Hepatocyte Transplants Improve Liver Function and Encephalopathy in Portacaval Shunted Rats

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Keywords

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SUMMARY

Aim: Rats with portacaval shunt (PCS) are useful experimental models of human hepatic encephalopathy in chronic liver dysfunction. We have previously shown that PCS modifies amine neurotransmitter systems in the CNS and increases voluntary alcohol intake by rats. Hepatocyte transplantation, used in acute liver failure, has recently also been applied to chronic liver diseases, which prompted us to investigate whether the altered brain amine system and the drinking behavior in long-term shunted rats could be normalized by hepatocyte transplants. **Methods:** Hepatocytes, isolated from syngeneic donors by collagenase digestion, were injected (3×10^6 cells/rat) into the pancreatic tail region, 6 months after PCS. Hepatic function was evaluated by measuring urine urea and plasma L-histidine concentrations. A free choice test with two bottles (tap water and 10% ethyl alcohol) was performed for 3 days to assess the rats' preference for alcohol. The rats were euthanized 2 months posttransplantation. Brain histamine and 5-hydroxyindoleacetic acid (5-HIAA) levels were measured by radioenzymatic assay and by HPLC-EC, respectively, *N*-*tele*-methylhistamine by GC/MS while MAOA and MAOB activities by isotopic procedures. **Results:** Portacaval shunt rats with hepatocyte transplants gave more urea than before transplantation, with lower plasma L-His levels and higher body weight versus the PCS counterparts. Also, those rats consumed less alcohol. The CNS amines and 5-HIAA concentrations, as well as MAO-B activity, being abnormally high in untreated PCS rats, significantly reduced after PCS hepatocyte treatment. **Conclusions:** The results support the therapeutic values of hepatocyte transplants in chronic liver diseases and the temporary character of PCS-exerted CNS dysfunctions.

Introduction

End-to-side portacaval shunt (PCS) diverts portal blood flow from the liver directly to systemic circulation and drastically reduces the participation of the organ in body metabolic processes. All substrates absorbed in the gastrointestinal tract, among them noxious ones, appear in systemic circulation. In humans, a consequence of PCS operation is the development of neuropsychiatric disturbances, termed hepatic encephalopathy (HE) [1,2]. HE also develops as a result of hepatic dysfunction; liver cirrhosis is the most common cause of HE [3–5].

There is a high degree of similarity in biochemical changes found in body fluids and/or organs of portacavally shunted mammals, with no species differences, allowing the use of animal models in research on human HE [3,6]. The rat PCS model is widely

used for the study of the effects of chronic liver disorders on brain function and metabolism. It reflects the B type of HE, according to the classification established in 1998 during the 11th World Congress of Gastroenterology [2,3]. PCS surgery in rat leads to liver atrophy without organ failure.

As a result of an enhanced concentration of aromatic amino acids (i.e., tryptophan, phenylalanine, histidine) in blood, which are actively transported to the brain [7] in the central nervous system (CNS) of PCS subjects, an excessive synthesis of biogenic amines occurs, which concerns both amine neurotransmitters (serotonin, 5-HT, dopamine, DA, histamine, HA) and trace amines (i.e., octopamine) [6,8,9].

We have demonstrated two mechanisms counteracting the amine excess in PCS rat brains—an increased catabolism and cellular deposition [10–13]. The first one is typical for 5-HT and DA,

as evidenced by increased cerebral indoleamine and catecholamine metabolite levels with no change in the parent compounds [10]. The second mechanism, cellular deposition, is unique for the histaminergic system [12,13].

In the mammalian brain, histamine is synthesized from L-histidine by histidine decarboxylase (HDC, EC 4.1.1.22) and metabolized exclusively by histamine N-methyltransferase (HNMT, EC 2.1.1.8) to *N*-*tele*-methylhistamine (*t*-MeHA), which is subsequently oxidized by the monoamine oxidase form B (MAO-B) to aldehyde intermediate and further oxidized to the histamine end product, *N*-*tele*-methylimidazoleacetic acid [14]. There is a huge rise in the central histamine concentrations in rats with PCS, especially in the hypothalamus, brought about by a better saturation of histidine decarboxylase with L-His substrate [6]. Neurons, but not mast cells, have been identified as cells which store excess histamine under these circumstances [12,13]. In addition to the deposition, activation of a histamine catabolic pathway occurs, as there is a rise in the cerebral *t*-MeHA concentration [10,12,13] and a parallel increase in MAO-B activity [11,12].

A characteristic feature of liver insufficiency is the development of an enhanced voluntary alcohol intake, as has been shown in two models of hepatic dysfunction, that is, PCS [15–17] and thioacetamide-induced liver cirrhosis [17–19]. Moreover, a correlation between an improvement of liver function and a decrease in the aberrant alcohol consumption has been demonstrated in cirrhotic rats after liver regeneration induced by partial hepatectomy [17], or following treatment with hepatotrophic factors [18]. All this, therefore, makes voluntary alcohol intake a suitable behavioral measure of liver function [17–18].

Hepatocyte transplantation is an efficient way of prolonging survival in humans and animals with acute liver failure and has been claimed to be of therapeutic value also in chronic liver diseases, including metabolic, inherited disorders [20–26]. Liver cell transplantation is an alternative technique for orthotopic organ transplantation, which is limited due to the great shortage of donated organs. Numerous studies performed on animal models clearly indicate the normal hepatic function of hepatocytes transplanted into different organs (spleen, pancreas, portal vein, mesentery) as well as showing that these cells can remain functional for the entire lifetime of the recipient [20–23]. There is an indication coming from experimental studies that hepatocyte transplantation may reverse the changes in brain metabolism and function caused by liver failure. The last postulate was based on the biochemical and behavioral studies' findings of neurological disorder prevention in portacavally shunted rats that had undergone hepatocyte transplantation [27,28].

Our preliminary data concerning the transplantation effects on the CNS aminergic systems in PCS rats [28,29] prompted further investigation on long-term shunted rats to determine whether the biochemical alterations and drinking behavior could be normalized in these animals.

Materials and methods

Animals and Treatment

All experimental procedures were performed in accordance with EU directives and approved by the local ethics committee.

Adult male inbred Lewis rats (body weight: 200–300 g) were used. End-to-side portacaval shunts were performed under ether anesthesia according to the method of Lee and Fisher, 1961 [30]. Sham-operated animals underwent laparotomy and occlusion of the portal vein for 10 min. Following surgery, the rats were housed in standard cages with a 12-h light/dark cycle (lights off at 7 p.m.) and had free access to food and water.

Hepatocytes were isolated from syngeneic donors by *in situ* liver collagenase digestion as described by Seglen [31] and administered to the animals with an opened abdomen under general anesthesia as a single injection (3×10^6 cells/rat) into tail region of the pancreas with 22 gauge needles, 6 months after PCS surgery.

Evaluation of Liver Function

Biochemical Markers

Urea concentration in urine was monitored as a biochemical marker of the liver function [32]. Urine collection was performed in metabolic cages (Techniplast Gazzada, Italy), where rats had free access to tap water and standard food (Animal Food Factory, Motycz, Poland). The fluid and feed consumption and urine output were recorded, and the mean values were used for daily values expression.

Plasma histidine concentration was assayed according to Ambrose *et al.* [33].

Behavioral Assessments

Voluntary alcohol intake was evaluated in free choice tests, as described earlier [17–19]. The tests were performed before and 3 weeks after hepatocyte transplantation. Rats were kept individually in metabolic cages, equipped with two bottles, one contained a solution of water with 10% ethyl alcohol and the other tap water. They also had free access to food. The test lasted 3 days. Each day, the volumes of fluids consumed were recorded.

PostMortem Analyses

The rats were euthanized by decapitation 2 days after the end of the final choice test. Truncal blood was taken on sodium citrate and plasma obtained by centrifugation. The brain structures, that is, cortex, hypothalamus, striatum, midbrain, medulla oblongata, and cerebellum, were dissected according to the method by Glowinsky and Iversen [34] and immediately frozen in liquid nitrogen. The dissected brain subregions included more than one distinct anatomical structure. The *Striatum* corresponds to the putamen, caudate, and globus pallidus nuclei. The *Cortex* contains the telencephalon without the *Striatum* and includes white and gray matter of the cerebral cortex. The *Midbrain* represents midbrain, hippocampus, thalamus, and subthalamus, whereas the *Medulla oblongata* corresponds to the medulla oblongata and pons.

The pancreas was excised for histology. It was fixed by overnight immersion in phosphate buffered with 10% formalin for further processing and fixed in paraffin wax. Three micrometer thin sections were cut from each paraffin block, and slides were routinely stained with hematoxylin and eosin dyes on glass slides

for histopathological evaluations [35]. For quantitative analysis, MultiScanBase v. 8.08 Image Analysis System (CSS, Ltd., Warsaw, Poland) was applied.

Activities of monoamine oxidase A and B forms were measured in the hypothalamus and cerebral cortex by radiometric methods. Labeled ^{14}C serotonin (fin conc. 200 μM) in the presence of MAO-B inhibitor, deprenyl (fin conc. 0.3 μM), was used for MAO-A, while ^{14}C β -phenylethylamine (fin conc. 20 μM) and clorgyline (fin conc. 0.3 μM), MAO-A inhibitor, were employed for MAO-B [36]. The enzyme activities are expressed as pmol/min/mg protein.

Histamine concentration was measured in all dissected brain regions using a radioenzymatic assay according to Taylor and Snyder [37], whereas indoles, serotonin, and 5-hydroxyindoleacetic acids were estimated by HPLC with electrochemical detection as previously described [10]. The injection, data collection, and sample analysis were carried out automatically by Millennium 2010 Chromatography Manager software (Waters, Milford, MA, USA). *N*-*tele*-methylhistamine concentration was estimated in brain structures as bis-heptafluorobutyl derivative with deuterated-methylhistamine used as an internal standard. The isolation, derivatization, and measurements were performed by the method of Hough et al. [38] modified by Tuomisto et al. [39]. GC/MS analyses were performed on a GCQ Finnigan MAT Instrument (ThermoQuest Finnigan Corporation, Austin, TX, USA).

Protein concentration was analyzed according to Lowry's method [40].

Statistical Analysis

The values are presented as mean $\pm$ SEM. Differences between groups were assessed with the paired *t*-test or one-way ANOVA, followed by the Student–Newman–Keuls test or multiple comparisons tests, as appropriate. All statistical analyses were performed using GraphPad 6.0 Prism program (GraphPad Software, Inc., San Diego, CA, USA). *P*-value of 0.05 or less was considered significant. In the table and figures, a single symbol always means $P < 0.05$, whereas two and three symbols mean $P < 0.01$ and $P < 0.001$, respectively.

Results

Liver Function and Voluntary Alcohol Intake in PCS Rat

The PCS surgery eliminates the liver from metabolic functions and reduces blood flow through the liver limiting the supply of hepatotrophic factors and, as a result, leads to atrophy of the organ. As can be seen from the results given in Figure 1, the shunted rats were characterized by decreased urinary urea output (over 50%) and those ones that had undergone hepatocyte transplantation (PCS + HEP group) tended to excrete more urea in the urine (0.05–0.10 g/24 h), compared to the results obtained before the treatment (0.04–0.08 g/24 h) (Figure 1B; paired *t*-test, $P < 0.05$). These rats also gained more weight than their untransplanted counterparts (data not shown).

The PCS rats had a significantly higher plasma L-histidine concentration (Table 1). It was increased by roughly half after

Figure 1 The effect of portacaval shunt (PCS) in rat and subsequent hepatocyte transplantation on liver function as measured by daily urinary excretion of urea. (A) Median (the line in the middle of box) and the range of values (whiskers) are given. Insert (B) shows urea concentration in the same PCS rats before- and after hepatocyte transplantation. One-way ANOVA and Tukey's multiple comparisons test, *** $P < 0.001$ versus control; paired *t*-test, # $P < 0.05$ versus before HEP. HEP, hepatocyte transplantation; wk, weeks.

Table 1 Plasma L-histidine concentration in PCS rat; the effect of hepatocyte transplantation

Group	Plasma L-histidine concentration (nmol/mL)
Control	33.0 $\pm$ 9.2
PCS	51.9 $\pm$ 8.2**
PCS + HEP	38.8 $\pm$ 6.5

PCS, portacaval shunted rat; HEP, hepatocyte transplantation. The values are mean $\pm$ SEM. One-way ANOVA and Tukey's multiple comparison test, ** $P < 0.01$ versus control.

portacaval shunting and returned to the control values in hepatocyte-transplanted rats when checked at the time of their euthanasia (i.e., 2 months posttransplantation).

Free choice tests revealed that voluntary alcohol intake displayed by portacavally shunted rats was significantly higher than that by sham-operated rats (Figure 2A; one-way ANOVA and Newman–Keuls multiple comparison test, $P < 0.001$). For example, PCS rats, 13 weeks after the operation, drank 8.48 ± 0.83 mL of 10% ethanol per 100 g of body mass daily, while control rats consumed <1 mL (0.89 ± 0.26 mL/100 g of body mass). The PCS rat also showed a significantly increased total fluid intake (12.91 ± 0.63 mL/100 g vs. 8.98 ± 0.51 mL/100 g body mass for control), and a higher proportion of this was ethyl alcohol. The contribution of ethanol to total fluid consumption was within the range 41–94% for the rats 13 weeks after shunting (average value: $67.17 \pm 9.08\%$) while only 0–21% for the control rats (average value: $9.91 \pm 2.61\%$) (Figure 2B). Transplantation of hepatocytes to PCS rats lowered their alcohol consumption as expressed in terms of percent contribution of ethanol to the total fluid intake (Figure 2B) as well as in mL of alcohol consumed (Figure 2A).

Figure 2 Voluntary alcohol consumption by Lewis rats with liver atrophy evoked by portacaval shunt surgery (PCS); the effect of hepatocyte transplantation. Fluid consumption was recorded daily. The bars represent means $\pm$ SEM, taken during the 3-day choice test. One-way ANOVA and Newman–Keuls multiple comparison test, $P < 0.001$: *** versus control, ### versus PCS 13 wk. ETOH, 10% ethanol, ***versus PCS 10 wk; HEP, hepatocyte transplantation; wk, weeks.

Histological and Biochemical Examinations

Histological examination of pancreases revealed hepatocyte-like cells in the organ (Figure 3).

Figure 3 Representative histology showing the presence of hepatocytes in the tail region of pancreas; hematoxylin and eosin (H&E)-stained section taken 2 months after transplantation, carried out using bright-field light microscopy. **(A)** Microphotograph of the H&E-stained paraffin section of the implantation site of hepatocytes in pancreas, showing cluster of hepatocytes in retroperitoneal space; original magnification: 40 $\times$. **(B)** Normal pancreas and hepatocytes with numerous bile dots in the neighborhood area; original magnification: 400 $\times$. **(C)** The peri-insular location of hepatocytes within the pancreas. Hepatocytes in a crescent around islet of Langerhans; original magnification: 400 $\times$. **(D)** The group of large hepatocytes and few scattered inflammatory cells, predominantly lymphocytes; original magnification: 400 $\times$.

Further confirmation that transplanted hepatocytes were metabolically active was obtained from the biochemical evaluation of the brain amine systems.

The changes in histamine, *N*-*tele*-methylhistamine, and 5-hydroxyindoleacetic acid concentrations evoked by portacaval shunt in various brain regions and the effect of hepatocyte transplantation are shown in Figure 4. To make comparison easier, as the structures varied in the concentrations of the compounds, the data were expressed as folds of the normal levels.

In all of the examined brain regions, PCS evoked statistically significant HA increases, visibly attenuated by hepatocyte transplantation (Figure 4A). In the hypothalamus, PCA rats exhibited *ca* 20-fold higher HA concentration compared with the control values, whereas PCS + HEP only *ca* 6-fold (one-way ANOVA and Tukey's multiple comparisons test, $P < 0.001$). Similarly, in the striatum and cerebral cortex, there was a 4- and above 2.5-fold increase in amine concentration after PCS, respectively, while in PCA + HEP rats, the corresponding values were about 3 and 1/3 above the control level (Figure 1A; $P < 0.01$ or $P < 0.001$ as appropriate); minor differences ($P < 0.05$) were recorded in medulla oblongata and cerebellum.

In the hypothalamus of the PCS + HEP group, the reduced concentration of histamine was accompanied by a statistically significant lower concentration of amine's metabolite, *N*-*tele*-methylhistamine (Figure 4B, $P < 0.01$). Hepatocyte transplantation, however, did not affect the t-MeHA concentrations in the cerebral cortex; in the PCS + HEP group, it still exceeded more than 1.5 times the control level.

The PCS rats had normal levels of serotonin (data not shown), but, in all examined brain regions, elevated concentrations of 5-hydroxyindoleacetic acids (*ca* 2- to 3-folds of control) were detected (Figure 4C). The rats subjected to hepatocyte transplantation exhibited a significantly lower 5-HIAA concentration in the hypothalamus, striatum, and midbrain ($P < 0.01$ or $P < 0.05$,

Figure 4 Histamine (HA), N-tele-methylhistamine (t-MeHA) and 5-hydroxyindoleacetic acid (5-HIAA) concentration in the CNS; the effects of portacaval shunt (PCS) and hepatic function improvement by syngeneic hepatocyte transplantation. The data, means $\pm$ SEM, were expressed as folds of the control levels. One-way ANOVA and Tukey's multiple comparisons test, * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$ versus PCS. HEP, hepatocyte transplantation; Med. obl., medulla oblongata.

respectively) with a tendency to decrease in the cerebral cortex and medulla oblongata.

Figure 5 presents the effects of PCS surgery and hepatocyte transplantation on brain monoamine oxidases A and B activity. Portacavally shunted rats had significantly higher MAO-B activity in both examined brain structures, that is, in the hypothalamus and cortex ($P < 0.05$), while the activity of MAO-A was not significantly different from control values. Hepatocyte transplantation in PCS rats normalized MAO-B activity in the cortex, while in the

Figure 5 The effect of portacaval shunt (PCS) and syngeneic hepatocyte transplantation on cerebral MAO-A and MAO-B activity. The values are mean $\pm$ SEM. One-way ANOVA and Tukey's multiple comparisons test, * $P < 0.05$ versus corresponding control, ## $P < 0.01$ versus corresponding PCS group. HEP, hepatocyte transplantation.

hypothalamus, only a downward tendency was noted (Figure 5, right panel). With respect to MAO-A activity, hepatocyte-treated rats (PCS + HEP) did not differ from their untreated counterparts (PCS rat) (Figure 5, left panel).

Discussion

Previous studies clearly demonstrated that transplanted hepatocytes perform synthesizing and detoxification functions, similar to the whole liver [20–26]. Various sites can house hepatocytes and they have been transplanted intraportally [41], into the peritoneal cavity [42], mesentery [20,21], spleen [22,23,42], lungs [43], and subcutaneously [44].

We chose the tail region of the pancreas, as this organ is a source of important hepatotrophic factors [18,45]. Indeed, histological examination confirms the presence of hepatocyte-like cells in pancreas tissue specimens (Figure 3). They must have been metabolically active as evidenced by an increase in urea output (Figure 1), the normalized plasma level of L-histidine (Table 1), and higher weight gain in PCA hepatocyte-transplanted rats in comparison with untreated PCS animals. These findings are in line with previous observations from experimental and clinical studies [22–26]. Hepatocyte transplantation was shown to reduce biochemical abnormalities evoked by different liver injuries caused by experimental procedures and to increase survival time. Restoration of the liver function has positively affected brain metabolism. For example, transplantation of hepatocytes into the spleen of cirrhotic rats resulted in normalization of prothrombin time, serum albumin, and bilirubin levels, prevented the development of hepatic encephalopathy as assessed by behavioral scoring and prolonged their survival while protecting against hyperammonemia in PCS rats [22,27]. Substantial reduction in blood ammonia levels and prothrombin time, and an improvement in HE score after intrahepatic or intrasplenic hepatocyte transplantation have also been found in humans with acute liver failure accompanied by severe encephalopathy (grade III to IV). This clinical improvement was verified by *postmortem* histopathological

examination, which showed the presence of liver cells at the site of transplantation [46].

Chronic hepatic dysfunction is accompanied by abnormalities in the amino acid profile in plasma. The increased concentrations of L-tyrosine and phenylalanine, aspartate and L-histidine are accompanied by decreased levels of leucine and isoleucine [6,7,47,48]. These changes in the plasma amino acids pattern, in addition to ammonia, are postulated to be involved in an imbalance in the CNS neurochemistry which is responsible for neuropsychiatric disturbances associated with hepatic dysfunction. Thus, alterations in multiple neurotransmitter systems have been implicated in brain disorders leading to the development of hepatic encephalopathy [1,3,8,9].

Portacaval shunt rats exhibited increased cerebral concentrations of serotonin, its precursor, tryptophan [49], and metabolite, 5-HIAA [17,49]. These biochemical changes were significantly correlated with the degree of liver insufficiency, that is, arterial ammonia levels [17,49].

Chronic liver failure is also associated with the dysfunction of the cerebral histaminergic system as amply documented by us and others. The increased brain concentrations of histamine and its metabolite, N-tele-methylhistamine, have been found in PCS rats [6,8,10–13,15,50] as well as in autopsied brains from cirrhotic patients who died in hepatic comas [51].

Normalization of plasma amino acid precursors of biogenic amines as evidenced here for L-His (Table 1) was associated with the lower concentration of histamine, N-tele-methylhistamine, and 5-hydroxyindoleacetic acid (Figure 4) as well as the activity of MAO-B (Figure 5, left panel) in the brain of hepatocyte-transplanted portacavally shunted rats.

Our results demonstrating a decrease in brain levels of the substances in question in PCS rats with hepatocyte transplants indicate that the changes evoked by portacaval shunt in the CNS aminergic system are potentially reversible.

We could confirm earlier findings that in advanced liver insufficiency (8 months after PCS surgery), (i) there is also a significant increase in N-tele-methylhistamine concentration, although not as dramatic as that of histamine, exceeding roughly 50% the normal t-MeHA level (Figure 4) and (ii) an increase in MAO-B activity (Figure 5, left panel).

Also, the present data, demonstrating an overconsumption of alcohol in PCS rats, are in agreement with our earlier studies in which we have shown a similar effect in rats with thioacetamide-induced liver cirrhosis [17–19].

Due to hepatocyte participation in body metabolic activity and the reinstatement of the amino acid profile, voluntary alcohol intake was also reduced. Our present results are consistent with the study by Martin et al. [52], who revealed that providing PCA rats with corn supplement or restricting these rats to a corn-only diet reduces or completely eliminates the overconsumption of ethanol by these animals. As restriction to the corn diet reduces brain concentrations of tryptophan and 5-hydroxyindoleacetic acid, these results confirm the hypothesis that the normalization of plasma branched-chain and aromatic amino acids profile reduces some of the abnormalities associated with hepatic dysfunction [52].

Experimental data suggest that hepatocyte transplantation is an effective therapeutic method in chronic liver failure [22, for review: 24]. The present findings are an extension of our former results and are in good agreement with them. We have previously observed that as long as the liver is able to control the concentration of plasma amino acids, as exemplified by L-histidine concentration and tested in mesocavally shunted rats [53], no alterations in CNS amine systems are present and voluntary alcohol intake is unchanged from the normal. In addition, we demonstrated also that liver regeneration is an effective way to diminish abnormal voluntary alcohol intake evoked by liver insufficiency associated with thioacetamide-induced liver fibrosis [17–18]. Thus, the other prominent effect of the improvement in liver function was the decreased interest in voluntary alcohol consumption.

Taken all together, the present results demonstrate that the alterations in the amine systems in brain after portocaval shunt are not permanent; they are potentially reversible, dependent on liver function improvement, which can be achieved by an appropriate therapy.

In this context, the present data support the proposed therapeutic value of hepatocyte transplants in chronic liver diseases. Hepatocyte transplantation is a good alternative to orthotopic liver transplantation and can provide new opportunities for the treatment of the neurological symptoms of hepatic encephalopathy.

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Conflict of Interest

The authors declare no conflict of interest.

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