Nitric Oxide donor drugs improve the distribution and engraftment of transplanted hepatocytes in the liver

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Abstract

Background : **Hepatocyte transplantation could be an alternative to liver transplantation for the treatment of metabolic diseases, however this therapy is still limited by the loss of transplanted cells in the portal radicles before their entry into the sinusoids to engraft. Therefore, we investigated the effect of glyceryl trinitrate on hepatic sinusoids and on the efficacy of cell engraftment in a syngenic mice model.**

Methods : **We first assessed the effect of GTN portal infusion on the parenchymal spreading of colored microspheres. Hepatocytes transplantation in a syngenic mice model was then performed concomitantly with GTN infusion. The distribution of transplanted hepatocytes and their ultimate engraftment were analysed.**

Results : **After GTN perfusion 27% of microspheres shifted from the portal to the sinusoidal zone. Transplanted hepatocytes distribution changed significantly in the portal and parenchymal zones from respectively 53 ± 2% and 46.8 ± 2% in control animals to 32.5 ± 2.4% and 67.5 ± 2.4% in GTN-treated animals. At days 7 and 15, we noted a significantly better engraftment in GTN group vs. controls (60 ± 4 vs. 37 ± 2 transplanted hepatocytes in 20 fields ×400).**

Conclusions : **Portal perfusion of GTN improved the access of microspheres and transplanted hepatocytes to the sinusoidal bed and also improved the percentage of cell engraftment in the liver. These results suggest that drug dilatation of portal pedicles prior to transplantation increases the efficiency of hepatocyte grafting.** (Acta gastroenterol. belg., **2012**, 75, **234-239**).

Key words : mouse model, liver, hepatocyte transplantation, vasodilatation, colored microspheres, drugs.

Abbreviations

GTN : glyceryl trinitrate
NO : Nitric Oxide

Introduction

Hepatocyte transplantation is a promising therapy for genetic metabolic liver diseases in which only hepatocytes are deficient. In this case, a selective replacement of hepatocytes would be therapeutic. Human allogeneic or genetically modified autologous hepatocytes have already been transplanted in patients, demonstrating the safety and feasibility of both approaches (1-13). However, these series of disparate cases failed to demonstrate that this strategy is able to provide a significant and sustained clinical improvement and highlighted the obstacles. Many animal models based on the injury of host liver giving a proliferative advantage to transplanted cells have been used and were able to achieve an important repopulation of the host liver (14-21). Although these remarkable models proved very useful to understand the mechanisms of integration and proliferation of transplanted cells, they still are not appropriate for humans. A simple approach would be to decrease the loss of transplanted hepatocytes in the distal portal branches by facilitating their delivery through the sinusoids. Hepatocyte transplantation is most effective when cells are deposited in hepatic sinusoids because transplanted cells can then integrate in liver plates (22). A large number of transplanted hepatocytes are entrapped in portal areas and cleared by macrophages during the first 16 hours after transplantation and less than 50% of the injected cells have access to liver sinusoids and ultimately engraft (23-25). Cells entry into hepatic sinusoids is driven by mechanical factors including the relationship between cell size and sinusoids diameter (26).

Nitric Oxide (NO) donating drugs are vasodilators with a greater effect on veins and have been shown to reduce the hepatic venous pressure gradient (27-30). The smooth muscle-relaxing properties of NO may be important in affecting sinusoidal hemodynamics modulating vascular tone (31). Nitroglycerin has been used in a rat model of hepatocyte transplantation and showed an important vasodilatory activity and improved cell engraftment (32,33). However, hepatocytes and nitroglycerine were both injected in the spleen and a local effect can not be excluded.

NO-donors are known to have a local activity on their infusion site. In order to avoid effects on the remaining mesenteric territory, we infused drugs, microspheres and cells directly in the portal trunk.

The aim of this study was to examine the effect of portal infusion of nitroglycerine on a) hepatic sinusoidal vasodilation and (b) the efficiency of hepatocytes engraftment in a syngenic mouse model.

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Material and methods

Animals

Adult DBA/2J syngenic male mice (IFFA-CREDO, L'Arbresle, France), 8 to 10 weeks-old (weight : 25-30gr) were used. Animals were housed at IFR Paris Sud, France, under a 12-hour light/dark cycle and provided standard laboratory mice chow. All procedures were performed within the guidelines for the humane care of laboratory animals.

Microspheres

Green-colored polystyrene microspheres (Ultraspheres, E-Z Trac Inc, Los Angeles, USA) were initially used to evaluate the degree of hepatic sinusoidal dilatation. E-Z Trac microspheres have been previously used in experimental studies of organ blood flow distribution, cardiac output distribution, organ ischemia and reperfusion (34-36). We chose to use 15 mm calibrated microspheres, diameter corresponding to the size of fetal hepatocytes.

Isolation and labeling of hepatocytes

Hepatocytes isolation was performed by a modified two-step collagenase perfusion, as previously described (25). The isolated cells were suspended in Hormonally Defined Medium (HDM) : DMEM/HAM F12 (Eurobio, Les Ulis, France), containing 10% foetal calf serum (FCS, Life Technologies, Cergy-Pontoise, France), 0.1% bovine serum albumine, 2 mM glutamine and antibiotics. Isolated hepatocytes were labeled with the Hoechst fluorescent dye. Briefly, cells were incubated for 30-45 min at 37°C (10^7cells/ml) in a serum-free culture medium containing 10 ng/ml of Hoechst (Hoechst 33258, Sigma, France). The dye solution was then replaced by medium supplemented with 10% serum ; cells were washed three times in PBS (Gibco) and resuspended in saline for transplantation. Cell viability after isolation and labeled was determined by Trypan blue dye exclusion and attachment to tissue culture plastic. Hepatocytes were used when viability exceeded 95% and they attached to culture dishes within 30 minutes.

Infusion of microspheres or hepatocytes : study Design (Fig. 1A, B)

The effect of Glyceryl trinitrate (Nitronal, France) portal infusion on parenchymal spreading of colored microspheres was at first assessed. Microspheres were infused exactly in the same conditions subsequently used for hepatocytes transplantation. In a second set of experiments, the effect of Glyceryl trinitrate portal infusion was evaluated on both hepatocytes parenchymal distribution and transplantation efficacy. Glyceryl trinitrate diluted in normal saline, infused into the portal vein (0.5 µg/Kg/min) at 0.3 ml/hr with a Harvard pump five minutes before microspheres or cell injection and ending 10 minutes later.

We used 20 male mice. Each recipient was anesthetized, the abdomen was incised and the left lateral lobe was immobilized gently. Saline solution at 37°C was suffused to prevent drying. 10^6 particles were diluted in 200 µl of normal saline and injected directly into the portal vein using a 29 gauge needle (Lymphangiography set PVC, Vygon, Ecouen, France).

Localization of microspheres or transplanted hepatocytes

Biopsies from all liver lobes (left, middle, and right lobe) of GTN-treated and control animals were taken 4 hours and 7 or 15 days after injection. Specimens from microspheres-receiving animals were fixed in formalin (10%), transferred through increasing concentrations of alcohol, incubated with histoclear (polylabo), before to be embedded in paraffin. Three-mm thick sections were deparaffinized through histoclear and alcohol then rehydrated through distilled water. They were stained with hematoxylin-eosin and mounted with an aqueous mounting medium (SuperMount, Alphelys, Plaisir, France). Frozen specimens from hepatocytes-receiving animals were used for quantification of Hoechst-stained hepatocytes. Five μm sections were examined under fluorescent microscope. Microspheres or transplanted cells were counted in the portal zone and centrolobular zone. This analysis concerned scoring of 20 fields (×400) of each liver lobe biopsy.

Engraftment of transplanted cells

In order to demonstrate the engraftment of transplanted hepatocytes within parenchymal plates, we evaluated the expression of connexin 32 seven days after cell transplantation. Five μm sections of frozen samples of each liver lobe were fixed in acetone for 10 min, rinsed in PBS and then incubated with a connexin 32 antibody (Santa Cruz Biotechnology, CA) (1:100 dilution) for 1 hour at room temperature. They were rinsed in PBS and incubated with the anti-goat FITC secondary antibody (Santa Cruz Biotechnology) for 45 min. Sections were finally rinsed and mounted with glycerol.

Statistical analysis

Values are presented as means ± SEM. Statistical significance between groups was analyzed using Mann-Whitney U test. P values less than 0.05 were considered significant.

Results

Parenchymal spreading of microspheres

In a first series of twenty mice (5 series of 4 animals), we infused colored microspheres. Analysis of the position of microspheres in the liver lobule in control animals showed that more than 50% of the microspheres were located in the portal veins (Fig. 2A). Microspheres

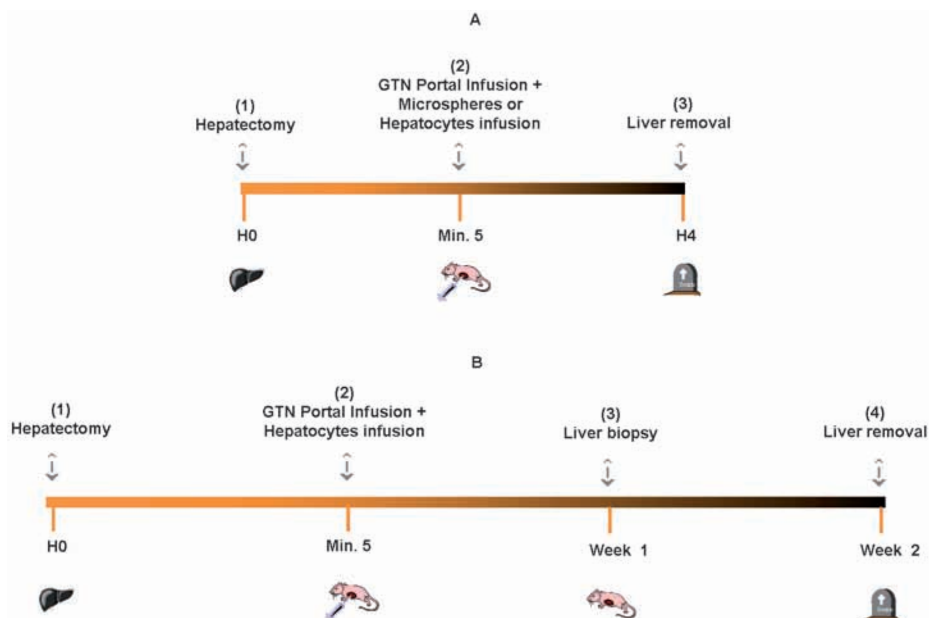

Fig. 1. — (A) Experimental drawing used to evaluate the effect of GTN portal infusion on microspheres parenchymal spreading and on the immediate distribution of transplanted cells.
(1) First a 40% hepatectomy is performed, (2) the portal perfusion of GTN is then followed 5 min later by the microspheres or cells infusion. (3) The liver is removed 4 hours later and samples from all liver lobes are analyzed.
(B) Experimental drawing used to evaluate the effect of GTN portal infusion on ultimate engraftment and functionality of transplanted cells.
(1) First a 40% hepatectomy is performed, (2) the portal perfusion of GTN is then followed 5 min later by hepatocytes infusion. (3) The liver is biopsied one week later and (4) removed 2 weeks later and samples from all liver lobes are analyzed.

percentages in the portal, intermediate and centrolobular zones were respectively 57 ± 5%, 21 ± 3% and 22.4 ± 5% (Fig. 2C).

The position of microspheres in the liver lobule changed in the GTN-treated animals where significantly more fluorescent spheres reached the centrolobular zone (Fig. 2B). The fraction of microspheres in portal veins declined proportionately and their distribution was respectively 34 ± 8%, 19 ± 4% and 46.8 ± 10% (Fig. 2C). Thus, in this model, microspheres distribution significantly changed in portal and centrolobular zones and remained unchanged in the intermediate zone, showing that the microspheres spread to parenchymal zones. After GTN infusion, the shift of microspheres was significant.

A further subdivision of the liver lobule in two zones : a portal zone, and a sinusoidal zone, showed that, in control animals, most of transplanted cells were entrapped intraportally whereas GTN portal infusion increased the entry of microspheres through the sinusoids. Microspheres increased significantly in the sinusoidal zone (43 ± 2, in controls ; vs. 66 ± 3 in GTN group) and decreased in the portal zone (57 ± 2 in controls ; vs. 34 ± 3 in GTN group) (Fig. 2 C).

Effect of GTN on hepatocytes transplantation

In a second set of experiments (5 series of 4 animals), we evaluated the short-term (4-hours biopsy) distribution of transplanted labeled hepatocytes within the liver zones (Fig. 3 A, B). Transplanted hepatocytes distribution in portal and centrolobular zones were respectively 53 ± 2% and 46.8 ± 2% in non-treated animals. This distribution was significantly different after GTN perfusion respectively 32.5 ± 2.4% and 67.5 ± 2.4% (Fig. 3C).

To assess whether enhanced delivery of transplanted hepatocytes by sinusoidal dilatation and greater entry in hepatic sinusoidal beds affected cell engraftment, we transplanted animals treated with GTN (n=10) and controls (n=10) and analyzed the number of engrafted hepatocytes integrated into liver plates on liver biopsies 1 and 2 weeks later. Engrafted cells were adjacent to portal veins and their number was limited in control animals (Fig. 4A). In GTN-treated animals, transplanted hepatocytes were found in centrolobular zone (Fig. 4B).

Nonetheless, more transplanted cells were engrafted in GTN-treated animals. The overall number of Hoechst-labeled hepatocytes (counted in 20 fields, 200×) on 1 week biopsy in control and GTN-treated animals was respectively 37 ± 2 and 60 ± 4 (Fig. 4C). A similar rate of grafted hepatocytes was found on 2 weeks biopsies.

Engraftment of transplanted cells

The expression of connexin 32, in transplanted mouse livers, 7 days after cell infusion by immunohistochemistry shows the presence of connexin 32 between

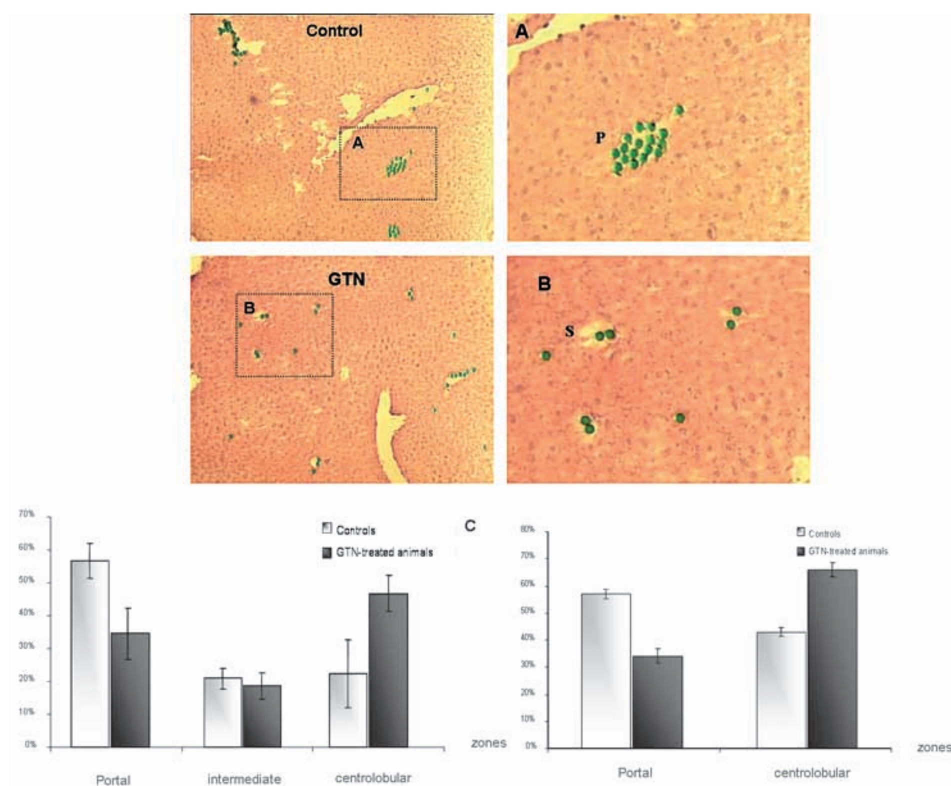

Fig. 2. — Parenchymal spreading of green colored microspheres with and without portal infusion of GTN. (A) Liver histological sections in control animals showing microspheres in clusters, trapped in portal branches (P). (B) Liver histological sections after GTN portal infusion showing microspheres spreading into sinusoids distally in the liver parenchyma (S). (C) Microspheres distribution in the liver lobule : portal and centroobular zones.

Hoechst-labeled and resident hepatocytes confirming the engraftment of transplanted cells. Connexin 32 marked punctuates structures on plasma membrane of hepatocytes to form channels between adjacent cells (Fig. 5).

Discussion

This study substantiates the effect of GTN, a vasodilatory drug into the mouse portal vein during, microspheres distribution and, more importantly, cell transplantation with, potential engraftment of transplanted hepatocytes within liver lobules.

In a preliminary study, we assessed the effect of portal GTN infusion on the distribution of microspheres, we show that infusion of GTN directly into the portal trunk allows microspheres to leave portal zones and spread towards innermost hepatic lobular zones, while fewer microspheres remained in portal vein radicles. The vasodilatory effect of GTN resulted to an important shift of microspheres localization from the portal area into sinusoids. Therefore, the percentage of microspheres in the centrolobular zones increased significantly.

The liver acinus was described by Rappaport (37). This result as well as our concern of identifying more easily the region of engraftment of transplanted hepatocytes led us to subsequently consider the liver lobule on the basis of two zones : (a) the portal zone ; portal spaces and portal radicles where cells could cause obstruction of the portal flow and generate vascular thrombosis ; and (b) the intra-parenchymal zone ; including sinusoids and liver plates and assembling the centrolobular zones, where transplanted cells can engraft and establish junctions with host cells. Our data reveal greater transplanted cell entry in sinusoids with a more distal distribution in GTN-treated mice. The presence of transplanted cells in the sinusoids enhanced their engraftment efficacy in intraparenchymal zones. This explains the improvement of the overall engrafted hepatocytes number after GTN perfusion. The last animal series confirmed that the transplanted cells redistribution was profitable to cell engraftment even if their percentage did not increase in the portal zone. This redistribution of transplanted cells decreased the number of cells trapped in portal veins and cleared by macrophages.

Another interesting finding of our study is that the percentage of engrafted hepatocytes did not increase during the second week after transplantation. This happened probably for two reasons. Firstly, hepatic regeneration after hepatectomy is almost completed by day 7 in mice and hepatocytes become quiescent later on (38,39). Secondly, it is very difficult to track all transplanted hepatocytes after two weeks because the Hoechst dye is not appropriate for such long period staining.

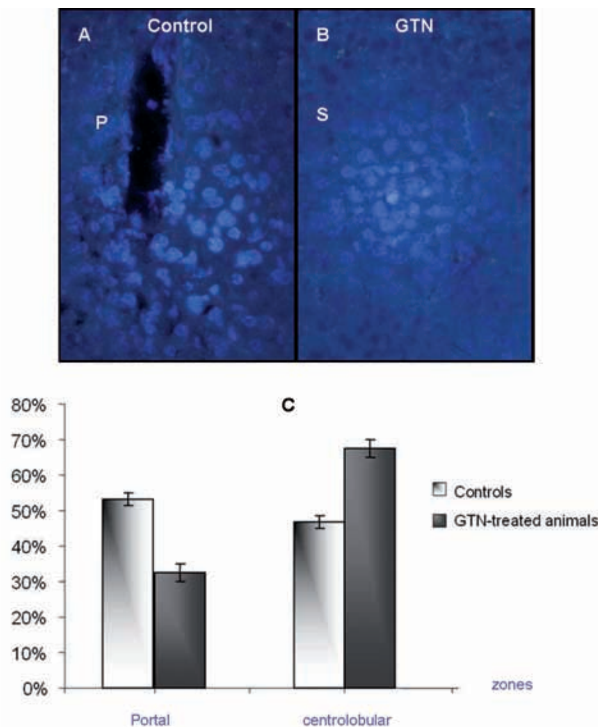

Fig. 3. — (A) Liver histological sections in control animals showing Transplanted hepatocytes in clusters, trapped in portal branches (P). (B) Liver histological sections after GTN portal infusion showing Transplanted hepatocytes spreading into sinusoids distally in the liver parenchyma (S). (C) Transplanted hepatocytes distribution in the liver lobule : portal and centrolobular zones showing their increase in the centrolobular zone in GTN treated animals.

NO-donors are known to have a local activity on their infusion site. In order to avoid effects on the remaining mesenteric territory, we infused drugs, microspheres and cells directly in the portal trunk. It was clear that this way of drug or cell infusion is possible in small animals and appropriate for further clinical applications. Shleria *et al.* (33) showed a similar effect of a NO-donor drug in a rat transplantation model in splenic sinusoidal beds. Even if the cell fraction retained in the spleen was identical in control and nitroglycerine-treated animals, hepatocytes entry in liver increased from 25 to 33%. Therefore, a local effect of nitroglycerine in the spleen cannot be excluded.

Furthermore, GTN could reveal beneficial as to the final percentage of engrafted hepatocytes after transplantation, since its vasodilatory effect increases the potential number of transplanted cells and reducing at the same time the risk of intraparenchymal portal radicle thrombosis due to cell entrapment in portal spaces. We believe that vasodilation by GTN or other agents could play an important role in the improvement clinical cell transplantation. However, in order to achieve this, further studies have to be undertaken in larger animals, such as primates.

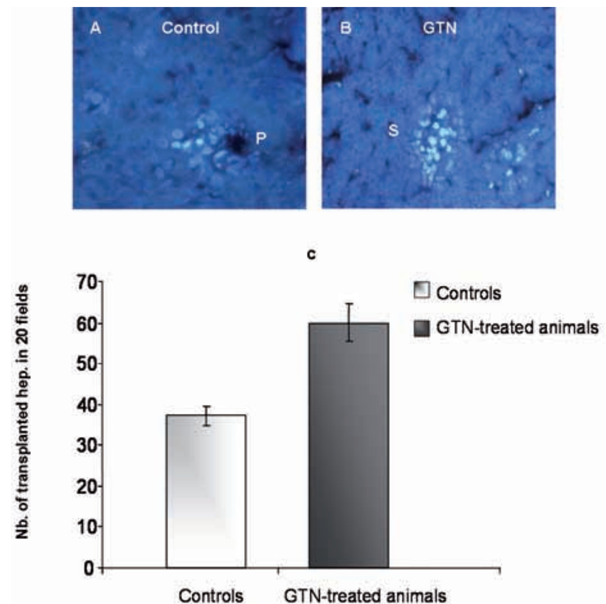

Fig. 4. — Transplanted hepatocytes stained with the Hoechst dye in fluorescent microscopy). (A) In controls, they are located periportally (P). (B) in GTN-treated animals they are located into sinusoids (S). (C) Transplanted hepatocytes number in 20 fields (400×).

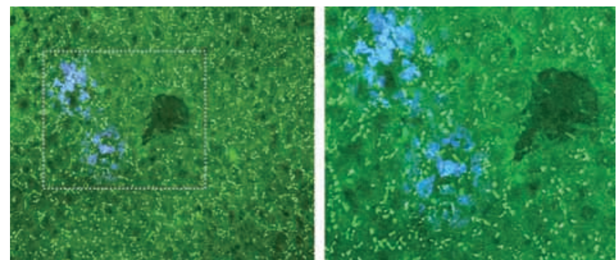

Fig. 5. — Engraftment of transplanted cells by Connexine 32 detection (yellow-green punctuates) showing connections between engrafted (blue) and native hepatocytes.

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