Aus der Klinik für Allgemein-, Visceral- & Transplantationschirurgie der
Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Transplantation von hepatozytenähnlichen Zellen (NeoHep Zellen) verbessert das Überleben in einem Modell akuten Leberversagens

zur Erlangung des akademischen Grades
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ZUSAMMENFASSUNG

ZIEL: Untersuchung der Effizienz der Implantation von von Monozyten stammenden, hepatozytenähnlichen Zellen (NeoHep Zellen) bei akutem Leberversagen. **HINTERGRUND:** Ausgedehnte Leberresektionen oder Teillebertransplantationen sind immer noch mit hoher Morbidität und Mortalität wegen der Gefahr einer postoperativen Leberinsuffizienz assoziiert. Mit Blick auf Leberunterstützungssysteme ist die Implantation von isolierten Hepatozyten oder hepatozytenähnlichen Zellen wie NeoHep Zellen zunehmend in der Diskussion.

METHODIK: 24 Stunden vor einer subtotalen Hepatektomie wurden Zellen unterschiedlichen Ursprungs (A: humane mononukleäre Zellen ($24x10^6$); B: NeoHep Zellen ($16x10^6$); C: NeoHep Zellen ($24x10^6$); D: Ratten Hepatozyten ($24x10^6$)) in die Milz von männlichen Wistar Ratten gespritzt. Während einer Beobachtungszeit von 5 Tagen wurden das Überleben, postoperative Gewicht und Zeichen der Enzephalopathie gemessen. Am Ende der Beobachtungszeit wurde Blut für Laboranalysen (Serumspiegel von ALT, Bilirubin und Albumin) abgenommen.

ERGEBNISSE: Die Transplantation sowohl von Ratten Hepatozyten als auch von NeoHep Zellen erbrachte ein statistisch signifikant höheres Überleben (72% in Gruppe D (p=.001), 50% in Gruppe C (p=.04), 36% in Gruppe B (p=.22)) verglichen mit der Kontrollgruppe (Gruppe A: 21%). Darüberhinaus zeigten die Tiere in diesen Gruppen seltener Zeichen einer postoperativen Enzephalopathie und wiesen eine schnellere postoperative Gewichtszunahme auf als in Gruppe A.

ZUSAMMENFASSUNG: Die Hepatozytentransplantation ist eine praktikable und erfolgreiche Behandlungsoption bei Vorliegen einer Leberinsuffizienz, da sowohl die

Implantation von primären Rattenhepatozyten als auch von NeoHep Zellen das Überleben deutlich verbessern konnten. Basierend auf den Ergebnissen dieser Studie halten wir eine Vorbehandlung von Patienten mit autologen, von Monozyten abstammenden hepatozytenähnlichen Zellen (NeoHep Zellen) durch ,,Eigen-Zell-Spende'' für ein wirksames Instrument, um die durch postoperative Leberinsuffizienz bedingte Morbidität und Mortalität deutlich zu reduzieren.

INTRODUCTION

History:

Transplantation of the whole liver, or a portion of the liver, has been remarkably effective in the treatment of end-stage liver disease and liver-based inherited metabolic diseases. However, organ shortage has impaired transplantation activity, causing crescent waiting list mortality everywhere. In view of this, many investigators have evaluated transplantation of isolated liver cells as a less invasive alternative to whole organ transplantation or as a 'bridge' for patients who are awaiting a donor liver. Extensive laboratory research work and limited clinical trials have shown that hepatocyte or stem cell transplantation may be useful both in bridging some patients to orthotopic liver transplantation and in dealing with metabolic disease in children [1-4].

As early as 1969, the technique of enzymatic digestion of liver to produce large numbers of metabolically active isolated hepatocytes was developed by Berry and Friend [5]. This innovation, together with improved techniques for handling isolated cells, stimulated a resurgence of interest in transplantation of isolated hepatocytes [6].

In 1977, Groth et al. reported for the first time the therapeutic effect in metabolic disease by hepatocyte transplantation in rat. The correction of hyperbilirubinemia was achieved in more than 50% in the glucuronyltransferase-deficient rat by intraportal hepatocyte transplantation [7].

Acute liver failure (ALF), which carries high morbidity and mortality (>80%) even in high-volume centres, is another target for hepatocyte transplantation. The most commonly used models include galactosamine-induced liver failure in rats, rabbits, guinea pigs and dogs, and thioacetamide-induced liver failure in rabbits and rats. In these experiments, hepatocyte transplantation has shown survival rates of more than 60 per cent [8].

The study by Mito and Kusano in 1992 was a landmark for taking hepatocyte transplantation into clinics because they were the first to attempt hepatocyte transplantation in cirrhotic patients. Hepatocytes were isolated from segments of the cirrhotic livers of the patients and transplanted by injection into the splenic pulp, splenic artery, splenic vein, or portal vein. There was indeed some evidence of improvement in encephalopathy, protein synthesis, and renal function, but the ultimate clinical outcome was not altered significantly [9]. Two years later, Habibullah et al. [7] published their hepatocyte transplantation experience in seven ALF patients presenting hepatic encephalopathy grade III and IV. All patients achieved complete recovery of hepatic encephalopathy, while in their control groups the survival rate was 50% in patients with encephalopathy grade III and 33% in those with encephalopathy grade IV [10].

The first report of hepatocyte transplantation in metabolic diseases was in 1998 by Fox and his colleagues. A child with Crigler-Najjar type I, suffering from dangerous hyperbilirubinaemia, was given $7.5x10^9$ allogenic donor hepatocytes by infusion via portal vein catheter and serum bilirubin levels was markedly reduced[11]. Treatment of other metabolic disorders such as ornithine transcarbamylase deficiency, α 1-antitrypsin deficiency, or glycogen storage disease type 1a was attempted by

hepatocyte transplantation as well [2, 12] (Table 1).

Table 1 Overview over hepatocyte transplantation studies in patients with inherited metabolic liver disease

Recipient	Year	Sex	Age	outcome
α 1-antitrypsin deficiency	1997 [13]	Male	18 w	OLT (d 2)
		Female	52 y	OLT(d 4)
Crigler-Najjar type I	1998 [11]	Male	10 y	OLT (3.5 y)
ornithine transcarbamylase deficiency	1999 [14]	Male	5 y	Normal ammonia level in 48 h; died after 43 days
	2003 [15]	Male	10 h	Normal protein intake possible; OLT (6 month)
glycogen storage disease type 1a	2002 [16]	Female	46 y	Improved for 3 ys
Refsum disease	2003 [17]	Female	4 y	Improved for 1 y
Factor VII defficiency	2004 [18]	Male	2y,11m	Improved and decreased requirement for recombinant factor VII
		Male	3 m	

OLT: orthotopic liver transplantation [12] .

Sources of hepatocytes for transplantation

Primary hepatocytes

Currently, first steps in hepatocyte transplantation are being taken to be used clinically with the intention of treating patients suffering from acute or chronic liver diseases [1, 12].

In this context, hepatocyte transplantation has been proposed as an assist and as an alternative to whole organ transplantation. Indeed, transplanted liver cells have already been used clinically to "bridge" patients to whole organ transplantation [9, 10, 13, 19]. Most of these experimental and clinical studies used adult primary hepatocytes as cell source for subsequent cell transplantation.

Usually, the primary human hepatocytes can be harvested from surgical samples, biopsies, or from liver grafts; however, their availability is limited. Cell extraction from surgically removed liver tissue e.g. due to primary liver cancer or secondary liver metastases contains the risk of contamination of the extracted cell fraction with occult tumor cells, which might result in de-novo cancer occurrence in the recipient after cell transplantation. Moreover, livers discarded from full-size liver transplantation are among the sources of hepatocytes for cell transplantation, but the most common reason for not using a liver graft for whole organ transplantation is liver fibrosis or severe steatosis. Thus, we are faced with the problem of trying to salvage useful cells from tumor-free livers that were judged as unsuitable for whole organ transplantation. In consequence, a wider use of hepatocyte transplantation will not be possible until appropriate cells for transplantation become more readily available. That is why hepatocyte transplantation is performed at relatively few medical centres, though this

approach seems to be a functioning alternative thinkable also in postoperative liver insufficiency. Despite positive reports, application of hepatocyte transplantation in humans is limited to less than 100 cases [12].

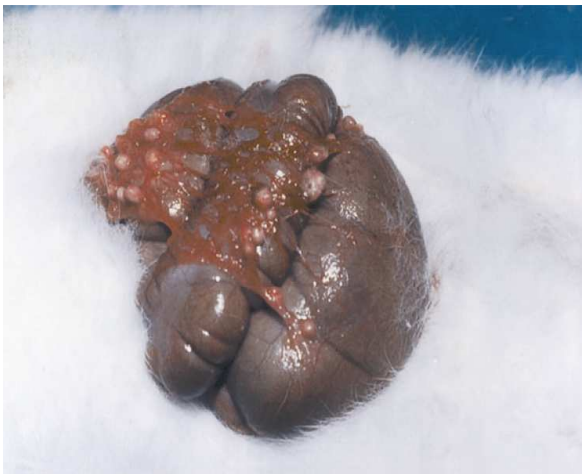
Fetal hepatocytes

Another source of hepatocyte transplantation is from fetal hepatocytes which have several characteristics that make them potentially suitable as donor cells. In contrast to adult hepatocytes, fetal hepatocytes are much more proliferative, which may facilitate engraftment and expansion of transplanted cell population. However, transplantation of fetal hepatocytes in patients with acute liver failure resulted in modest clinical improvement regarding signs of hepatic encephalopathy. Systematic evaluation of this important clinical resource is clearly warranted but ethical concerns need to be addressed first [10, 20].

Xenogenic hepatocytes

Xenogenic hepatocytes as an alternative source can be applied in both extracorporeal bioartificial liver support system and isolated hepatocyte transplantationl. Khan et al. recently reported on xenogenic transplantation of microencapsulated rat hepatocytes in rabbits with D-galactosamine fulminant hepatic failure. Rat hepatocytes were microencapsulated in alginic acid poly-l-lysine membrane to prevent immune destruction and transplanted intraperitoneally in rabbits (*Figure 1*). All animals that did not receive hepatocytes died within 36 hours. The animals that received encapsulated hepatocytes experienced a better survival rate (73%) compared to only 25 per cent of those that received non-encapsulated hepatocytes [8].

***Figure 1**. Encapsulated rat hepatocytes in the peritoneum of rabbit [8].*



Xenogeneic hepatocytes might be an unobtrusive alternative to hepatocyte transplantation. Clinical studies in acute or chronic liver failure, as well as in metabolic disorders, have also been undertaken in some centres and have shown encouraging results. Their major advantage consists in their continuous cell availability (from porcine, rabbit, canine), however, there are concerns about the use of xenogenic donors in view of transmission of zoonosis and immunogenicity [20].

Stem cells in liver and extrahepatic stem/precursor cells

Hepatic oval cells have been demonstrated to share with hematopoietic stem cells CD34, Thy-1, and c-kit mRNA and protein as well as flt-3 receptor, previously reported to be restricted to hematopoietic stem cells. Therefore, they are regarded as hepatic stem/progenitor cells [20]. Oval cells appear and expand in the liver when hepatocyte proliferation is compromised. They have been recently proved to be valuable candidates for liver cell therapy. EpCAM(+) oval cells are bipotential adult hepatic epithelial progenitors and display a mixed epithelial/mesenchymal phenotype

[21].

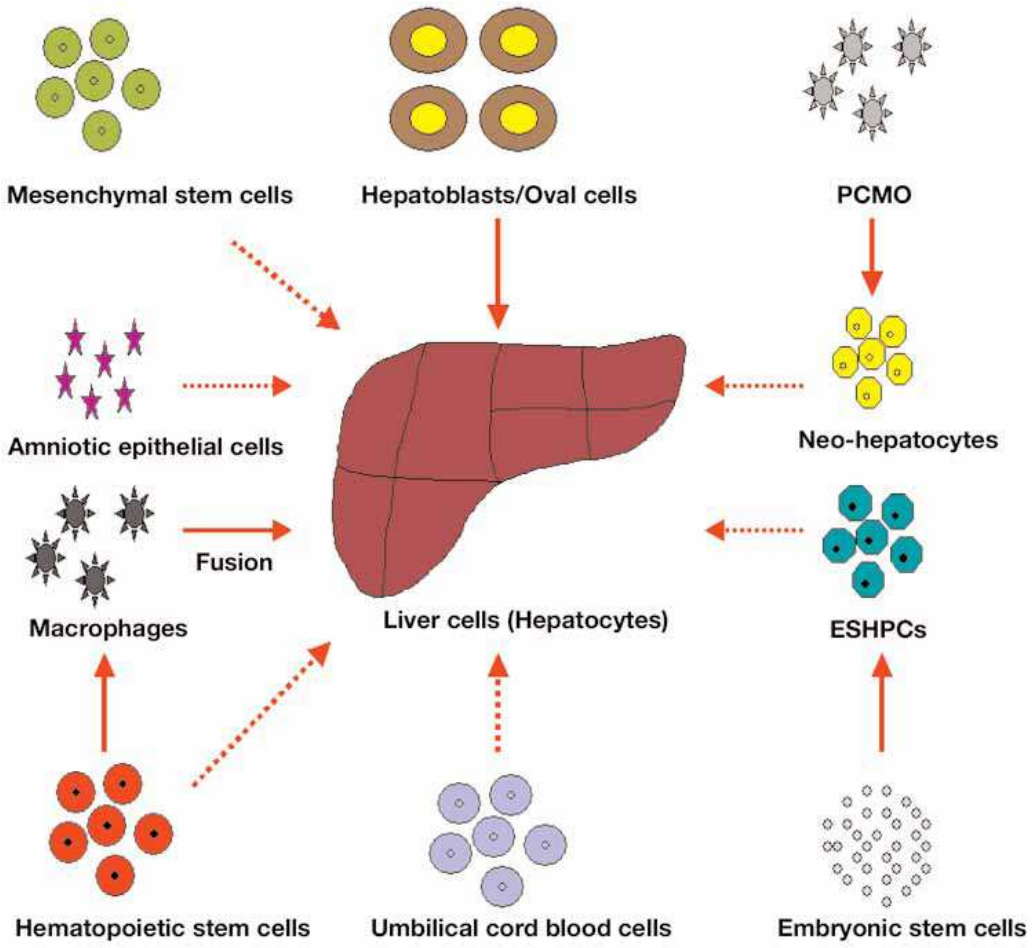
Stem cells from bone marrow are capable of migrating throughout the body and of differentiating into various cell types, including liver cells. Since 1999, numerous articles have reported the generation of hepatocytes from different types of extrahepatic stem or precursor cells. This seems to open exciting new possibilities for cell transplantation therapy. However, preclinical data are not yet sufficient to justify clinical studies. Further information about the generated hepatocyte-like cells is needed and so are reproducible results in preclinical animal models of human liver disease [12].

Recently, hepatocyte-like cells from terminally differentiated peripheral blood monocytes were made available by culturing them under conditions which promote hepatocyte-like cell differentiation [22]. These so called NeoHep cells resemble primary human hepatocytes with respect to morphology, expression of hepatocyte markers, various secretory and metabolic functions, and drug detoxification activities [23].

NeoHep cells seem to present an ideal cellular system suitable for therapy in acute liver failure. The option to support a de-compensated liver after liver resection or split liver transplantation by transplanting these hepatocyte-like cells would be an excellent chance to improve the therapeutic options in the case of postoperative liver insufficiency.

Possible sources of hepatocyte transplantation are shown in *Figure 2*.

Figure 2*.Various potential sources for the generation of hepatocytes.*



Among hepatic stem cells, hepatoblasts (during embryonic development) and oval cells (facultative adult liver stem cells) give rise to hepatocytes. Hematopoietic stem cells (HSC), macrophage amniotic epithelial (AE) stem cells, mesenchymal stem cells (MSC), programmable cells of monocytic origin-derived neo-hepatocytes, umbilical cord blood cells (UCB), and embryonic stem cells-derived hepatocyte-like cells have been reported to form hepatocyte-like cells. Formation of hepatocytes from hepatoblasts/ oval cells and macrophage fusion with hepatocytes has been shown convincingly in vivo. Continuous arrows represent convincingly reported pathways, whereas discontinuous arrows illustrate concepts under intensive research but not

yet convincingly proven. In spite of several reports suggesting the hepatic differentiation of HSC, MSC, multipotent adult progenitor cells, UCB cells, embryonic stem cells, and monocytes there is not even a single convincing report, which describes long-term functional engraftment and repopulation of liver. Therefore, in vivo utility of these in vitro differentiated hepatocyte-like cells remains as a challenging task in liver stem cell biology [24].

Aims of the present study

As discussed above, hepatocyte transplantation has been considered an optimal option to treat various liver diseases. But limited availability of hepatocytes has become the major hurdle in the path to its wide application. Much effort has been put into seeking new recourses. Among them, NeoHep cells which can be easily obtained and harvested from peripheral blood, present an ideal cellular system suitable for a therapy in acute/chronic liver failure and other disease as well.

Using an experimental model of subtotal hepatectomy in rats, we investigated the therapeutic efficacy of NeoHep cells acute postoperative liver failure.

MATERIALS AND METHODS

Animals and surgical procedure

In the study we used 65 male Wistar rats, Fa. Harlan-Winkelmann, Borchen, Germany, weighing between 232-333g (mean 276 ± 20g), which were housed in our animal facility. The data of specification are listed in *Table 1*. Rats were maintained with commercial standard laboratory rat chow, a 12-hour light/dark cycle, constant temperature of 25℃ and relative humidity of approximately 40%. They were acclimatized to laboratory conditions for one week prior to the experiments.

Acute liver failure was induced by subtotal hepatectomy following a varied protocol of the method of Higgins and Anderson, developed by Eguchi et al. [25]. Left and median liver lobes were removed after central ligature with 4-0 absorbable, synthetic, braided thread. The right upper and lower lobes were rendered necrotic by ligature of the common right liver lobe pedicles using braided silk thread. Both omental liver lobes and parts of the liver tissue surrounding the intrahepatic portion of the inferior vena cava remained, together representing approximately 10% of the total liver mass. With this procedure highly reproducible symptoms of fatal hepatic failure including severely impaired ability of the residual liver tissue to regenerate may be achieved [25, 26].

Before and after intervention, rats were allowed free access to food and tap water. Postoperatively, 5% dextrose was offered ad libitum. Analgesia was performed with tramadol s.c. adapted to body weight after surgical procedure. Immunosuppression

was not applied, since the observation period was only 5 days and rejection does not occur within this period in a model of xenotransplantation.

All procedures were reviewed and approved by the local government (Senator für Gesundheit und Soziales, Berlin, Germany) and carried out according to the European union regulations for animal experiments.

Treatment groups

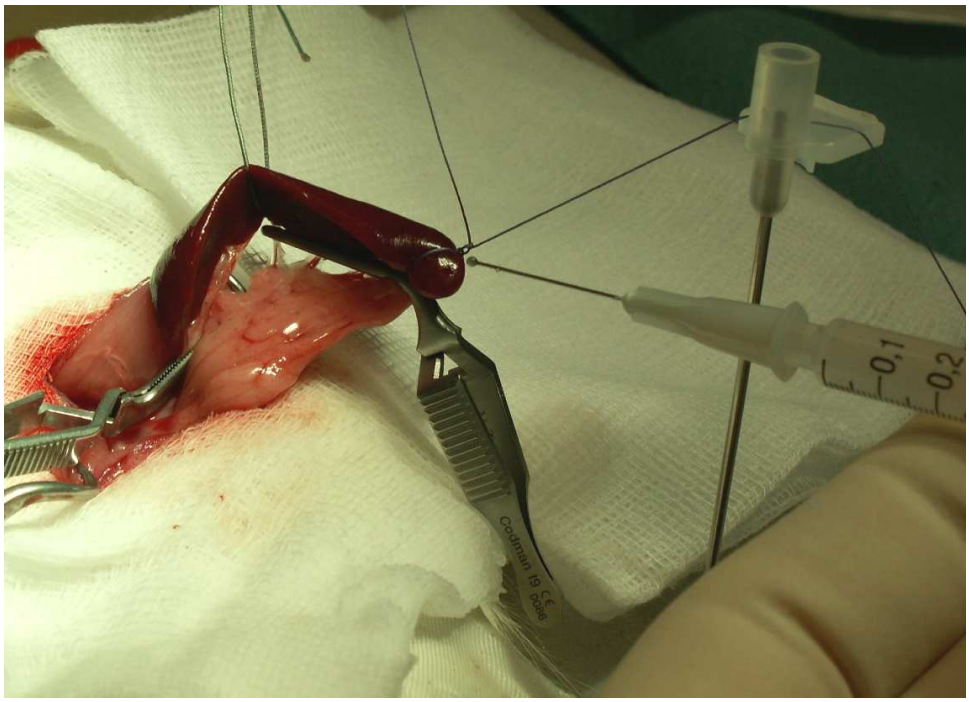
Extension of liver resection and observation time of 5 days after liver resection was constant in all groups. Cell implantation was done one day before liver resection as recently described by Gaebelein et al. [26].

Hepatocyte implantation was performed by slow injection of 0.7 ml in PBS suspended isolated rat hepatocytes by using a 25-gauge needle connected to a 1 ml syringe. Blood flow in both splenic arteries and veins was clamped before injection and remained occluded for a further 5-7 min to avoid immediate passage of cells into the portal vein (*Figure 3*). The injection site was ligated to prevent cell leakage and bleeding. Instead of hepatocytes, a saline solution (0.9% sodium chloride of analogous volume) was used in each series for appropriate controls.

Four study groups were built (*Table 2*). In the first group, human mononuclear cells (group A) were used for controls since these cells represent peripheral blood monocytes prior to differentiation to hepatocyte-like cells (NeoHep cells). In other words, these cells are the (undifferentiated) pre-stage of (differentiated) NeoHep cells and are without metabolic function in terms of synthesis or detoxification. The therapy groups were loaded with $16x10^6$ or $24x10^6$ of NeoHep cells (group B and C, respectively) suspended in 0.7ml PBS. One group of animals was treated with $24x10^6$

primary rat hepatocytes (group D), suspended in 0.7ml PBS, too, in order to compare NeoHep cells with the ‘optimal’ cell source.

Figure 3: *Hepatocyte transplantation was performed by injection into the spleen [26].*



Encephalopathy score based upon a coma scale described by Nataga et al. [27] was used to assess grade of encephalopathy in the postoperative course. Description of behaviour: (5 points) spontaneous and interested ramble; (4 points) reserved spontaneous activity; (3 points) temporary activity after disturbing excitability; (2 points) erecting after laying down in a lateral position; (1 point) no activity, weak life signs, but able to drink; (0 points) positive corneal reflex as single reaction. Moreover, this score was also used to decide premature harvesting (in the case of 0-1 points) due to incompatibility with survival [27].

At the end of observation time surviving animals were harvested on day five. Blood and tissue samples were taken. Laboratory values such as serum albumin, serum

bilirubin, and serum ALT were determined using commercially available reaction kits. Measurements were performed at the Institute of Laboratory Medicine, Charité, Campus Virchow Klinikum, Universitätsmedizin Berlin, Germany. Histological analysis of liver, lung, and spleen samples was performed on formalin fixed sections, which were stained with haematoxylin and eosin according to standard procedures. Moreover, a human-DNA-hybridisation was performed in order to detect cells of human origin (NeoHep cells) in rat tissue.

NeoHep cells

NeoHep cells were generated by EUFETS GmbH (Idar Oberstein, Germany) according a recently published protocol by Ruhnke et al. [22, 23].

The mononuclear cell fraction was isolated from peripheral blood by density gradient centrifugation. Cells were cultured in RPMI 1640 (Cambrex) containing 10% human serum (local blood bank), 2 mM glutamine and 100 U/mL penicillin, and 100 mg/mL streptomycin (all from Invitrogen). After 2 hours, nonadherent cells were removed by gentle washing with phosphate-buffered saline. Cells were then cultured for 6 days in a RPMI 1640-based medium with 140 mM-mercaptoethanol, 5ng/mL macrophage-colony stimulating factor (M-CSF,R&DSystems), and 0.4 ng/mL human interleukin (IL)-3 (R&D Systems). These monocytes, now termed PCMO, were cultured for another 14 days with 3 ng/mL fibroblast growth factor (FGF)-4 (Sigma) to induce hepatic differentiation. Monocytes that were cultured in the absence of M-CSF or IL-3 during the initial culture or in hepatocyte differentiation medium without FGF-4 did not differentiate into NeoHep cells. Cells were cultured in differentiation medium for 17 days before being injected into the

spleen of animals. Cells were trypsinized, washed twice with PBS and then diluted according to the experimental protocol with PBS. The final cell suspension volume for each animal was 0.7 ml PBS.

Cell isolation and implantation technique

Primary rat hepatocytes isolated using a two-step collagenase perfusion technique according to the standard procedure of our laboratory were used as cell source for implantation [28]. Rat hepatocytes were isolated using a modification of the in situ collagenase (sigma) perfusion technique of Seglen [29]. Hepatocytes were separated from nonparenchymal cells by differential centrifugation at 50g and then passed over a 30% percoll (Pharmacia, Piscataway, NJ) gradient at a concentration of 10^6 hepatocyte/mL Percoll to obtain a highly purified cell population. Hepatocyte purity in all three species, assessed by microscopy, was greater than 98%.

Determination of viability was done by trypan blue method counted in a Neubauer Chamber. Viability range of all isolated cells was between 67 and 88%. The cells were suspended in 0.7 ml of PBS. A 0.9% sodium chloride solution of analogous volume was used in the control groups.

Cell implantation was performed by slow injection using a 25-gauge needle connected to a 1ml syringe. For intrasplenic injection, blood flow in both splenic arteries and veins was clamped before cell injection and remained occluded for a further 5 min to avoid immediate cell passage into the portal vein. The injection site was then ligated to prevent cell leakage and bleeding [26].

Histology and DNA-hybridization

Liver tissue samples were stained with hematoxylin and eosin. In situ hybridization was performed based on a method originally described by Hengstler et al. [30]. Briefly, NeoHep cells were marked with regard to their human DNA content using human Alu DNA probe sets for in situ hybridization (Innogenex©). The hybridization leads to a complementary bond between human sequences in the tissue and the corresponding gene probe, which is FITC-marked. After incubation either with a biotinylated anti FITX antibody or with streptavidin HRP, the complementary bond colour changes to brown.

Statistics

Results were expressed as means ± SEM. Significance of differences was determined by using the Anova test. Survival was tested by log-rank test and plotted as Kaplan-Maier curve. Box-and-Whisker plots were used to describe graphically plasma levels of laboratory parameters. In all instances, $p \leq 0.05$ was considered statistically significant. Statistics were performed using the software package SPSS 13.0 (SPSS Inc., Chicago, Ill., USA).

RESULTS

Survival

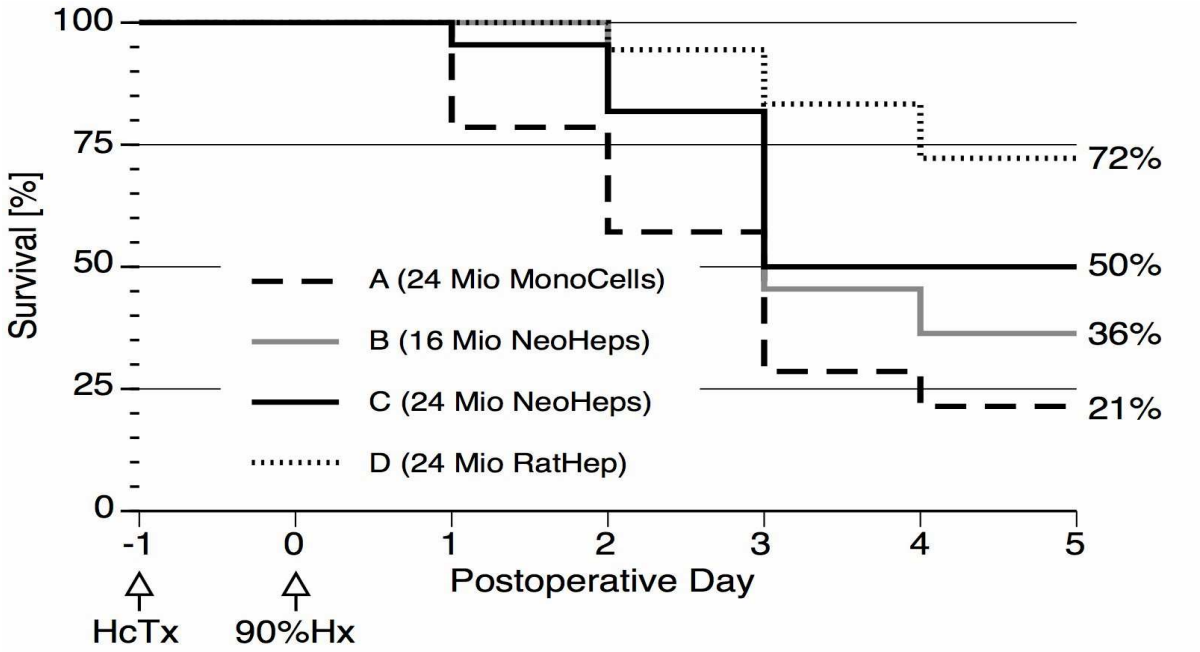
As shown in *Table 2*, the extent of resected liver mass was comparable between the groups, reaching about 7.2 to 8.3 g (*Table 2*). Following subtotal hepatectomy, 5-day survival was 21% in control animals transplanted with mononuclear cells, while mean survival was 3 days in this group (group A). Survival was considerably improved in animals treated with NeoHep cells (group B: 36%; group C: 50%, respectively), reaching the level of significance for group C ($24x10^6$ NeoHep cells; p=0.04) but not for group B ($16x10^6$ NeoHep cells; p=0.22) when compared to group A, indicating a dose-dependent effect.

Table 2: *Study groups; a subtotal hepatectomy was performed at day 0 to induce acute postoperative liver failure (n = group size; BW = body weight; ns. = not significantly different compared to group A). * = resected liver mass when compared to group A*

Groups	**Transplanted cells: amount & origin**	**n**	**Mean body weight [g]**	**Mean resected liver mass [g]**	**Relative resected liver volume [vol%]**	**Significance *p*= ***
A	24 x10^6 human mononuclear cells	14	269 ± 28	7.4 ± 0.5	2.8	
B	16 x10^6 human NeoHep cells	11	253 ± 20	7.3 ± 0.7	2.9	.97
C	24 x10^6 human NeoHep cells	22	232 ± 19	7.2 ± 0.5	3.1	.85
D	24 x10^6 rat hepatocytes	18	287 ± 13	8.3 ± 0.6	2.9	.91

Implantation of $24x10^6$ autologous rat hepatocytes (group D) revealed the best outcome, reaching 72% of survival (*Figure 4*). The significantly increased survival rate in this group (p=0.001 vs. group A) confirmed the potential of hepatocyte transplantation during acute (postoperative) liver failure as suggested by many authors [31, 32].

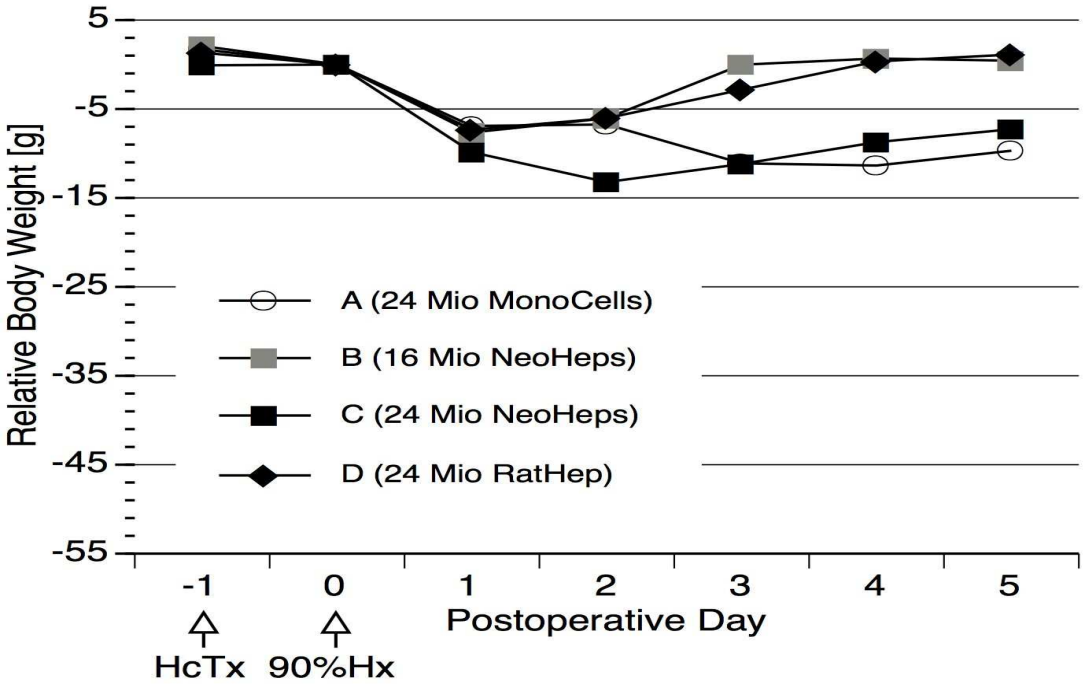
Figure 4: *Kaplan-Meier curve of 5-day survival after subtotal hepatectomy (HcTx=hepatocyte cell transplantation; 90%Hx=subtotal hepatectomy).*



Body weight

Postoperatively, all animals lost body weight when compared to preoperative levels. Although no statistically significant differences were recorded between any of the groups, we observed that animals treated with rat hepatocytes (group D) or $16x10^6$ NeoHep cells (group B) gained weight already after the second postoperative day, and reached almost their original weight at the end of the observation period, indicating improved postoperative recovery. Although less distinctive, a similar observation was made for group C ($24x10^6$ NeoHep cells), while animals in group A (only mononuclear cells) did not show any increase of body weight until the end of the observation time (*Figure 5*).

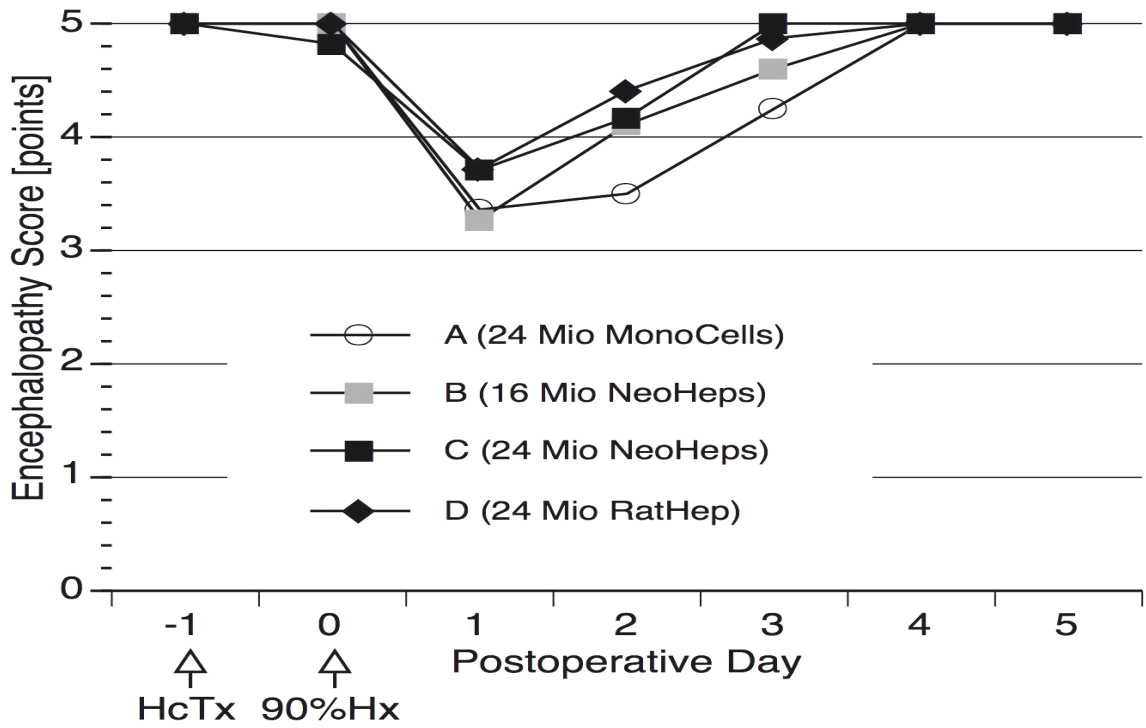
Figure 5: *Body weight relative to weight prior to subtotal hepatectomy (HcTx=hepatocyte cell transplantation; 90%Hx=subtotal hepatectomy)*.



Encephalopathy score

In all groups a descent of the encephalopathy score was noticed after liver surgery. While negative peaks occurred at postoperative day 1 in all groups, animals loaded with NeoHep cells (groups B and C) or rat hepatocytes (group D) experienced higher scores at days 2 and 3 when compared to group A (*Figure 6*). However, no significant differences were observed.

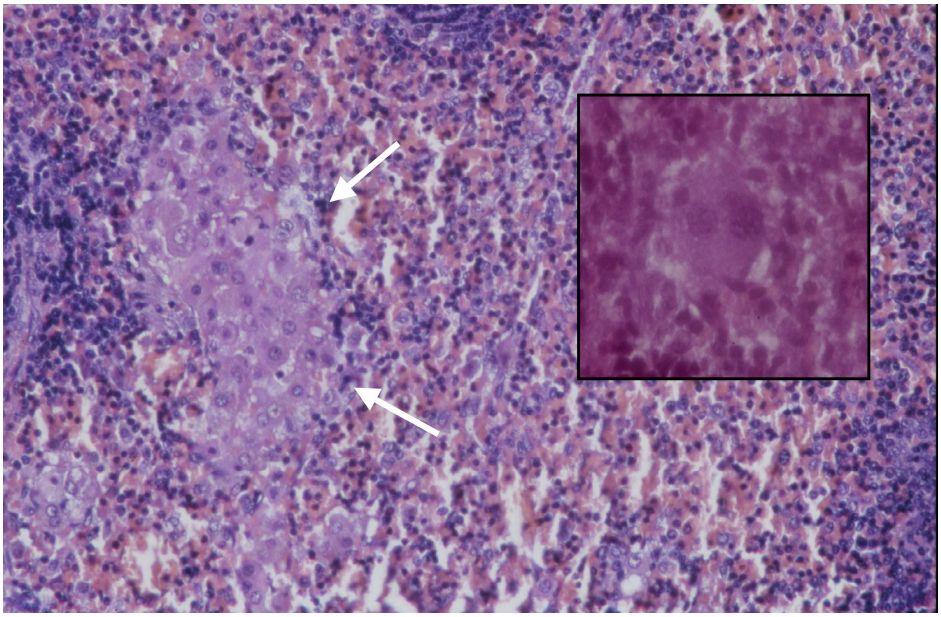
Figure 6: *Encephalopathy score after subtotal hepatectomy (HcTx=hepatocyte cell transplantation; 90%Hx=subtotal hepatectomy).*



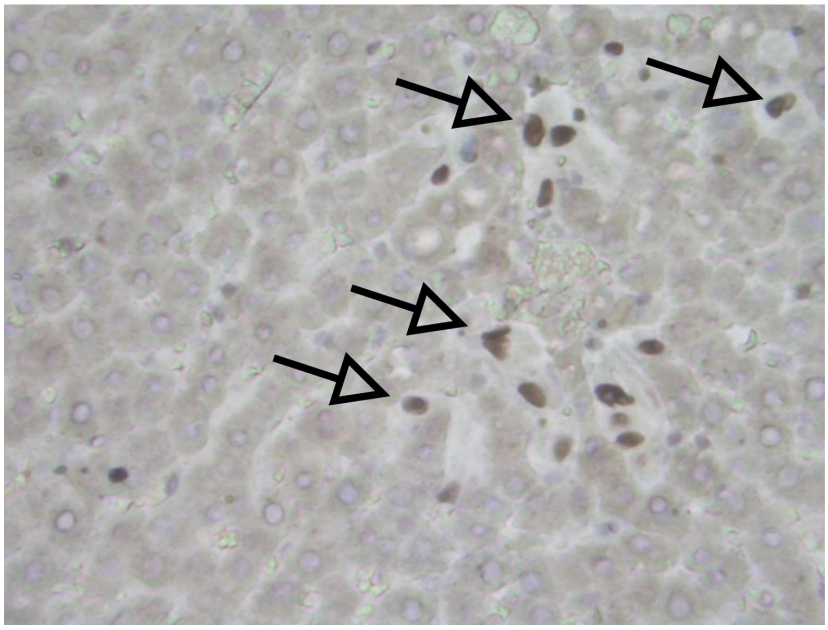
Histology

Cells morphologically characterized as hepatocytes were found within the spleen of survived animals (*Figure 7: rat hepatocyte in Group D*). In order to identify these hepatocyte-like cells as cells of human origin (NeoHep cells), DNA-hybridisation was performed. As demonstrated in *Figure 8 and Figure 9*, human DNA was found in the liver until postoperative day 1 and in the spleen until day 3 after surgery. Moreover, NeoHep cells were also found in the lung until one day after cell implantation into the spleen (*Figure 10*).

Figure 7: *Hepatocytes in the spleen 6 days after implantation of 24x10^6 primary rat hepatocytes in spleen pulpa, and 5 days after subtotal hepatectomy (HE staining, 200x and 400x).[26]*



.***Figure 8 A+B:*** *Proof of human allosequences after injection of 24x10^6 NeoHep cells into rat spleen one day prior to subtotal hepatectomy. A: group with NeoHep cells; B: control group (Liver until POD 1)*



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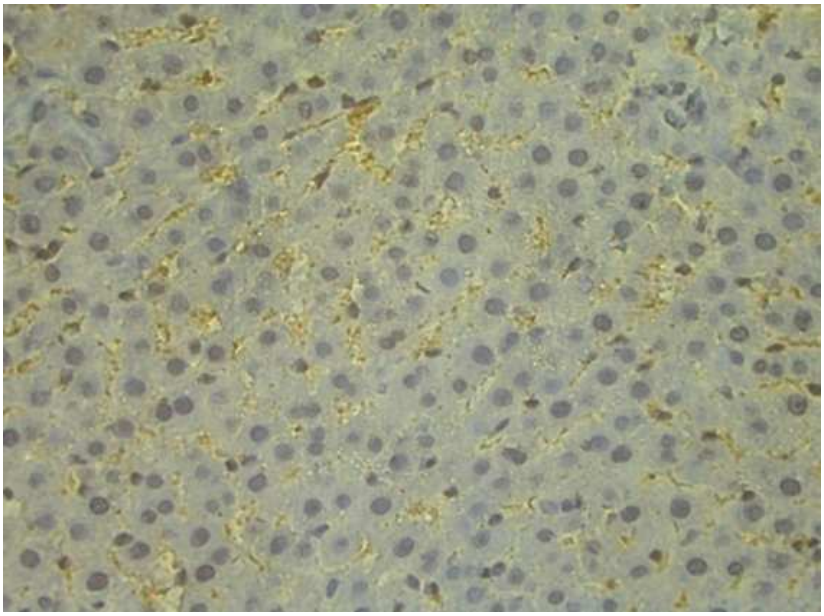
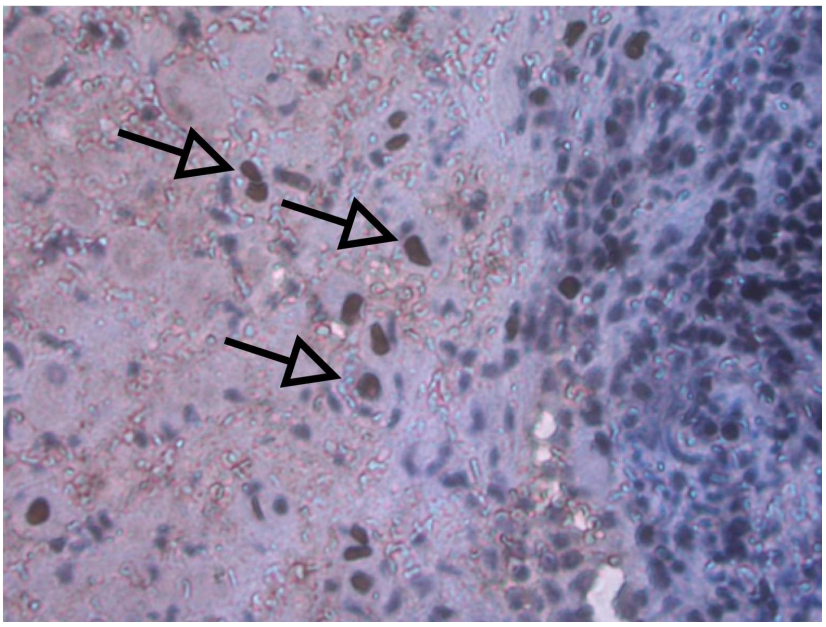


B

Figure 9 A+B: *Proof of human allosequences after injection of 24x10^6 NeoHep cells into rat spleen one day prior to subtotal hepatectomy. A: group with NeoHep cells; B: control group (Spleen until POD 3)*



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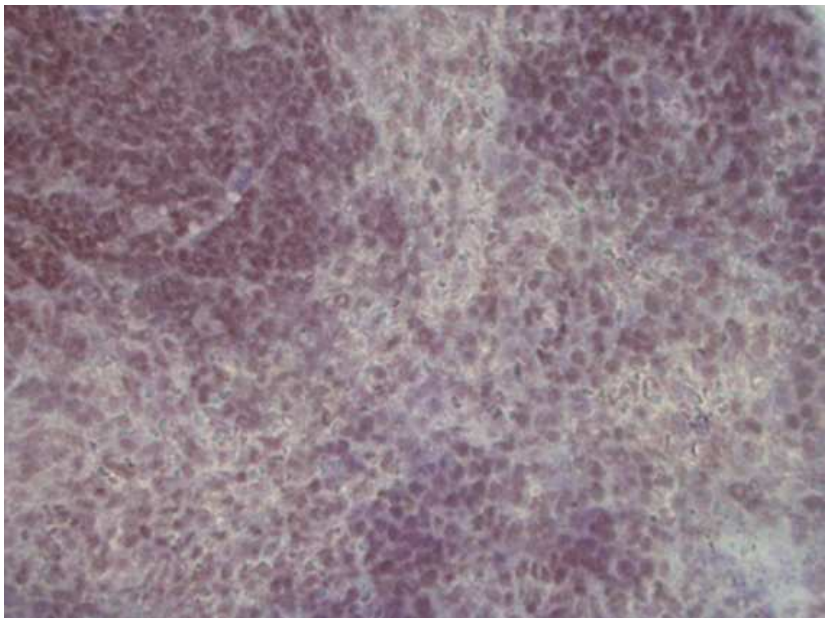
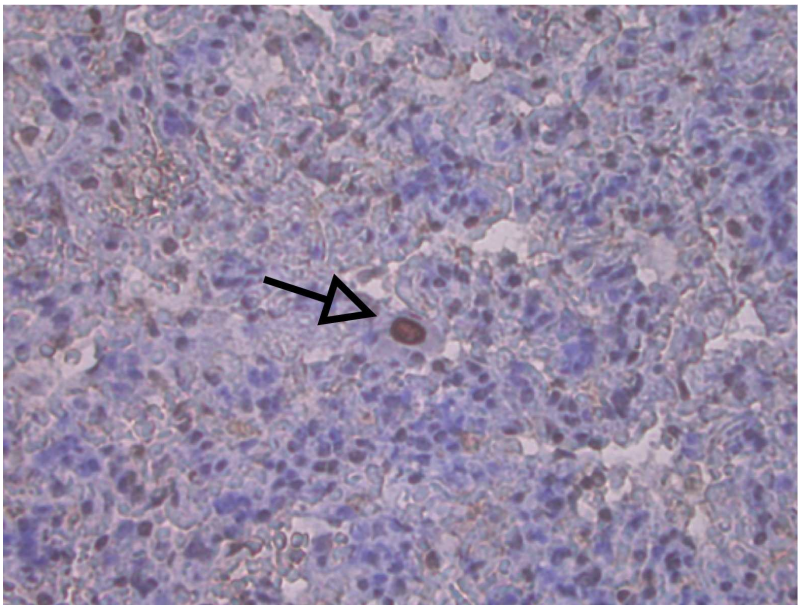
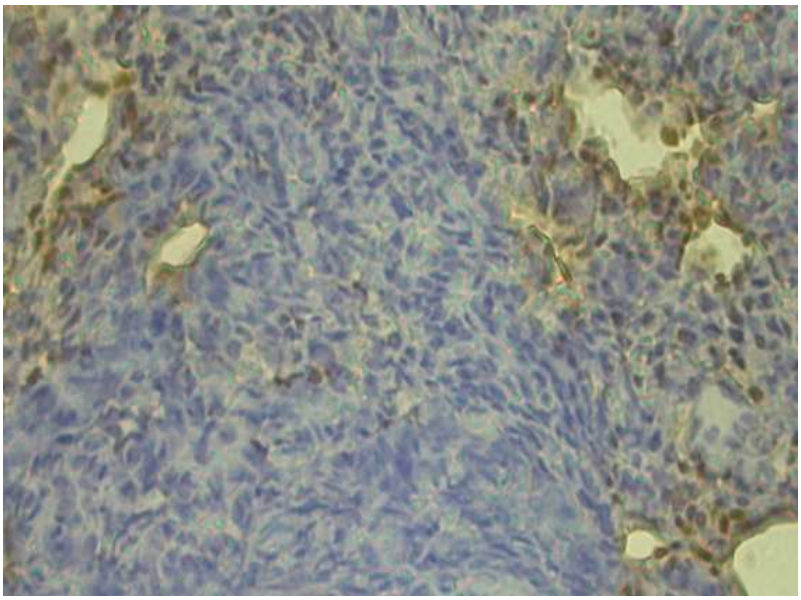


B

Figure 10 A+B: *Proof of human allosequences after injection of 24x10^6 NeoHep cells into rat spleen one day prior to subtotal hepatectomy. A: group with NeoHep cells; B: control group (Lung until POD 0)*



A

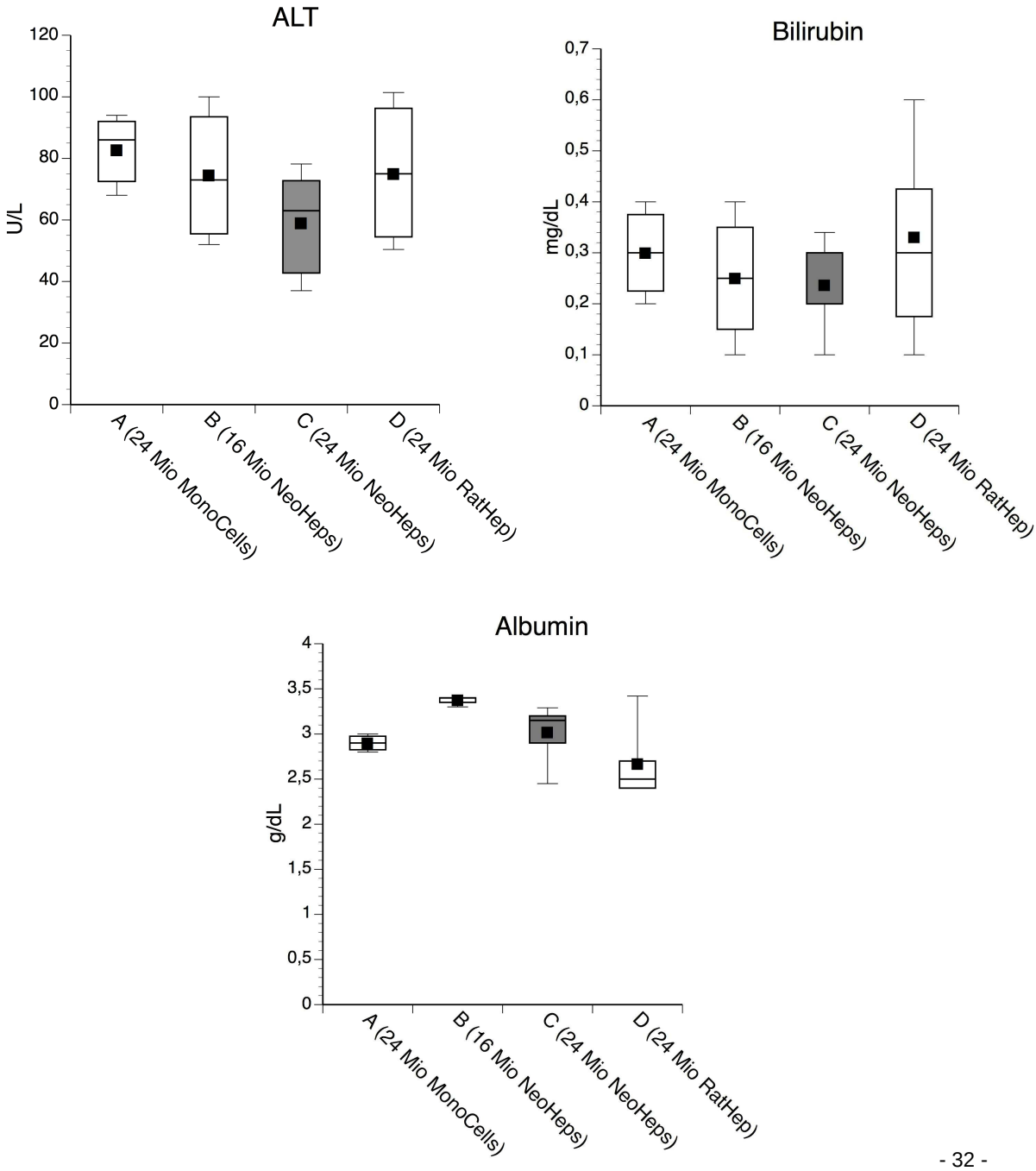


B

Laboratory findings

After the observation time the survived animals were harvested. In these animals, no statistically significantly differences were observed regarding laboratory levels such as ALT, albumin or bilirubin *(Figure 11)*.

Figure 11: *Laboratory findings of the survived animals.*



DISCUSSION

Whole or split liver transplantation has been remarkably effective in the treatment of postoperative liver failure and end-stage liver disease. However, the demand for transplantable livers is progressively outpacing the supply of donated cadaveric organs, resulting in longer waiting times and increased mortality for prospective transplant recipients [33]. Transplantation of isolated liver cells has already been considered as a 'bridge' for patients who are awaiting a donor liver [12, 31, 34, 35]. But also here, the limit is set by the critical shortage of suitable livers for isolation of liver cells. Thus, the wider use of hepatocytes to treat liver disease will not be possible until hepatocytes for cell transplantation become more readily available.

To address this issue, a novel source of hepatocytes needs to be investigated. The use of hepatocytes of xenogenic origin such as porcine hepatocytes appears to be an attractive therapeutic option because of an unlimited source of mature hepatocytes [27, 36]. However, reports of porcine endogenous retrovirus, which can infect human cells, halted the development so far [37]. Ongoing exciting areas of investigation involve the study of fetal hepatocytes, liver stem cells isolated from adult livers, embryonic or umbilical cord stem cells, and hepatocytes conditionally immortalized by gene transfer [12, 32, 38-40].

Monocyte-derived hepatocyte-like cells were first generated from terminally

differentiated peripheral blood cells and named NeoHep cells. Transformation of in-vitro-modified human peripheral blood monocytes into these hepatocell-like cells (NeoHep cells) established a new cell source that expresses several hepatocyte-specific markers [22]. Moreover, initial results have proved the detoxification and synthesis of various liver specific factors of NeoHep cells comparable to those of primary hepatocytes [22, 23]. NeoHep cells might represent an important alternative to liver-bridging devices, allowing patients to benefit from the regenerative potential of this virtually unlimited cell source (monocytes) and in particular from the simple and rapid technology to procure sufficient amounts of these cells, and rejection will not occur in planned operations [22]. Thus, the theoretical consideration in this study was to give proof of the concept of implanting NeoHep cells instead of primary hepatocytes to evaluate *in vivo* their efficacy in terms of postoperative liver support in the presence of acute liver failure.

Using a model of subtotal hepatectomy we first confirmed that hepatocyte transplantation per se is beneficial in order to prevent death from postoperative liver failure. The survival rate was significantly increased reaching 72% compared to resected, non-treated control animals (21%). With respect to the use of NeoHep cells, we secondly observed that implantation of this cell composition was very effective in prevention of postoperative death. Indeed, the implantation of NeoHep cells significantly increased animal survival up to 50% in a dose-dependent matter. The higher level of survival in an autologous

cell transplant setting was not unexpected since no immunological components were involved.

Using DNA hybridisation, cells of human origin were detectable until day 3 after surgery, or day 4 after implantation of NeoHep cells, respectively. Morphologically, cells analogous to hepatocytes were present in the spleen until day 5 after surgery. Although we have not found differences in liver function of the surviving animals, we believe that this was not surprising, since laboratory analyses were performed only in the survivors. Nevertheless, the data showed a significant increase of survival, which is one of the most important criteria.

Overall, we observed that NeoHep cells tremendously improved the short-term outcome after surgically induced liver failure, while transplantation with undifferentiated monocytes showed no change in survival. In the same line of evidence, Hutchinson et al. recently suggested that monocyte-derived cells could directly complement lost hepatic functions [41]. Although speculative since the exact mechanisms are still unknown, he suggested that the beneficial effects are perhaps generated by acquiring metabolic capabilities usually only associated with hepatocytes, or directly by contributing to the liver parenchyma, either by a process of transdifferentiation or by fusion with hepatocytes. Alternatively, monocyte-derived cells may exert an indirect effect through the suppression of inflammatory and fibrotic processes, by supporting hepatocyte

regeneration or by promoting angiogenesis [41].

Circulating CD14+ monocytes which originate from hematopoietic stem cells in the bone marrow, consist of 5 to 10% of circulating white blood cells in humans. They are a heterogeneous population in terms of surface markers, phagocytic capacity, and differentiation potentials, but are committed precursors in transit from the bone marrow to ultimate sites of activity. Circulating monocytes have the capacity to differentiate into a variety of phagocytes, including macrophages, dendritic cells, osteoclasts, microglia in the central nervous system, and Kupffer cells in the liver [42]. Furthermore, it was demonstrated that human monocytes can differentiate into chondrocyte-like cells [43] or endothelial-like cells [44]. Seta et al. reported a primitive human cell population called monocyte-derived multipotential cells (MOMC), which has a fibroblast-like morphology and a unique phenotype positive for CD14, CD45, CD34, and type I collagen. This novel cell type exhibits mixed morphologic and phenotypic features of monocytes, endothelial cells, and mesenchymal cells. These cells were demonstrated to contain progenitors with capacity to differentiate into a variety of non-phagocytes, including bone, cartilage, fat, skeletal and cardiac muscle, neuron, and endothelium [42].

Irrespective of underlying mechanisms or cellular interactions, monocyte-derived cells have already been shown to improve cardiac function after myocardial infarction in a rat model [45], and to mitigate

streptozotocin-induced diabetes in mice [23]. Recently, Yan et al. reported for the first time that an infusion of a monocyte-enriched suspension of autologous peripheral blood mononuclear cells via the hepatic vein into two patients with HBV-related cirrhosis was associated with a sustained improvement in the clinical condition of both individuals [46].

All these observations indicate that circulating monocytes are more multipotential than previously thought. Additionally, transplantation therapies using cells derived from circulating monocytes are a potential approach for tissue regeneration and might be applied in many kinds of such related disease because of their easy availability.

Nonetheless, the question of natural cell death in stable and live hosts arises since our model was equal to xenogenic transplantation. Beside the need for additional application of immunosuppressants, several further questions such as cell trafficking, homing, optimal amount of cells transplanted into the host, or site of cell implantation have to be answered before NeoHep cells may be implemented to clinical situations.

Undoubtedly, if the beneficial effect of Neohep cell transplantation is confirmed by more extensive studies, its clinical application would have a prosperous future.

In conclusion, the mighty possibilities which are gained from monocytes cannot be overrated. Monocyte-derived cells such as NeoHep cells may become

available precisely at the time of need, for example by cryopreservation techniques. ‘Own cell donation’ prior to surgery, analogous to (already well clinically established) own blood donation, best characterizes the potential of autologous monocyte-derived hepatocyte-like cells. They may theoretically serve as temporary bridging for liver transplantation, they may serve as an alternative treatment in acute liver failure due to various intoxications or even after extended liver resection, or they may above all serve as a true alternative to orthotopic liver transplantation.

SUMMARY

OBJECTIVE: Investigation of the efficacy of implantation of monocyte-derived hepatocyte-like cells (NeoHep cells) in acute liver failure.

SUMMARY BACKGROUND DATA: Extended liver resection or split liver transplantation is still associated with high morbidity and mortality due to postoperative liver insufficiency. In view of liver support systems, implantation of isolated hepatocytes or hepatocyte-like cells such as NeoHep cells is increasingly under discussion.

METHODS: 24 hours prior to subtotal hepatectomy, cells of different origin (A: human mononuclear cells ($24x10^6$); B: NeoHep cells ($16x10^6$); C: NeoHep cells ($24x10^6$); D: rat hepatocytes ($24x10^6$)) were injected into the spleen of male Wistar rats. Following an observation period of 5 days, animal survival, postoperative weight, and signs of encephalopathy were recorded. At the end of the observation period, blood was collected for laboratory analysis (serum levels of ALT, bilirubin, albumin).

RESULTS: Transplantation of both rat hepatocytes and NeoHep cells significantly improved animal survival when compared to control animals (group A: 21%), reaching 72% in group D (p=.001), 50% in group C (p=.04), and 36% in group B (p=.22). Moreover, animals in these groups postoperatively experienced less frequently signs of encephalopathy, they also showed an

earlier weight increase when compared to group A.

CONCLUSION: Hepatocyte transplantation is a practicable and successful treatment option in the case of liver insufficiency since implantation of NeoHep cells or primary rat hepatocytes had an improving effect on survival. Based on this study, we believe that pre-treatment of patients with autologous monocyte-derived hepatocyte-like cells (NeoHep cells) by ‘own cell donation’ may represent an effective tool to markedly reduce morbidity and mortality due to postoperative liver failure.

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CURRICULUM VITAE

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

DECLARATION (ERKLÄRUNG)

„Ich, Baomin Shi, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema:

Transplantation of Monocyte-derived Hepatocyte-like Cells (NeoHep cells) Improves Survival in A Model of Acute Liver Failure

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