

# Intraportal Injection of Porcine Multipotent Mesenchymal Stromal Cells Augments Liver Regeneration after Portal Vein Embolization

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**Abstract.** *Portal vein embolization (PVE) can be used prior to liver surgery to increase the volume of the remaining liver tissue after an extensive resection. However, the application of PVE is limited and new strategies to augment liver regeneration by cellular therapy are promising alternatives. Materials and Methods: The influence of syngeneic multipotent mesenchymal stromal cells (MSC) on liver regeneration was analysed after the ligation of the right portal vein branches in a porcine model, closely mimicking the situation of human surgery. Liver regeneration was monitored by ultrasonography, immunohistological analysis and serum biochemistry. Results: The volume of the contra-lateral, non-ligated liver lobe increased in all piglets after portal vein ligation. This hyperplasia occurred earlier and was more pronounced in those piglets receiving MSC infusions as compared to non-treated controls. Biochemical liver function was stable in all pigs. Only solitary transplanted MSC were detected in recipient livers two weeks after the infusion. Conclusion: The infusion of porcine MSC into the portal vein in a setting of liver regeneration after surgical resection leads to accelerated and augmented hyperplasia. This effect is most likely due to bystander effects of the transplanted MSC.*

There has been significant progress in liver surgery during the last two decades, especially through the development of new surgical techniques and the introduction of modern peri-

and postoperative intensive care. Nevertheless, many patients with primary or secondary liver malignancies are not suitable for radical surgical therapy, which could potentially result in the definitive cure of the underlying malignant disease. One of the main problems of extensive surgical resection is the risk of postoperative liver failure due to a lack of functional liver remnant in the early postoperative phase – before the onset of significant parenchymal regeneration. Portal vein embolization (PVE) can be used to increase the volume of the future liver remnant volume (FLRV) before resection. However, the technical applicability of PVE is limited and an unsuccessful increase of the FLRV or progression of malignant disease are not uncommon (1, 2). Thus, new strategies to augment liver regeneration by cellular therapy are needed.

Multipotent mesenchymal stromal cells (MSC) are a fraction of the adult bone marrow stem cell compartment. They contain a subpopulation of mesenchymal stem cells that can differentiate into mesenchymal adult tissues under specified conditions. Differentiation into adipocytes, chondrocytes, osteoblasts and myocytes has been demonstrated *in vitro* and *in vivo* (3). Whether and how bone marrow-derived stem cells, and MSC in particular, can contribute to liver regeneration is not entirely clear. Initial reports outlined that bone marrow-derived (stem) cells can transdifferentiate into liver cells or their progenitors after bone marrow transplantation (4-7). Later investigations clarified and complemented these observations, outlining that bone marrow cells can also fuse with resident liver cells (8, 9). In addition to direct cellular effects, transplanted bone marrow cells can also contribute to liver regeneration by bystander effects, such as the provision of a beneficial proliferative cytokine milieu or antiapoptotic effects (10). In the current report, the influence of syngeneic multipotent mesenchymal stromal cells on liver regeneration after the ligation of portal vein branches is analysed in a

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porcine model closely mimicking the clinical situation after liver resection. It is hereby outlined that portal venous infusion of MSC stimulates the speed and extent of liver regeneration and could potentially increase the FLRV in a clinical setting of hepatic resection.

## Materials and Methods

**Preparation of MSC.** Bone marrow cells from 6 week-old piglets were obtained by bone marrow aspiration under general anesthesia (azaperon 1.0 mg/kg body weight (BW), ketamin 5-10 mg/kg BW). After bone marrow aspiration, aspirates were plated in 175 cm<sup>2</sup> flasks in expansion medium (low glucose DMEM with GlutaMAX, without pyruvate, Biochrom, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom), 1% penicillin/streptomycin and 1% glutamine. After 24, 48 and 72 hours, non-adherent cells were removed by changing the culture medium. Adherent cells were then trypsinized (0.5% trypsin-EDTA), harvested and re-plated into new flasks, each time when cell confluency reached 60% to 80%. To histologically identify transfused MSC within the recipient liver, cultured MSC were labeled with 5-bromo-2-deoxyuridine (BrdU) 60 hours before cell application.

**Surgical procedure.** All animal experimentation was conducted in accordance with European Union legislation and animal care guidelines of the Medical Faculty of Pilsen, Charles University Prague. N=15 piglets were included into this study (n=6 into the MSC-treated group, n=9 into the control group). Anesthesia was administered continually through a central venous catheter (azaperon 1.0 mg/kg BW, thiopental 10 mg/kg BW/hour, ketamin 5-10 mg/kg BW and fentanyl 1-2 µg/kg BW/hour). Muscle relaxation was achieved by a bolus of pancuronium (0.1-0.2 mg/kg BW at the beginning of surgery. All animals were intubated and mechanically ventilated. Portal vein branches for the caudate, right lateral and right medial lobes were ligated (50-60 per cent of the liver parenchym). MSC were then applied into the non-occluded portal vein branches (8.75, 14.0, 17.0, 17.5, 43.0 and 61.0×10<sup>6</sup> MSC respectively). Normal blood flow in the hepatic artery and the occlusion of the ligated portal vein branches were controlled by ultrasonography (Medison Sonoace 9900). The border between atrophic and hypertrophic parenchyma was marked by titanium staples for later identification by ultrasonography. Ultrasonographic controls were undertaken immediately after the operation and on postoperative days (p.d) 3, 7, 10 and 14. All piglets were examined clinically each day and culled on the 14th day under deep general anaesthesia.

**Immunohistology and biochemistry.** Histological specimens from both atrophic and hypertrophic parenchyma were examined after staining with hematoxylin-eosine and PAS. The proliferation activity was examined using antibodies against Ki67 (MIB 1 MW, 1:1000, DakoCytomation, Denmark). Cells were analyzed by immunohistochemical staining with antibodies against CD117 (1:150, Dako Glostrup, Denmark), CK7 (1:200, Dako Glostrup, Denmark) and CK19 (1:1000, Neo Markers Westinghouse, USA). Primary antibodies were visualized using the streptavidin-biotin-peroxidase method (DakoCytomation). The length of 20 lobuli was measured and binucleated hepatocytes were counted in 20 microscopical fields. Hepatocyte length was assessed by an eyepiece micrometer (Olympus).

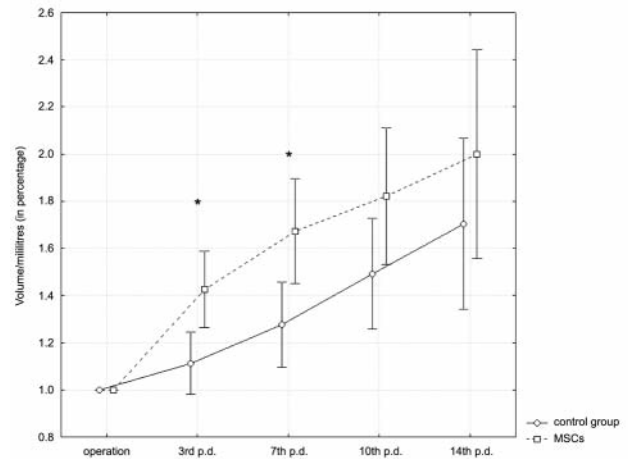


Figure 1. Volumes of non-ligated liver lobes: Volumes of the non-ligated liver lobes are depicted for the control and the MSC-treated group. Liver volume was assessed by 3D ultrasonographic measurements. Error bars show standard deviation. Statistical difference ( $p < 0.05$ ) is indicated by an asterisk (\*). Growth acceleration in the MSC-treated group was at its maximum on the 3rd post operative day.

Blood samples were collected from a central venous catheter before the operation, after ligation of the last portal branch, during the application of MSC or placebo, 2 hours after the application of MSC, and on p.d. 1, 3, 7, 10 and 14. Serum bilirubin, urea, creatinine, alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), cholinesterase (CHE), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin were quantified on a clinical biochemical analyzer (Olympus 2700). Serum levels of C-reactive protein (CRP), cytokines TNF- $\alpha$  and IL-6, as well as growth factors HGF, TGF- $\beta$ 1 and IGF were measured by an enzyme-linked immunosorbent assay (ELISA) on an Auto-EIA II Analyzer (Lasystems Oy Helsinki, Finland). Immunoassay kits were purchased from Biosource California (HGF, KAC2212/KAC2211, TGF- $\beta$ 1, KAC1688/1689 and TNF- $\alpha$ , KSC3012/KSC3011), Immunodiagnostic Systems Britain (IGF, Octeia IGF-1), RD Systems Minnesota (IL-6, Quantikine) and Tridelta Development Ireland (CRP, Phase Range).

**Statistical analysis.** Statistical analysis was performed using CRAN 2.4.0<sup>®</sup> and Statistica 98 Edition<sup>®</sup> software. Non-parametric tests (Wilcoxon) were used to analyze differences between groups. Parameter relations were examined by Spearman rank correlation coefficients. The comparison of variables in time, between groups and the interaction of examined factors was analyzed by the parametric ANOVA repeated design test.

## Results

**Functional liver remnant volume.** To assess the overall functional effect of MSC infusions after partial portal vein ligation in the porcine model, the volume of the occluded and non-occluded liver lobes was analyzed by ultrasonography. Repeated ultrasonographic assessments were carried out on

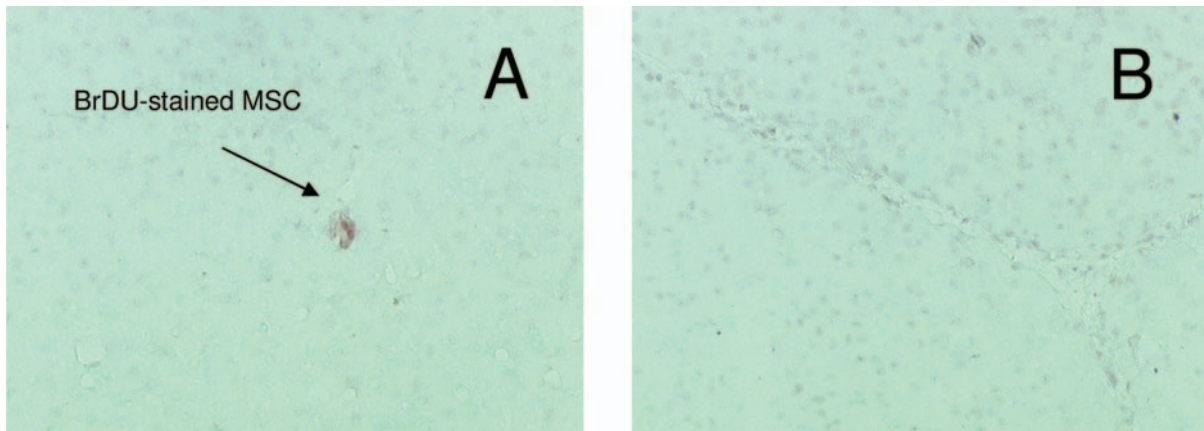


Figure 2. Serum biochemistry during the postoperative period: Blood was drawn from piglets before and during the procedure, as well as on postoperative days 1, 3, 7, 10 and 14. Liver function tests and other biochemical markers were measured on a clinical analyzer and are depicted as mean±standard deviation. (ALB=serum albumin, ALT=alanine aminotransferase, CHE=cholinesterase, BI=serum bilirubin and growth factors TGFβ1, IGF and HGF)

p.d. 0, 3, 7, 10 and 14. The volumes of both the ligated right, and the non-ligated left liver lobes were computed by a standard three-dimensional ultrasonographic approach and were confirmed by physical examination during necropsy on day 14. All experimental animals were clinically examined on each p.d. Neither surgical complications nor side-effects of the MSC infusion occurred during the postoperative course. The volume of the hypertrophic lobe increased more rapidly after MSC application in the treatment group when compared to the control group (Figure 1). The control group did not show any change in the volume of the hypertrophic liver lobes within the first three days after surgery. The growth acceleration of the hypertrophic liver lobes in the MSC group was at its maximum between the 3rd and 7th p.d. ( $p < 0.05$  versus control). However, this stimulating effect slowed down during the second week and there was no statistically significant difference in the size of the hypertrophic liver lobes on day 14. However, the average volume of the non-occluded liver lobe was increased by 30% in the MSC group in contrast to the control group at the end of the experiment (Figure 1).

**Biochemistry.** To outline the functional liver capacity after the infusion of MSC and to understand possible systemic side-effects, biochemical parameters, as well as cytokine and growth factor levels were analyzed in sera of both animal groups during and after the intervention. All parameters were measured on a clinical analyzer before and during the procedure, as well as on p.d. 1, 3, 7, 10 and 14 (Figure 2). Serum levels of AST and ALT increased after portal vein ligation, whereas serum cholestatic markers bilirubin, ALP and GGT remained stable. Synthetic liver function was unchanged with normal levels of CHE. There was no difference between the MSC-treated and the control group regarding these

markers. Serum CRP levels were elevated to the same extent in all animals during the postoperative period. Kidney function was stable in all piglets with physiological levels of creatinine and urea. Overall, all treated animals of both groups tolerated the treatment well with neither apparent differences in the clinical course or the biochemistry markers assessed. To describe the regenerative micromilieu within recipient livers, the secretion of growth factors HGF, IGF, TGFβ1 and TNFα was analysed in sera of all animals over time (Figure 2). Both groups expressed the same pattern of TGFβ1, IGF and TNFα in the periphery with a tendency towards decrease of TGFβ1 and IGF in the early phase and stable TNFα. HGF expression was slightly higher in the control group early after the procedure. Nevertheless, there were no proved statistical differences between studied cytokines and growth factors in particular time points. Overall, the cytokine expression pattern in the periphery indicates that differences in the extent of postoperative liver hypertrophy between the groups are most likely due to micromilieu changes within defined anatomical spaces in the liver.

**Immunohistology.** Histological examination of liver biopsies was carried out to outline differences in the proliferative response and the composition and size of liver cells between the groups. Screening for a possible involvement of resident progenitor cells was also performed to try and identify the infused population of MSC. The analysis was performed at the time of necropsy on the 14th p.d.. No overall difference in the length of liver lobuli was detected between the groups on H&E staining. However, a tendency towards longer lobuli was demonstrated in the MSC-treated group, potentially indicating a bystander effect of the infused MSC on the size of liver lobuli. Although lobuli length was slightly increased,

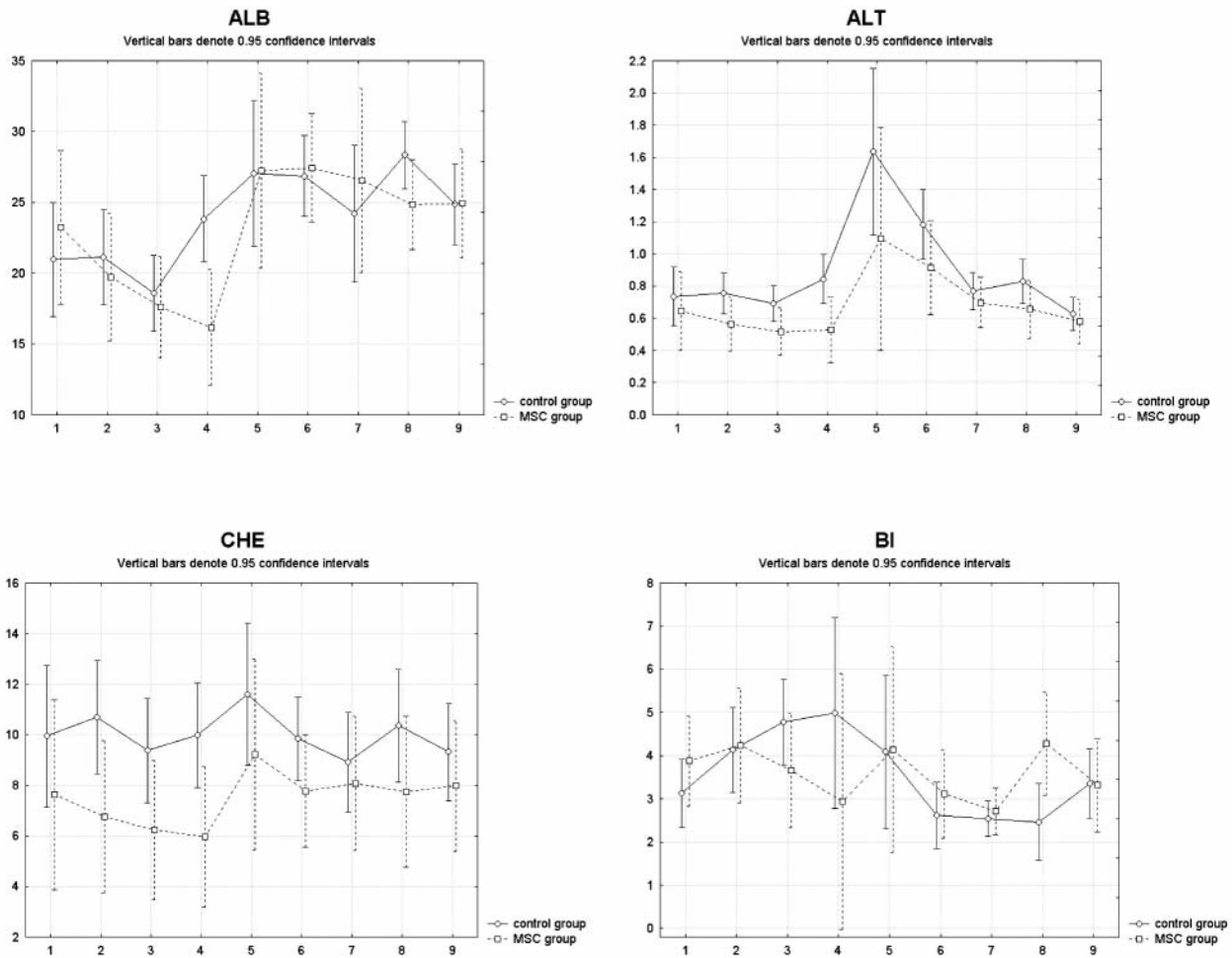


Figure 3. Identification of infused MSC: Solitary BrdU-stained MSC could be identified in the treatment group on day 14 (a). No BrdU-positive cells were identified in control animals (b).

the size of the individual hepatocytes did not prove to be different between the groups and the number of binucleated hepatocytes was equal in both groups. Two weeks after the intervention, the intrinsic proliferative activity was low in both groups and the Ki67 proliferative index was practically the same as in normal liver tissue. Neither progenitor-type cells of bone marrow nor hepatic origin were detected using immunohistochemical staining by anti- CD117, CK7 or CK19 antibody on atrophic and hypertrophic lobes respectively. Only solitary BrdU-stained transplanted MSC were detected within the hypertrophic liver parenchyma (Figure 3).

## Discussion

In the present work the effect of syngeneic multipotent mesenchymal progenitor cells on liver regeneration was analyzed in a porcine model of right portal vein branch

ligation. This model closely mimics the situation of human oncological liver surgery, in which portal vein embolization can be utilized to create a larger future liver remnant volume before resection. The clinical goal of portal vein embolization is to increase the number of patients that can undergo extensive liver resections. This porcine experimental model of liver regeneration is especially applicable to the clinical situation since pigs closely resemble human liver anatomy and physiology. After portal venous infusion of MSC in this model, augmented and accelerated regeneration of the non-occluded liver lobes is outlined. This effect was strongest between the third and seventh postoperative day and slowly weaned off thereafter. However, there was no overall volume difference of statistical significance between both groups at the end of the observation period. This result might have been expected, though, since the body weight to liver weight ratio is tightly controlled in most species.



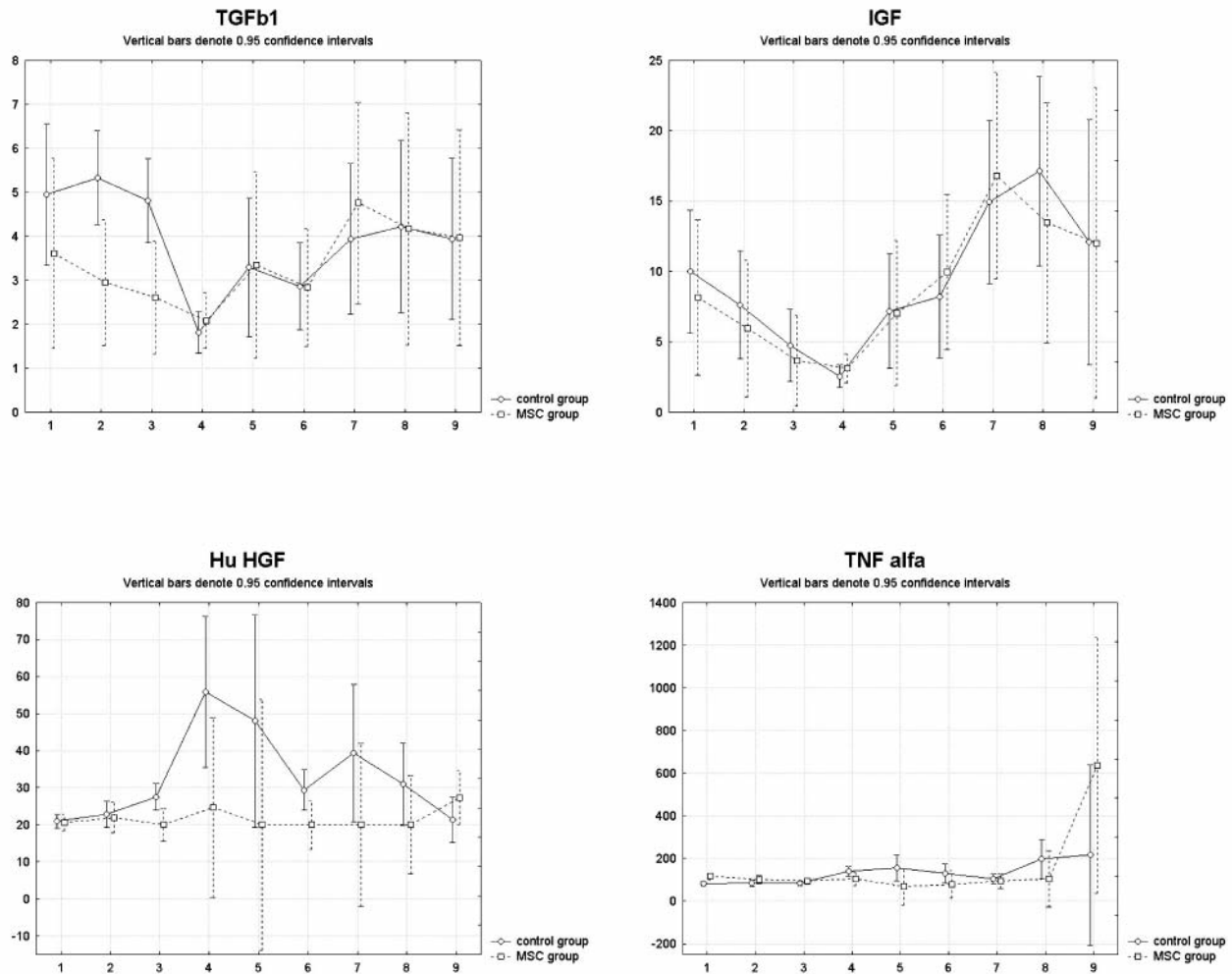


Figure 4. Serum cytokine expression in the periphery: Blood was drawn from piglets before and during the procedure, as well as on postoperative days 1, 3, 7, 10 and 14. Serum growth factors TGFb1, IGF, TNFa and HGF were determined by ELISA.

Previous studies have tried to clarify the potential contribution of MSC to liver regeneration in rodent models. Most of these approaches used toxic models, in which the intrinsic self-renewing capacity of the recipient liver was suppressed (11, 12). Di Bonzo observed only 0.2% human hepatocytes after transplantation of human MSC into immunodeficient mice with chronic liver injury (13). Overall, whether MSC can significantly contribute to liver regeneration *in vivo* remains controversial (14-16), although MSC can show some features of hepatic differentiation in culture or under immunoprivileged conditions (17, 18). Chamberlain observed differentiation of human MSC into hepatocytes after infusion of MSC into foetal sheep (19) and transplantable hepatocytes were also obtained from heavily growth factor-treated MSC (20, 21). Despite the fact that it was not possible to identify transplanted MSC within recipient livers in this model after 14 days, liver hypertrophy was markedly

augmented. It is therefore likely that bystander effects of the transplanted MSC accelerated regeneration in this model. This effect may well be mediated through paracrine effects of the infused MSC on the microenvironment in the periportal regions, where parenchymal regeneration is initiated. Aldeguer *et al.* suggested that increased production of IL-6 by bone marrow-derived cells, for example, can stimulate intrinsic liver regeneration (10). In addition to being effective in terms of an accelerated regenerative response, the infusion of syngenic MSC into the portal vein also proved to be safe in the preclinical model. Probable side-effects of MSC infusions include immunological problems (hypersensitivity, immune complex reactions), metabolic dysregulation and emboli. None of these problems occurred in the presented study cohort.

All biochemical parameters, including cytokine levels, that were assessed during the observation period, did not outline any significant differences between the MSC-infused and the

control group, indicating that both groups were systemically stable throughout the regenerative period. Concerning the role of cytokines it may also be assumed that these act on a more local level within the liver parenchym and differences cannot be observed in the periphery. No histological differences were observed after 14 days between the groups (number of mitotic figures, binucleated hepatocytes, length of lobuli, hepatocyte size), indicating that the initial phase of liver regeneration had been completed in both groups and there was no influence of the infused MSC on liver architecture. In addition, no CD117 positive cells with stem like properties were detected in either group. Using BrdU staining, only very few of the infused MSC could be detected. Thus, injected MSC do not contribute to liver regeneration through proliferation, but most likely help to establish a micromilieu supportive for intrinsic proliferation of resident hepatocytes. Results acquired by this experimental study confirm the experiences of Furst and Esch, who have previously shown that cell grafts of bone marrow origin (CD133 positive cells) applied after PVE in human surgery can increase the FLRV (22, 23). From the present experimental data, however, it cannot be concluded with certainty that the application of stem cells, including MSC, does not support the growth of liver malignancies to the same extent that it supports liver regeneration. Assuming a bystander effect of MSC on the micromilieu makes this even more likely (24). Thus, further animal investigation is necessary before optimized MSC therapies can be applied in the setting of human medicine (25).

In conclusion, it has been shown that the intraportal infusion of syngeneic porcine MSC after PVE in a setting of liver regeneration led to an accelerated and augmented compensatory liver hypertrophy. This effect is most likely due to bystander effects of the transplanted MSC.

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