committee for Research involving Biological Agents and Genetically Modified Organisms (CEIAB) (ref. number: M30/2018/022) of the University of the Basque Country (UPV/EHU).

Consent for publication

Not applicable

Availability of data and material

Please contact author for data requests

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors have contributed equally to this article. All authors read and approved the final manuscript.

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Figures

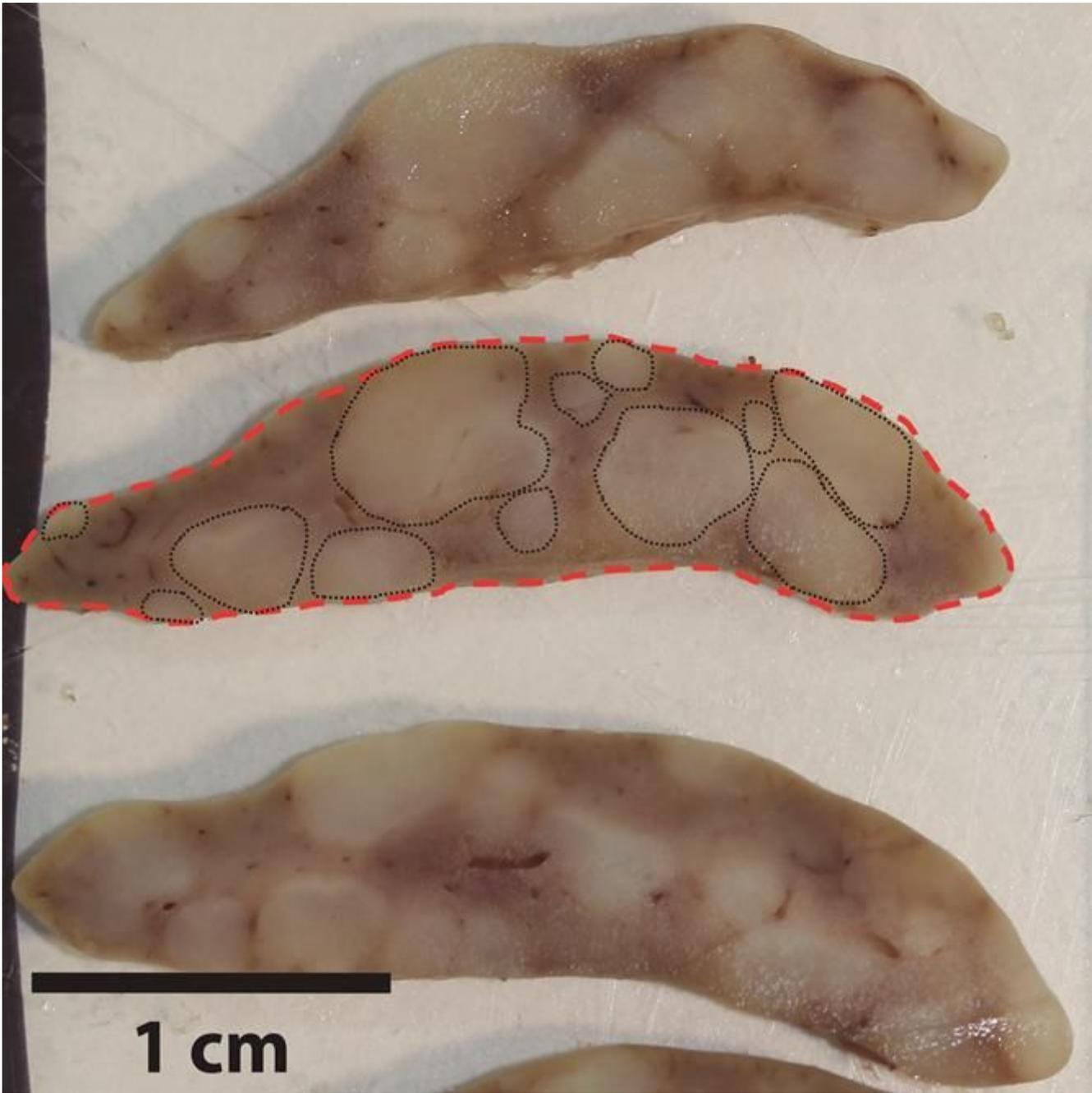


Figure 1

Measurement of absolute tumour surface area (ATSA) (encircled by discontinuous black lines) and absolute liver surface area (ALSA) (shaded green and encircled by a discontinuous red line) in each slice using Image J Software.

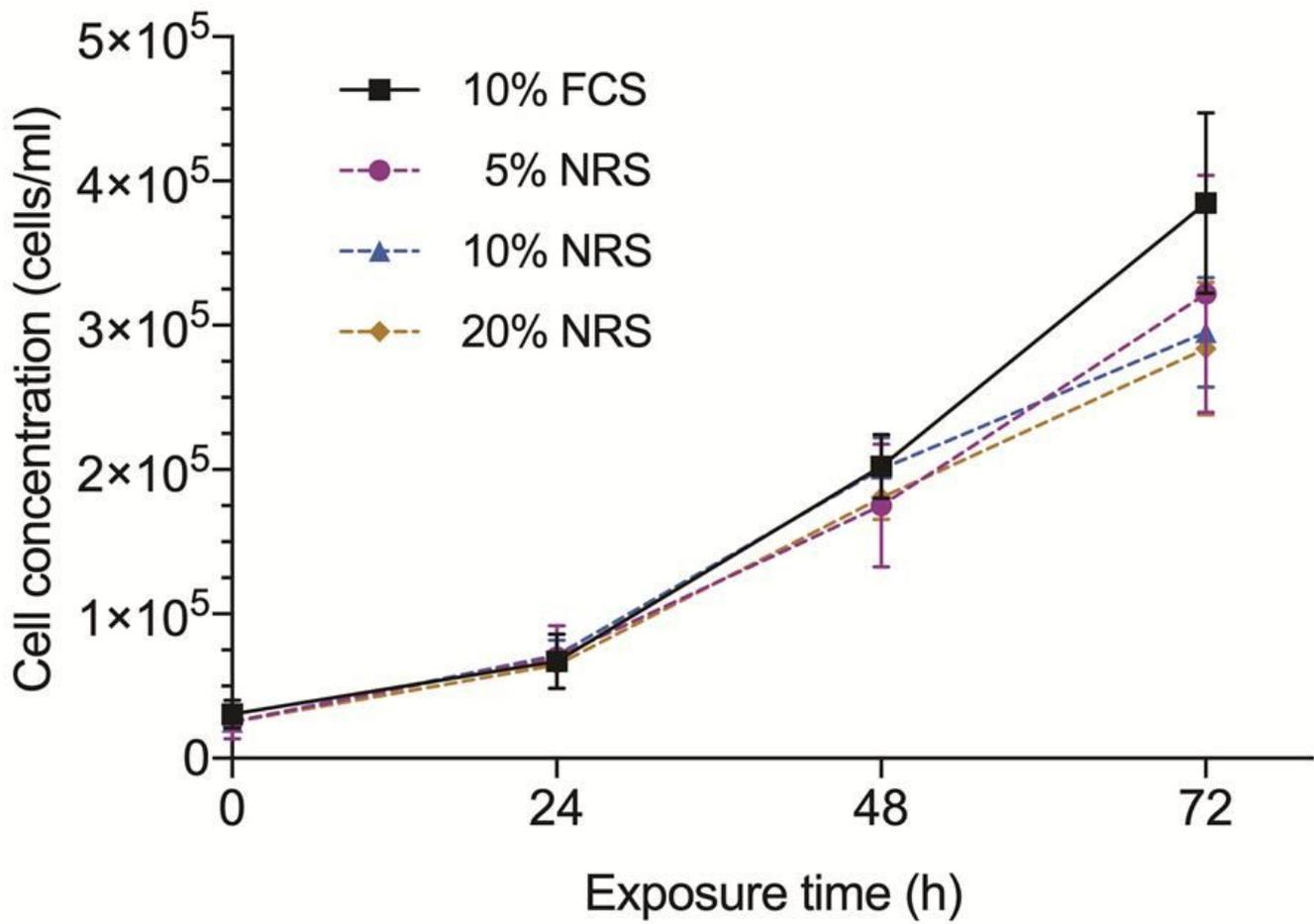


Figure 2

Proliferation curve of CC531 cell cultures. Cell concentration after 24, 48 and 72 hours of exposure to different experimental conditions. Control (10% FCS) and normal rat serum (5%, 10%, 20%).

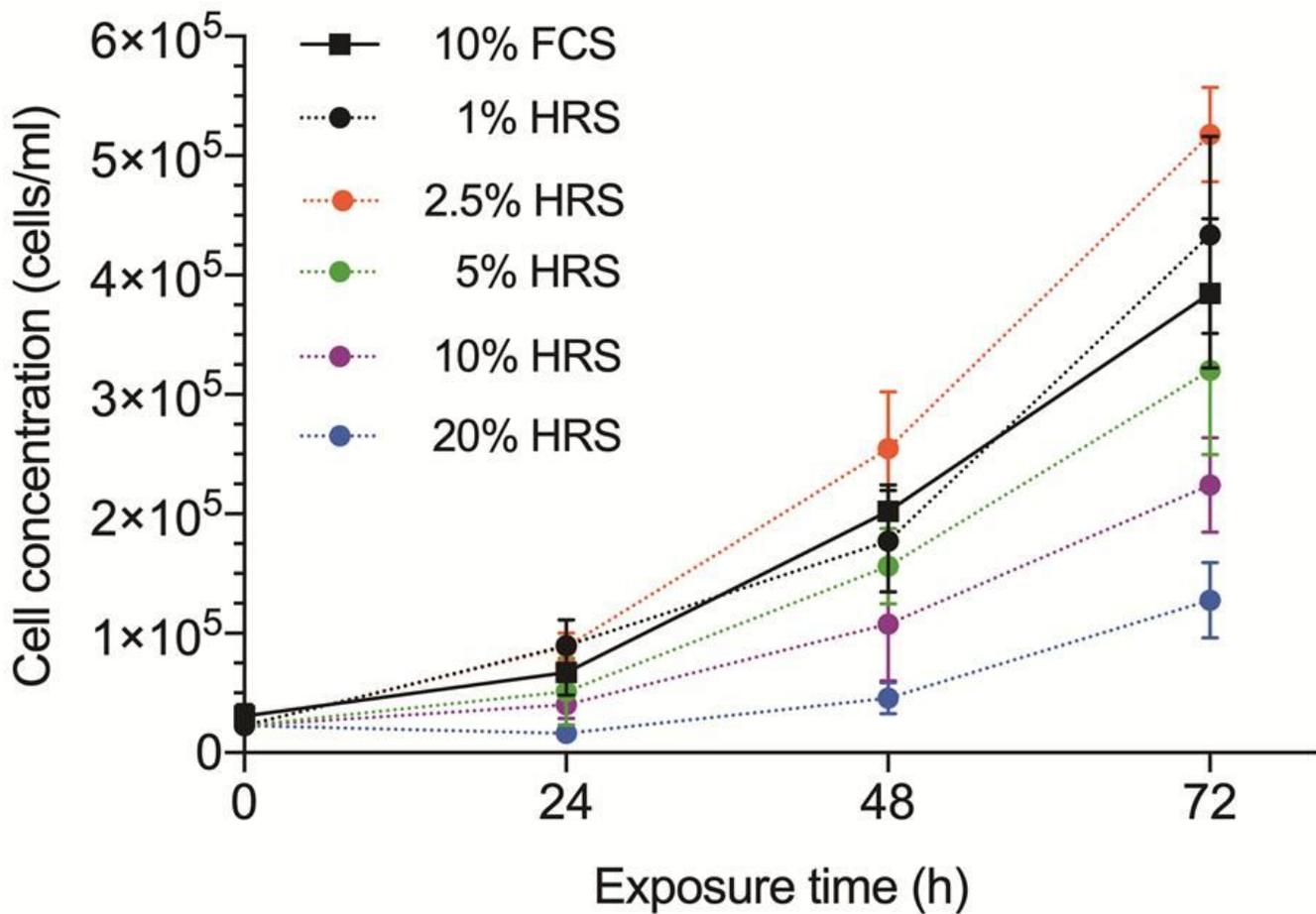


Figure 3

Proliferation curves of CC531 cell cultures enriched with serum from hepatectomized rats. Control (10% FCS) and hepatectomized rat serum (HRS; 1%, 2.5%, 5%, 10%, 20%).

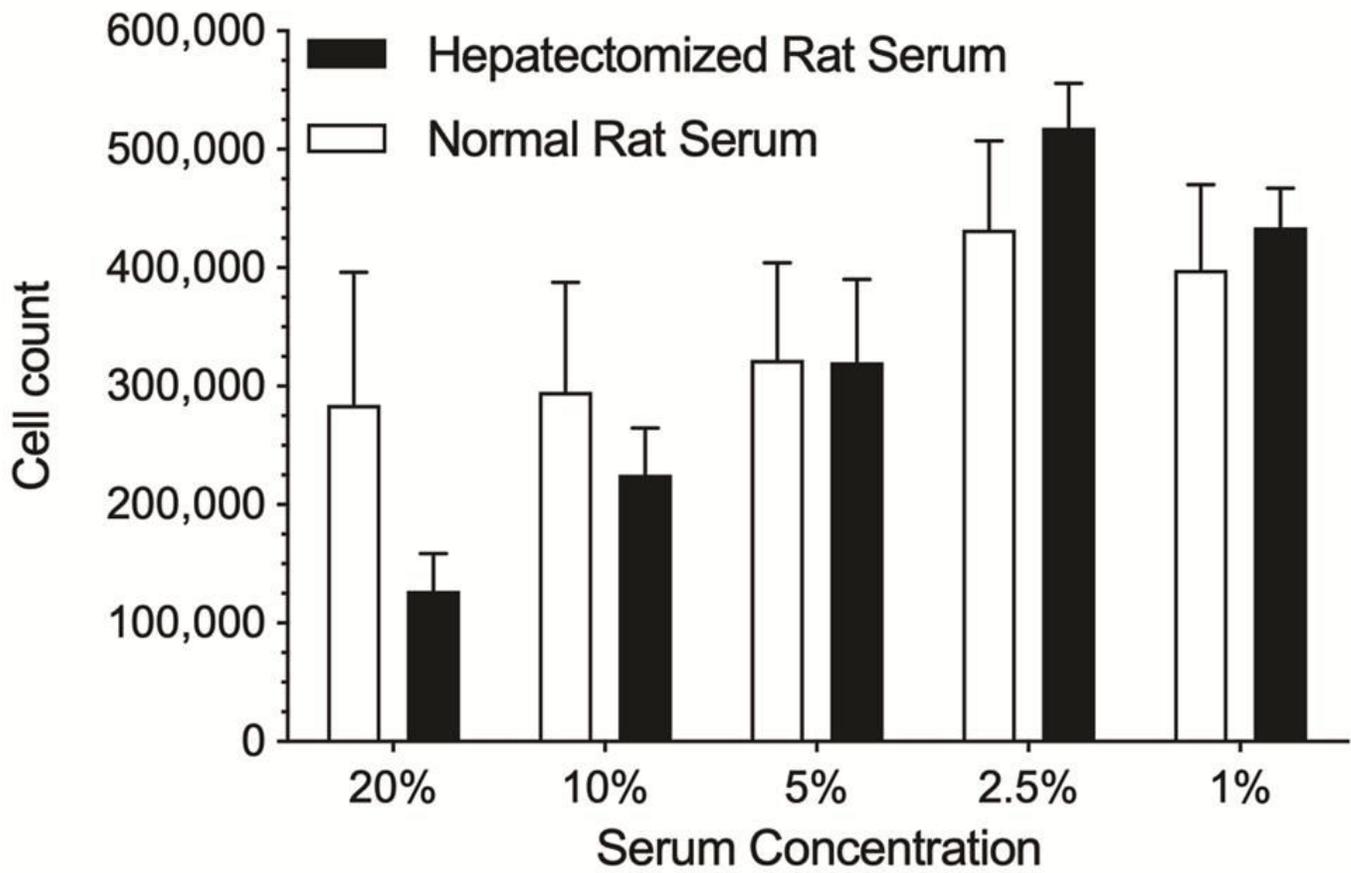


Figure 4

Proliferation curves of CC531 cell cultures enriched with low concentration of rat serum (Normal rat serum: white; Hepatectomized rat serum: black).

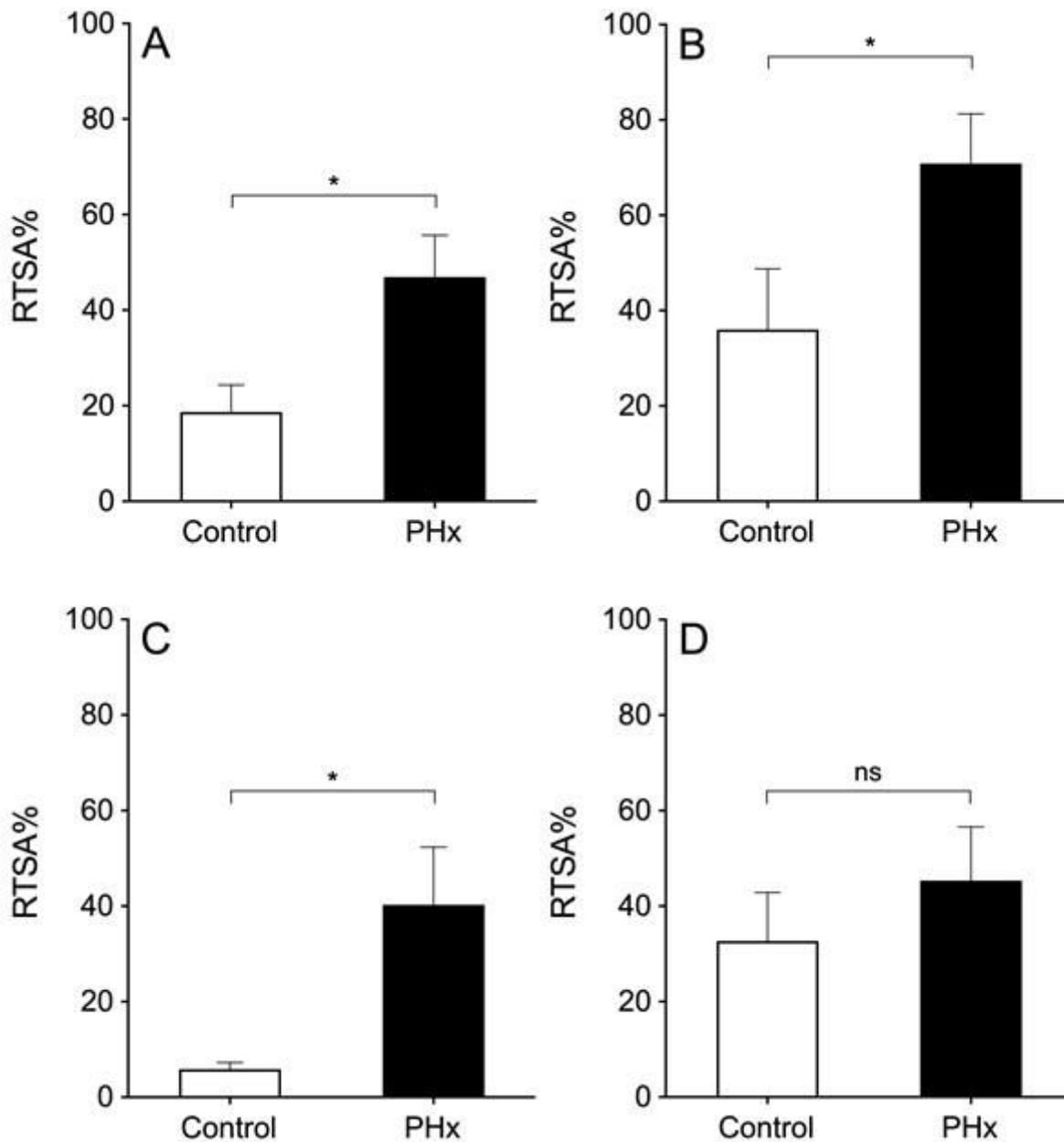


Figure 5

Relative tumour surface area percentage (RTSA%). Bar graphs of total liver mass RTSA% (A), caudate lobe RTSA% (B), paramedian lobe RTSA% (C) and right lateral lobe RTSA% (D) of both control (non-hepatectomized animal; white) and PHx (hepatectomized animal, black) groups.