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Influence of Mesenchymal Stem Cell Administration on The Outcome of Partial Liver Resection in a Porcine Model of Sinusoidal Obstruction Syndrome

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Abstract. *Background: In patients with colorectal liver metastases, the possibility for radical liver resection can be limited by oxaliplatin-induced sinusoidal obstruction syndrome (SOS). This study investigates the potential of mesenchymal stem cells (MSC) to improve the outcome of liver resections in pigs with SOS. Materials and Methods: SOS was induced in all animals (n=20) on day 0. Animals in the experimental group (n=8) received allogeneic MSC on day 7. Liver resection was performed in all animals on day 14 and the animals were observed until day 28. Ultrasound volumetry, biochemical analysis and histological examination of liver parenchyma was performed during the follow-up period. Results: Six animals from the control group died prematurely, while all animals survived in the experimental group. According to histology, biochemical analysis and ultrasound volumetry, there were no significant differences between the groups documenting the effect of MSC. Conclusion: Single dose allogeneic MSC administration improved survival of animals with SOS undergoing partial liver resection. Further experiments with different timing of liver resection and MSC administration should be performed to investigate the effect of MSC in more detail.*

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More than 50% of patients with colorectal cancer develop liver metastases (1, 2) and the only potentially curative therapy for these patients is radical liver resection (3). However, the extent of resection is limited by the future liver remnant (FLR) volume as well as the FLR functional and regenerative capacity (2, 4, 5). The quality of liver parenchyma may be compromised by pre-existent liver diseases such as liver steatosis, cirrhosis or chemotherapy-associated hepatotoxicity (4, 6). Irinotecan is responsible for an increased risk of steatohepatitis, and oxaliplatin-based chemotherapy can cause sinusoidal obstruction syndrome (SOS) (7-9).

SOS, also known as hepatic veno-occlusive disease, is caused by toxic injury to the endothelium of liver sinusoids by certain exogenous toxins and can be a life-threatening condition (10). Typical clinical symptoms of SOS are painful hepatomegaly, jaundice, fluid retention with ascites and in severe cases even multiorgan dysfunction or failure (11, 12). SOS can present in acute, subacute or chronic forms (13). The first-identified agents of SOS were pyrrolizidine alkaloids synthesized by certain plants and even today, ingestion of herbal teas and herbal medicine preparations containing these alkaloids can be a cause of SOS (10, 14). Most often, however, SOS is reported in patients after hematopoietic stem cell transplantation (HSCT) due to preconditioning by high-dose chemotherapy or hepatic irradiation (10, 11, 15).

Experiments on rats have confirmed that SOS is a risk factor increasing morbidity and mortality after liver resection (16). A similar impact of SOS has been documented in the clinical setting. Patients with SOS had decreased long-term survival and earlier intrahepatic recurrence compared to patients without SOS. Therefore, it is apparent that the presence of SOS can have a major impact on the outcome of patients with colorectal liver metastases, and strategies to prevent or treat this disease are needed (17).

Many animal models of SOS have been established to study the pathophysiology, diagnostic markers and potential prevention or treatment of this syndrome. We have analyzed these studies in more detail in our review (18). Rat models of SOS induced by pyrrolizidine alkaloid monocrotaline helped to characterize the pathophysiology in detail (19-21). A reactive metabolite of monocrotaline binds to F-actin in sinusoidal endothelial cells (SEC) and causes its depolymerization, which results in a change of the shape of SEC and formation of gaps between them. Red blood cells, leucocytes and cellular debris then penetrate through the gaps into the space of Disse, thus causing subsequent dissection of whole sinusoidal lining and embolization of SEC with obstruction of liver sinusoids (19-21). Moreover, the F-actin depolymerization increases the expression and activity of matrix metalloproteinase-9 (MMP-9) in SEC. Increased MMP– 9 activity is responsible for breakdown of the extracellular matrix in the space of Disse and facilitates the dissection of SEC (22).

De Leve *et al*. described the development of histological changes of liver parenchyma in their rat model during the first 10 days after monocrotaline administration. Based on this, they defined *early stage* changes (typically days 3-5, characterized by coagulative necrosis) and *late stage* changes (typically days 6-7, characterized by subendothelial and adventitial fibrosis) (22).

Harb *et al*. showed in an experiment with monocrotaline-induced SOS in rats that bone marrow-derived endothelial cell progenitors are able to replace SEC and central venous endothelial cells after injury (23). Furthermore, this study demonstrated the ability of monocrotaline to suppress endothelial cell progenitors in bone marrow as well as in circulation, indicating that the SOS is probably caused by 2 mechanisms, *i.e*. the toxic injury to SES and toxic injury to bone marrow progenitors. This finding is in accordance with the fact that the most severe acute cases of SOS are caused by HSCT. High-dose chemotherapy preconditioning before HSCT involves the bone marrow as well as SEC (11).

SOS therapy is limited and includes both preventive strategies and treatment of already- present disease. Prevention is focused on consideration of patient-related or transplant-related risk factors before HSCT as well as on pharmacologic prophylaxis (24). Many agents have been tested to verify their prophylactic effect in animal and also clinical studies, but the evidence is limited and none of them has been assigned indication criteria for clinical use (18, 25). Most of them prevent hemocoagulation and obstruction of sinusoids (*e.g*. heparine, prostaglandin E1, soluble thrombomoduline, cilostazol, *etc*.) (26-29) or inhibit MMP-9 (sesame oil, doxycycline, sorafenib, regorafenib, *etc*.) (20, 21, 30).

Some of the agents tested for their treatment effect act through the same mechanisms as pharmacologic prophylaxis (MMP-9 inhibition and anti-inflammatory effect) (31, 32) or as anticoagulants (33). However, the only treatment of SOS with approved indications in clinical medicine is defibrotide, which protects endothelial cells and causes hydrolysis of fibrin clots (34).

Harb *et al*. used bone marrow-derived endothelial cell progenitors and proposed another mechanism of potential treatment: the replacement of injured SEC by bone marrow-derived cells (23). This method probably corresponds to the mechanism by which the organism itself reverses SEC injury in normal conditions when the bone marrow is not injured. His study demonstrated a therapeutic effect of whole bone marrow administration in rats with monocrotaline-induced SOS (23). In another experiment on mice, infusion of endothelial progenitor cells successfully prevented SOS development after HSCT (35). As it was shown that the disruption of hepatic endothelial barrier during SOS facilitates transplanted cell engraftment, the principle of cell treatment sounds reasonable (36).

In this study we decided to focus on the enhancement of liver regenerative capacity in SOS and the potential treatment of this disease. According to the results of our previous studies, mesenchymal stem cells (MSC) augment liver regeneration in pigs and humans (37, 38). A promising role of MSC in the treatment of liver failure has been shown in animal experiments as well as in clinical settings (39). The aim of this study was to evaluate whether administration of MSC can improve the outcome of animals after partial liver resection in SOS. The effect of MSC administration on the course of monocrotaline-induced SOS was also evaluated.

Materials and Methods

This study was conducted under the oversight of the Ministry of Agriculture of the Czech Republic. All the procedures using animals were approved by the Commission for Work with Experimental Animals at the Charles University, Faculty of Medicine in Pilsen, and the whole study was conducted in accordance with the laws of the Czech Republic, which are compatible with the legislation of the European Union.

Experimental animal and structure of the experiment. The Prestice black-pied pig was chosen as the experimental animal. All pigs were obtained from the agriculture company Mladotice (Mladotice, Czech Republic). Both males and females were included (16 females, 5 males) aged 3-4 months, with an average weight of 34.2 kg (range=23-44 kg, median 34 kg). In all 21 animals, monocrotaline was administered to induce SOS on day 0. The animals were sorted into the experimental (n=8) and control (n=12) groups on day 7.

Animals in the experimental group received MSC intravenously on day 7. No treatment was administered to animals in the control group. All the surviving animals underwent partial liver resection on day 14 and were sacrificed on day 28. One of the animals was not included in the analysis because of its premature death during the first week of the experiment.

Premedication and general anaesthesia. All invasive procedures were performed under general anaesthesia, while blood draws and ultrasound examinations under analgosedation. Intramuscular premedication before the first induction of general anaesthesia was done with 10 mg/kg of ketamine (Narkamon – Spofa, Prague, Czech Republic), 5 mg/kg azaperone (Stresnil – Jannssen Pharmaceutica NV, Beerse, Belgium) and 1 mg of atropine (Atropin Biotika – Hoechst Biotika, Martin, Slovak Republic). The same mixture in lower doses (1-2 mg/kg ketamine, 0.5-1 mg/kg azaperone, 1 mg atropine) was administered intravenously *via* the ProPort system to provide analgosedation during ultrasound examinations and blood draws and as premedication before the second general anaesthesia. General anaesthesia was induced and maintained by intravenous (IV) administration of propofol (1% mixture 5-10 mg/kg/h Propofol, Fresenius Kabi Norges, Halden, Norway). IV administration of fentanyl (1-2 μg/kg/h Fentanyl Torrex, Chiesi cz, Prague, Czech Republic) was used for continuous analgesia. Airways were secured by endotracheal intubation and pigs were mechanically ventilated. Physiological functions were monitored throughout the surgery. During the procedure pigs received infusions and volume substitution when needed: Hartmann's Solution (B.Braun Melsungen AG, Melsungen, Germany) and Plasmalyte (Baxter Healthcare Ltd., Compton, UK). Amoxicillin and Clavulanic acid (Augmentin 1.2 g per pig, GlaxoSmithKline Slovakia., Bratislava, Slovak Republic) were used as antibiotic prophylaxis at the beginning of the procedure; the dose was repeated after 2 hours.

SOS induction. The ProPort Plastic Venous Access System with a PolyFlow polyurethane catheter (Deltec, Smiths Medical, USA) was implanted and introduced through the jugular vein into the superior vena cava in all animals on day 0 of experiment. This venous access simplified the drug and fluid administration in the postoperative period as well as premedication and blood drawing. Subsequently, a midline minilaparotomy was performed and a biopsy from the periphery of left lateral liver lobe was taken; the portal vein was cannulated and a solution with 36 mg of monocrotaline per 1 kg of animal weight was administered over a period of 20 min. The dosage of monocrotaline was established in our previous pilot study (40). To prepare the solution of monocrotaline, the crystals of monocrotaline (Oakwood Products, Inc., SC, USA) were dissolved in 3-4 ml of 1M hydrochloric acid in a warm water bath (40˚C). Sterile PBS was added in a sufficient amount to neutralize the pH (approximately 20 ml) and finally distilled water was added to decrease the osmolarity of the solution. The final volume of the solution was approximately 70 ml, depending on the absolute dose of monocrotaline. After monocrotaline administration the minilaparotomy was closed in anatomical layers, animals were extubated, recovered and each pig was placed in a separate pen. They were monitored daily to evaluate their clinical status and detect any surgical complications. During the first postoperative days the doses of granular feed were reduced but the access to water was not limited. Ten ml of Hartmann's Solution per 1 kg of animal weight, the same volume of 5% glucose (B. Braun Melsungen AG, Melsungen, Germany) and 40 mg of pantoprazole (Nolpaza KRKA Slovensko, Bratislava, Slovak Republic) was administered daily *via* the ProPort system during the first postoperative days. Blood samples were drawn regularly according to the follow-up scheme described below.

MSC cultivation and administration. Bone marrow was harvested by aspiration from the tibia of healthy pigs, matched in age and weight with the animals included in this experiment. Following a puncture with a sterile needle, 15-25 ml of bone marrow was aspirated into a solution of 2 ml saline and 2,500 IU of heparine (Heparine – Zentiva, Prague, Czech Republic). To isolate MSCs, gradient centrifugation (440 × *g*, 30 min) was performed on Ficoll-Paque Plus (GE Healthcare, North Richland Hills, TX, USA). The layer of mononucleated cells was washed with phosphate buffer saline and plated on a culture flask (75 cm^2; TPP, Troy, NY, USA). The culture media consisted of α-MEM cell culture media (Thermo Fisher Scientific, Waltham, MA, USA) with 10 % fetal bovine serum (Thermo Fisher Scientific), 1 mM L-glutamine (Biochrom, Cambridge, UK), 6.0 mg/ml penicillin, 10 mg/ml streptomycin (Biosera, Nuaille, France), and 0.25 mg/ml gentamicin (Biosera). The culture media were changed every other day. After 10 days, MSCs were harvested by EDTA/Trypsin 1x (Biosera) and separated into three culture flasks (75 cm^2; TPP). Culture media were again changed every other day and after 10 days, MSCs were harvested by the same method and cryopreserved in liquid nitrogen (1×106 cells/cryotube). MSCs were thawed 4 weeks before transplantation and plated on flasks (150 cm^2; TPP) in 20 ml of the same culture media and cultured for 4 weeks to get ~50 million cells with one passage cycle, maintaining their stem cell properties. The stem cell phenotype of transplanted cells was identified by flow cytometric detection of CD90, CD73, and CD44. Differentiation ability of MSC was evaluated by their differentiation into adipo-, chondro- and osteo-genic lineages. On the day of administration, MSC were harvested as described above, counted, and re-suspended in 100 ml of physiological solution (B Braun) (37˚C, 10^6 cells/kg of pig weight) per pig. The solution was administered through the ProPort system by slow infusion (30 min) on day 7 of the experiment in all animals from the experimental group (n=8). Infusion of 5ml/kg of Hartmann's Solution and the same volume of 5% glucose followed the MSC administration. Subsequently, animals were monitored according to the follow-up scheme.

Liver resection. On day 14 of the experiment, a midline laparotomy was performed partly at the location of the previous incision but prolonged cranially and caudally, and the left lateral liver lobe was resected using electrocautery. Blood loss was minimized by the Pringle manoeuvre. The vessels and bile ducts for the left lateral liver lobe were ligated and bipolar forceps were used to eliminate bleeding from the parenchyma at the resection area. Samples of the resected left lateral liver lobe were taken and stored in 10% formaldehyde for subsequent histological examination. The laparotomy was closed, animals were extubated, recovered and monitored during a postoperative period of 2 weeks. The food restriction and intravenous administration of crystalloids followed the same scheme as post-monocrotaline administration.

Follow-up (biochemistry and ultrasound examination). Throughout the experiment, regular blood samples were taken in all animals, on day 0 before monocrotaline administration (1), day 3 (2) and day 7 (for the animals in the experimental group, blood was drawn before MSC administration) (3), day 10 (4), day 14 before (5) and right after (6) liver resection, day 14 (2 hours after liver resection) (7), day 15 (8), 17 (9), 21 (10), 24 (11) and day 28 before sacrifice (12). B-mode and

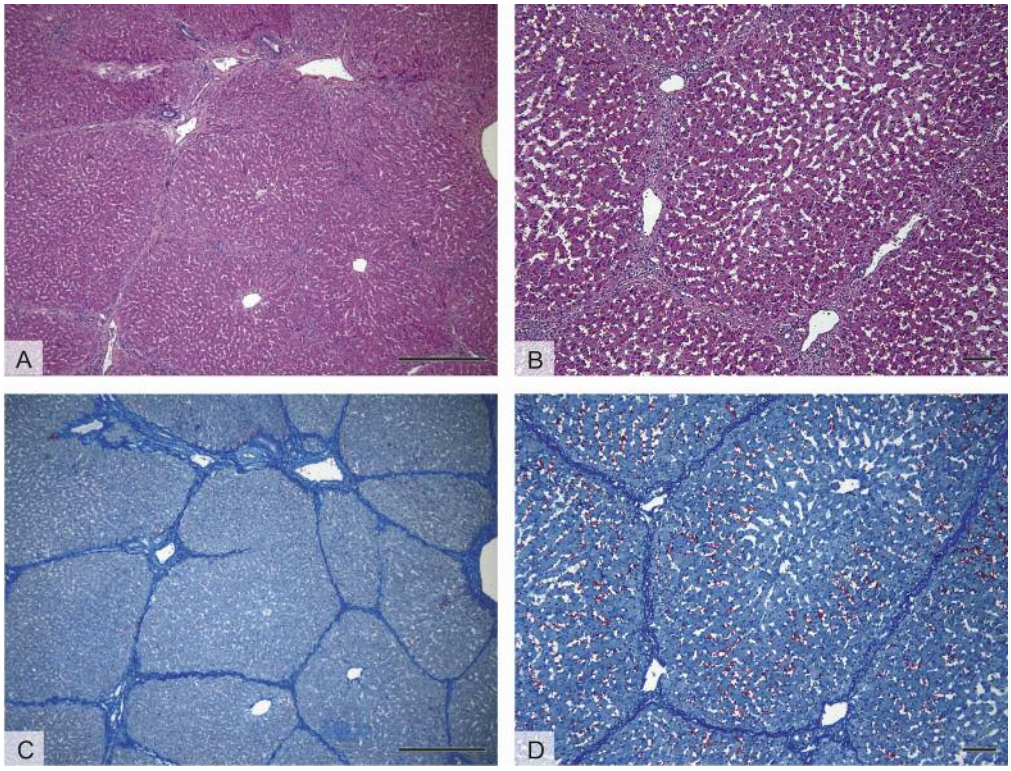

Figure 1. *Examples of histological staining, healthy porcine liver. (A, B) Hematoxylin and eosin, (C, D) anilin blue with nuclear fast red used for collagenous connective tissue detection. Scale bars: A and C=500 μm, B and D=100 μm.*

Doppler ultrasound examination (Ultrasound Scanner Pro Focus 2202, BK Medical, Herlev, Denmark) was regularly performed in order to assess the toxic liver injury and to follow potential regeneration after the resection. The diameter of the extrahepatic part of the portal vein, maximal blood flow velocity at this area, thickness of the gallbladder wall and liver volume were also measured. For liver volumetry, the liver was measured in B-mode in the axial, sagittal and coronal planes and the volume was calculated using an ultrasonographic formula (axial × sagittal × coronal/2) according to our previous publication (41). Ultrasound examination followed this scheme in all animals: day 0 before monocrotaline administration (1), day 7 (for animals in the experimental group, ultrasound was performed before MSC administration) (2), day 14 before (3) and after liver resection (4), day 17 (5), 21 (6), 24 (7) and day 28 before sacrifice (8). Samples of liver parenchyma for histology examination were also taken on day 28 at the end of the experiment or during autopsy, in the case of premature death. The animals were then sacrificed by intravenous administration of T 61 solution (MSD Animal Health, Kenilworth, NJ, USA) under general anaesthesia.

Histology. Liver tissue blocks were fixed with 10% formalin, embedded in paraffin wax and cut into 4 μm-thick sections using an automatic microtome (RM2255, Leica Biosystems, Germany). After dewaxing and rehydration, one randomly selected section per tissue block was stained by hematoxylin-eosin and another randomly selected section was stained with 0.5% aniline blue (Merck KGaA, Darmstadt, Germany) and nuclear fast red (Waldeck GmbH, Münster, Germany) (Figure 1).

Evaluation of histopathological changes of liver tissue was based on a semi-quantitative scoring system previously published by DeLeve *et al.* (19) and Schiffer *et al.* (42). The histopathological evaluation included a qualitative description of changes in liver architecture, namely the presence of haemorrhagic necrosis, blood congestion within sinusoids, presence of inflammatory infiltration of lobules and interlobular septa, presence of reactive fibrotic changes and presence of steatosis (Figure 2). In order to quantitatively describe the severity of liver injury, we quantified two further parameters: volume density of centrilobular haemorrhagic necrosis V_V (necrosis, liver) and volume density of viable liver parenchyma V_V (parenchyma, liver).

The V_V (necrosis, liver) and the V_V (parenchyma, liver) were quantified in 6 necrosis and 8 parencyhma microphotographs per section stained with hematoxylin and eosin, captured with 10x (the necrosis quantification), and 40x objectives (the parenchyma quantification) on an Olympus BX41 microscope (Olympus, Hamburg, Germany). Each of the photomicrographs was taken in a systematic uniform random manner. All quantitative estimates were calculated using well established stereological methods, specifically the point-counting method (43). Ellipse software (ViDiTo, Košice, Slovakia) was used for the quantification (Figure 3).

Statistical analysis. Standard frequency tables and descriptive statistics were used to characterize the sample data set. Quantitative histological parameters were evaluated using repeated measures ANOVA (with the 'Group' factor at two measurement levels of 'Time point' and their interaction). In order to isolate the differences

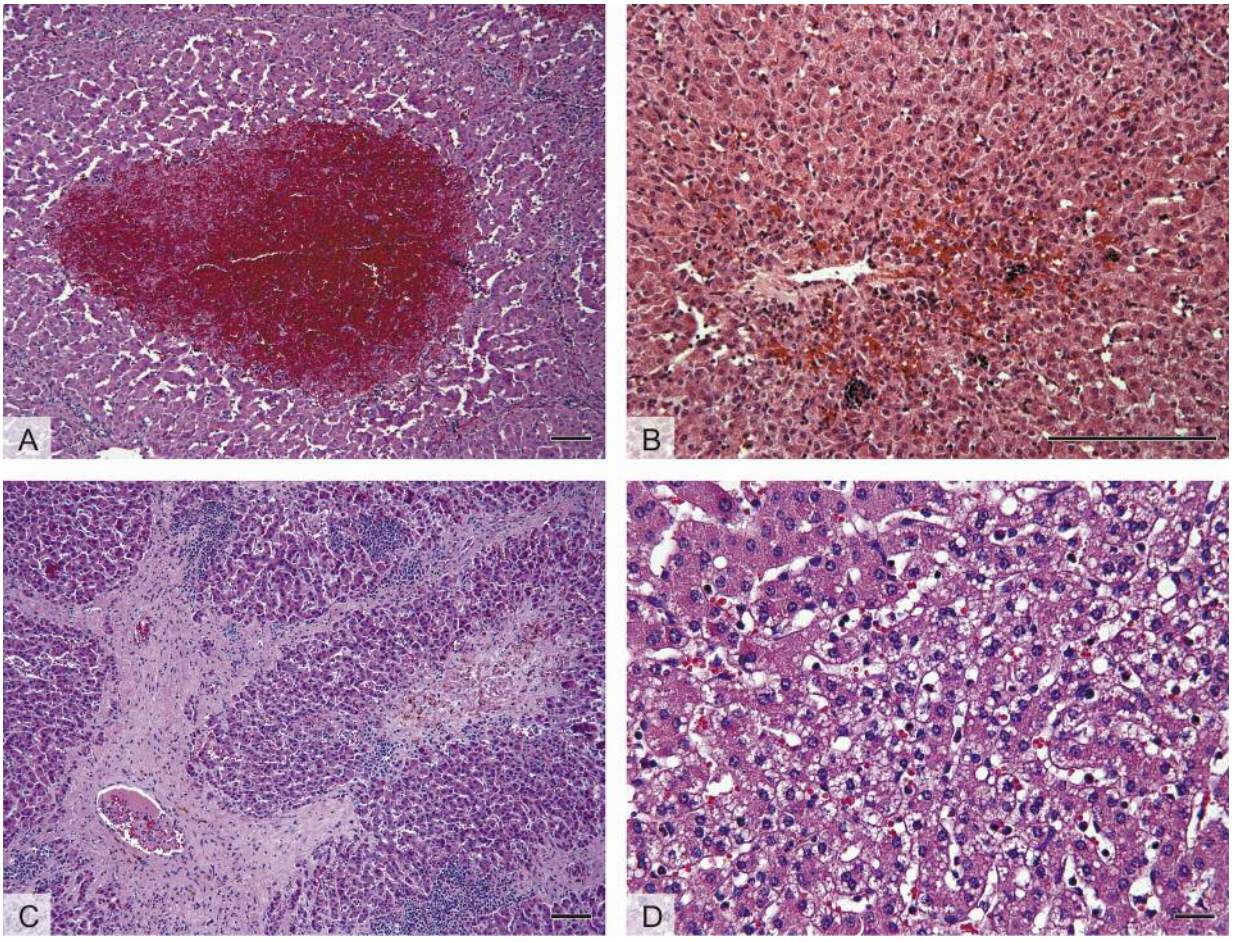

Figure 2. *Histopathological changes in liver parenchyma found 14 days (A, B) and 28 days (C, D) after monocrotaline administration. (A) Centrilobular hemorrhagic necrosis in liver sample 14 days after monocrotaline administration. The necrosis represents one of the most important histopathological hallmarks of sinusoidal obstruction syndrome. (B) Congestion of blood causing dilation of hepatic sinusoids. (C) Reactive fibrotic changes that distort normal hepatic architecture. (D) Steatosis in the center of hepatic lobules. Hematoxylin and eosin, scale bars=100 μm.*

in the progression of biochemical variables and ultrasound measurements caused by the application of MSCs from the baseline values that varied randomly among the pigs, the following normalization procedure was carried out. For each variable, the average value before the point of MSC application (or non-application in the case of the control group, *i.e.* the first 3 time points for biochemistry and the first 2 time points for ultrasound measurements) was subtracted from all the subsequent variable values for each pig. These normalized values from the time points after the separation of the groups were subsequently statistically compared using two-way ANOVA (with factors of 'Group', 'Time point' and their interaction). Raw, non-normalized data from all time points were also analysed (using repeated measures ANOVA) and are also shown. Only the pigs that survived the whole duration of the experiment were included in the statistical analysis. All reported *p*-values are two-tailed and the level of statistical significance was set at α=0.05. Statistical processing and testing were performed using the STATISTICA data analysis software (Version 12; StatSoft, Inc., Tulsa, OK, USA).

Results

Survival. Six out of the 12 pigs in the control group died prematurely during the experiment. The deaths occurred before the liver resection in 3 cases (day 10 and in two instances on day 13) and after the liver resection in 3 cases (day 22, 24 and 27). One out of the 6 deaths was not directly caused by liver parenchyma injury but by peritonitis due to fasciitis of the wound after laparotomy. This animal died on day 22. However, in this case the liver injury also undoubtedly had an impact on the overall condition of the animal. The pathological findings in the other 3 animals revealed not only liver injury but also signs of pneumonia. One animal presented with hemopericardium and because the possibility of traumatic or iatrogenic injury was ruled out, the cause was classified as coagulopathy due to the severe liver injury. Only 1 pig showed signs of liver injury without any other pathological findings.

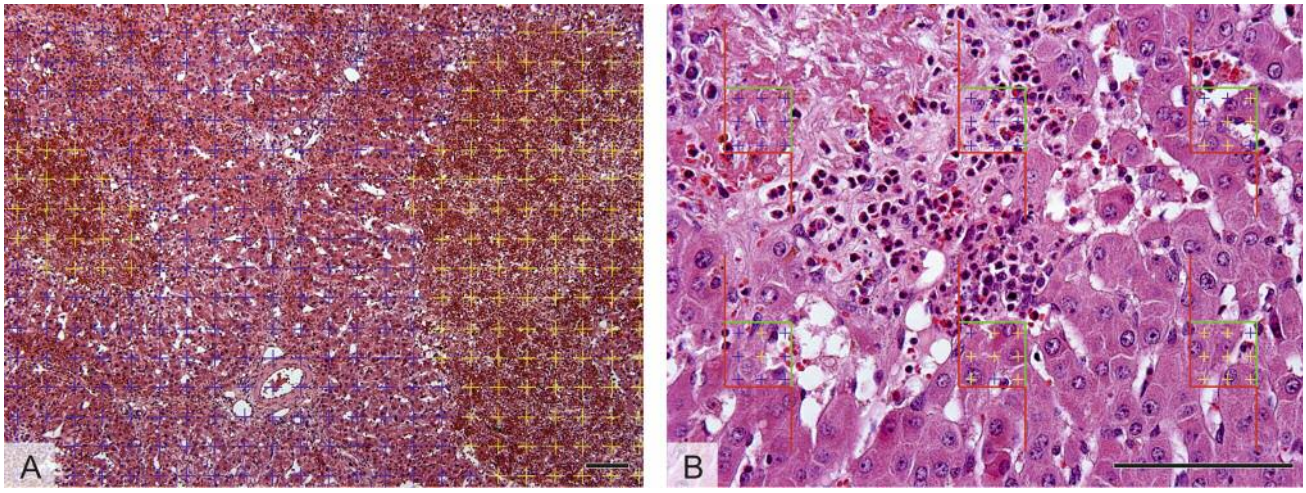

Figure 3. *Quantitative analysis of volume density of centrilobular hemorrhagic necrosis V_V (necrosis, liver) and of volume density of viable liver parenchyma V_V (parenchyma, liver). (A) Stereological point grid used for quantification of hemorrhagic necrosis. Points hitting the necrosis are highlighted in yellow. (B) Stereological point grid used for quantification of viable liver parenchyma. The viable parenchyma was defined as hepatocytes organized into one cell thick plate. The hepatocytes had undamaged cytoplasmatic membrane and clear, eosinophilic cytoplasm. Points hitting the viable liver parenchyma are highlighted in yellow. Scale bars=100 μm.*

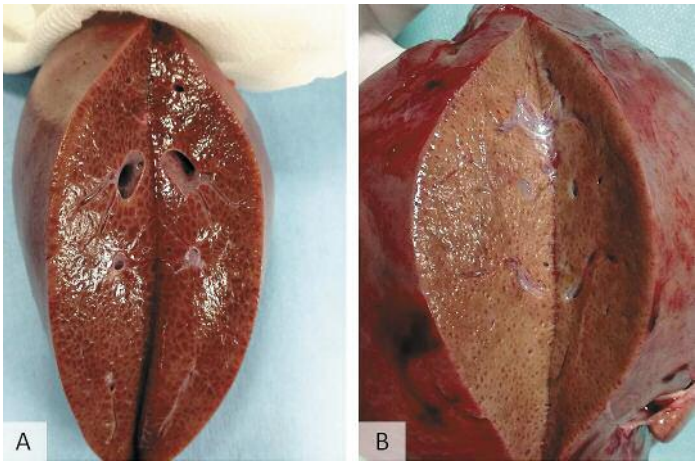

Figure 4. *(A) Appearance of resected liver parenchyma on day 14. The dark lesions inside liver lobules correspond to centrilobular necrosis documented by histology. (B) Liver parenchyma at the end of experiment on day 28 when the lesions were smaller but still present.*

During autopsy or liver resection, the macroscopic appearance of the liver parenchyma was altered. This is documented in Figure 4. All the animals that died prematurely were discarded from the biochemical analysis, evaluation of the ultrasonography data and from quantitative histological analysis. However, all the liver parenchyma samples underwent qualitative histological analysis.

Evaluation of MSC phenotype and differentiation ability. The stem cell phenotype of transplanted MSC was evaluated by flow cytometry. MSC were transplanted after the third passage to ensure both minimal loss of stem cell characteristics and minimal differences between MSC groups. Transplanted MSC were positive for $CD44^{pos}$ (99.1%), $CD73^{pos}$ (96.2%), and $CD90^{pos}$ (99.1%), and negative for $CD45^{pos}$ (0.44%) (Figure 5A). The differentiation ability of transplanted MSC was evaluated by differentiation into adipo-, chondro- and osteo-genic lineages. After 21 days of differentiation, MSC were able to accumulate lipid droplets, detected by oil red staining (Figure 5C) as a marker of adipogenic lineage differentiation. They could produce glycosaminoglycans, detected by alcian blue staining (Figure

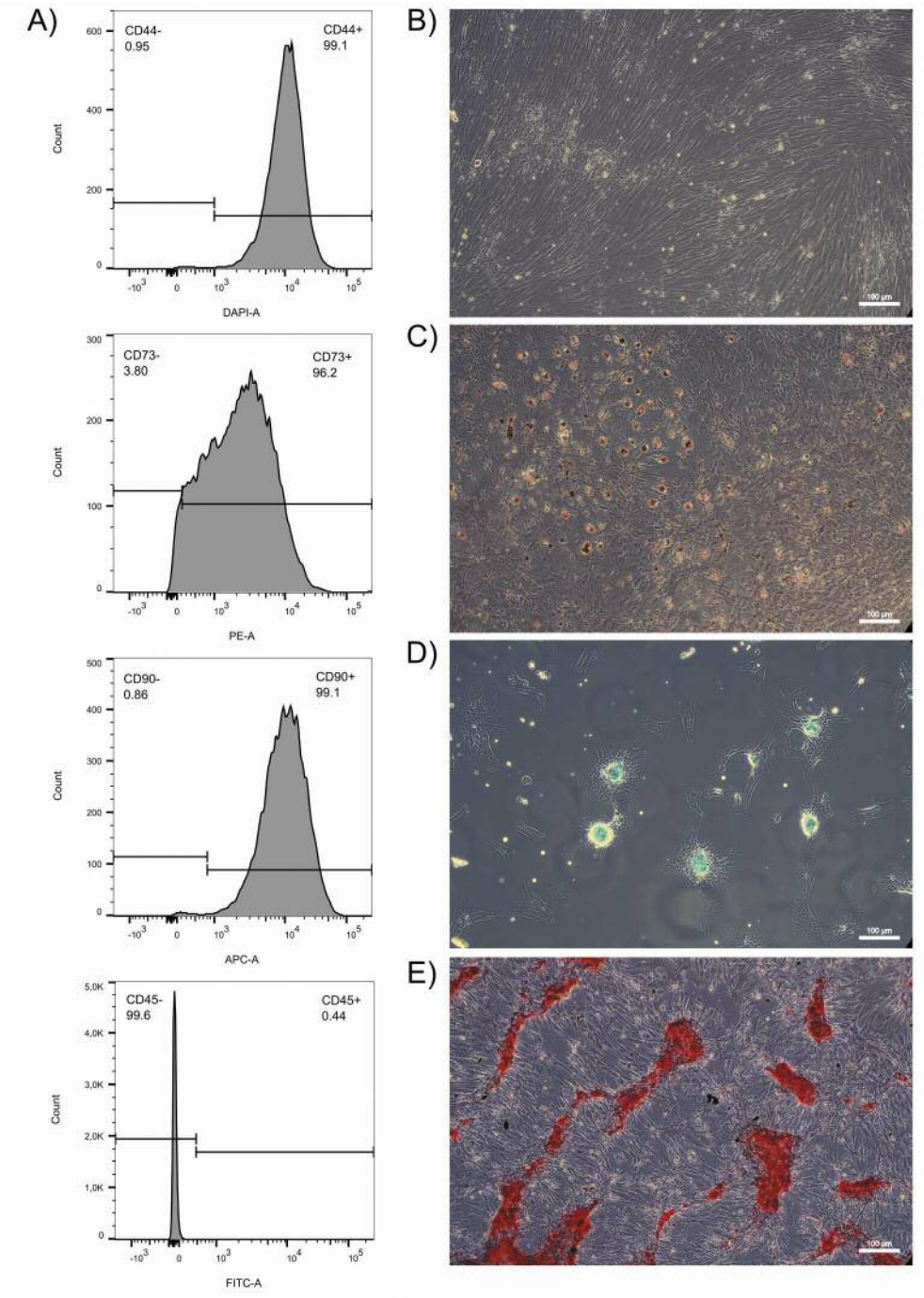

Figure 5. *Evaluation of MSC phenotype by flow cytometry and examination of differentiation potential. (A) Representative histograms show that MSCs were negative for CD45 and positive for CD90, CD73 and CD44. Differentiation potential of transplanted MSCs was evaluated by their differentiation in to adipogenic (C), chondrogenic (D) and osteogenic (E) lineages, compared with untreated controls (B). Scale bars=100 μm.*

5D) as a marker of chondrogenic lineage differentiation, and deposits of calcium cations could be identified detected by alizarin red staining (Figure 5E), as a marker of osteogenic lineage differentiation (Figure 5B).

Histology. At the time of liver resection (day 14), we found extensive centrilobular haemorrhagic necrosis in most of the pigs, which is typical of SOS. In two pigs there were no signs of necrosis [one pig from the control group (pig C7) and one

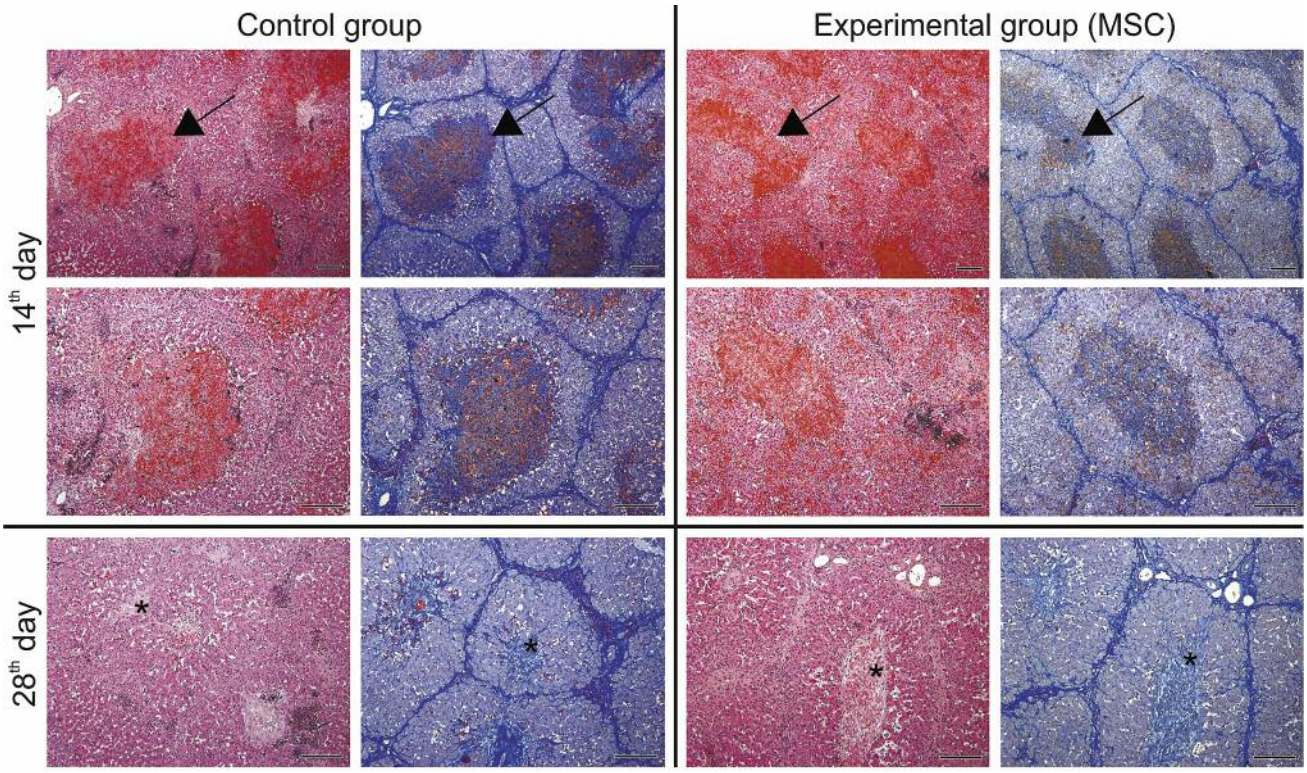

Figure 6. *Comparison of liver injury in control and experimental groups 14 and 28 days after monocrotaline administration. 14 days after monocrotaline administration, the liver injury was characterized by denudation of sinusoidal lining, obstruction of the sinusoidal lumina and hemorrhage into the liver parenchyma, leading to extensive centrilobular hemorrhagic necrosis (arrows). After 28 days, the samples showed parenchymal extinction lesions with fibrotic and stenosed hepatic veins (asterisks). Control and experimental groups did not show any significant differences regarding the character and extent of liver injury. Hematoxylin and eosin (1st and 3rd column) and anilin blue with nuclear fast red (2nd and 4th column) were used for staining. Scale bars=200 μm.*

from the experimental group (pig E4)]. The liver parenchyma in these two pigs showed changes which were also observed in the other animals – denudation of SEC and obstruction of the lumen of sinusoids leading to their dilation, as well as inflammatory infiltration. Figure 6 shows the histological changes at the time of liver resection. All the results of qualitative histology for this time point are summarized in Table I.

At the end of the experiment (day 28), haemorrhagic necrosis was no longer present in the majority of animals and was replaced by centrilobular fibrotic lesions. Steatosis was found in the centre of the hepatic lobules. Figure 6 shows the histological findings and Table II the qualitative results.

The most severe change, nodular transformation of the parenchyma, occurred in two animals from the control group that did not survive the experiment. The overview of the liver histology of the animals with premature death is summarized in Table III. The quantitative analyses did not show significant differences in the volume density of haemorrhagic necrosis and viable liver parenchyma between the experimental and control group at the time of liver resection or at the end of the experiment (Figure 7, for more details see Table IV). The morphology of liver injury was comparable in both groups for both time points. The viable liver parenchyma was chosen as a marker for evaluation of the functional capacity of the liver because the volume of parenchyma was influenced not only by the extent of necrosis but by reactive fibrotic changes, dilation of hepatic sinusoids and by inflammatory infiltration as well.

Biochemistry. The observed levels of biochemical markers of liver and renal functions correspond to severe toxic liver injury (Figure 8). Elevation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin and ammonium were observed in both groups. The first peak was observed after monocrotaline administration and the second peak after liver resection. The level of AST returned to the normal range by the end of the experiment. The level of ALT also dropped during the last week of follow-up and was even lower than the initial value, but still within the normal range. The level of ammonium and bilirubin remained elevated until the end of experiment. The level of urea dropped during the first three days after monocrotaline administration and elevation was observed one day after liver resection.

Table I. *Overview of qualitative histological findings at the time of liver resection (day 14). E, Animals from the experimental group; C, animals from the control group.*

	Control group				Experimental group			
	Animal	Hemorrhagic necrosis	Inflammatory infiltration	Other findings	Animal	Hemorrhagic necrosis	Inflammatory infiltration	Other findings
Liver resection	C1	Yes	Yes	Steatosis in centre of lobules	E1	Yes	Yes	
	C5	Yes	No		E2	Yes	Yes	
	C7	No	Yes	Without hemorrhagic necroses, almost normal appearance of liver parenchyma	E3	Yes	No	
	C10	Yes	Yes		E4	No	Yes	Without hemorrhagic necroses, dilated sinusoids
	C11	Yes	Yes	Small centrilobular hemorrhagic necroses	E5	Yes	Yes	
	C12	Yes	Yes		E6	Yes	Yes	Severe inflammation
					E7	Yes	Yes	Severe inflammation
					E8	Yes	Yes	

Table II. *Overview of qualitative histological findings at the end of the experiment (day 28). Animals from the experimental group are labeled E, animals from the control group are labeled C.*

	Control group					Experimental group				
	Animal	Hemorrhagic necrosis	Inflammatory infiltration	Fibrotic centrilobular reactive lesions	Other findings	Animal	Hemorrhagic necrosis	Inflammatory infiltration	Fibrotic centrilobular reactive lesions	Other findings
End of experiment	C1	No	Yes	Yes		E1	No	Yes	Yes	Steatosis
	C5	No	Yes	Yes	Severe inflammation	E2	No	Yes	Yes	
	C7	No	No	No	Almost normal appearance of liver parenchyma	E3	No	Yes	Yes	
	C10	Yes (minimally)	Yes	Yes	Severe inflammation	E4	No	Yes	Yes	Severe inflammation
	C11	Yes	Yes	Yes	Severe inflammation	E5	No	Yes	Yes	
	C12	No	Yes	Yes		E6	No	Yes	Yes	Steatosis
						E7	No	Yes	Yes	
						E8	Yes	Yes	No	Centres of lobules with bleeding surrounded by fibrotic tissue

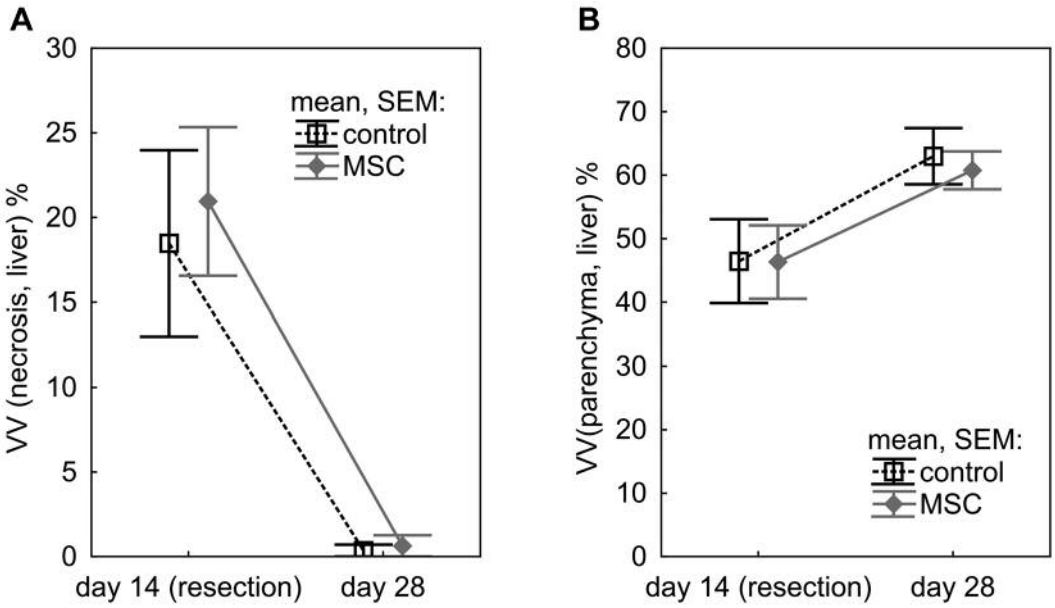

Figure 7. *Results of quantitative histological analysis in the experimental (MSC) and control group (control). Neither the volume density of necrosis (A) nor the density of viable parenchyma (B) differed bewween experimental (MSC) and control groups. The volume density of necrosis was smaller at day 28 than at day 14 (resection). The volume density of viable parenchyma was greater at day 28 than at day 14 (resection). Repeated measures ANOVA was used for quantitative histological analysis.*

Table III. *Histological findings in samples of liver parenchyma taken during autopsy in animals that died prematurely.*

Death during the experiment				
Animal	Sample taken	Hemorrhagic necrosis	Inflammatory infiltration	Other findings
C2	Death (Day 10)	Yes	Yes	
C3	Resection	Yes	Yes	
	Death (Day 24)	No	Yes	Almost normal appearance of liver parenchyma
C4	Death (Day 13)	Yes	No	
C6	Resection	Yes	Yes	
	Death (Day 22)	Yes	Yes	Nodular transformation of liver parenchyma, sporadic centrilobular necroses
C8	Resection	Yes	Yes	
	Death (Day 27)	Yes	Yes	Nodular transformation of liver parenchyma, sporadic centrilobular necroses
C9	Death (Day 13)	Yes	Yes	

To compare the levels of biochemical markers in the two groups the values were normalized according to the average value before day 7. The comparison of the course of biochemical markers after the day of administration of MSC seemed to be more accurate than comparison of absolute values. However, the analysis of the results for the whole period of follow up without any data normalization was also performed.

The level of ALT and creatinine was significantly higher in the control group during the period after day 7 (p=0.001 for both markers). In contrast, the level of bilirubin and ammonium was significantly higher in the experimental group during the same time period (p=0.040 and 0.0001, respectively). The values of the other markers were comparable between the experimental and the control group.

The analysis for the whole follow-up period did not show any significant differences in biochemical markers between the two groups.

Ultrasound. For the ultrasonography analysis the same data normalization was performed and the groups were compared for the period after day 7. There was no significant difference in the diameter of portal vein or in the blood flow velocity in the

Table IV. *Results of quantitative histological analysis of samples taken at the time of liver resection and at the end of the experiment. Volume density of necrosis - V_V (necrosis, liver). Volume density of viable liver parenchyma - V_V (parenchyma, liver).*

	Control group			Experimental group		
	Animal	V_V (necrosis, liver) %	V_V (parenchyma, liver) %	Animal	V_V (necrosis, liver) %	V_V (parenchyma, liver) %
Liver resection	C1	30.0	29.2	E1	23.3	49.3
	C5	22.8	46.1	E2	38.2	27.8
	C7	0.3	73.1	E3	23.7	45.6
	C10	20.0	35.4	E4	0.0	77.5
	C11	4.5	56.7	E5	19.4	40.7
	C12	33.4	38.2	E6	17.7	45.6
				E7	10.2	57.4
				E8	35.2	26.6
End of experiment	C1	0.0	47.5	E1	0.0	72.7
	C5	0.0	61.6	E2	0.0	60.6
	C7	0.0	74.5	E3	0.0	50.0
	C10	0.0	76.2	E4	0.0	67.8
	C11	2.1	60.9	E5	0.0	63.4
	C12	0.0	57.4	E6	0.0	47.9
				E7	0.0	64.8
				E8	5.0	58.8

portal vein during the follow-up period between the different groups (Figure 9A, B). The time course of these parameters was not significantly influenced by the SOS induction and by the liver resection. The diameter of the gallbladder wall had a significant tendency to increase throughout the follow-up period (p=0.004) and did not differ between the groups (Figure 9C). The volume of liver parenchyma increased during the first week after the monocrotaline administration and dropped after day 7 and after the liver resection (Figure 9D). A smaller increase in liver volume occurred during the first days after the resection and was followed by another period of volume loss. For the period after day 7, the relative liver volume was significantly higher in the control group (p=0.023). The ultrasonography detected hyperechogenicity of the liver parenchyma in all animals after day 7 compared to normal echogenicity on day 0. In some animals, reversed flow in the portal vein was observed on day 7 onwards.

Discussion

The study aimed to assess the potential of MSC administration to enhance liver regeneration in SOS and/or its potential to attenuate it. The SOS was induced by intraportal monocrotaline administration in pigs which were then divided into two groups. One of the groups was treated with one intravenous dose of MSC one week after monocrotaline administration, while the second group was a control without any treatment. The survival of animals, levels of biochemical markers, ultrasonography data and histology of liver parenchyma were compared between the groups to verify the severity of SOS and thus the treatment effect. The capability of liver regeneration after partial liver resection in animals with SOS was also compared to evaluate the effect of MSC.

Induction of SOS. Data from biochemistry, histology and ultrasonography show that SOS was successfully induced in pigs by intraportal monocrotaline administration in a single dose of 36 mg monocrotaline per 1 kg of body weight.

Hepatomegaly and hyperbilirubinemia are mentioned in all diagnostic criteria for SOS used in clinical medicine (44). In this study, the animals showed an increase of liver volume and elevation of bilirubin in the first days after monocrotaline administration. Liver enzymes are also usually elevated in patients with SOS, which corresponds with the finding of elevated AST and ALT during the first days after SOS induction in this study (13, 44). The decrease in the level of urea after monocrotaline administration can correspond with attenuation of liver functions (45, 46). The induction of SOS was also confirmed by histological findings. The denudation of SEC, obstruction of the lumen of sinusoids and hemorrhagic necrosis observed in this study correspond to changes described in rodent models of SOS and in humans (19, 47, 48).

Ultrasonography helped to not only to detect hepatomegaly but also revealed a changed echogenicity of the liver parenchyma, altered flow in the portal vein and increasing

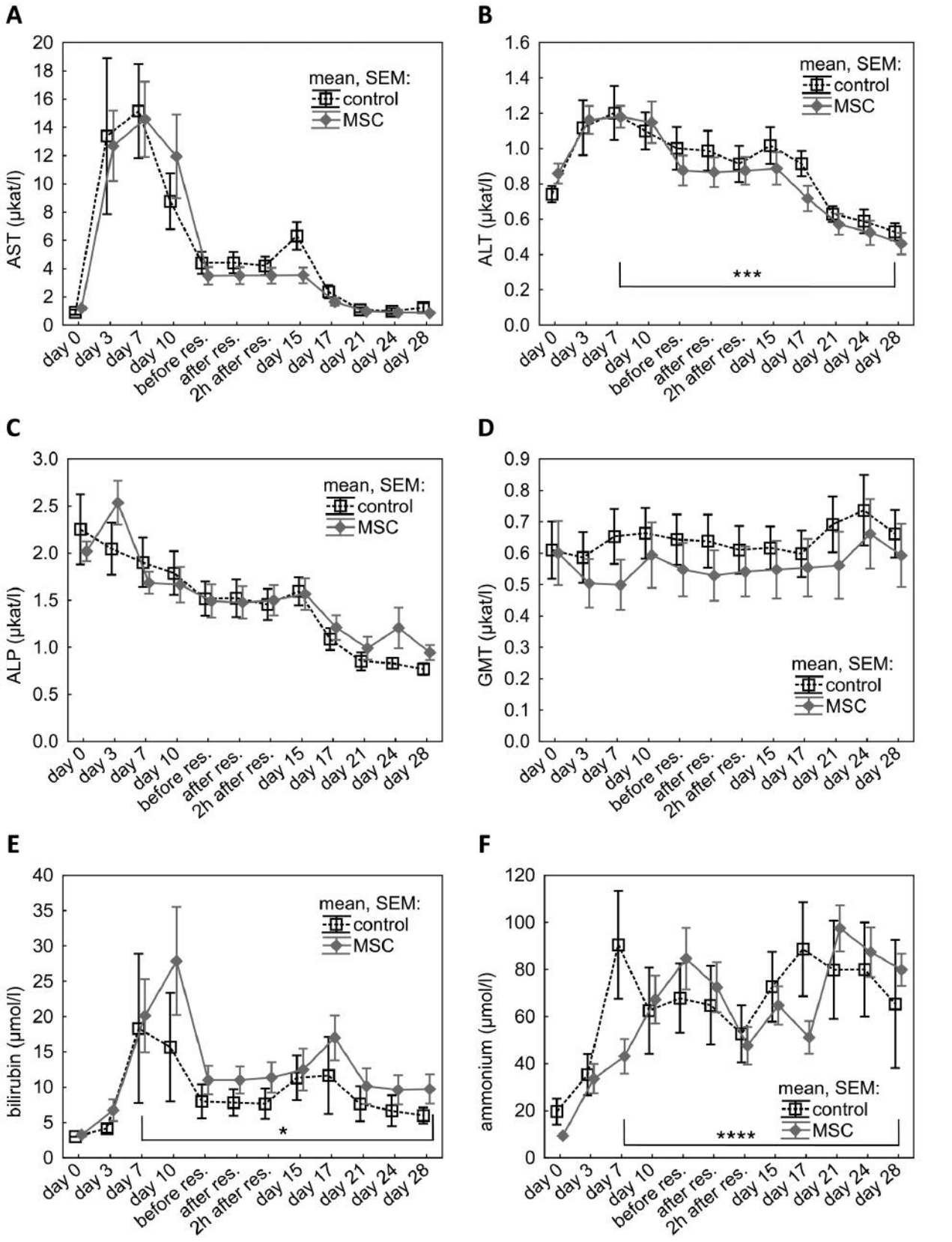

Figure 8. *Continued*

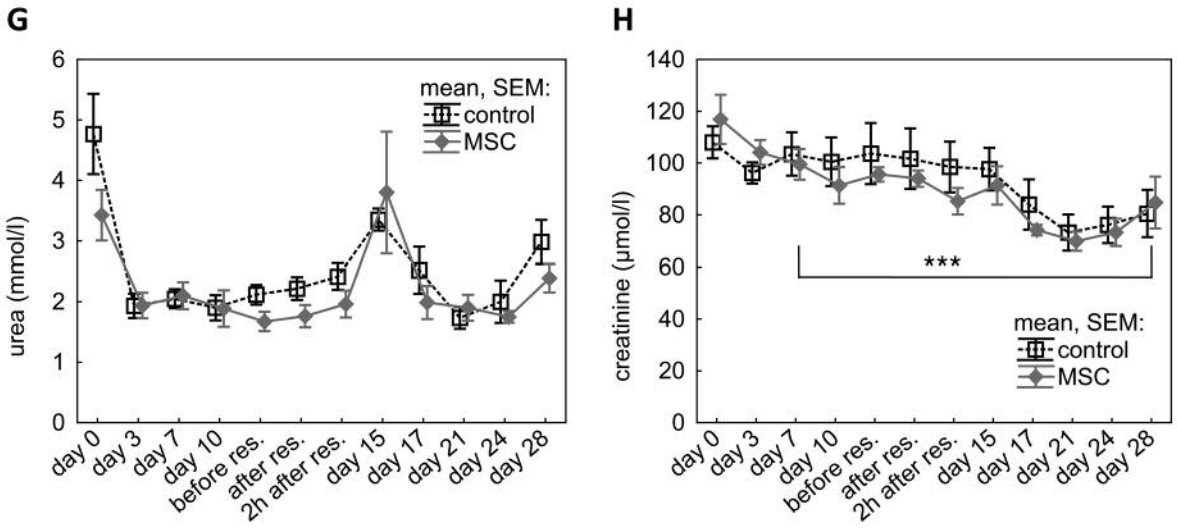

Figure 8. *Results of biochemical analysis in the experimental (MSC) and control group (control) throughout the experiment. Significant differences between the groups were observed in the levels of ALT (B) and creatinine (H) (higher in control group after day 7) and in the levels of bilirubin (E) and ammonium (F) (higher in experimental group after day 7). The other markers did not show any significant differences. Two-way ANOVA was used for biochemical analysis after data normalization which is described in methods. The figure shows data before normalization. *p≤0.05, ***p≤0.001, ****p≤0.0001.*

thickness of the gallbladder wall. Imaging methods such as ultrasonography, computed tomography and magnetic resonance imaging are now starting to be used for the diagnosis of SOS in clinical medicine (44). The thickening of the gallbladder wall is a typical finding in patients with SOS (14, 49, 50), and retrograde flow in the portal vein is also mentioned (51). According to our results, ultrasonography seems to be a suitable method to help confirm the diagnosis of SOS in large animal models.

Finally, the macroscopic appearance of the liver parenchyma 2 weeks after monocrotaline administration (or earlier in case of premature death) with apparent hemorrhagic necrosis also supports the diagnosis of SOS. It is important to mention that two animals showed only minimal alterations in the levels of biochemical markers and negligible histological changes of liver parenchyma compared to the other pigs. Because one of these animals was from the experimental and one from control group, we decided not to exclude these animals with less severe injury.

Evaluation of the effect of MSC. Data from the control and experimental groups were compared to verify the effect of MSC administration on the course of SOS and on liver regeneration after resection in animals with SOS.

To avoid the confounding effect of potentially different values between the groups before day 7, we decided to focus on the course of biochemical markers after the day of treatment. The same step was taken in evaluation of ultrasonography data to control for differences in body weight.

According to the biochemical analysis, the experimental group had significantly higher levels of bilirubin and ammonium compared to the control group; in contrast, ALT and creatinine were higher. In this experimental setting the level of bilirubin and ammonium reflects liver function (13, 52). ALT indicates injury of hepatocytes and the elevation of creatinine levels is a mark of worse overall status of the animal and can suggest development of hepatorenal syndrome (57, 58). This suggests deteriorating liver functions in animals from the experimental group compared to the surviving animals from the control group. It is important to mention that in the latter, 5 animals with the most severe liver injury died prematurely due to liver failure and were not included in the statistical analysis. In contrast, all animals survived in the experimental group. Therefore, for the statistical evaluation, the animals with most severe liver injury from the experimental group were included. This suggests a positive effect of MSC administration. However, the effect was limited, as the data show significantly better survival in the experimental group but not improved liver function and histological parameters.

MSC have been used in several animal experiments to verify their ability to treat liver failure of different etiologies. They have been shown to have a positive effect on acute liver injuries in rodents. The bone marrow derived MSC acted through reduction of fibrosis and improvement of immunoregulatory mechanisms (55, 56). We assume that these mechanisms probably do not play a dominant role in the early phase of SOS, which may explain the limited effect of MSC administration in our study.

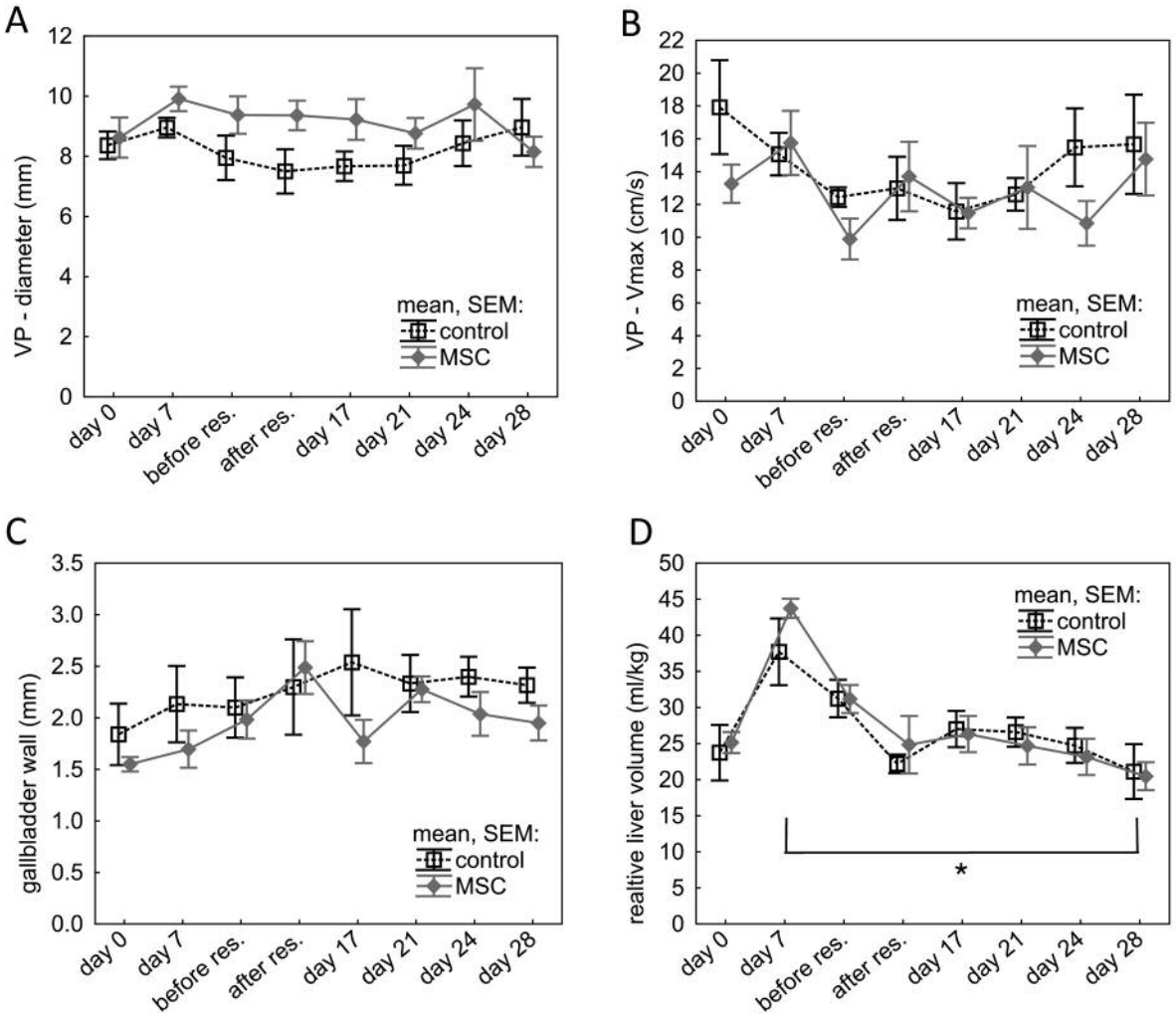

Figure 9. *Results of the ultrasound examination in the experimental (MSC) and control group (control). VP stands for portal vein (A), VP – Vmax stands for maximal blood flow velocity in the extrahepatic part of the portal vein (B). The only significant difference was observed in the relative liver volume which was higher in the control group after day 7 (D). Two-way ANOVA was used for evaluation of ultrasound examination after data normalization which is described in methods. The figure shows data before normalization. *p≤0.05.*

Moreover, the antifibrotic effect of MSC is probably caused by induction of MMP-9 expression (57). As increased MMP-9 activity plays a role in the pathogenesis of SOS, it is possible that the MSCs could also have a partially negative effect on the course of SOS, at least in the early phases of the disease.

Because liver fibrosis and inflammatory infiltration was apparent in later phases, it is possible that MSC administration later than day 7 would have a major effect.

However, we also chose this treatment timing according to other functions of MSCs. MSCs are able to reduce hepatocyte apoptosis and promote hepatocyte proliferation. Cytokines and growth factors (*e.g.* epidermal growth factor) secreted by MSC are responsible for this effect (58). The idea was to attenuate liver failure by promotion of liver regeneration and therefore enable the animals to undergo liver resection with a better outcome. That is why day 7 was chosen as the most appropriate for MSC administration.

The ability of MSC to alleviate liver failure after resection by promotion of regeneration was confirmed by Ding *et al.* (59). They found improved survival after 90% hepatectomy in rats with a healthy liver after MSC administration via portal vein. They also observed a major increase in liver volume after MSC administration. The impact on survival of animals is in accordance with our results. However, the liver volume results are opposite to those in our experiment. In our study, ultrasonography showed smaller liver volume in MSC-treated animals. This finding cannot be simply explained as attenuated liver regeneration because animals in our study did not have a healthy liver parenchyma. In studies of resection of healthy liver parenchyma, the subsequent regeneration of liver functions

correlates with increasing liver volume (60). This relationship is not present in cases of acute SOS where the liver volume is increasing because of developing hepatomegaly while liver functions are decreasing (11). The liver volume after resection in this experiment was undoubtedly influenced by decreasing hepatomegaly. The liver volume increased only over the first three days after resection and then decreased until the end of the follow-up. The gradual remission of hepatomegaly started after day 7 and was not completed before the liver resection. The smaller liver volume in the experimental group therefore does not have a clear interpretation and can be even a sign of earlier remission of acute SOS in MSC-treated animals.

A model of the chronic or subacute type of SOS would be more accurate for correlation with clinical patients who develop SOS during chemotherapy and require liver resection because of liver metastases. Therefore, further experiments with liver resection postponed to later phases of monocrotaline-induced SOS should be performed to verify the effect of MSCs. In such an experimental setting, repeated administration of MSCs with the second dose at the time of resection would be informative. MSCs are usually administered right after liver resection, which enables them to act more effectively and support liver regeneration (59).

Conclusion

SOS was successfully induced in the pig by intraportal monocrotaline administration. Biochemical analysis, histological findings and ultrasonography confirmed the development of SOS. Intravenous administration of allogeneic MSC one week after induction of SOS improved animal survival. The liver volume after partial resection has to be evaluated with consideration of the hepatomegaly induced by acute SOS. Further experiments with different timing of liver resection and MSC administration should be performed to verify the positive effect of MSC.

Conflicts of Interest

None to be declared.

Authors' Contributions

Study conception and design: RP, VL, AM, LV; Acquisition of data: RP, JR, KB, HM, ZT, OB, AK, LB, VT; Analysis and interpretation of data: RP, VL, AM, PH, LV; Drafting of manuscript: RP, VL, AM, LV; Critical revision of manuscript: VL, PH, VT, VM, LE. All Authors read and approved the final manuscript.

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