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Bevacizumab Does Not Inhibit the Formation of Liver Vessels and Liver Regeneration Following Major Hepatectomy: A Large Animal Model Study

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Abstract. *Background/Aim: Patients with unresectable liver colorectal cancer metastases are treated with neoadjuvant chemotherapy often accompanied by biological therapy aimed at reducing the mass of metastases and thus increasing the chances of resectability. Bevacizumab comprises an anti-VEGF (vascular endothelial growth factor) humanized IgG monoclonal antibody that is used for biological therapy purposes. It acts to inhibit angiogenesis, thereby slowing down the growth of metastases. Due to its being administered systematically, bevacizumab also exerts an effect on the surrounding healthy liver parenchyma and potentially limits the process of neovascularization and thus regeneration of the liver. Since the remnant liver volume forms an important factor in postoperative morbidity and mortality following a major hepatectomy, we decided to study the effect of bevacizumab on vascular and biliary microarchitecture in healthy liver parenchyma and its ability to regenerate following major hepatectomy. Materials and Methods: We performed an experiment employing a large animal model where a total of 16 piglets were divided into two groups (8 piglets in the control group and 8 piglets in the experimental group with bevacizumab). All the animals were subjected to major hepatectomy and the experimental group was given bevacizumab prior to hepatectomy. All the animals were sacrificed after 4 weeks. We performed biochemical analyses at regular time intervals during the follow-up period. Histological examination of the liver tissue was performed following sacrifice of the animals. Results: No statistical difference was shown between groups in terms of the biochemical and immunohistochemical parameters. The histological examination of the regenerating liver tissue revealed the higher length density of sinusoids in the experimental group. Conclusion: Bevacizumab does not act to impair liver regeneration following hepatectomy.*

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Key Words: Bevacizumab, anti-VEGF, hepatectomy, liver regeneration.

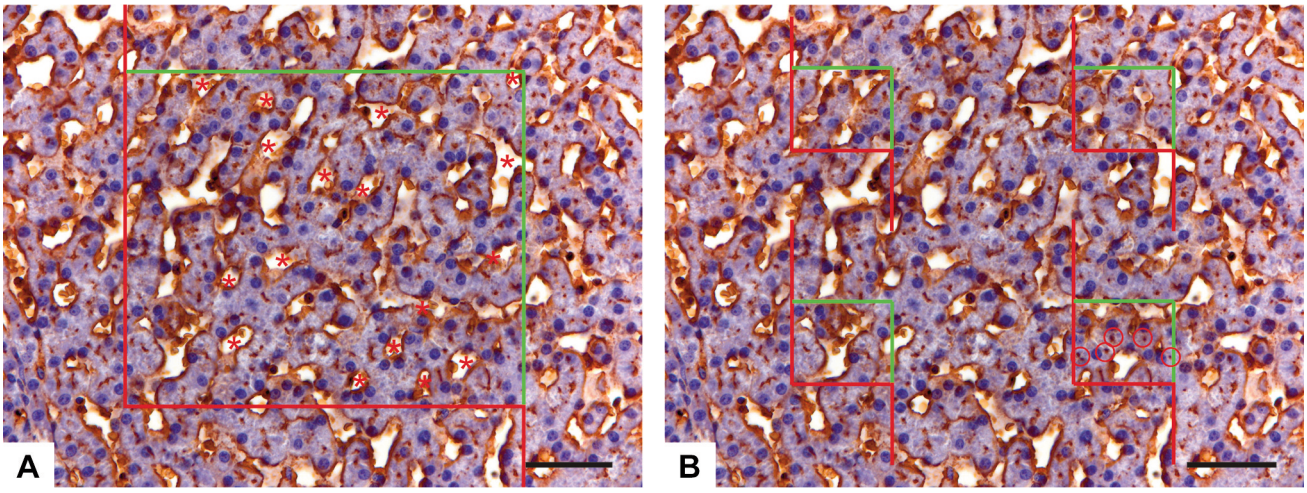

Figure 1. *Quantification of the length density of the sinusoids L_V (sinusoids, liver) and length density of the bile canaliculi L_V (bile ducts, liver). (A) The length density of the sinusoids was estimated using an unbiased counting frame applied over the microphotographs as captured by an objective with a magnification of 40×. We counted the blood vessel profiles that lay within the counting frame or crossed the green inclusion lines but did not cross the red exclusion lines (red asterisks). (B) The same rules were applied when estimating the length density of the bile canaliculi. An example of the counting of the bile canaliculi is provided at the bottom of the right counting frame (the counted canaliculi are marked by red circles). The sinusoids and bile canaliculi were marked based on the brown staining of their inner linings and their spatial relationships with the hepatocytes – among the hepatocyte trabeculae (sinusoids) or between individual hepatocytes (bile canaliculi). RCA lectin histochemistry staining. Scale bars=50 μm.*

Colorectal cancer is one of the most frequently diagnosed cancers worldwide. At the time of diagnosis more than one-third of patients evince the advanced stage of the disease, with at least 25% of patients developing colorectal liver metastases during the course of their illness (1). The liver is the most common site for colorectal cancer metastases (2). The only curative method for liver metastases is surgical resection; however, primary surgery is not suitable for the majority of patients. In cases where metastases appear to be unresectable, neoadjuvant chemotherapy with or without biological-targeted therapy is advised, aimed at reducing the size of the tumor and thus increasing the chances of resectability. The standard combination of neoadjuvant chemotherapy comprises 5-fluorouracil with oxaliplatin or irinotecan (FOLFOX, FOLFIRI or FOLFOXIRI) (3).

Bevacizumab, a biological-targeted therapy, comprises a humanized IgG monoclonal antibody targeted at vascular endothelial growth factor A (VEGF-A) (4) accompanied by chemotherapy for patients with colorectal liver metastases (CRLM). The addition of bevacizumab to chemotherapy leads to an improved response rate and is thought to increase the chances of resectability (5). Both progression-free survival and overall survival have been found to be higher following application of bevacizumab (6). Although the benefits of bevacizumab are known to be significant and its use is increasing, its effect on the regenerating liver parenchyma following the resection of metastatic disease has not yet been studied in detail. The remnant liver volume and its ability to regenerate following a major hepatectomy is crucial in terms of further morbidity and mortality (7, 8). Therefore, it is important to study the effect of this agent on healthy liver parenchyma.

To date, only a small number of studies have considered the effect of bevacizumab on the liver parenchyma following resection in humans. Millet *et al.* studied the effect of bevacizumab on liver regeneration after major hepatectomy by calculating the volumetric gain by means of computed tomography volumetry. No statistically significant difference was determined between the groups treated with chemotherapy with or without bevacizumab, thus suggesting that the liver regeneration capacity is not impaired by bevacizumab (9). Margonis *et al.* compared early and late liver regeneration rates (2 and 9 months following a hepatectomy) in patients who received neoadjuvant chemotherapy with or without bevacizumab and patients who had not had preoperative chemotherapy. The results of this study suggested a higher liver restoration rate following the application of bevacizumab (10).

Although studies in humans suggest that bevacizumab does not impair liver regeneration or that it even enhances regeneration following hepatectomy, an experiment performed in a rabbit model revealed the decreased proliferation of hepatocytes following a hepatectomy in animals treated with bevacizumab compared to the control group treated with saline (11). Conversely, a study in a rat model demonstrated an increase in the regeneration rate postoperatively following a major hepatectomy in animals

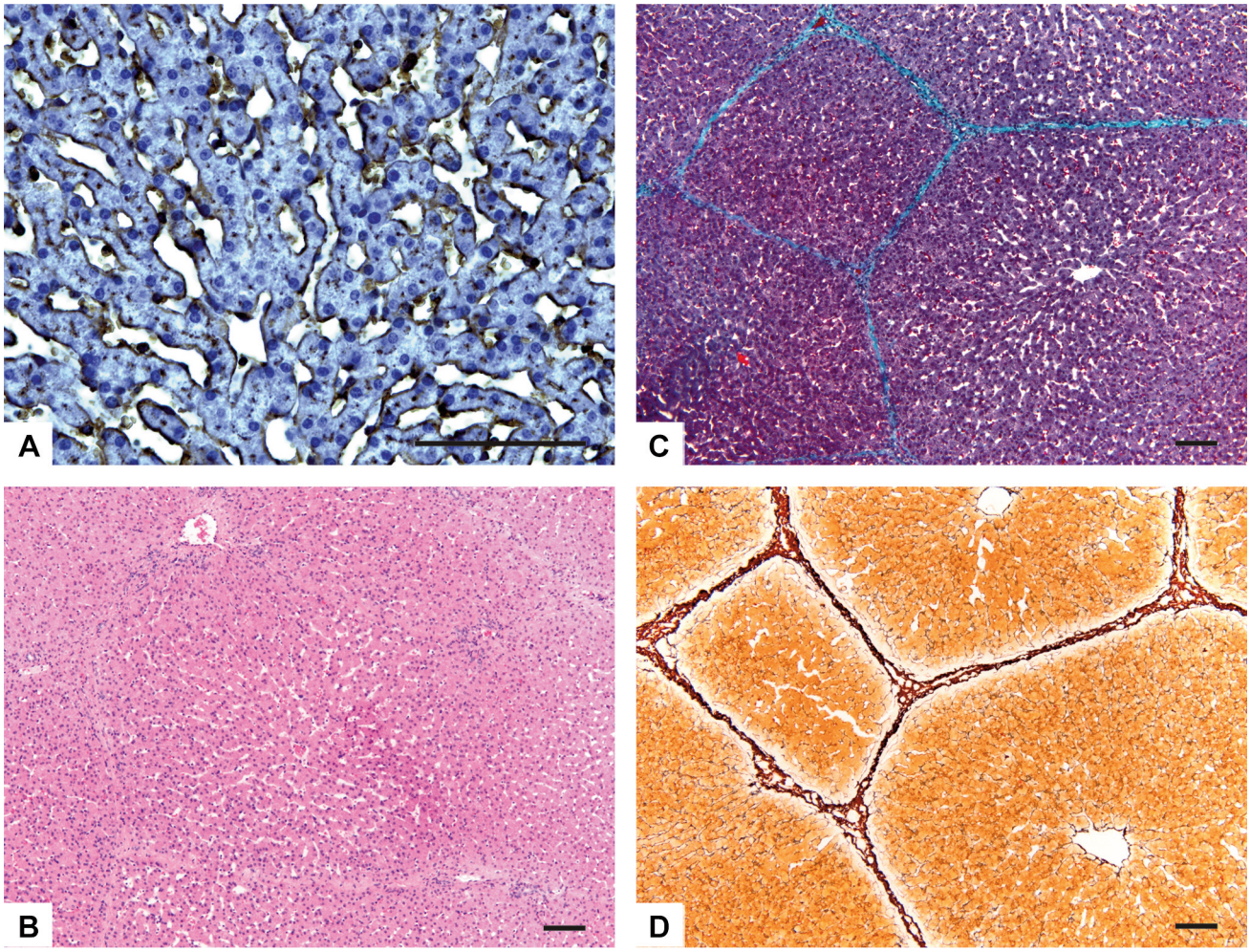

Figure 2. *Examples of the histological staining methods. (A) RCA lectin histochemistry counterstained with hematoxylin used for the visualization of the hepatic sinusoids and bile canaliculi by staining the glycocalyx on the cell borders of the hepatocytes and endothelial cells. (B) Hematoxylin-eosin staining. (C) Green trichrome staining. (D) Reticulin staining for the visualization of collagen III fibers (black). Scale bars=100 μm.*

pretreated *via* the intraperitoneal application of bevacizumab compared to the control group (12).

In view of the variability of results of previous studies on the effect of the administration of bevacizumab on liver regeneration, we decided to conduct an experiment employing a large animal model. We chose pigs as the most suitable model due to their similar anatomical and physiological features to those of humans. Most studies to date have tended to focus solely on the overall outcome of patients and the volumetric analysis of the regenerating liver, and no studies have yet focused on the angiogenesis process, which forms a crucial part of the liver regeneration process. A further key aspect of the functional capacity of the liver comprises the production of bile, which can be determined *via* the biochemical monitoring of the bilirubin level in the peripheral blood of patients or by means of describing the morphology of the hepatic bile ducts.

Therefore, our aim was to assess the effect of the administration of bevacizumab on porcine liver regeneration by evaluating the microarchitecture of the regenerated liver, *i.e.*, the length density of hepatic sinusoids and bile canaliculi.

Materials and Methods

Ethical approval. All the experimental procedures were approved by the Animal Welfare Advisory Committee of the Ministry of Education, Youth and Sports of the Czech Republic (approval ID MSMT - 2084/2020-3) and conducted under the supervision of the Animal Welfare Advisory Committee of the Charles University Faculty of Medicine in Pilsen.

Study design. Both female and castrated male pigs (a total of 16 animals) of the Prestice breed were used in the study with weights of 20-30 kg at the outset of the experiment. The animals received standard care according to EU directive 2010/63/EU, were fed twice

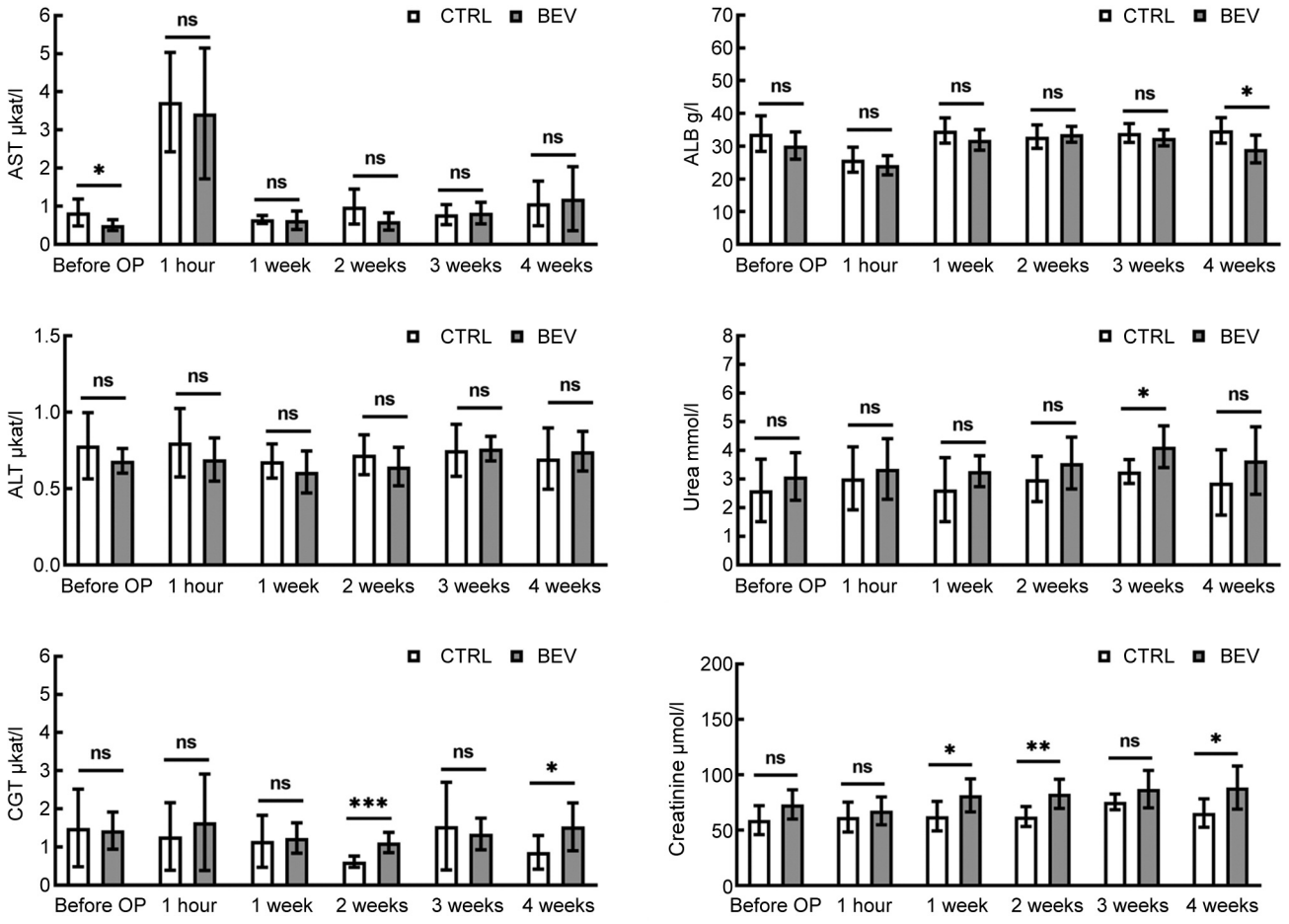

Figure 3. *Biochemical analysis of blood serum liver function markers. Data are expressed as the mean±SD by Unpaired t-test, ns: not significant, *p<0.05, **p<0.01, ***p<0.001. The control group (CTRL) contained 8 experimental pigs (N=8) and the treated group (Bevacizumab, BEV) contained 7 experimental pigs (N=7).*

a day with a complete feed mixture (Fink Nezvestice, Czech Republic) and had free access to water. The intake of feed was recorded daily. The light/dark cycle was 12 h/12 h. The pigs were housed individually in pens with floors that were tempered and covered with a layer of rubber. The pens were cleaned daily.

The animals were divided into 2 groups of 8 pigs; the control group and the experimental group with the administration of bevacizumab.

Firstly, operations were conducted on the control group of pigs. After one week of acclimatization, the animals were subjected to a partial hepatectomy that involved the removal of part of both left lobes and part of the right medial lobe, *i.e.*, approximately 30% of the liver. Samples of the livers were preserved in formaldehyde solution for the subsequent histological examination. Blood samples were collected for biochemical and immunochemical examination purposes during the immediate preoperative and postoperative periods and at regular time intervals until the animals were sacrificed. Furthermore, ultrasonographic examinations of the liver areas were performed prior to surgery, following surgery and subsequently at weekly intervals aimed at evaluating the volume of liver tissue. Due to the inaccuracy of the results, this method was performed only for the control group, *i.e.*, this method was not applied to the experimental group and not included in the study. Following the sacrificing of the animals, liver tissue samples were again preserved for histological examination purposes.

Once the treatment of the control group was concluded, hepatectomies were performed on the experimental group, to which bevacizumab was administered systemically intravenously *via* infusion immediately prior to the start of the operation procedure. The rest of the experiment, with the exception of the ultrasonography, was identical to that conducted for the control group.

Operation procedure. Following a 12-h fasting period, the animals were premedicated *via* the intramuscular injection of Tiletamine-zolazepam (5 mg/kg), Xylazine (2 mg/kg) and Atropine (0.02 mg). The pigs were then subjected to continuous total venous anesthesia and analgesia (2% Propofol 2 mg/kg and Nalbuphine 1-5 mg/h). In addition, all the experimental animals were fitted with a central venous catheter positioned in the internal jugular vein, as described previously (13). The animals were laid supine on the operating table and abdominal access was achieved through a midline laparotomy. Hepatectomies were performed for both groups. Parts of the left

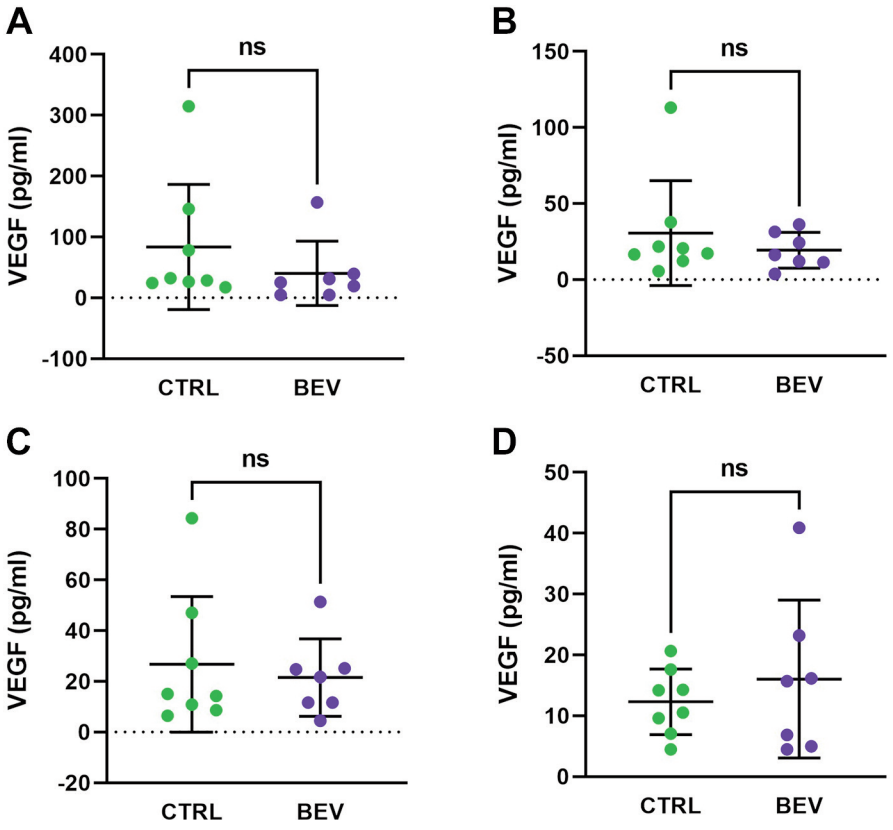

Figure 4. *Concentrations of VEGF in the plasma samples. Data are expressed as the mean±SD. (A) Prior to operation, Mann Whitney (p<0.05). (B) 1 day following operation, Mann Whitney (p<0.05). (C) 3 days following operation, Mann Whitney (p<0.05). (D) 28 days following operation, unpaired t-test (p<0.05). ns: Not significant. The control group (CTRL) contained 8 experimental pigs (N=8) and the treated group (Bevacizumab, BEV) contained 7 experimental pigs (N=7).*

medial and lateral lobes and part of the right medial lobe were removed. Cholecystectomies formed a standard part of the operation procedure for all the animals. The extent of the resected liver mass was between 250 and 300 g, *i.e.*, approximately 30% of the total liver mass. All the animals were treated with prophylactic antibiotics (amoxicillin 10 mg/kg and clavulanic acid 2.5 mg/kg twice a day for 5 days) during the postoperative period. With respect to the second group, *i.e.*, with the administration of bevacizumab, following the induction of general anesthesia and the installation of the central venous catheters, the pigs were intravenously administered with Avastin (250 mg/ml) at a dose of 10 mg/kg body weight *via* infusion for 30 min.

All the animals were sacrificed 4 weeks following surgery under deep general anesthesia conditions. One pig from the bevacizumab group died due to complications caused by intubation. The rest of the animals survived the experimental period with no major complications.

Ultrasonography. Follow-up sonographic examinations were performed for the measurement of the volume of the liver during the immediate preoperative and postoperative periods and, subsequently, at regular weekly intervals until the animals were sacrificed. All the examinations were performed by the same experienced radiologist. Due to the inaccuracy of the results, only the control group was examined, *i.e.*, the group with bevacizumab was not subjected to this form of examination. The control group results revealed that the ultrasonography technique was inadequate for the examination of the liver volume of the pigs. Therefore, we adopted the CT scanning of the abdomen as a more suitable method for the examination and measurement both of the liver volume and potential postoperative complications.

Biochemistry. Blood samples were collected prior to operation and 1 h and 1, 2, 3 and 4 weeks following operation. The biochemical serum parameters were assessed with respect particularly to the monitoring of the influence of bevacizumab on the functioning of the liver. The serum levels of albumin, urea, creatinine, AST, ALT, ALP and GGT were assessed using a Cobas 8000 (Roche Diagnostics). The control group (CTRL) contained 8 experimental pigs (N=8) and the treated group (with Bevacizumab, BEV) contained 7 experimental pigs (N=7).

VEGF, IL6 and IL10 plasma concentrations. Blood samples were collected prior to the operation procedure and at 1, 3 and 28 days following operation. VACUETTE® LH Lithium Heparin tubes were used for collection purposes. The blood samples were centrifuged at 2,000×*g* for 10 min at 4˚C, the plasma was collected and the samples were then stored at –80˚C until the day of analysis. The

Table I. *Quantitative analysis – results. LV (sinusoids, liver) – length density of hepatic sinusoids; LV (bile ducts, liver) – length density of bile canaliculi; resection – sample collected from the resected liver tissue; regeneration – sample collected from the regenerated liver tissue.*

Liver	Group	Area	LV (sinusoids, liver) (mm^{-2}) resection	LV (sinusoids, liver) (mm^{-2}) regeneration	LV (bile ducts, liver) (mm^{-2}) resection	LV (bile ducts, liver) (mm^{-2}) regeneration
A	Control	Periphery	694.62	998.78	3,847.15	3,797.83
A	Control	Center	1,043.99	826.15	3,781.39	3,304.60
B	Control	Periphery	1,150.86	1,122.08	4,110.20	4,373.25
B	Control	Center	998.78	1,183.74	4,652.75	4,619.87
C	Control	Periphery	1,080.98	957.68	5,195.29	3,912.91
C	Control	Center	1,278.27	776.83	4,389.69	4,439.02
D	Control	Periphery	1,196.07	990.56	4,817.16	4,882.92
D	Control	Center	1,179.63	928.91	4,833.60	4,586.98
E	Control	Periphery	1,035.77	912.46	4,800.71	4,537.66
E	Control	Center	1,167.30	883.69	5,310.38	4,225.29
F	Control	Periphery	1,130.31	1,150.86	4,981.56	4,028.00
F	Control	Center	1,249.50	904.24	4,850.04	3,978.67
G	Control	Periphery	1,093.31	850.81	4,258.17	3,929.35
G	Control	Center	933.02	887.80	3,830.71	4,439.02
H	Control	Periphery	830.26	1,015.22	4,291.05	3,912.91
H	Control	Center	NA	859.03	NA	3,912.91
I	Bevacizumab	Periphery	1,093.31	1,159.08	4,208.85	4,488.34
I	Bevacizumab	Center	1,418.02	1,335.82	4,998.00	3,863.59
J	Bevacizumab	Periphery	957.68	1,233.06	4,373.25	5,737.84
J	Bevacizumab	Center	990.56	1,159.08	4,323.93	5,425.47
K	Bevacizumab	Periphery	764.50	1,080.98	4,307.49	4,110.20
K	Bevacizumab	Center	1,183.74	1,150.86	4,570.54	3,797.83
L	Bevacizumab	Periphery	1,237.17	1,076.87	4,340.37	4,521.22
L	Bevacizumab	Center	1,085.09	1,035.77	4,258.17	4,554.10
N	Bevacizumab	Periphery	974.12	1,023.44	4,307.49	4,340.37
N	Bevacizumab	Center	887.80	1,072.76	3,896.47	3,781.39
O	Bevacizumab	Periphery	739.84	990.56	4,734.95	4,570.54
O	Bevacizumab	Center	1,052.21	974.12	4,323.93	3,929.35
P	Bevacizumab	Periphery	842.59	998.78	4,521.22	4,504.78
P	Bevacizumab	Center	941.24	1076.87	4,537.66	4,225.29

concentrations of VEGF, IL6 and IL10 in the blood plasma were determined using the ELISA method. All the samples were undiluted. Commercially available kits with pig specificity were employed, particularly Pig Vascular Endothelial cell Growth Factor, VEGF ELISA Kit (cat. number CSB-E12053p) with a detection range of 4.69-300 pg/ml, Pig Interleukin 6, IL-6 ELISA Kit (cat. number CSB-E06786p) with a detection range of 1.25-80 pg/ml, and Pig interleukin 10, IL-10 ELISA Kit (cat. number CSB-E06779p) with a detection range of 6.25-400 pg/ml, all of which were supplied by Cusabio Technology LLC (Houston, TX, USA). The protocols recommended by the manufacturer were followed in all cases and the signal was detected using a Synergy™ HT microplate reader (BIO-TEK Instruments Inc., Winooski, VT, USA). Gen5 BIO-TEK software was used for the construction of the calibration curves and the calculation of the concentrations of VEGF, IL6 and IL10 in unknown samples. The control group (CTRL) contained 8 experimental pigs (N=8) and the treated group (Bevacizumab, BEV) contained 7 experimental pigs (N=7).

Histological processing and sampling. Each of the porcine livers was represented by 4 tissue samples. 2 of the samples represented the peripheral and central regions of the liver (14); these were collected at the time of the resection (resection), while the other 2 samples were collected at the end of the experiment (regeneration). The tissue blocks were dehydrated and embedded in paraffin. Three 3 μm-thick sections were obtained from each of the tissue blocks. The orientation of the section plane was randomized in view of the unknown isotropy/anisotropy status of the hepatic sinusoids and bile canaliculi in the porcine livers. Randomization was ensured by applying the orientator scheme (15). The RCA lectin histochemistry counterstained with hematoxylin was used to visualize the glycocalyx and, consequently, the cell borders of the hepatocytes and endothelial cells (Figure 1A). Hematoxylin-eosin staining, Mallory trichrome staining and reticulin staining for the visualization of collagen III fibers (Reticulin kit, BioGnost Ltd, Zagreb, Croatia) were applied for the qualitative analysis (Figure 2) (16).

The quantitative analysis included the evaluation of the microstructure (17) and the presence of pathological patterns such as inflammatory infiltration, microarchitecture disruptions, necrosis and other pathological patterns (18).

Quantitative analysis of histological samples. The quantitative analysis was based on 708 microphotographs; 12 fields of view (FOV) for each of the tissue blocks. The microphotographs were taken in a systematic uniform random manner using a 40× objective. The length density of the hepatic sinusoids L_V *(sinusoids, liver)* and

Table II. *Quantitative analysis – results. LV (sinusoids, liver) – length density of hepatic sinusoids; LV (bile ducts, liver) – length density of bile canaliculi; resection – sample collected from the resected liver tissue; regeneration – sample collected from the regenerated liver tissue.*

All samples	Group	Mean	SD	Minimum	Maximum	Median	Lower quartile – Upper quartile
L_V (sinusoids, liver) (mm^{-2}) resection	Control	1,070.8	157.6	694.6	1,278.3	1093.3	998.8-1,179.6
L_V (sinusoids, liver) (mm^{-2}) resection	Bevacizumab	1,012.0	185.6	739.8	1,418.0	982.3	887.8-1,093.3
LV (sinusoids, liver) (mm^{-2}) regeneration	Control	953.0	117.9	776.8	1,183.4	920.7	871.4-1,007.0
L_V (sinusoids, liver) (mm^{-2}) regeneration	Bevacizumab	1,097.7	101.2	974.1	1,335.8	1,076.9	1,023.4-1,159.1
LV (bile ducts, liver) (mm^{-2}) resection	Control	4,530.0	400.6	3,781.4	5,310.4	4,652.7	3,912.9-4,488.3
L_V (bile ducts, liver) (mm^{-2}) resection	Bevacizumab	4,407.3	259.4	3,896.5	4,998.0	4,332.2	4,307.7-4,537.7
L_V (bile ducts, liver) (mm^{-2}) regeneration	Control	4,180.1	400.6	3,304.6	4,882.9	4,126.6	3,915.9-4,488.3
L_V (bile ducts, liver) (mm^{-2}) regeneration	Bevacizumab	4,417.9	574.8	3,781.4	5,737.8	4,414.4	3,929.4-4,554.1
Periphery	**Group**	**Mean**	**SD**	**Minimum**	**Maximum**	**Median**	**Lower quartile – Upper quartile**
L_V (sinusoids, liver) (mm^{-2}) resection	Control	1,026.5	173.7	694.6	1,196.7	1,087.1	933.0-1,140.6
L_V (sinusoids, liver) (mm^{-2}) resection	Bevacizumab	944.2	179.6	739.8	1,237.2	957.7	764.5-1,093.3
L_V (sinusoids, liver) (mm^{-2}) regeneration	Control	999.8	99.8	850.8	1,150.9	994.7	935.1-1,068.7
L_V (sinusoids, liver) (mm^{-2}) regeneration	Bevacizumab	1,080.4	89.0	990.6	1,233.1	1076.9	998.8-1,159.1
L_V (bile ducts, liver) (mm^{-2}) resection	Control	4,537.7	474.4	3,847.1	5,195.3	4545.9	4,184.2-4,899.4
L_V (bile ducts, liver) (mm^{-2}) resection	Bevacizumab	4,399.1	175.5	4,208.8	4,734.9	4340.4	4,307.5-4,521.2
L_V (bile ducts, liver) (mm^{-2}) regeneration	Control	4,171.9	384.2	3,797.8	4,882.9	3978.7	3,912.9-4,455.5
L_V (bile ducts, liver) (mm^{-2}) regeneration	Bevacizumab	4,610.5	521.2	4,110.2	5,737.8	4504.8	4,340.4-4,570.5
Center	**Group**	**Mean**	**SD**	**Minimum**	**Maximum**	**Median**	**Lower quartile – Upper quartile**
L_V (sinusoids, liver) (mm^{-2}) resection	Control	1,121.5	131.0	933.0	1,278.3	1,167.3	998.8-1,249.5
L_V (sinusoids, liver) (mm^{-2}) resection	Bevacizumab	1,079.8	178.0	887.8	1,418.0	1,052.2	941.2-1,183.7
L_V (sinusoids, liver) (mm^{-2}) regeneration	Control	906.3	121.8	776.8	1,183.7	885.7	842.6-916.6
L_V (sinusoids, liver) (mm^{-2}) regeneration	Bevacizumab	1,115.0	116.4	974.1	1,335.8	1,076.9	1,035.8-1,159.1
L_V (bile ducts, liver) (mm^{-2}) resection	Control	4,521.2	560.8	3,781.4	5,310.4	4,652.7	3,830.7-4,850.0
L_V (bile ducts, liver) (mm^{-2}) resection	Bevacizumab	4,415.5	339.9	3,896.5	4,998.0	4,323.9	4,258.2-4,570.5
L_V (bile ducts, liver) (mm^{-2}) regeneration	Control	4,188.3	442.9	3,304.6	4,619.9	4,332.2	3,945.8-4,513.0
L_V (bile ducts, liver) (mm^{-2}) regeneration	Bevacizumab	4,225.3	598.2	3,781.4	5,425.5	3,929.4	3,797.8-4,554.1

the length density of the bile canaliculi *L_V (bile ducts, liver)* were assessed stereologically using unbiased counting frames applied over the FOVs in Ellipse software (ViDiTo, Kosice, Slovak Republic) (Figure 1). We counted the number of vascular profiles per area of the counting frames for QA (sinusoids) and QA (bile ducts). The *L_V (sinusoids, liver)* and *L_V (bile ducts, liver)* were calculated as follows:

$$L_V=2Q_A$$

where L_V is the length of the vessels per unit volume and Q_A is the number of intersections of the plane with the vessels per area (19).

Statistical analysis. The data was expressed as the mean±SD. The control group (CTRL) contained 8 experimental pigs (N=8) and the treated group (Bevacizumab, BEV) contained 7 experimental pigs (N=7). The statistical analysis was performed using GraphPad Prism 9.1.3 (GraphPad Holdings LLC, CA, USA). The data normality was determined *via* the Shapiro-Wilk test ($p<0.05$). The difference between the control and the treated groups, assuming normal data distribution, was tested *via* the unpaired *t*-test ($p<0.05$), and the difference between the control and the treated groups (not assuming normal data distribution) was tested *via* the Mann Whitney test ($p<0.05$).

Results

Biochemistry. The concentration of the AST, ALT, GGT liver enzymes and of albumin, urea and creatinine were monitored in the blood serum for all the experimental animals prior to and 1 h and 1, 2, 3 and 4 weeks following the operations. None of the liver markers indicated significant continuous changes in the blood serum following the operations (Figure 3), thus indicating the safety of the application of bevacizumab with respect to the functioning of the liver.

VEGF, IL6 and IL10 plasma concentrations. The concentration of IL6 and IL10 in the plasma samples was under the detection limits of the applied kits; IL6 <1.25 pg/ml and IL10 <6.25 pg/ml. Although the concentration of VEGF in the plasma samples was within the detection range of the applied kit, no statistical difference was observed between the control and treated groups at any of the time

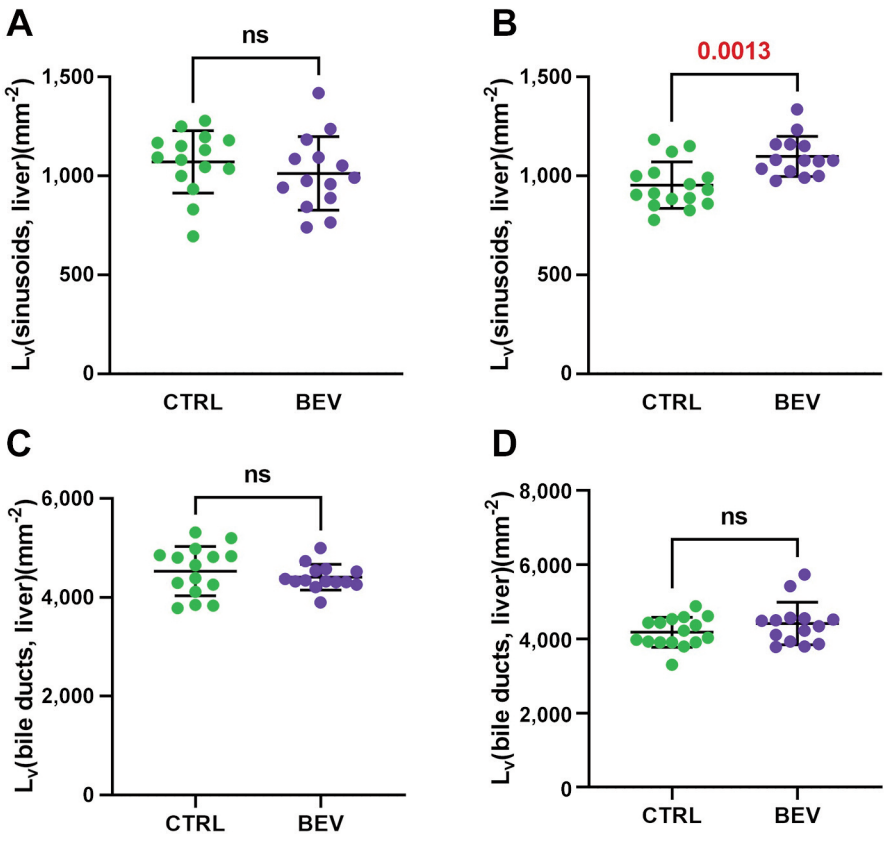

Figure 5. *Quantitative histological analysis of the length density of the sinusoids (A, B) and the length density of the bile canaliculi (C, D) in the resected (A, C) and regenerated (B, D) liver parenchyma. The data are expressed as the mean±SD. Mann Whitney was used. Ns: Not significant or reached p-values. The control group (CTRL) contained 16 analyzed liver samples (N=16) and the treated group (Bevacizumab, BEV) contained 14 analyzed liver samples (N=14), i.e., 2 samples (peripheral and central) per one experimental pig.*

points (Figure 4). Prior to operation (Figure 4A) the concentrations were 83.51±103.00 pg/ml for the control group and 40.27±52.90 pg/ml for the treated group. One day following operation (Figure 4B) the concentrations were 30.61±34.55 pg/ml for the control group and 19.38±11.73 pg/ml for the treated group. Three days following operation (Figure 4C) the concentrations were 26.71±26.78 pg/ml for the control group and 21.53±15.25 pg/ml for the treated group, and 28 days following operation (Figure 4D) the concentrations were 12.30±5.41 pg/ml for the control group and 16.03±12.98 pg/ml for the treated group.

Histology. The results are summarized in Table I and Table II. The length density of the sinusoids estimated in the samples collected after 4 weeks of regeneration L_V (sinusoids, liver) was higher in the bevacizumab group than in the control group (Mann-Whitney *U*-test, $p<0.05$). No significant difference was detected in the length density of the bile canaliculi L_V (bile ducts, liver) between the control group and the bevacizumab group (Mann-Whitney *U*-test, $p>0.05$). No significant difference was detected in the length density of either the sinusoids or the bile canaliculi at the time of resection (Mann-Whitney *U*-test, $p>0.05$) (Figure 5). No correlation was determined between the level of VEGF in the blood and the length density of the sinusoids in the liver (Spearman's correlation).

The parenchyma of the samples taken from the resected part of the liver was observed to be organized into well-defined hepatic lobules with no pathological patterns (Figure 6A and C). Undisrupted microarchitectures composed of hepatic lobules were also detected in the majority of the samples taken during the regeneration period (Figure 6B and D). Remnants of the suture material were occasionally detected surrounded by reactive tissue in the samples taken during the regeneration period (Figure 7).

Discussion

VEGF represents an angiogenic factor that promotes both the proliferation and migration of endothelial cells and vascular

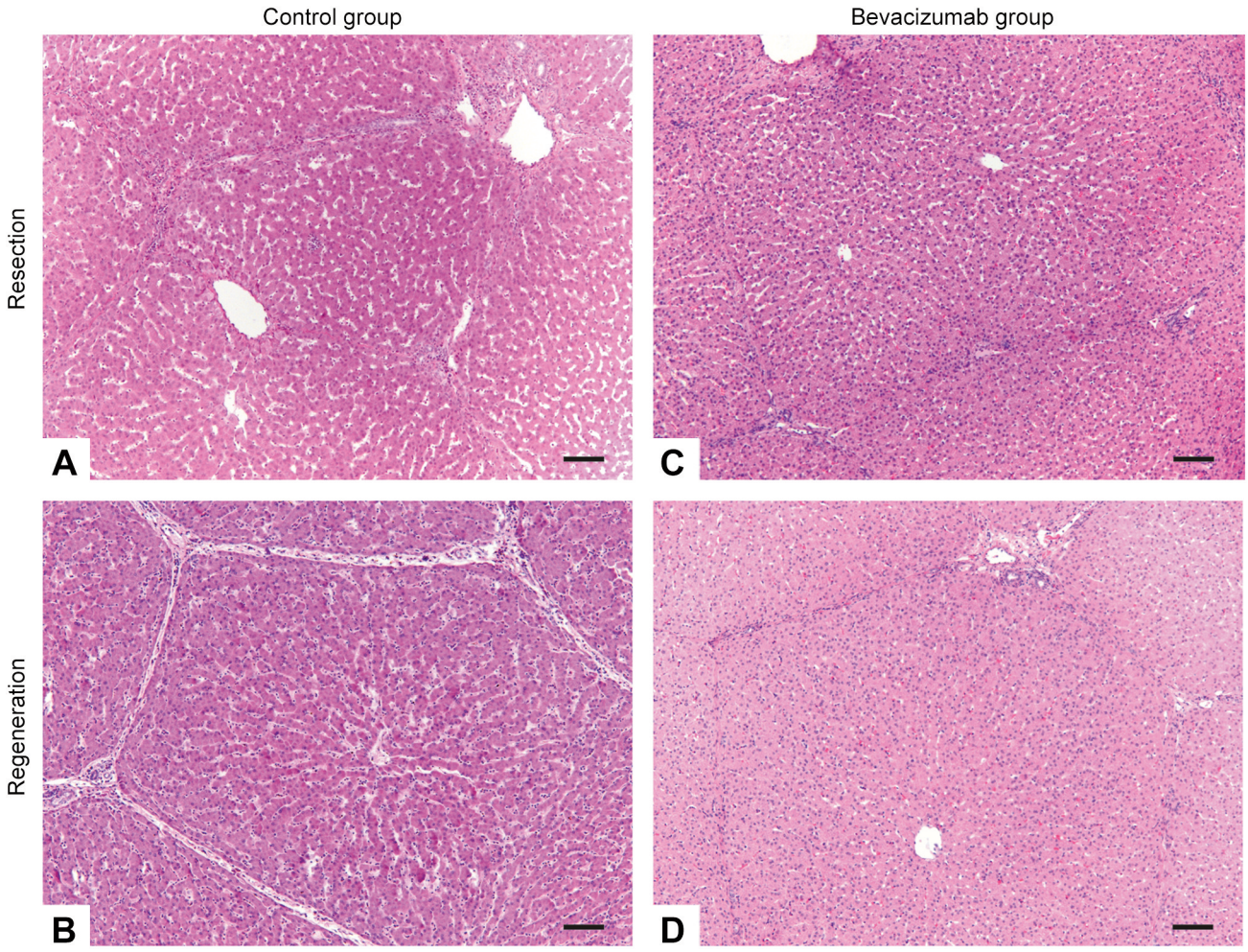

Figure 6. *The microscopic structure of the porcine liver samples. The parenchyma of the liver in both the control and the Bevacizumab groups was organized in well-defined polygonal hepatic lobules with a vein in the center. The lobules were clearly demarcated by connective tissue septa containing the hepatic triad. No excessive pathological patterns such as necrosis or significant inflammatory infiltration were observed in the majority of samples taken at the time of regeneration. Hematoxylin-eosin staining. Samples A1_center (A), A2_center (B), I1_center (C), I2_center (D). Scale bars=100 μm.*

permeability. During the liver regeneration proliferation phase following hepatectomy, hepatocytes up-regulate the expression and secretion of VEGF, which peaks at 72 hours following the procedure (20). Bevacizumab binds to VEGF-A and prevents it from binding to VEGFR (vascular endothelial growth factor receptor) resulting in inhibition of the pathways that promote neovascularization (21). Supposedly, inhibiting the VEGF factor should exert a negative impact on liver regeneration due to impaired endothelial and, thus, vascular proliferation; nevertheless, some studies have demonstrated the opposite. In an experiment on rats, the liver regeneration rate following hepatectomy was higher in the group that was pretreated with bevacizumab (12). The administration of bevacizumab along with chemotherapy preoperatively was associated with enhanced liver volume restoration (10). The effect of bevacizumab on postoperative liver regeneration remains controversial.

Only a small number of studies have, to date, considered the effect of Bevacizumab on liver regeneration following a major hepatectomy, all of which have involved experimentation employing small animal models (10-12), *i.e.*, no studies have previously been performed employing a large animal model. We decided to study the effect of bevacizumab on liver regeneration with concern to the pig liver, which has similar anatomical and physiological features to that of humans (which is not the case of small animals). The pig and the human livers have the same number of liver segments and a common vascular system, thus providing an ideal analog for the effects of liver surgery

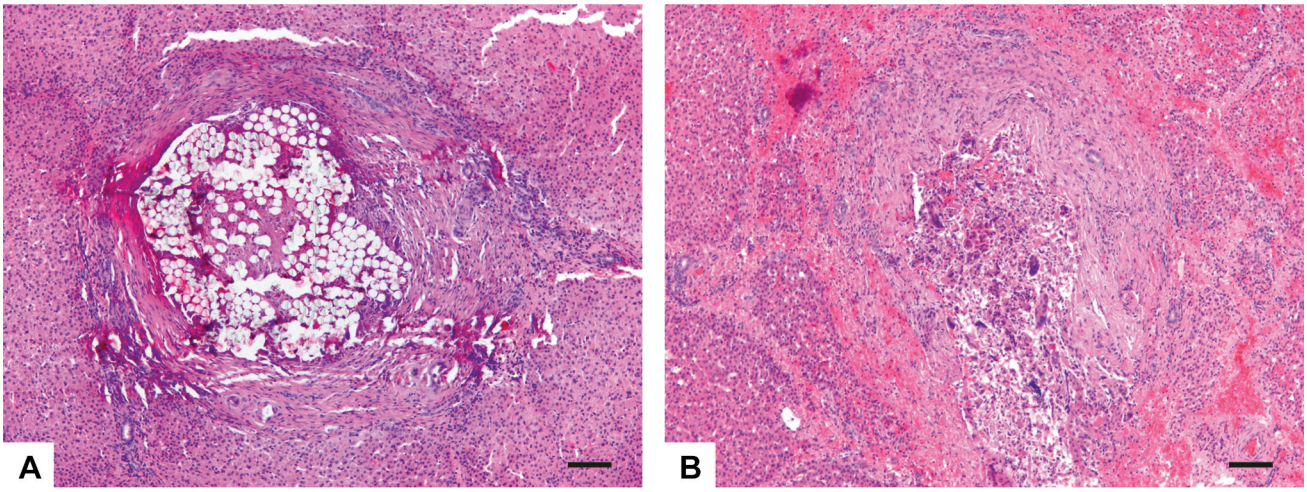

Figure 7. *The Bevacizumab group during the regeneration period. The majority of samples were composed of hepatic lobules surrounded by connective tissue septa. Remnants of the suture material were occasionally detected surrounded by reactive tissue. The nearby tissue contained dilated sinusoids with blood congestion. Hematoxylin-eosin staining. Samples I2_periphery (A), K2_periphery (B). Scale bars=100 μm.*

(22). Thus, in terms of both the concept and choice of animal model, our study is unique and provides valuable data concerning both the field of liver surgery in general and the treatment of the advanced stage of colorectal cancer.

We performed a major hepatectomy on pigs immediately after the intravenous administration of bevacizumab and compared the results with those of a control group that was not pretreated with bevacizumab. We collected blood samples at regular intervals for the evaluation of the levels of VEGF, IL6 and IL10 in plasma. Liver regeneration is a complex mechanism that is influenced by a host of cytokines and growth factors that are either produced at the site of injury or reach the site *via* circulation (23). The levels of the observed factors did not evince any statistically significant differences, thus suggesting that bevacizumab does not lead to the disruption of the regeneration process by means of any imbalance in inflammatory phase. We also considered the standard liver and kidney biochemical function parameters (ALT, AST, GGT, Albumin, Urea and Creatinine); again, no significant difference was observed between the groups. Based on the results of the biochemical examination, we suggest that the systemic application of bevacizumab does not cause damage to the liver and kidneys which would lead to their functional deterioration.

The histological examination revealed that the length density of the sinusoids was higher in the bevacizumab group than in the control group – the application of bevacizumab does not act to decrease angiogenesis in the liver tissue during regeneration. In addition to VEGF, neoangiogenesis in the liver is influenced by other factors and forms a part of a complex and reciprocal process. The construction of new sinusoids occurs *via* cross-talk between hepatocytes, LSECs (liver sinusoidal endothelial cells) and HSCs (hepatic stellate cells), following the activation of which, hepatocytes act to enhance the proliferation of LSECs *via* VEGF. LSECs are also activated by angiopoietins 1 and 2 that are produced by the HSCs. LSECs in return upregulate the proliferation of both hepatocytes and HSCs (24).

In our study, a single dose of bevacizumab did not cause a negative effect on formation of sinusoids, adversely. As mentioned before, neoangiogenesis in the liver is a complex process consisting of many overlapping mechanisms. It is therefore open to discussion, to what extent do other angiogenic factors affect this whole process and whether elimination of one of these factors (in our case VEGF) leads to an over-expression of others and therefore substitution of its function.

No significant difference was detected in terms of the length density of the bile canaliculi between the control and the bevacizumab groups – the restoration of the biliary tree is essential for the proper functioning of the organ. It appears that the attainment of accelerated biliary regeneration following hepatectomy occurs *via* the proliferation of adult liver parenchymal cells once they re-enter their cell cycle (25). The results suggest that the biliary tree was restored to a sufficient extent together with the hepatic parenchyma. Since the length density of the sinusoids did not correlate with the level of VEGF in the blood, we suggest that liver regeneration and angiogenesis is influenced more by the expression of VEGF within the hepatocytes than the level of VEGF in the peripheral blood.

It is usual in clinical practice to allow a therapy-free period between the administration of the final dose of biological therapy and a hepatectomy so as to reduce the

toxicity of the agent and to avoid any negative effects on the regeneration of the liver. It is recommended that bevacizumab be withheld for 6 to 8 weeks before resection, thus respecting the 3-week half-life of this agent (26). Our experiment involved the administration of bevacizumab immediately prior to the surgical procedure. Since this did not impair the regeneration ability of the liver, and the animals did not show any more signs of postoperative complications that those in the control group, it is open to discussion as to whether such a therapy-free period (or at least the length thereof) is necessary.

However, since our study involved administration of only one dose of bevacizumab, it would be advisable going forward to initiate the study of the effect of the lasting and repeated administration of bevacizumab on liver regeneration. Bevacizumab usually forms only a part of the complex neoadjuvant therapy process for CRLM patients. In order to obtain more detailed knowledge on the effect of bevacizumab on liver regeneration, it will be necessary to compare groups treated with chemotherapy and bevacizumab and treated solely with bevacizumab.

Conclusion

We studied the effect of a single intravenous dose of bevacizumab on liver vascular microarchitecture and liver regeneration capacity following hepatectomy. Our study revealed the higher length density of sinusoids in the liver following the preoperative administration of bevacizumab compared to the control group. Based on these results, we suggest that bevacizumab does not negatively affect the neoangiogenesis process in the liver following a major hepatectomy; thus indicating that it does not exert a negative impact on physiological liver regeneration.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in regard to this study.

Authors' Contributions

Ondrej Troup: performance of the experiment (operation), investigation, data collection, writing of the article. Adam Skalicky: methodology, performance of the experiment (operation), data collection, data analysis, writing of the article. Lucie Vistejnova: methodology, performance of the immunological examination, data analysis, statistical analysis, writing of the article, resources. Pavel Klein: methodology, perioperative care, writing of the article. Anna Maleckova: performance of the histological examination, data analysis, statistical analysis, investigation, writing of the article. Blanka Florova: data collection. Tomas Malkus: performance of ultrasonographical examination. Jiri Molacek: supervision, methodology, writing of the article, resources. Vladislav Treska: supervision, methodology. Miroslav Kriz: performance of general anaesthesia. Jan Zeman: performance of the experiment (operation), samples collection. Tomas Skalicky: supervision, methodology, resources.

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