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VEGF inhibition by bevacizumab in liver regeneration: study in rats

Inibição do VEGF pelo bevacizumabe na regeneração hepática: estudo em ratos

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ABSTRACT

Introduction: Extensive resections and transplantations of liver parts are possible due to their regenerative capacity, which is dependent on the activation of vascular endothelial growth factor (VEGF), which also regulates angiogenesis. Bevacizumab (BV), a monoclonal antibody that inhibits VEGF, is a neoadjuvant widely used in the treatment of cancers, restricting microvascular density and may cause undesirable effects on postoperative healing and liver regeneration.

Objective: To evaluate liver regeneration after 70% hepatectomy under the action of 1 dose (5 mg/kg) of BV in a murine experimental model for 15 days.

Method: Thirty-two Wistar rats submitted to partial hepatectomy were used and separated into 2 groups according to the control treatment (C) treated with saline solution and BV treated with bevacizumab, subdivided according to the observation time (48 h and 15 days). Its effects on liver regeneration were evaluated in weight assessments and liver function. In the remaining liver, mitotic activity, microvascular density (angiogenesis), nuclear proliferation, and histopathology were evaluated to investigate fibrosis, edema, and congestion.

Result: On the 15th day of treatment with BV, there were no differences in weight assessments, liver function, and mitotic indices. However, there was a decrease in cell viability, microvascular density, and worsening in the occurrence of histopathological changes such as fibrosis, edema, and hepatic congestion.

Conclusion: The single dose of bevacizumab did not cause changes in weight, serum biochemistry, or mitotic indices on the 15th day. However, it induced a decrease in microvascular density, cell viability, and hepatic histopathological changes such as edema, congestion, and fibrosis.

KEYWORDS: Hepatic regeneration. Angiogenesis. Bevacizumab, VEGF.

RESUMO

Introdução: extensas ressecções e transplantes de partes do fígado são possíveis, graças à sua capacidade regenerativa que é dependente da ativação do fator de crescimento vascular endotelial (VEGF) o qual também regula angiogênese. O bevacizumabe (BV), anticorpo monoclonal inibidor do VEGF é neoadjuvante amplamente utilizado no tratamento de cânceres restringindo a densidade microvascular, podendo causar efeitos indesejáveis na cicatrização pós-operatória e na regeneração hepática.

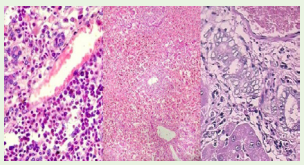
Objetivo: Avaliar a regeneração hepática, pós-hepatectomia a 70%, sob ação de 1 dose (5 mg/kg) de BV em modelo experimental murino, durante 15 dias.

Método: Utilizaram-se 32 ratos Wistar submetidos à hepatectomia parcial (HP), separados em 2 grupos conforme o tratamento controle (C) tratados com solução fisiológica e BV tratados com bevacizumabe, subdivididos conforme o tempo de observação (48 h e 15 dias). Os efeitos dele na regeneração hepática foram avaliados nas avaliações ponderais e função hepática. No fígado remanescente avaliaram-se atividade mitótica, densidade microvascular (angiogênese), proliferação nuclear, histopatologia para investigar fibrose, edema e congestão.

Resultado: No 15º dia e tratamento com BV, não ocorreram diferenças nas avaliações ponderais, na função hepática e nos índices mitóticos. Porém, houve diminuição na viabilidade celular, densidade microvascular e piora na ocorrência de alterações histopatológicas como fibrose, edema e congestão hepáticas.

Conclusão: A dose única de bevacizumabe não causou ao 15º dia alterações ponderais, bioquímicas séricas e dos índices mitóticos. Contudo induziu diminuição da densidade microvascular, viabilidade celular e alterações histopatológicas hepáticas como edema, congestão e fibrose.

PALAVRAS-CHAVE: Regeneração hepática. Angiogênese. Bevacizumab. VEGF.



Histological sections after 15 days of bevacizumab-treated liver: A) hemorrhage and intraparenchymal inflammation; B) intraparenchymal congestion; C) intense ductal proliferation

Central Message

Biological and biotechnological products have occupied a central place in the scope of research and therapeutic development, and pharmacology has reinvented itself with the emergence of products developed by a new route, biotechnology, which aims to offer options aimed at personalized medicine and targeted therapy for tumor cells. This study shows results on bevacizumab in liver recovery.

Perspective

The present study contributes to some important and promising questions regarding liver regeneration during treatment with bevacizumab, which is currently little explored in the specialized literature. Another future approach would be to extend the observational period longer than 15 days so that the evaluations of cell viability and angiogenesis can be defined in a longer period and whether the fibrosis observed here would occur.

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INTRODUCTION

Biological and biotechnological products have occupied a central place in the scope of research and therapeutic development, especially since the 1980s, when pharmacology reinvented itself with the emergence of products developed by a new route, biotechnology, aiming to offer options aimed at personalized medicine and targeted therapy for tumor cells.

In Brazil, the National Health Surveillance Agency (ANVISA) distinguishes 6 main groups of biological products: vaccines, hyperimmune sera, blood products, biomedicines obtained from biological fluids, tissues of animal origin or from biotechnological procedures, medicines containing live, attenuated or dead microorganisms and monoclonal antibodies (mABs). mABs are antibodies derived from the same B lymphocyte clone, whose cloning and propagation are carried out in continuous cell lines and under high technological sophistication. Many mABs are produced to identify and bind to specific antigens of certain types of tumor cells and to selectively exert cytotoxicity on them, preserving non-tumor cells, which is the great advantage when compared to conventional chemocytotoxic therapies. With the advent of these biological drugs, patient survival has increased, also influenced by the improvement of surgical techniques and modern systemic therapies.[1]

Among the biologics applied in anticancer therapy, bevacizumab (BV), a recombinant humanized monoclonal IgG1 antibody, with 93% amino acid sequence of human origin and 7% of murine origin, targets vascular endothelial growth factor receptor (VEGF) isoforms, thus being classified as an anti-angiogenic drug.[2]

The well-established role of VEGF in the promotion of tumor angiogenesis and in the pathogenesis of human tumors served as a stimulus for BV, an anti-VEGF receptor capable of inhibiting angiogenesis and consequently tumor growth, to be incorporated into the treatment of patients, adjuvanting other chemotherapy options in preoperative treatments. Among the oncological surgical indications for the liver, resections of metastases in patients with colorectal carcinoma stand out.

Progress in anti-VEGF therapy has gradually revealed its possible adverse effects related to inadequate blood supply to tissues, which has led to the indication of discontinuation of its use before or after surgical interventions. However, these issues have not yet been fully elucidated for the different bodies. The effects of angiogenesis inhibition on the complex phenomena inherent to liver regeneration are not very clear.[3,4]

It is not surprising that the signaling pathways activated during liver regeneration strongly resemble those of wound healing, seen in other tissues. The difference with the classic wound healing process is that the changes observed in the liver occur throughout the organ and that some of the signs may be derived in part from the peripheral circulation. In a typical wound healing scenario, tissue injury results in the rupture of capillary vascular networks and blood leakage, accompanied by local release of coagulation factors, platelets, growth factors, among others. This is clearly not the case after 2/3 hepatectomy in which 3 hepatic lobes are surgically removed without damaging the 2 remasnescents. Even if there is no damage to the remaining tissue, there are major changes in blood flow patterns. There is considerable literature suggesting that early hemodynamic changes after hepatectomy are important and, even in the absence of extravasation of whole blood, hemodynamic changes after hepatectomy induce a global spectrum of events throughout the liver that resembles a scarring response.

The arterial component of the blood supply per unit of liver tissue does not change after removal of 2/3 of the liver; The distribution of portal blood by unit tissue, however, triples and the portal vein continues to carry the entire flow of the stomach, intestine, spleen and pancreas. The integral flow now needs to traverse a capillary bed whose cross-section is mathematically up to 1/3 of the original. Hepatic capillaries have fenestrated endothelial cells that allow direct access from plasma through endothelial cells to hepatocytes.

Considering that clinical studies do not allow precise analysis of liver regeneration and the scarcity of experimental studies in this specific domain, the present study was developed with the objective of evaluating the influence of liver regeneration. BV at a dose of 5 mg/kg in hepatic regeneration in rats hepatectomized at 70%.

METHOD

Experimental design

This study was evaluated and approved by the Animal Research Ethics Committee (CEPA) of the Angelina Caron Hospital and Maternity according to protocol No. 015/16 CEPA/HAC. The norms contained in Federal Law No. 11,794 were complied with and theEthical Principles in Animal Experimentation of the Brazilian College of Animal Experimentation (COBEA-2000) were observed.

A total of 32 Wistar rats (Rattus norvegicus albinus, Rodentia mammalia) were used, aged between 112 and 125 days and weighing $201.^{8} \pm 9.^{27}$ g, from the Vivarium of the Federal University of Paraná and kept during the experiment in the premises of the Teaching and Research Coordination of the Angelina Caron Hospital and Maternity (Table).

TABLE — Demonstration of the organization of the study groups and procedures performed

Groups	Sub	Procedures
Control (C) n=16	Control 48 n=8	Partial hepatectomy, treatment with saline solution and maintenance for 48 h
	Control 15 n=8	Partial hepatectomy, treatment with saline solution and maintenance for 15 days
Bevacizumab (BV) n=16	BV 48 n=8	Partial hepatectomy, treatment with bevacizumab and maintenance for 48 h.
	BV 15 n=8	Partial hepatectomy, treatment with bevacizumab, and maintenance for 15 days.

The rats were screened through coat inspection to investigate ectoparasites, signs of diarrhea and skin lesions. They were kept in a specific environment for laboratory animals with forced air exhaust (negative pressure), controlled temperature between 19-23 ºC and lighting cycles automatically regulated every 12 h. After the inspection, they were separated into groups of 4 per box with tillage changed every 24 h and received specific feed for the species and water ad libitum. They were weighed at 3 moments, on the day of the beginning of the experiment (T0), 48 h of evolution (T48) and 15 days of evolution (T15).

The experimental procedures were performed in a single cycle of activities, following the protocols for anesthesia and hepatectomy. On the date of the procedures, the rats were weighed on an analytical scale (Coleman® model SK280251) and separated into the sub-groups shown in Table 1.

In anesthetic induction, the association of ketamine hydrochloride at a dose of 80 mg/kg and xylasine hydrochloride at a dose of 10 mg/kg was used. Prior to anesthetic induction, the rats were submitted to sedation by inhalation of isoflurane in a closed circuit. Then, the anesthetics were injected separately, intramuscularly, into both pelvic limbs. Trichotomy of the thoracic region and ventral abdominal wall was performed, fixation with adhesive tapes on the surgical clipboard, antiseption of the trichotomized area with iodinated alcohol, and placement of sterile fenestrated drapes.

Subcostal laparotomy was performed in planes, release of the hepatic ligaments, and partial hepatectomy of approximately 70%.[5] Ligations of the hepatic pedicles were performed with polyglecaprone 3.[0] thread and resection of the median and left lateral lobes, followed by laparorrhaphy in 2 planes, with continuous sutures with polypropylene 6.[0] thread. After closure, the surgical wound was cleaned with sterile saline solution and iodinated alcohol was applied again.

Treatments

Immediately after the surgical procedure and still under anesthetic plan, morphine sulfate was administered intraperitoneally for postoperative analgesia, at a dose of 2 mg/kg and 10 ml of saline solution subcutaneously in the dorsal region, for hydration. The rats in the BV group were also administered intraperitoneally with 5 mg/kg of bevacizumab (Avastin-Roche® 100 mg/4 ml) and the same volume of saline solution as those in the control group. The animals were kept warm until anesthetic recovery. Subsequently, they were returned after identification to the boxes where they remained in a normal diet.

On the dates scheduled for the measurements (48 h and 15 days of evolution), the animals were weighed and then euthanized when an anguinactive puncture was performed, with the collection of 8-10 ml for serum biochemical evaluations. After death was confirmed, a wide median laparotomy and resection of the remaining hepatic lobes were performed, which were placed in identified vials and kept in a 4% paraformaldehyde solution, at PBS pH 7.[4,] for 24 h and sent for histopathological and immunohistochemical evaluations.

Evaluations

The evaluations used were biochemical measurements to evaluate liver function, histopathological measurements to evaluate the microscopic characteristics of the regenerating hepatic parenchyma, and immunohistochemical to evaluate cell viability and microvascular density through specific markers.

Serum biochemical evaluations were performed for albumin levels, total, direct, and indirect bilirubins, transaminases, aspartate aminotransferase determinations, and platelet counts.

Histopathological evaluations were performed in perpendicular sections with 5µm thickness with the sections fixed in slides and stained by the H&E method evaluating edema, hemorrhage, congestion, ductal proliferation, inflammation and also to establish the mitotic index by microscopic counting of the mitosis figures; the Gomori trichrome method for the occurrence of fibrosis, and immunohistochemistry - with specific monoclonal antibodies - to evaluate the microvascular density with anti-CD34 monoclonal antibody and cell viability by anti-cell proliferation nuclear antigen (PCNA) monoclonal antibody. The determination of the mitotic index was obtained by counting mitosis figures on slides with histological sections of samples in H&E and counted under light microscopy. The H&E slides were evaluated by 2 independent pathologists, and the number of mitosis figures was counted in 10 random fields (Figures 1 and 2).[6]

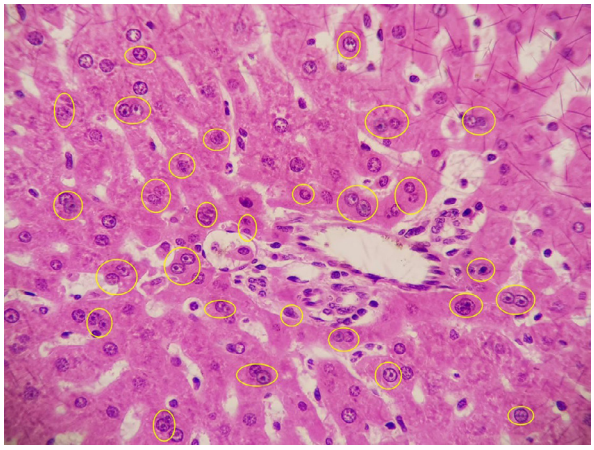

FIGURE 1 — Histological section (H&E, 40x) of the liver after 48 h of partial hepatectomy, demonstrating in detail the figures of mitosis

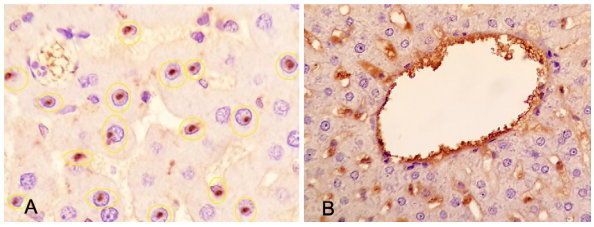

FIGURE 2 — Histological sections (100x) by immunohistochemistry of the liver after 48 h demonstrating positive nuclei: A) anti-PCNA monoclonal antibody; B) anti-CD34 monoclonal antibody.

Evaluation of the occurrence of fibrosis in the hepatic parenchyma was performed by Gomori's trichrome, and fibrotic areas characterized by the presence of collagen (bluish-green, Figure 3) and the other tissue components in red/orange shades were evaluated.[7]

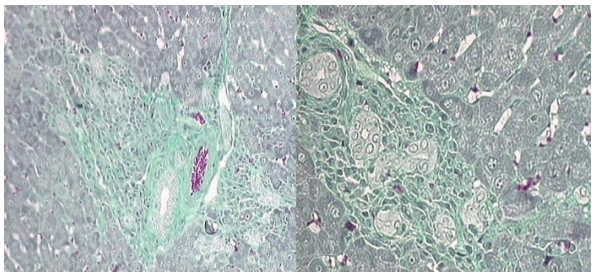

FIGURE 3 — Gomori's trich-trim sections (40x) of the liver treated with bevacizumab 15 days after hepatectomy, demonstrating areas of fibrosis

For the histopathological evaluation of the regenerating hepatic parenchyma, H&E was used and the analyses were performed by 2 trained evaluators. The following histological characteristics were evaluated: absence of alterations, percentages of occurrences of edema, hemorrhage, congestion, ductal proliferation (Figure 4).

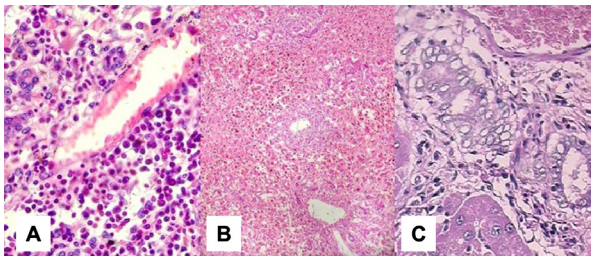

FIGURE 4 — Histological sections after 15 days of the liver treated with bevacizumab: A) hemorrhage and intraparenchymal inflammation; B) intraparenchymal congestion; C) intense ductal proliferation

Statistical analysis

ANOVA and Student's t-tests were used for comparisons between the groups using the GraphPad InStat® software. To obtain the results, the individual readings were tabulated in an Excel spreadsheet and submitted to validation by the application of statistical criteria, and if the values of the 3 different readings did not present a significant difference ($p > 0.05$), they were considered valid and the average of the readings of that criterion was calculated in that group of rats. Next, the means and 95% confidence intervals of each subgroup were calculated and statistically compared using the Student's t-test.

RESULT

No differences were observed in weighing between the subgroups at the beginning of the study (T0, $p = 0.7941$). There were no deaths during the experiment and there was variation in weights according to the sub-groups. in the control group, subgroup C48, there was no significant difference between T0 and T48 ($p = 0.6757$). In C15, when comparing T0 and T48, there was no significant difference ($p = 0.8642$), but there was a difference in the comparisons between T0 and T15 ($p = 0.0003$) and between T15 and T48 ($p = 0.0004$). In the group treated with bevacizumab, BV48, there was no significant difference between T0 and T48 ($p = 0.4252$). In BV15, there were no significant differences when comparing T0 and T48 ($p = 0.6430$), T0 and T15 ($p = 0.4452$) and between T15 and T48 ($p = 0.7597$). There were no differences between C15-T15 and BV15-T15 ($p = 0.0691$).

There were no statistical differences in albumin levels between the subgroups evaluated at T48 and T15, as well as for total, direct and indirect bilirubins. However, there was a statistically significant decrease in glutamic-oxaloacetic transaminase (GOT) levels between groups C48 and C15 ($p = 0.0002$). In glutamic-pyruvic transaminase (TGP) dosages, there was a significant increase in C15 compared to C48 ($p = 0.0169$). In the determination of aspartate-aminotransferase/platelet indices, there was a significant difference between subgroups C48 and C15 ($p = 0.0031$), C48 and BV 15 ($p = 0.0135$) and C15 and BV 48 ($p = 0.0415$); however, there were no significant differences in the comparisons between groups C48 and BV48 ($p = 0.6782$), C15 and BV 15 ($p = 0.5814$), BV48 and BV15 ($p = 0.1001$).

In the determination of the mitotic index, there was a significant difference between the subgroups C48 and C15 ($p = 0.0016$), C48 and BV 15 ($p = 0.0002$), C15 and BV 48 ($p = 0.0001$) and BV48 and BV15 ($p = 0.0000$). However, there were no significant differences in the comparisons between groups C48 and BV 48 ($p = 0.7816$), C15 and BV 15 ($p = 0.1576$). In the evaluation of cell viability by PCNA immunohistochemistry, there was a significant difference between the subgroups C48 and C15 ($p = 0.0000$), C48 and BV 15 ($p = 0.0001$), C15 and BV 48 ($p = 0.0000$), C15 and BV 15 ($p = 0.0000$) and BV48 and BV15 ($p = 0.0000$). However, there were no significant differences in the comparisons between groups C48 and BV 48 ($p = 0.0561$). In the evaluation of microvascular density by CD34, there was no significant difference between the subgroups C48 and C15 ($p = 0.8049$) and C15 and BV48 ($p = 0.8644$), C15 and BV 48 ($p = 0.6853$). However, there were comparisons between groups C15 and BV 15 ($p = 0.0162$), BV48 and BV15 ($p = 00080$). In the evaluation of the occurrence of fibrosis in the hepatic parenchyma, it was found in all rats of the BV15 subgroup.

In the histopathological evaluation of the hepatic parenchyma, it was demonstrated that they differed between the subgroups, as follows: 1) in C48 there was 37.5% of edema, 25% of congestion and 12.5% of ductal proliferation; 2) in C15, there was no edema, but there was 15% congestion and 37.5% ductal proliferation; 3) in BV48, there was 62.5% of edema, 100% of congestion and 12.5% of ductal proliferation; and 4) in BV15 there was 87.5% edema, 100% congestion and 37.5% ductal proliferation.

DISCUSSION

The experimental model

The rat was chosen for this study because it is a small animal adapted to the laboratory environment, accessible acquisition and easy handling in multiple housing and species-specific feeding, as well as the possibility of standardization of variables such as breed, age and sex.[8] The importance of partial hepatectomy models in rodents has been highlighted and widely used because they provide a more convenient approach to the study of this process, given their great similarity with the human situation, such as the absence of immediate tissue injury or inflammation and precise definition of the initial time point when liver regeneration is triggered.[9] In the present study, 32 Wistar rats, aged between 112 and 125 days and weighing 201.8 ± 9.27 g, were used, separated into 4 groups with similar weights (p = 0.7941), which demonstrates homogeneity at the beginning of the experiment. Partial hepatectomy was performed in all rats in a single cycle of activities by a team trained for the purpose, and the procedures were performed under sterile conditions. The principles of animal welfare, including pain control and the euthanasia procedure, were applied in accordance with the regulations of the Federal Council of Veterinary Medicine. It was decided to evaluate the regenerating livers after BV treatment, in the periods of 48 h and 15 days after partial hepatectomy based on a similar study that evaluated at 0, 1, 2, 3, 5, 7, 10 and 14 days.

The BV dose used was 5 mg/kg of weight, and there were references to experiments in different animals and also different doses.[10] The dose of 2.5 mg/kg was used to evaluate the induction of intra-abdominal adhesions in an experimental model of abrasion in the cecum, and they observed less severity of adhesions. In rabbits, they used doses of 50 mg/kg BV, higher than those recommended in humans, in a healing model of intestinal anastomosis and concluded that the preoperative use of BV can negatively affect the resistance of the intestinal anastomosis.[11]

Angiogenesis and liver regeneration

Despite decades of research dedicated to liver regeneration, the understanding of the sequence and coordination of the events that control the process has been considered imprecise, but even so, its importance is recognized as vital for the investigation of possibilities for obtaining clinical benefits.[12]

Liver regeneration after experimental partial hepatectomy at 70% is associated with angiogenesis, as a consequence of increased vascular density. Inhibition of angiogenesis leads to delayed liver regeneration, confirming that it is at least partially dependent on angiogenesis as evidenced in studies with antiangiogenic treatments with angiostatin and vascular endothelial growth factor (VEGF).

The recovery of the original liver mass and function after partial hepatectomy at 70% is very important after injuries, resections and transplants of living donors. It requires dynamic interaction of several cell types with especially critical emphasis on angiopoietin-2 (Ang-2)-secreting hepatic endothelial cells (LSEC). Ang-2 was found to be down-regulated in the early phase of liver regeneration (days 0-3) with recovery of Ang-2 levels during the later phase of liver regeneration (days 4 to 8). Since the initial phase corresponds to the proliferation of hepatocytes and the later phase of non-parenchymal cell regeneration (LSEC, Kupffer cells, and stellate cells) and angiogenesis, the authors hypothesized that LSEC-derived Ang-2 may have dual actions and mechanisms during both phases of liver regeneration. In the angiogenic phase, Ang-2 upregulation elicits VEGFR-2 signaling to induce proliferation of nonparenchymal cells. Thus, a single molecule, Ang-2, can regulate liver regeneration through specific temporal and cellular effects. It is becoming increasingly recognized that angiogenesis – the formation of new microvasculature from pre-existing blood vessels – is essential for liver regeneration to proceed to completion.

In the present study, the immunohistochemical method was used by immunostaining the CD34 antigen with anti-CD34 monoclonal antibody to evaluate the microvascular density (angiogenesis) of the hepatic parenchyma after partial hepatectomy in the proposed periods. CD34 is considered one of the markers of neovascularization, the expression of CD34-positive endothelial cells can play an important role in understanding the angiogenesis process.[13]

CD34 has high specificity, despite having some lymphatic and stromal staining, however, other marker antibodies can be used to identify microvascularization, such as anti-CD31, anti-FVIII and anti-CD105. In the present study, the evaluations of immunostaining with anti-CD34 monoclonal antibody did not differ (p = 0.1276) between the groups evaluated at 48 h, C48 and BV48, after hepatectomy, and it can be concluded that BV does not exert an antiangiogenic effect in an early manner. However, on the 15th day of follow-up, there was a significant difference (p = 0.0162) in immunostaining between the groups treated with BV, with the mean BV15 being lower than that of BV48, which allows us to infer that there was an antiangiogenic effect.

Cell viability, angiogenesis and liver function

The evaluation of PCNA is consecrated in the evaluation of cell viability, considering regeneration as a whole, that is, including parenchymal cells. Gaglio et al.[14] evaluated liver regeneration in primates comparing Ki-67 and PCNA and the results were similar. They used laparoscopic biopsy samples on days 1, 2, 7, 14, 30 and 60 in 5 animals with 5 years of life, submitted to 30-60% hepatectomy. In the same study, they reported that between the 14th and 20th days there was a significant increase in marking, with PCNA maximums, which they concluded coincided with maximal liver regeneration.

There is a study with rats after hepatectomy at 0, 3, 6, 12, 24, 48 and 96 h, with an abrupt increase in 48 h of PCNA protein levels marked by Western blotting, considered 4 times higher than the reference values, remaining up to 72 h after hepatectomy Assy et al.[15]

An increase in PCNA expression has already been demonstrated at 24 h, with a maximum peak 36 h after hepatectomy with hepatectomies at 35%, 68% and 85% by Hashimoto and Sanjo.[16]

In the present study, there was a significant increase in cell viability assessments in the control group, subgroup C15 (p = 0.0000) when compared to C48, which is considered the standard of normality for the partial post-hepatectomy without treatment. In the groups treated by BV, there was the opposite behavior in this evaluation, as BV15 showed a significantly lower mean than C48 (p = 0.0000), which can be considered as an effect of BV treatment.

When comparing the evaluations between the BV48 and BV15 subgroups in relation to the evaluation of cell viability by immunostaining with anti-PCNA and angiogenesis by anti-CD34, the same trend of these measurements could be observed, i.e., the BV15 and BV48 subgroups showed a decrease when compared in both. It is known that VEGF blockade induces complete suppression of hepatocyte proliferation as well as endothelial cells after partial hepatectomy in rats, which supports the results discussed above.

The functions of the liver are multiple and essential for homeostatic regulation and balance. Some biochemical tests can be used to evaluate some of these functions, such as ALT measurement, AST, AST/ALT ratio, gamma-GT, alkaline phosphatase, prothrombin time, albumin, and bilirubins. However, even in severe liver disease, it can still present normal levels of liver enzymes, or cause changes 1.5 to 3 times above reference levels. Silva et al.[17] demonstrating, in addition to the hepatectomy tissue injury, that there were different degrees of other lesions caused by hypertension that the remaining livers suffered, confirmed by the levels of all serum analyses. In a reflection cited by Wu et al.[18] in 2017, where they mention that, even when patients with normal AST, considered a serum marker in the prediction of inflammation, they may still have a significantly aggravated risk or progression of liver disease, which supports the observation in the present study that even if BV had significantly altered regeneration, assessed by all methods between the groups, Normal parameters could have been found for biochemical laboratory evaluations. The most surprising thing about the regenerative process, in addition to the proliferative capacity of the hepatocyte, is the fact that these cells simultaneously maintain all the fundamental functions for maintaining homeostasis, such as the regulation of the glycemic level, synthesis of plasma proteins and coagulation factors, bile secretion, urea cycle and biodegradation of toxic compounds.

Histopathological findings, immunohistochemistry and liver regeneration

Histopathological evaluations of the hepatic parenchyma showed that the percentages of occurrences of the findings edema, congestion and ductal proliferation varied according to the subgroups. Regarding the percentages of occurrence of edema, C48 presented 37.5%, BV48 62.5% and BV15 87.5%. The BV15 sub-group stands out, in which no edema was evidenced, so it would be inferred that in the control group, parenchymal repair occurred before the 15th day in this group and in BV15 there was still a high percentage of edema. Regarding the occurrence of intrahepatic congestion, there was a decrease between C48 (25%) and C15 (12.5%), which can be considered that in the course of regeneration in the control group there was an improvement in relation to the occurrence of congestion.[7] In the same respect, the groups treated by BV did not show improvement, since the percentage of 100% occurrence of congestion remained in the BV48 and BV15 sub-groups. Similar results were observed in a study on the effects of allourinol on hepatic ischemia when they concluded that transient hepatic normothermic ischemia causes significant histopathological changes in the livers of rats. and allopurinol exerted a beneficial effect in relation to hepatocyte necrosis, which reinforces the involvement of the enzyme xanthine oxidase in the damage resulting from hepatic ischemia-reperfusion.

The histopathological findings previously reported may be justified by the fact that, after partial hepatectomy, portal flow is the main factor contributing to the regenerative response of the liver. The increase in hepatic perfusion is due to a pressure difference between the portal vein and the hepatic artery in the remaining part, inducing a form of blood infusion in the spaces ajuzuing to the vessels of the remaining lobes, which may explain the occurrence of congestion and edema after partial hepatectomy.[19]

Also reinforcing the arguments of Petroianu et al.[19] It was defined that 70% partial hepatectomy is recognized as a sufficient stimulus to increase portal blood flow by 2 to 3 times the basal volume, a fact that leads to microcirculation injury within 10 min after resection of the median and left lateral hepatic lobes. With the increase in portal blood flow in a reduced vascular bed - which causes an increase in vascular resistance - there is an increase in portal vein pressure by 30-40%, characterizing transient portal hypertension after hepatectomy, which characterizes hyperplasia.[7]

Wu et al.[18] in 2017, in a study to evaluate liver fibrosis and biochemical alterations in patients with chronic hepatitis B, they applied the APRI test and measured TGP, TGO, GGT, bilirubins, and albumin in 323 patients. Changes in this laboratory routine were not significant, even in patients who were classified as having greater inflammatory intensity or in patients with different degrees of fibrosis. Thus, significant changes were not expected in the present study, since the experimental reports that in 70% hepatectomy tests remain within normal limits.[7]

Within the same line of investigation, the results of this study regarding the expressive occurrence of liver fibrosis in the BV 15 group, observed by the histochemical method with Gomori's trichromic staining, are not related to the results obtained in the APRI method, when in this method there was no significance in the BV15 group in comparison with C48, C15 and BV 48 and thus the lack of robustness of serum AST measurement to diagnose liver inflammation could be indicated Isolated.

The APRI test is able to confirm the existence of significant liver fibrosis.[20] Hematopoietic growth factor, which is most important in regulating megaricyte development and platelet production, is produced in the liver and kidney, so it is not expected that diseased hepatic states or those who suffer physical injuries will

have the quantity and function of platelets preserved at all times, considering their short half-life. Therefore, the statistical insignificance when applying the APRI method in the BV 15 group, contrasting with Gomori staining, is a fact that deserves further exploration.

In a study conducted by Zhang et al.[21] in 2016, where, in addition to comparing non-invasive methods, they evaluated the application of the APRI and FIB-4 method for severe fibrosis and cirrhosis in patients infected with the hepatitis B virus, they concluded that the clinical utility of FIB-4 and APRI for fibrosis needs further external validation in a large population, before being applied to predict fibrosis in patients with hepatitis B virus infection. Murata et al.[22] report that direct contact between platelets and hepatocytes initiates the transduction of activation signal of growth factors such as VEGF; Lesurtel et al.[23] also reported an increase in the rate of liver regeneration due to the presence and homogeneity of platelets; and Wai et al.[24] mention that APRI is able to confirm the existence of significant fibrosis and exclude cirrhosis, which makes it available as an alternative in clinical practice. Thus, in the present study, the correlation of platelets with liver regeneration by the application of the APRI method seems to conflict with histochemistry by Gomori trichrome staining.

Hepatic fibrosis is a dynamic process that results from an imbalance between the production and dissolution of the extracellular matrix. In angiogenesis, there is a tightly controlled balance between angiogenic factor signaling, MMP activity/signaling, endogenous angiogenesis inhibitors, and MMP inhibitors. This may explain fibrosis profiles in angiogenic disorders, since there is no balance.[25]

A VEGF inhibitor was used and liver fibrosis was observed in the BV15 group, as well as significant alterations for immunostaining with anti-PCNA between groups C48 and BV48 ($p = 0.0561$) and between groups C15 and BV15 ($p = 0.0000$). It is observed that in this context there is a break in the balance of angiogenesis and most likely an imbalance in the behavior of the matrix through the activities of MMPs and MMps inhibitors, resulting in liver fibrosis in the BV group15.

Perspective

The present study contributes to some important and promising questions regarding liver regeneration during treatment with BV, which is currently little explored in the specialized literature. Among the results obtained, the decrease in immunostaining with anti-CD34 and anti-PCNA stands out, which showed that BV at 15 days of evolution exerted an anti-angiogenic effect and decreased cell viability. Could the occurrence of fibrosis in the rats of this sub-group be related to the previous results? This question can be considered as a perspective for a future proposal based on this study, addressing the possibility that the regenerated liver in the presence of compromised angiogenesis could suffer oxidative stress and metalloproteinase activation as causes of alterations such as fibrosis and also how to avoid this condition with the use of antioxidants or inhibitors of oxidative stress activating enzymes. Another future approach would be to extend the observational period longer than 15 days so that the evaluations of cell viability and angiogenesis can be defined in a longer period and whether the fibrosis observed here would occur. Good results do not end the themes, they only direct us to more studies!

CONCLUSION

The single dose of BV did not cause weight changes in serum biochemistry (albumin, bilirubins and transaminases) and mitotic indices on the 15th day of follow-up. However, it induced a decrease in microvascular density, cell viability, and hepatic histopathological alterations such as edema, congestion, and fibrosis.

Authors' contributions

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