## 1. INTRODUCTION

Today, major hepatic resections and living related transplantations are routinely and safely performed for malignant and benign disease, due to a better understanding of the anatomy, refinement of operative techniques and the ability of the liver to regain its functional mass in a matter of days to weeks. However, liver failure following partial hepatectomy still occurs from time to time and remains incompletely understood. Predisposing factors to consider include functional mass of remnant liver, age of the patient, and presence of pre-existing liver disease such as cirrhosis, chronic hepatitis and fatty liver disease. Each of these conditions interferes with the normal regenerative pathways and/or initiates apoptotic pathways, resulting in a net loss of functioning hepatocytes in which surviving hepatocytes cannot maintain adequate metabolic functions. This represents either a failure to regenerate after partial hepatectomy or accelerates destruction of hepatocytes from necrosis or apoptosis. In the clinical setting paying attention to factors that can reduce chances of liver failure following partial hepatectomy, especially provision of adequate liver remnant, avoidance of major hepatectomy in the cirrhotic patient, and caution when embarking on resections in the steatotic liver might improve the outcome. Preoperative portal venous/hepatic arterial embolisation and two-stage hepatectomies have been successfully applied to increase the number of patients who qualify for extended liver resection (trisegmentectomy). However, as previously described, a clinical small-for-size syndrome following liver transplantation and extended hepatectomy still significantly contribute towards a high morbidity and mortality after liver resection (1).

Over the past 50 years much has been learned about what initiates liver regeneration, how it is sustained, and what inhibits it. Post-hepatectomy liver regeneration is a complex, but well orchestrated series of events initiated by a number

of autocrine, paracrine and endocrine hepatotrophic factors. Regeneration was studied quite easily in the rat or mouse partial hepatectomy model or in cell culture. Based on the increasing understanding of the mechanisms underlying hepatic regeneration, a variety of strategies have been developed in animal studies to bolster liver regeneration, including injection of several growth factors and preconditioning methods (2-4). Currently there is no clinically established treatment which effectively shows the increasing regeneration capacity of remnant liver thereby improving the outcome of patients undergoing major hepatectomy.

Erythropoietin is a glycoprotein hormone, which has been used in the clinical setting for over 30 years. It has been proven in various animal studies that recombinant human erythropoietin (rHuEPO) protects renal, cardiac and neuronal as well as hepatic tissue from ischemia, and promotes regeneration of adult CNS neurons and wound healing. These attributes of a positive effect of rHuEPO could be helpful in accelerating liver regeneration after hepatectomy. Therefore, to date no data are available on whether rHuEPO has the ability to stimulate liver regeneration after liver resection. This is the first study investigating the effect of erythropoietin on rat liver regeneration capacity after major liver resection.

# 2. LITERATURE REVIEW

## 2.1 Ancient history of liver regeneration

*And now the last recess of the Black Sea opened up and they [the Argonauts] caught sight of the high crags of the Kaukasos, where Prometheus stood chained by every limb to the hard rock with fetters of bronze, and fed an eagle of his liver. The bird kept eagerly returning to its feed. They saw it in the afternoon flying high above the ship with a strident whirr. It was near the clouds, yet it made all their canvas quiver to its wings as it beat by. For its form was not that of an ordinary bird: the long quill-feathers of each wing rose and fell like a bank of polished oars. Soon after the eagle had passed, they heard Prometheus shriek in agony as it pecked at his liver. The air rang with his screams till at length they saw the fresh–devouring bird fly back from the mountain by the same way as it came.*

*Argonautica 2.1238f*

***Caucasian Eagle & Prometheus***

**Figure 1.The regenerative power of the liver. As punishment for stealing fire from Zeus, Prometheus was bound to a rock where each day a bird ate out his liver, which would then regenerate each night while the bird slept.**

The regenerative power of the liver was first described by the Greeks more than 2,500 years ago (5). Greek mythology gives perhaps the most infamous reminder of the liver's regenerative powers with the mythological account of Prometheus, who stole fire from Zeus and gave it to humankind. As a punishment, he was chained to one of the Caucasian Mountains, where every day a voracious eagle feasted upon his liver, causing excruciating pain (Figure 1). Each night, while the bird slept, the damaged liver would mend so the eagle could begin anew, illustrating the incredible capacity of the liver to regenerate. Regeneration is a common feature in invertebrates (e.g., worms), but is very limited in the majority of vertebrates. The mechanism of liver regeneration remains poorly defined, but the process is central to success of surgical resections and live donor transplantation.

After a 70% hepatectomy in rats, as originally described by Higgins and Anderson (6), the weight of the remnant liver increases to constitute approximately 45% and 70% of the original liver weight at 24 and 72 hours after the operation. During the next week, the weight increases by less than 5%, but approximately 2 weeks after partial hepatectomy, it reaches the original weight of the preoperative liver (±10%). Two key properties of the process should be noted:

1) The lobes removed at surgery do not grow back, but the lobes of the hepatic remnant expand to reach the original liver weight by tissue hypertrophy. It does not regenerate in the true sense, as in reptiles that can regrow a limb or tail.

2) The growth of the liver remnant is regulated even after repeated partial hepatectomy.

These properties indicate that liver regeneration is not regeneration in the strict sense of the word, but instead a process of compensatory growth regulated by restoration of function, rather than form (7). Interestingly, work on orthotopic partial liver transplantation in humans has revealed that the transplanted graft regenerates rapidly if it is small, while conversely it atrophies if it is too large for the recipient. Either way, an optimal liver-to-body mass ratio is the result (8). Important clues of understanding the mechanisms of liver regeneration may be obtained by searching for potential linkages between liver cell proliferation and hepatic function. This illustrates the incredible capacity of the liver to regenerate; the only solid organ able to do so and to maintain the normal function of metabolic homeostasis.

**2.2 Factors affecting regeneration in liver resection.**

Although much is known about normal liver regeneration in animal models, no systematic molecular and functional studies have been done on human livers after resection. Many of the cytokines and growth factors discovered in animal models have been found to be expressed after liver resection in human serum, however, there is only little data available on the molecular level.

However, there is remarkable similarity between the process of hepatic regeneration in laboratory animals and humans, and it is reasonable to assume that mechanisms that regulate regeneration may be fairly similar among species, and the knowledge gained from studies of the liver regeneration in rats and mice is applicable to the human liver.

***2.2.1 Chronic liver disease states***

Patient with preexisting hepatic dysfunction, such as cirrhosis, biliary obstruction and cholestasis, or non-alcoholic steatohepatitis, have a significantly increased risk of liver insufficiency after resection (9). The molecular aspects of abnormal liver regeneration

are unclear; however, it has been observed that there is increased cell death, impaired cell division, and delayed return of normal hepatic function in these disease states.

### *2.2.2 Age*

Liver age is a significant factor in hepatocellular regeneration. Older livers do not regenerate as quickly as younger ones and show delayed regeneration after acute injury. Rodent models have shown reduced and delayed thymidine kinase uptake in older animals after partial hepatectomy, and there is a striking difference in the magnitude of DNA synthesis and timing between young and old livers (10). Aging has been shown to be associated with a progressive decline in growth hormone secretion and Foxm 1B expression. Treatment of old mice with growth hormone can restore hepatocyte proliferation with increased Foxm 1B and cyclin B1 expression as well as significant reduction in p27 protein levels (11).

### *2.2.3 Steatosis*

Hepatic steatosis affects regeneration on several molecular levels. Lipid accumulation has been associated with hepatocyte mitochondrial damage caused by free radical injury. Steatotic livers in rats show delayed mitosis and increased mortality after partial hepatectomy, which may be due to abnormal TNF and IL-6 signaling (12). Hepatocyte mitochondrial damage associated with lipid accumulation is caused by free radical injury from fatty acid oxidation. Abnormalities in induction of cytochrome P-450 may be one mechanism in the pathophysiology in fatty livers and may contribute to poor regeneration (13).

### *2.2.4 Ischemic injury*

Warm ischemic injury is a possible component during extensive liver resection. After prolonged warm ischemia of liver, there is an initiation of the cell cycle pathways with upregulation of markers of liver regeneration. When ischemic injury is significant, there is a great expression and activation of cytokines, transcription factors, and immediate

early genes, with an increase in hepatocellular replication. The liver can tolerate ischemic injury only to a certain “point of no return”, after which the damage is too extensive and the liver is unable to maintain functional homeostasis and regenerative capabilities, which results in dysfunction and liver failure (14).

### *2.2.5 Minimal liver mass*

Small-for-size liver syndrome (SFSS) is a recognizable clinical syndrome, which occurs if there is a reduced mass of liver insufficient to maintain normal liver function. The syndrome is characterized by postoperative liver dysfunction with prolonged cholestasis and coagulopathy, portal hypertension, and if severe with ascites. The continuing liver dysfunction predisposes to further complications including sepsis, and gastrointestinal bleeding. These features can persist for several weeks, with improvement if the liver recovers satisfactorily. The biochemical profile includes cholestasis with elevated conjugated bilirubin, mild to moderate elevation of transaminases, and prolonged prothrombin time. Histologic features include cholestasis with bile plugs, and areas of regeneration and ischemia with patchy necrosis.

Tanaka et al. were able to show that graft to recipient weight ratios of less than 0.8% in living donor liver transplantation were associated with poor patient survival of less than 50% at one year (15). Death was invariably from sepsis in association with liver dysfunction. This report and the recognition of SFSS has led to a better understanding of the concept of what an adequate liver mass and the factors influencing liver regeneration and recovery after major liver resection and partial liver transplantation are. In comparison, extended resection of 80% of functional parenchyma can be performed in the absence of chronic liver disease for hepatobiliary malignancies (16). Recommended minimal functional remnant liver volume following extended hepatectomy is ≥25% in a normal liver, and ≥40% in an ‘injured’ liver, with moderate to severe steatosis, cholestasis, fibrosis, cirrhosis, or following chemotherapy (17, 18)

### *2.2.6 Other factors*

There is some clinical and experimental evidence suggesting that several other factors may influence the regenerative response after transplantation. Increased portal venous flow has been implicated in more rapid regeneration, and poor hepatic venous drainage has been shown to inhibit regeneration. However, the changes in liver volume that occurred during regeneration in the donor and recipient patients differed after adult right lobe living donor liver transplantation. Although both groups demonstrated immediate rapid increases in liver volume in the early postoperative period, liver regeneration was significantly faster and reached a higher peak in recipients than in donors. Peak liver volume in donors was at 6 months (74% increase), whereas the peak volume in recipients was at 2 months (120% increase). The increase in liver volume is probably related to the increase in portal blood flow after partial hepatectomy (19, 20). The difference in growth rate of same-source hepatocytes in donors and recipients suggests that liver regeneration is probably regulated by factors other than the hepatocytes.

## 2.3 Mechanisms of extrarenal erythropoietin production

The liver is the primary site of both erythropoietin production and erythropoiesis in fetal and neonatal animals. Studies show significantly increased hepatic erythropoietic factor activity in the serum of young rats during the second to fifth weeks of life. Negligible activity was detected in rats over five weeks of age. It has been reported that the kidney's of rats begin producing erythropoietin by the third week of life, and by the eighth week the kidney is the major site of synthesis with liver production significantly diminishing (21). Thus, the findings show a temporal relation between hepatic erythropoietic factor activity in the serum and the reported transition from liver to kidney production of erythropoietin (22).

This factor, originally termed hepatopoietin and more recently called the hepatic erythropoietic factor (HEF), is detectable in higher concentration in the hepatic venous blood than in other blood draining organs, thus supporting its hepatic origin. Production of the hepatic erythropoietic factor is best related to hyperactivity of the Kupffer cells and not to the parenchymal cells. Liver regenerating after partial hepatectomy produces significant quantities of erythropoietin in response to hypoxia. Erythropoietin production is also related to the mass of regenerating liver with peak erythropoietin production occurring during times of greatest tissue proliferation.

**2.4 A site of erythropoiesis in the regenerating liver**

The marked reduction in liver mass caused by subtotal hepatectomy is associated with a lowering in hepatic blood flow. An increased perfusion rate per gram of hepatic tissue is accompanied by only a 25-30% rise in portal venous pressure (23). Hepatic extraction efficiency is dependent on hemodynamic factors, but it also depends on both the number and activity of the liver phagocytes (21, 24). Technetium sulfur colloid counts/cm² increase gradually to a peak at 72 hours after hepatectomy and decline to slightly elevated levels 96-288 hours post-hepatectomy. Relative Kupffer cell numbers as well as Kupffer cell: parenchymal cell ratios as well as technetium sulfur colloid counts were highest at 72 hours after hepatectomy. Kupffer cell relative cytoplasmic areas were also greatest at this time. This coincides with the period of peak erythropoietin production in regenerating liver after nephrectomy and hypoxia (25) and with peak globulin synthesis.

Hypoxia is the primary stimulus for both renal and extrarenal erythropoietin production (26, 27). Plasma levels of this factor are barely detectable in the hepatectomized anephric rat kept at room pressure, but increase considerably when hypoxia is applied. The decrease in relative cytoplasmic areas of the Kupffer cells

corresponds to the appearance of erythropoietin in the circulation of these animals following hypoxia. This is consistent with the hypothesis that hypoxia causes not only the production of erythropoietin but also its release. The Kupffer cell is a likely site of synthesis and/or storage of this principle. Single membrane bound lipid-like droplets have been seen in Kupffer cells 24 - 120 hours post-hepatectomy (Figure 2).

**B. A. Naughton et al. (1979)**

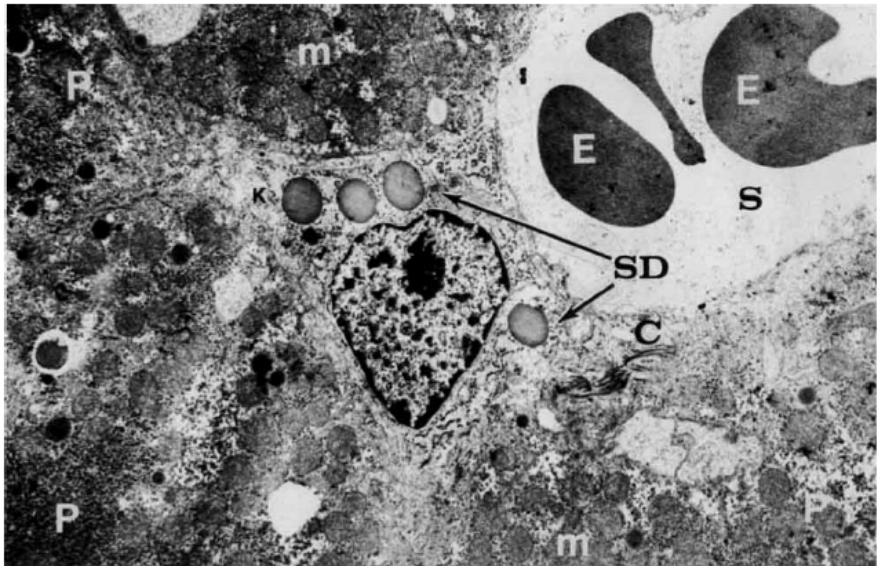



**Figure 2. Electron micrographs of hepatic cells at various time intervals after subtotal hepatectomy. A Kupffer cell (k) lining a sinusoid (S) is surrounded by parenchymal cells (P). Secretion droplets (SD) are noted in the Kupffer cell cytoplasm. Parenchymal cell mitochondria (m) are numerous and collagen fibers (C) are also seen in the P cells. Erythrocytes (E) are also present in the sinusoid. (72 hours post-hepatectomy magnification,*4800).**

Subtotal hepatectomy also evokes the production of a renal inhibitory factor (RIF), which reduces the ability of the liver to function as an extrarenal source of erythropoietin. This inhibitor is found in renal venous blood and not in blood draining from other organs. It is suggested that the RIF reduces the hepatic erythropoietin response to hypoxia by diminishing the production and/or activity of the HEF. The RIF possesses no anti-erythropoietin activity, and its appearance and actions are not influenced by accumulation of metabolic wastes (as in the nephrectomized or ureterally-ligated rat). Levels of HEF are significantly higher in rats 24 to 72 hrs after

hepatectomized whereas its antagonist, the RIF, is not detectable until approx. 48 to 72 hrs after hepatectomy. Inhibition of RIF of the hepatic erythropoietin response is nearly complete at 96 hrs following surgery. In previous studies an increase in the hepatic erythropoietin response to hypoxia was noted from 24 to 72 hrs after hepatectomy. Peak hepatic erythropoietin levels occurred at 72 hrs post hepatectomy and declined thereafter, manifesting near normal hepatic erythropoietin levels at 96 hrs after hepatectomy. The present study provides an explanation for these findings by demonstrating that production of erythropoietin by the hepatectomized animal is dependent on the relative levels of a stimulatory (HEF) and an inhibitory (RIF) factor. Erythropoietin production varied with the amount of liver tissue removed prior to nephrectomy and exposure to hypoxia. Serum erythropoietin levels 72 hours after 80-90% hepatectomy were significantly higher than in the 30-40% hepatectomized rats. Decreased hepatic regenerative potential and erythropoietin production were noted following multiple hepatectomies (22).

Fetal liver erythropoiesis requires, to some extent, the reticular or "nurse" cell (28). The presence of erythropoietin on the membrane of the Kupffer cell might facilitate its function as a ‘‘nurse cell” for the developing erythroblasts that occur at various foci in regenerating liver tissue. Erythropoietin was also associated with the membranes of these erythroblasts. These cells support erythropoiesis by transferring ferritin to erythroblasts (28), and by phagocytizing defective erythroblasts and the pyknotic nuclei extruded from mature erythroid elements. They are also essential to both the medullary and hepatic hematopoietic inductive microenvironments (29). It has been reported that reticular cells of erythroid islands are indistinguishable from Kupffer cells after the initiation of hepatic erythropoiesis by phenyl hydrazine in the adult mouse (30). The initiation of erythropoiesis in the regenerating liver may be dependent on the production and local diffusion of erythropoietin produced by these phagocytic cells in response to

the changes undergone by the hepatic hematopoietic inductive microenvironment. The hepatic erythropoiesis observed in these rats is effective. Thus the numbers of reticulocytes in the peripheral blood increase markedly as regeneration progresses (31).

### 2.5 Recombinant Human Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone (30.5kDa), which is a cytokine for erythrocyte precursors in the bone marrow, also called hematopoietin or hemopoietin. It is primarily produced by peritubular capillary endothelium cells in the kidney, where its production is stimulated by low oxygen levels in the blood, also know as hypoxia (32). Secondary amounts of the hormone are synthesized in the liver hepatocytes of healthy adults. In premature as well as full-term infants, the liver is the primary site of EPO production. EPO is the primary hormone regulating erythropoiesis, stimulating growth, and preventing apoptosis (33). Erythropoietin is available as a therapeutic agent produced by recombinant DNA technology in mammalian cell culture. The EPO receptor belongs to the cytokine receptor superfamily. Erythropoietin binds to its receptors on the surface of erythroid progenitors in bone marrow, contributing to their survival, proliferation, and differentiation, which in turn produces an increase in red blood cell (RBC) and hemoglobin concentrations (34). Conversely, an excessive increase in RBC mass suppresses erythropoiesis to prevent blood from becoming more viscous and increasing the possibility of thrombosis and stroke. Although the primary role of EPO is to regulate the red cell production, EPO and its receptors have been identified in various non-hematopoietic tissues as well as in the central nervous system, solid tumors, the liver and the uterus (35). Recombinant human erythropoietin (rHuEPO) has been used to reduce the requirement of packed red cells in elective liver resections in humans (36). rHuEPO improves the quality of life of patients with end-stage renal disease undergoing hemodialysis (37). Erythropoietin therapy also improves anaemia associated with

rheumatoid arthritis, acquired immunodeficiency syndrome, prematurity and malignancy. Besides these effects, rHuEPO has been demonstrated to protect several tissues like kidney, heart, liver and the central nervous system from hypoxic damage and promote tissue repair (38). The results of these studies suggest that EPO may also participate in the beneficial effects of ischemic preconditioning in several organs. Additionally, EPO may have several clinical applications with its anti-ischemic property, such as assisting in organ transplantation.

### 2.5.1 Efficacy of erythropoietin on multiple organs in previous studies

#### *2.5.1.1 Kidney*

EPO protected ischemia-reperfusion injured rat kidneys, at least partially, via upregulation of HIF-1α protein (39). EPO administration clearly increased EPO-R mRNA levels in ischemia-reperfusion injury of the kidneys. Several authors have suggested that EPO bound to erythropoietin-receptor (EPO-R) and the EPO/EPO-R complex or activated EPO-R are involved in the suppression of apoptosis, the promotion of proliferation, and/or differentiation of renal cells (40, 41).

#### *2.5.1.2 Heart*

Cardiomyocytes were markedly prevented from apoptosis by EPO treatment in an in vitro model; in vivo a 50% reduction of cardiomyocyte loss following coronary ischemia reperfusion could be detected. Additionally, EPO has been shown to stimulate angiogenesis in the ischemia-damaged heart as markedly as VEGF. This effect has been attributed to upregulation of matrix metalloproteinase-2 (42). Importantly, cardioprotective effects of EPO were seen without an increase in hematocrit (eliminating oxygen delivery as an etiologic factor in myocyte survival and function), demonstrating that EPO can directly protect the ischemic and infarcted heart.

#### *2.5.1.3 Liver*

The results in the model of liver I/R injury strongly support the concept that EPO is a tissue-protective cytokine, which seems to be true for all the organs expressing the EPO receptor. M Schmeding, et al. (43) were able to show that apoptosis rates were dramatically reduced in animals pretreated with EPO while mRNA of tumor necrosis factor-α and STAT-3 were upregulated. Intraportal venous injection displayed superiority to subcutaneous preconditioning. Transaminases were significantly reduced among the EPO-treated animals after 6 and 12 h. The data confirm that pretreatment with rHuEPO reduced the activity of caspase-3, ultimately reducing the apoptosis. The fundamental concept that emerges from previous studies is that rHuEPO induces a tolerance of the liver and other organs to a subsequent insult with ischemic reperfusion, and this induction seems to change MAPK expression in ways that promote cell survival (44).

#### *2.5.1.4 Brain*

In the brain, EPO has demonstrated impressive capacities in reducing infarct size by directly effecting neurons as well as astrocytes and microglia. EPO-R upregulation is induced by hypoxia and the consecutively activated hypoxia-inducible factor 1 (45-47). The goal of a therapy against potential damages induced by hypoxia, such as those following a stroke, is to preserve neural function either by rescuing neurons from death or by replacing dead neurons. In this respect, hypoxia-inducible factors, and particularly EPO for its cytoprotective and regenerative properties and its ability to pass through the blood-brain barrier, are of interest in cerebral ischemia. Although EPO has been well studied clinically and is a well-tolerated compound, treatment with EPO has also been associated with side effects due to its erythropoietic properties. For instance, EPO increases blood viscosity, which can lead to perfusion deficits in the brain during the treatment of cerebral ischemia. Consequently, alternative strategies have been developed using derivatives of EPO to reduce erythropoietic activity and potential toxicity. For example, asialoerythropoietin, which was generated by enzymatic

desialylation, possesses a very short plasma half-life, resulting in no erythropoietic activity, and exerts a neuroprotective effect in the brain (48). In the same way, derivatives of EPO such as carbamylated EPO have been developed and have shown neuroprotection against stroke and several other brain disorders (49).

Many authors have mentioned erythropoietin's role as a neuroprotective agent both in the central and the peripheral nervous system (50, 51). Marios G. Lykissas. et al. (52) have demonstrated that systemic EPO administration stimulates axonal regeneration after axonotmesis.

#### *2.5.1.5 Wound*

Administration of rHuEPO has been effective in reversing the impaired parameters in ischemic wounds. The increased VEGF content caused by rHuEPO administration correlated well with the wound-healing parameters, including collagen content and formation of new capillary vessels. Angiogenesis is central to granulation tissue formation, because the in-growth of newly formed vessels is needed to ensure the supply of oxygen and nutrients to the regenerating tissue. EPO has a direct stimulatory activity on the angiogenic factor and ability to stimulate endothelial cell mitosis. Administration of rHuEPO helps to improve wound healing by stimulating neovascularization and dermal regeneration (53).

### 2.5.2 Erythropoietin Biology in Cancer

There are still concerns about the possibility that rHuEPO may influence tumor growth via the induction of angiogenesis and cell proliferation (54). The expression of erythropoietin (EPO) and the EPO receptor (EPOR) has been recognized in a variety of human cancers, including breast, prostate, colon, ovary, uterine, cervical, glioblastoma, and head and neck squamous cell carcinoma (HNSCC). rHuEPO has been widely used in the prevention and treatment of cancer-related anemia, leading to increased

hemoglobin levels, reduction of RBC transfusion requirements and improvement of quality of life. Despite these beneficial effects and some studies suggesting an improved survival trend in EPO-treated patients with cancer, two recent prospective, randomized clinical trials involving head-neck and breast cancer patients have raised concerns over potential adverse effects of rHuEPO in cancer patients. The expression of EPOR in cancer cells has suggested the possibility that exogenous rHuEPO may exert direct effects on tumor cells associated with stimulation of proliferation, inhibition of apoptosis, or modulation of sensitivity to chemoradiation therapy. The presence of an autocrine-paracrine EPO-EPOR system in tumors and possible effects of EPO on tumor microenvironment and angiogenesis are consistent with a complex biology for EPO-EPOR signaling in cancer that requires further research. The overall direct effect of EPO-EPOR signalling is clearly not a straightforward one, since signalling can potentially activate several pathways important to tumor behaviour and response to treatment. Furthermore, the expression of EPOR alone is not always sufficient to modulate these pathways as shown by the variable effect of rHuEPO on chemoradiation sensitivity and the absence of a consistent proliferative effect in different types of cancer cells (55). These findings may reflect a possible absence of intracellular signalling after ligand-receptor interaction, low EPOR density, or nonfunctional EPOR at the cell surface in tumor cell lines. Of most physiological relevance are in vivo tumor studies, some of which have demonstrated that treatment with erythropoiesis-stimulating agents (ESAs) reduced tumor growth through radiosensitization, reduced hypoxia, or enhanced tumor immunity. Most noteworthy is that, at the time this manuscript was written, no animal in vivo tumor study has demonstrated that ESAs treatment alone enhances tumor progression or decreases survival. The on-going, well-controlled clinical trials will clarify whether the use of ESAs in anemic cancer patients is safe (56).

### 2.5.3 The dosage and route of administrations of erythropoietin on multiorgan in previous studies

These attributes of rHuEPO's efficiency could be helpful in accelerating liver regeneration after major liver surgery. EPO doses and route of administration were chosen in accordance with previously published protocols. However, this is a first relationship study in erythropoietin-accelerated liver regeneration and the dosage of our study should correspond to the previously ischemic-reperfusion study in multi-organ, specifically, in rodent heart, brain, kidney and liver ischemic-reperfusion studies.

Patel et al. (57) have reported that the pretreatment of mice with EPO (1000 IU/kg/day for 3 days) also reduced renal injury, dysfunction and inflammation caused by bilateral renal artery occlusion in the mouse. The degree of protection against injury, dysfunction and inflammation afforded by the pretreatment regimen with EPO was greater than the one afforded by the acute administration of EPO upon reperfusion. There are several potential explanations for this finding (52).

First, the pretreatment protocol has resulted in favorable systemic or renal hemodynamic effects. However, although acute administration of EPO (in the rat) had no effect on blood pressure or on cortical and pyramidal perfusion, the repetitive administration of EPO for up to 1 week caused a small reduction in cortical blood flow (58). This would argue against hemodynamic changes affecting preinsult glomerular filtration rate, but might contribute to delayed pre-conditioning.

Second, as the half-life of EPO is ~ 10 hours in the rodent, it is possible and likely that the repetitive administration of 1000 IU/kg subcutaneously per day for 3 days (pretreatment protocol) resulted in a higher steady-state plasma concentration, when compared to the peak concentration achieved from a single dose administration prior to reperfusion (1000 IU/kg prior to reperfusion).

Third, pretreatment of mice with EPO for 3 days may well have resulted in the up-regulation of protective genes, including endothelial nitric oxide synthase (eNOS), magnesium superoxide dismutase (Mn-SOD) and heat shock protein 70 (HSP70), which in turn could have contributed to the observed protective effect, mimicking the effects of delayed preconditioning (59).

In the rat kidney, preconditioning with EPO (3000 U/kg) 24 hours before ischemia was associated with reduced injury, mediated in part by the up-regulation of HSP70 and the antiapoptotic protein Bcl-2 (60). In this study, rHuEPO-induced HSP70 was dose-dependent (500→1000→3000 U/kg) and time-dependent (6→12→24 h).

Fourth, there is evidence that the pretreatment protocol of EPO used here results in enhanced levels of circulating endothelial progenitor cells. Most notably, it has been suggested (61) that the increase in circulating endothelial progenitor cells by EPO contributes to the beneficial effects of EPO in animal models of ischemic-reperfusion. It is, however, not known whether enhanced circulating levels of endothelial cell progenitors protect the kidney against ischemic reperfusion injury or improve recovery of renal function after an acute ischemic event.

The degree of protection was dependent on the timing of administration of EPO, with pretreatment for 3 days proving to be more effective than a single treatment at the time of reperfusion.

Edward J. Sharples et al. demonstrated that a single intravenous bolus injection of recombinant human EPO (300U/kg), either during preischemia or just before the onset of reperfusion, attenuates renal ischemic reperfusion injury in rats, *via* the inhibition of proapoptotic caspase activation. In contrast to the previous studies, Anthony J. et al (62) confirmed that EPO was able to limit the cardiac infarct size in a model of reperfusion injury when administered after a period of ischemia both in an in vivo and in an isolated perfused heart model. Lipsic et al. (63) detected a more potent effect of EPO on

cardiomyocytes when administered after ischemia. However, in vivo administration EPO 5000 U/kg at different times protects the myocardial structure and preserves cardiac function during ischemic-reperfusion. The cardioprotective effect of EPO extends beyond the start of reperfusion, providing a broad “window of opportunity” for the potential treatment of acute coronary syndromes.

The clinical benefit of non-erythropoietic effects of EPO has been implicated by Ehrenreich et al. (64) in a pilot, double-blind, randomized clinical trial investigating the acute effects of EPO treatment in patients with ischemic stroke. There, administration of EPO within 8 hours after stroke reduced brain infarct size and improved the clinical outcome. This short-term therapy with a high dose of EPO proved to be both safe and well tolerated. The serum EPO levels achieved in these patients (4000–6000 mIU/ml) were well comparable with those measured in Lipsic et al. (63) study (5000U/kg).

B. Sepodes, et al. (44) have shown that administration of rHuEPO 5 min before ischemia was able to reduce the biochemical evidence of liver injury; however, this protection was not evident when rHuEPO was administered 5 min before reperfusion. Mechanistically, early administration of rHuEPO was able to reduce the oxidative stress and caspase-3 activation, suggesting the subsequent reduction of apoptosis.

M. Schmeding et al. (43) were able to show that EPO application prior to warm ischemia of 45 min leads to a significant reduction in liver enzyme levels (ALT, GLDH). While reduction of apoptosis was dramatic in EPO subcutaneously pretreated animals (from 62.10% in controls to 0.54%), it was even greater among animals treated with intraportal venous EPO injection (apoptosis rate 0.06%). Transaminase levels were lowest when EPO was applied directly into the portal vein 30 min before ischemia.

However, most of the liver studies reporting protective effects of EPO on ischemic-reperfusion damage use drug application prior to tissue injury. Regarding the recently published data on liver ischemia-reperfusion, studies have demonstrated a benefit of

pretreatment with intraportal erythropoietin injection. rHuEPO application in this route simulates the production of hepatic erythropoietic factor during liver regeneration. The most beneficial dosage in most studies lies between a low dosage of 300 U/kg and a high dosage of 3000-5000 U/kg.

## 3. PURPOSES OF THE STUDY

Liver surgery, including partial hepatectomy and liver transplantation is the only curative treatment for benign and malignant including primary and metastatic liver disease. However, major resections and split liver transplantations are limited by the remnant functional liver parenchyma. A variety of strategies based on the increase in understanding of the mechanisms underlying hepatic regeneration have been developed to counteract postoperative liver insufficiency (65, 66). The main approaches are preoperative portal venous embolization and two-stage hepatectomies, which have been successfully applied to increase the number of patients who qualify for liver resection (79). However, postoperative liver insufficiency still significantly contributes to morbidity and mortality after liver surgery, particularly in patients with liver disease (67). Currently, no clinically established treatment strategy is available which effectively increases the regeneration capacity of the remaining liver after liver resection for tumour or split liver transplantation, thus improving the outcome of patients undergoing major liver surgery and transplantation.

Erythropoietin production is directly related to the process of the hepatic regeneration after liver resection. rHuEPO administration is also effective in ischemic protection and regeneration in multiple organs. However, it has not been proven yet that rHuEPO administration is able to promote proliferative liver cells on rat liver regeneration and enhance survival after major hepatectomy.

The aim of our study is to investigate if exogenous administration of rHuEPO in rats leads to stimulated liver regeneration after 70% partial hepatectomy and if treatment with rHuEPO may confer a survival advantage after 90% subtotal hepatectomy.

### 3.1 STUDY QUESTIONS.

1) To compare the capacity of liver regeneration between groups of pretreated single bolus rHuEPO intraportal venous application (4000 U/kg body weight) and subcutaneous applications of three doses of rHuEPO postoperatively (1333 U/kg body weight during three consecutive days, total dose 4000 U) after 70% hepatectomy in Wistar rats. Saline treated animals served as controls.

2) To compare survival of Wistar rats after 90% liver resection among the three groups (pretreated intraportal group, postoperatively subcutaneous group and control group)

## 4. MATERIALS AND METHODS

### 4.1 Animals and surgical procedure

Male Wistar rats with a body weight of 230–270 g (Charles River Laboratories, Sulzfeld, Germany) were used for the experiments. Animals were housed in standard animal laboratories with a 12 hour light-dark cycle with free access to water and standard laboratory chow ad libitum. The experimental design was reviewed and approved by the local government (Senator fur Gesundheit und Soziales, Berlin), and carried out according to the European Union regulations for animal experiments and the 'Guide for the Care and Use of Laboratory Animals' [DHEW Publ. No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, Md., USA]. Rats fasted overnight, however, with free access to tap water.

The first group served as controls. The second group received a cumulative dose of 4000 IU/ kg rHuEPO subcutaneously on 3 consecutive days starting immediately after surgery (1333 IU per dose). Thirty minutes prior to resection the third group received a single dose of 4000 IU / kg rHuEPO injected into the portal vein with a small No. 30 needle which was compressed with gel foam for a minute after removing the needle, (Figure 3). All injections were diluted with sterile saline and adjusted to a final concentration of rHuEPO in 1.0 cc. Control animals received an equivalent volume of saline as portalvenous bolus injection 30 minutes prior to hepatic resection.

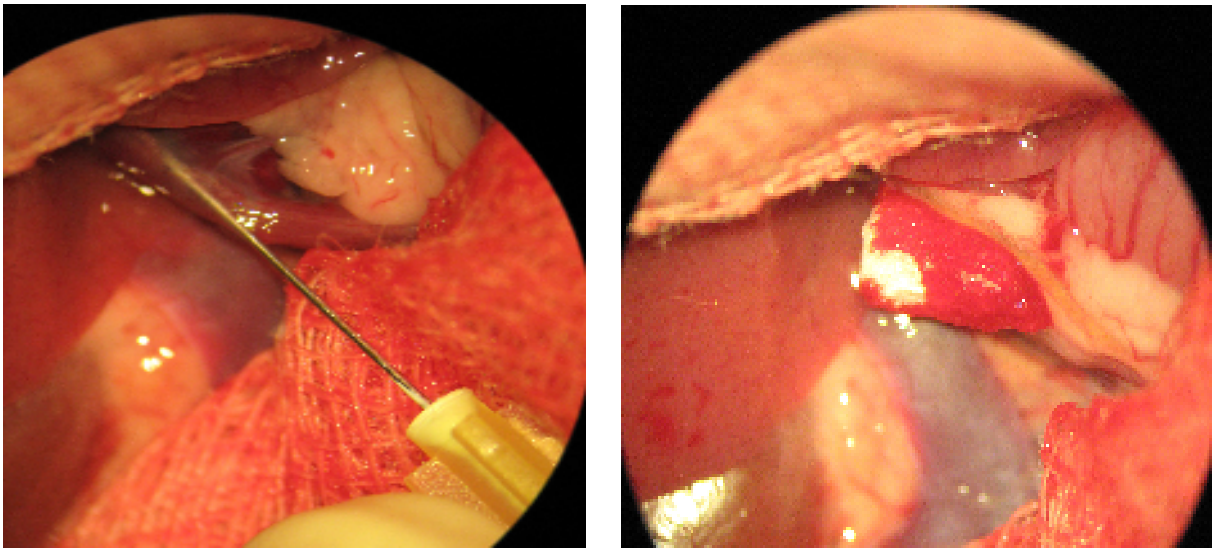


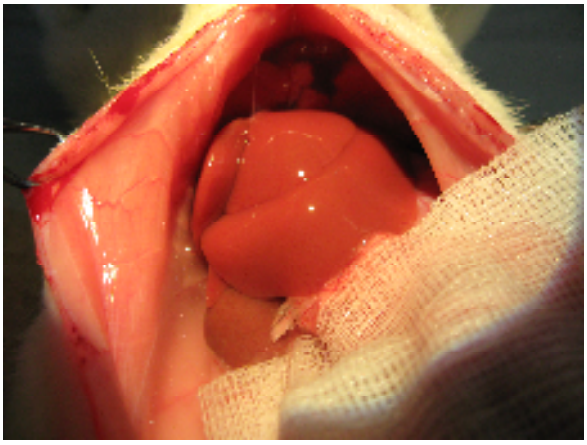
**Figure** 3. **Intraportal venous injection was performed with a small needle (a) with small gel foam packing at the puncture site afterward (b).**

A partial 70% hepatectomy resecting the left and median lobes of the liver was performed according to the method of Higgins and Anderson (6) to study the histology and immunohistochemistry (ki-67, PCNA) of the regenerative capacity. PCR was also performed to measure TGF-β, HIF, STAT-3 and VEGF.

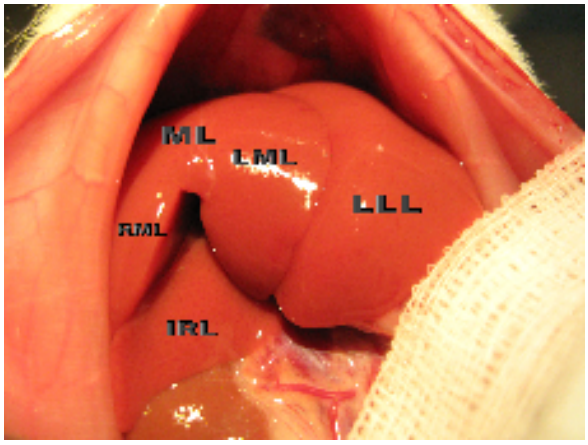
In the case of a 90% hepatectomy the right lateral lobe was additionally excised for the survival analysis (n=10 in each group).

### 4.2 Operative technique

Animals under isoflurane 1.5-3% and oxygen flow 0.6L/min inhalative anesthesia were placed on a pad in a supine position. Long midline incision from xyphoid process to waistline was performed and retracted to open the abdominal cavity with tower clips (Figure 4). The small intestine was wrapped up in moist gauze and then folded to the left side of the animal. The falciform ligament was then dissected, and the left infraphrenic vein was ligated and divided. The next step was the double ligation and dissection of a small arterial branch arising from the left hepatic vein which supports the lower esophagus.



a.

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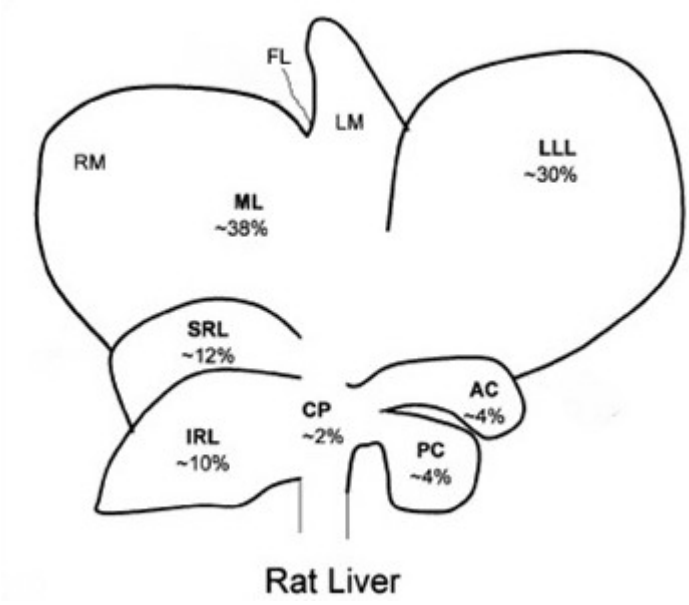



c.

**Figure 4. Gross anatomy of the liver: (a) and (b) The ventral view of the intraoperative situs; (c) The visceral surface of the rat liver shows the lobes and their mean relative weight. The liver mass of each lobe in the rat is quite constant. The caudate lobe (CL) is formed by the caudate process (CP), anterior caudate lobe (AC) and posterior caudate lobe (PC). The right liver lobe is formed by the superior right lateral lobe (SRL) and inferior right lateral lobe (IRL), and the medial lobe formed by the left portion of the middle lobe (LM) and right portion of the middle lobe (RM). The left lateral lobe (LLL) is a rhomboid shape.**

### *4.2.1 Surgical technique of liver resection*

In 90% hepatectomy, the left lateral, median and both right lobes were resected. The left lateral lobe was removed after clamping and ligating the narrow pedicle with a 6-0 silk

and 7/0 Prolene suture. After placing the Mosquito clamp around the base of the respective lobe, the liver tissue was dissected just above the branches of the instrument. Piercing sutures (4 for the median lobe, 4–5 for the right superior lobe), penetrating the whole parenchyma were placed below the clamp. Then the clamp was released. Thus, a plain cutting surface with only a thin layer of necrosis was achieved. The right inferior lobe was resected using two sutures. The right inferior lobe was resected using two ligatures. Only the caudate lobe of the liver was in place after 90% resection (Figure 5).

In 70% hepatectomy, the left lateral and median were resected according to the above technique, but the right (superior and inferior lobes) and caudate lobe remained intact (Figure 6).

Bleeding was stopped with the bipolar coagulator. The bowel was rearranged in the proper position, and the abdominal wall was closed with two layers of Prolene 3/0 continuous suturing technique (Figure 7).

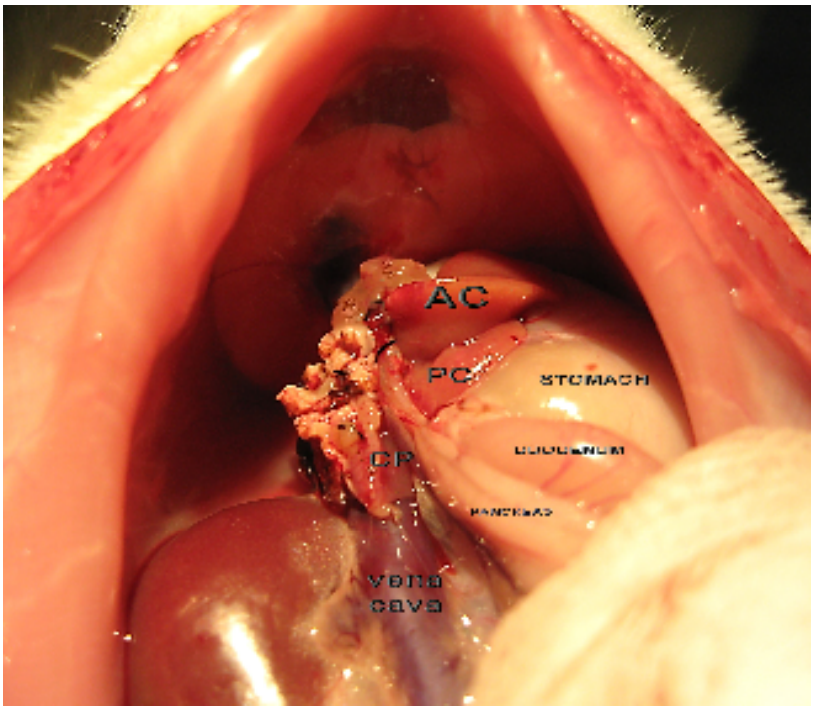



**Figure 5. 90% liver was resected. The caudate lobe comprises 8-10% of liver weight and is divided into two portions: The paracaval portion (caudate process) (CP), which accounts for 2-3% of the liver mass, and Spiegel lobe, which has an anterior (superior) (AC) and a posterior (inferior) (PC) portion in the form of disc, each representing 4% of the liver mass.**

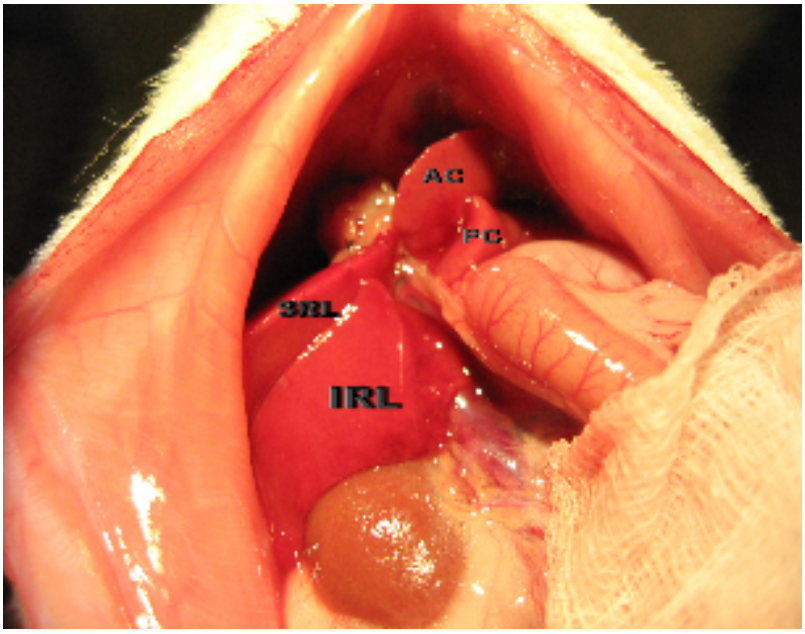



**Figure 6. 70% hepatectomy: the median lobe (ML) is the largest, accounting for approximately 38% of the liver weight. The left lateral lobe (LLL) has a rhomboid shape, is flattened and has approximately 30% of the liver weight. Both lobes were resected.**

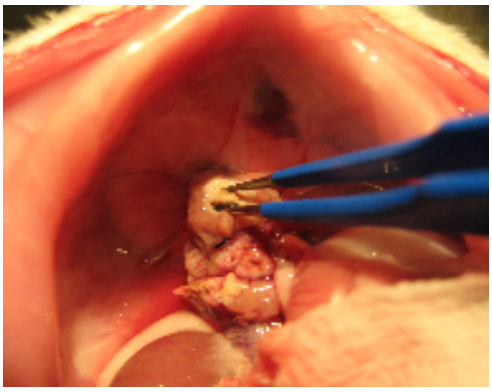
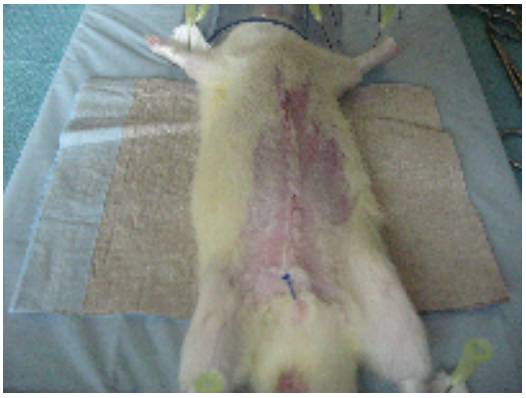
a.  b. 

**Figure 7. a) Bipolar coagulator was used to stop bleeding on the raw cut surface of the liver after resection. b) Abdominal wall was closed with two layers suturing technique.**

Postoperatively animals recovered from surgery with access to food and water ad libitum. All animals were supplied with analgesic treatment of tramadol 10mg/ kg applied directly into the peritoneal cavity before closing the abdomen.

### 4.3 Animal Sacrifice and Organ Harvest

All animals received complete anaesthesia at 3 hr, 6 hr, 12 hr, 24 hr, 2 days, 4 days and 7 days after liver resection. The abdomen was opened again and a clamp was placed on the infrarenal aorta. Blood was drawn from the aorta until circulation stopped completely. The heart was incised to secure animal death; liver tissue was harvested and forwarded for histological (paraffinized and cryo-preserved) and PCR evaluation. Blood samples were taken and stored for enzymatic and biochemical analysis. (n=6 in each time and in each group).

### 4.4 Survival Analysis

Ten rats in each group with a 90% hepatectomy were used for the survival study. Rats which lived for more than 10 days after hepatectomy were considered survivors.

### 4.5 Histology

Remnant liver tissue was fixed in 4% phosphate-buffered formalin for 2–3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 5 µm sections were cut and stained with hematoxylin and eosin (HE). To evaluate hepatocyte replication, mitotic figures were counted in 2,000 hepatocytes (200-fold magnification) and given as mitotic index (number of mitotic figures per 1,000 hepatocytes). All slides were judged by the same investigator who was blinded to the corresponding study group.

### 4.6 Measurement of hepatocyte proliferation (Ki-67 positive cells, PCNA, mitotic index)

The labeling index of Ki-67 reacting with the equivalent Ki-67 (DakoCytomation, Glostrup, Denmark) protein to detect all active parts of the cell cycle and PCNA (Caltag Laboratories, Burlingame, CA  USA) was determined immunohistochemically using an indirect enzyme-linked antibody method (68). The proliferative capacity was expressed in terms of labeling indices, determined as the number of labeled hepatocytes per 2.000 hepatocyte nuclei (high power field; x200). Data are expressed as the mean percentage of Ki-67 and PCNA positive cells. The number of mitotic hepatocytes (mitotic index) was evaluated by counting 2,000 cells in hematoxylin-eosin stained tissue sections.

### 4.7 Quantitative real-time polymerase chain reaction (RT-PCR):

#### *4.7.1 TGF-ß1, STAT-3, and HIF-1α investigation*

**Liver tissue samples of sacrificed animals were investigated for mRNA of TGF-ß1, STAT-3, and HIF-1α with GAPDH as internal control (Table 1):**

Total RNA was directly extracted from cell layers using the RNApure® reagent (Peqlab Biotechnologie, Erlangen, Germany). Reversely transcribed complementary DNA templates were amplified by quantitative real time-polymerase chain reaction (Roche

Diagnostics, Mannheim, Germany) using fluorogenic probes labeled with 6-carboxy-fluorescein and 6-carboxy-tetramethylrhodamin (Sigma-Genosys, Steinheim, Germany), and primer pairs (Invitrogen, Karlsruhe, Germany). To normalize for differences in RNA amounts and variable efficacy of the reverse transcription reactions, the housekeeping gene glyceraldehyde-phosphate dehydrogenase (GAPDH) was quantified in a parallel reaction.

**Real-time RT-PCR** Real-time RT-PCR was performed as described (69). Briefly, total RNA was isolated from the cell lysates or liver homogenates using RNApure (PeqLab, Erlangen, Germany) and 0.5 g of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) with 50 pmol of random hexamer and 100 pmol of oligo (dT) primers (Promega, Mannheim, Germany). Relative mRNA transcript levels were quantified on a LightCycler (Roche Applied Science) using the TaqMan technology. The housekeeping gene GAPDH was amplified in a parallel reaction for normalization. TaqMan probes and primer sets were designed using the Primer Express software (PerkinElmer Life Sciences) based on published sequences as summarized in Table 1. All TaqMan probes, such as spanning exon-exon boundaries of corresponding genes, were positioned to exclude co-amplification of genomic DNA. Sense and antisense primer (each at 0.5 µM) and 0.125 µM probe, labeled and phosphorylated at its 5'-end with 6-carboxyfluoresceine (6-FAMTM) and at the 3'-end with tetramethylrhodamine, were synthesized at MWG Biotech (Ebersberg, Germany) and validated using conventional RT-PCR and agarose chromatography (69). Relative mRNA transcript levels were expressed in arbitrary units as n-fold versus untreated controls (mean±S.E.) after normalization to GAPDH mRNA.

**Table 1.**

| Target | Oligonucleotide sequence (5'-3') | | |
|---|---|---|---|
| | Probe* | Primer sense | Primer antisense |
| TGF-β1 | ACCGCAACAACGCAATCTATGACAAAACCA | AGAAGTCACCCGCGTGCTAA | TCCCGAATGTCTGACGTATTGA |
| STAT3 | TGAAAGCAGAGGGCTGGGGG | GTCTTTGGGCAGTCTGGGTA | GGCGGACAGAACATAGGTGT |
| HIF-1α | CGTGAACAAATACATGGGGTTGACTCA | TATCTGAAAGCCCTGGATGG | CATGGTCACATGGATGGGTA |
| GAPDH | TGGTGAAGCAGGCGGCCGAG | CCTGCCAAGTATGATGACATCAAGA | GTAGCCCAGGATGCCCTTTAGT |

*All probes were labeled 5' with 6-carboxy-fluorescein and 3' with 6-carboxy-tetramethylrhodamine.

***4.7.2 VEGF investigation.***

**VEGF- mRNA was measured by non-competitive semiquantitative RT-PCR with ß-actin as internal control:**

After isolation, as described above (69), we used a TRIZOL© reaction kit. All samples were incubated with desoxypolymerase I (DNAse) for 15 minutes to exclude DNA contamination thereafter. After photometric determination of RNA purity and concentration, samples were stored in aliquots of 10µl at -80˚C. RNA was amplified using a RT-PCR kit (Boehringer, Germany), which combines reverse transcriptase and Taq polymerase activity in a final reaction volume of 50µl. Controls using no RNA or only Taq polymerase without reverse transcriptase activity were performed. Amplification products were analyzed for size and quantity by gel electrophoresis (agarosis 2%). In addition to cytokines, ß-actin was also determined for each sample to assess differences in RNA concentration. Primers for ß-actin were designed to anneal in two different exons to further control possible DNA contamination, which would result in a bigger size PCR product including the intron in-between. Finally, the amplification

products of all cytokines were analyzed for correct sequence by restriction analysis. The sequences for primers used were as follows:

ß-actin

5`-ACC CAC ACT GTG CCC ATC TA-`3

5`-CGG AAC CGC TCA TTG CC-`3

VEGF

5`-GGT GTG GTG GTG ACA TGG TTA ATC-`3

5`-CCA GCA CAT AGG AGA GAT GAG CTT C-`3

**4.8 Statistical analysis**

Results were expressed as mean ± standard error of the mean (SEM). After proving the assumption of normality and equal variance across the three groups, differences between groups were assessed using Mann-Whitney-U test. Kaplan-Meier Analysis was performed to identify differences in the survival figures. Significance was tested by log-rank test. In all instances $p < 0.05$ was considered statistically significant.