ORIGINAL PAPERS

# First approaches for the transplantation of hepatocytes from Wistar rat preneoplastic livers into healthy recipients

Marina Cecilia Vera[1], María Eugenia Biancardi[2], Hebe Bottai[3], María Cristina Lugano[2], Ariel Darío Quiroga[1,2], Gerardo Bruno Pisani[2] and Alejandra Beatriz Quintana[2]

*[1]Institute of Experimental Physiology (IFISE-CONICET), [2]Morphology and [3]Statistic and Data Analysis. Biochemical and Pharmaceutical Faculty. Universidad Nacional de Rosario. Santa Fe, Rosario. República Argentina*

**Received:** 22/07/2018 · **Accepted:** 26/10/2018

**Correspondence:** Alejandra Beatriz Quintana. Morphology, Physiology Department. Biochemical and Pharmaceutical Faculty. Universidad Nacional de Rosario. Suipacha, 535/579. S2002LRK Rosario, Santa Fe. República Argentina. **e-mail:** aquintan@fbioyf.unr.edu.ar

## ABSTRACT

**Background:** the shortage of donors of hepatocyte transplantation therapy led to the use of so-called marginal donors. Some donors may have a hepatic illnesses that is associated with hepatic preneoplasia with foci of altered hepatocytes (FAH).

**Aims:** to determine whether recipients developed FAH upon transplantation with hepatocytes from a preneoplastic liver and whether FAH progresses to a preneoplastic hepatocyte-derived tumor (PHDT), up to 60 days after transplantation.

**Material and methods:** male Wistar adult rats were used as donors and recipients. Donors underwent a 2-phase model of liver preneoplasia for hepatocyte isolation. Recipients underwent a partial two thirds hepatectomy and received 150,000 hepatocytes. Recipients were euthanized seven and 60 days after transplantation. The number of FAH per liver area, percentage of liver occupied by FAH, the hepatic enzymatic profile, the percentage of prothrombin time (PT), the proliferative index (PI) and liver morphology were analyzed.

**Results:** recipients developed few and very isolated FAH. No statistical differences were found between hepatic enzyme activities and PT. There were no differences between the groups with regard to the number of FAH per liver area and percentage of liver occupied by FAH after 60 days. The PI decreased on day 60 compared to day seven. No morphological alterations were found.

**Conclusions:** recipients developed few FAH that did not increase in number or size, nor did they progress to PHDT and had normal plasma biochemical features and liver morphology up to 60 days post-transplant. Additional studies are needed to determine whether FAH development constitutes a risk for recipients while waiting for whole organ transplant.

**Key words:** Hepatocarcinogenesis. Rat liver preneoplasia. Hepatocyte transplantation. Foci of altered hepatocytes. Marginal donors.

## INTRODUCTION

Hepatocyte transplantation (HT) therapy is the best alternative therapeutic approach for whole-organ transplantation (1,2). An adequate number of human differentiated hepatocytes are transplanted into a liver with acute hepatic failure. Thus, the liver can survive and restore the normal hepatic function based on the liver regeneration capability. Nowadays, this procedure is in the experimental phase. It has also been used as a temporary bridge until the whole-organ transplantation can be performed in patients with liver-based inborn errors in metabolism and acute or chronic liver failure (3).

Nowadays, the number of patients suitable for liver transplantation is progressively increasing. This has given rise to a growing imbalance in the number of candidates on the waiting list and the number of donors (4). This situation has prompted scientists to search for alternative approaches in order to increase the number of liver grafts. These include the use of "marginal" or "extended donors" for whole-organ transplantation or to obtain isolated hepatocytes for HT. Although the definition of an extended donor has not been thoroughly established, most agree that it conveys a higher risk of either physiologic dysfunction or infectious/metabolic disease transmission. Extended criteria can be separated into two groups: donor-related risk factors and surgical technique-related issues. Donor-related risk includes donation after cardiac death, advanced age, increased cold ischemia time, ABO incompatibility, steatosis, previous malignancies in the donor, hepatitis C virus or hepatitis B virus infection, human T-cell lymphotropic virus type I/II infection or other active infections. These extend-

Vera MC, Biancardi ME, Bottai H, Lugano MC, Quiroga AD, Pisani GB, Quintana AB. First approaches for the transplantation of hepatocytes from Wistar rat preneoplastic livers into healthy recipients. Rev Esp Enferm Dig 2019;111(5):351-357.

**DOI: 10.17235/reed.2019.5830/2018**

ed criteria donors can generally be accepted or declined by the transplant team during evaluation of the allograft (5). Some diseases of marginal donors may be associated with undetectable subclinical hepatic preneoplasia such as patients with non-alcoholic fatty liver disease and non-alcoholic steatohepatitis, which could progress to a preneoplastic hepatocyte-derived tumor (PHDT) in the absence of cirrhosis (6).

Since hepatic preneoplasia is an early, clinically undetectable stage of the HCC, it is necessary to study the risks associated with the transplantation into healthy recipients of hepatocytes isolated from livers that could have a subclinical neoplasia. These are characterized by the presence of foci of altered hepatocytes (FAH), without cirrhosis. For this purpose, a rat model was used and donor livers were induced to develop hepatic preneoplasia in order to mimic a potential transplant with a pool of hepatocytes that may contain some preneoplastic hepatocytes. The specific marker to determine the presence of FAH in rats was used in order to perform these experiments. We hypothesized that recipient rats transplanted with hepatocytes obtained from a preneoplastic liver could develop FAH, which may constitute a potential risk for patients.

The present study determined whether recipient rats developed FAH upon transplantation with hepatocytes from a preneoplastic rat liver. The number of FAH growth and percentage of liver occupied by foci, the proliferative index and the potential progression of FAH into PHDT up to 60 days after transplantation were evaluated.

## MATERIAL AND METHODS

### Animals

Two male adult Wistar rats (300-350 g body weight) were maintained per cage on a constant 12 hour light/dark cycle under controlled temperature and humidity conditions. They had free access to tap water and were fed with standard rat pellets *ad libitum.* All the experimental protocols were performed according to the NIH "Guide for the Care and Use of Laboratory Animals" (7) and approved by the "Guide for the Care and Use of Laboratory Animals Committee", Biochemical and Pharmaceutical Faculty, UNR, Resolution No. 6109/012. All surgeries were performed under a ketamine-xilazine anesthetic combination (70 mg/kg body weight and 2.1 mg/kg body weight, intraperitoneal, respectively).

### Reagents

Diethilnitrosamine (DEN), 2-actylaminofluorene (2-AAF) and collagenase type IV of *Clostridium histolyticum* were obtained from Sigma Chemical Co. (St Louis, MO). Rabbit polyclonal anti-GST3/GST-p antibody was purchased from Abcam® (AB106268, Boston, USA). The anti-proliferating cell nuclear antigen (PCNA) antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The biotinylated goat anti-rabbit secondary antibody and horseradish-peroxidase-conjugated streptavidin (HRP CytoScan Detection Kit) were obtained from Cell Marque (CMD302, USA). The DAB Substrate Kit was purchased from Cell Marque (957D-20, USA). Reagents for enzymatic determinations were provided by Wiener Laboratories S.A.I.C. (Rosario, Argentina).

### Pre-neoplasia induction in donors (group D)

Animals of group D (n = 3) were subjected to a 2-phase model (initiation-promotion) of chemical hepatocarcinogenesis to induce hepatic pre-neoplasia based on the Solt-Farber method (8) and adapted by Álvarez et al. (9). This model mimics the early events of the latent period of clinically undetectable human carcinogenesis (10). The initiation stage was performed by the administration of two intraperitoneal necrogenic doses of the initiator agent DEN (150 mg/kg body weight), two weeks apart. Administration of the promoter drug 2-AAF (11,12) was performed one week after the last injection of DEN. The 2-AAF was dissolved in dimethyl sulfoxide and then suspended in corn oil to a final concentration of 8 mg/ml. Rats received 20 mg/kg body weight of 2-AAF by gavage for four consecutive days per week for three weeks. After six weeks of treatment, the livers from group D developed FAH (9). Animals that received the vehicles of the drugs instead of DEN+2-AAF were used as the vehicle controls (n = 3).

### Hepatocyte isolation

Hepatocytes from group D were isolated by liver collagenase perfusion followed by mechanical disruption, as previously described by Seglen (13). Briefly, a laparoscopic excision was performed in the abdominal cavity after rats were anesthetized and a 14 G catheter was introduced into portal vein. An open perfusion (non-recirculating) of the liver was started for two minutes, with calcium-free Ringer solution (NaCl 137 mM; $NaHCO_3$ 26 mM; $Na_2HPO_4$ 0.6 mM; KCl 5.4 mM; glucose 5.6 mM) supplemented with HEPES (3 g/l) and EGTA (0.24 g/l), to deplete endogenous calcium (14). For tissue digestion, the liver was perfused in a non-recirculating system with the same Ringer solution without EGTA, with the addition of $MgSO_4$ (1 mM), collagenase type IV (specific activity: 249 U/mg, perfused for nine minutes with a final concentration in the perfusion media of 4,300 U/l) and $CaCl_2$ (2.5 mM). The liver was removed and submerged in the solution with collagenase and disrupted mechanically. The isolated hepatocytes were subsequently filtered through a 40 µm-nylon membrane. Non-parenchymatic and other cells were separated by a low-speed centrifugation (30 g, two minutes) and the pellet was washed three times with media without collagenase. Perfusion solutions were bubbled with oxygen for 15 minutes at pH = 7.40-7.50, with an osmolarity of 295-305 mOsm. Cell viability was evaluated via trypan blue exclusion (15).

### Partial hepatectomy of recipients

Recipients (group R) underwent a two-thirds hepatectomy by the removal of the central and the right lateral lobes, as originally described by Higgins and Anderson (16). Partial hepatectomy is used in many models of HT as an exogenous factor to enhance hepatocyte division in the recipient liver (17). All the surgeries were performed between 10 a.m. and 2 p.m. in order to avoid alterations due to circadian rhythm.

**Hepatocyte transplantation**

Group R received 1.5 x $10^5$ hepatocytes in 200 µl of Ringer solution. Hepatocyte solutions were injected directly into the splenic pulp using a tuberculin syringe with a G21 (0.8 x 25 mm) needle (18) immediately after partial hepatectomy; 1.5 x $10^5$ hepatocytes were used, based on bibliographic references (19) to ensure that a portal thrombosis did not occur. In addition, a simulation of the transplant was performed by injecting the animals (n = 6) with only the vehicle used in the hepatocyte solution (group Sham). Six recipients were used for each rat from group D. Animals from group R and the Sham group were euthanized seven and 60 days after transplantation (n = 3 for each time) and the livers were removed and processed. These times were chosen as the liver is still in the regeneration process at day 7 (20) and day 60 in order to determine whether FAH developed in recipient livers augmented or progressed to a PHDT.

**Post-transplant studies**

***Histological analysis***

Pieces of all hepatic lobules were fixed in 10% v/v formaldehyde and paraffin embedded for histological studies. To analyze liver morphology and fibrosis, 4 µm sections were stained with hematoxylin-eosin (H&E) and Direct Red 80, respectively.

***Immunohistochemical studies***

FAH quantification

To investigate the presence of FAH, sections of all samples obtained were mounted and immunohistochemical detection was performed using an antibody against the placental form of rat glutathione S-transferase (rGST-Pi). This isozyme has been described as the best effective marker of hepatic preneoplasia in rats (21). Briefly, deparaffinized tissue sections were treated with 3% $H_2O_2$ in methanol for ten minutes to remove endogenous peroxidase and were then microwaved in 10 mM buffer citrate solution for ten minutes at 96 °C for antigen retrieval. Normal serum was applied to the slides to block nonspecific binding and the slides were subsequently incubated with a rabbit polyclonal antibody against rGST-Pi, diluted 1/250, at 4 °C overnight. The slides were incubated with biotinylated goat anti-rabbit secondary antibody and then with horseradish-peroxidase-conjugated-streptavidin. Signals were detected with the DAB substrate kit, followed by hematoxylin counterstaining.

After immunohistochemistry, the number of FAH per $cm^2$ of hepatic tissue and the percentage of liver occupied by FAH were calculated. To determinate the first parameter, the area occupied by the hepatic tissue was calculated using a NIH imaging analysis system (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA) and the number of FAH was counted in the entire casted tissue using light field microscopy. To determinate the percentage of liver occupied by foci according to the modified Saltykov's method (22), a representative number of field sections (usually 1-1.5 $cm^2$ of tissue per animal) from recipient tissue samples were collected using a digital camera (Olympus D-360, Tokyo, Japan) attached to a light field microscope (Olympus, U-MDOB model). The images were processed using the corresponding analysis software (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). The detection of rGST-Pi positive FAH was also performed in group D (lobe removed prior to hepatocyte isolation) and in the negative control group. FAH quantification was not performed in group D.

Determination of proliferative index

To investigate differences in the proliferation activity among the experimental groups, liver slices were examined by immunohistochemical staining with anti-proliferating cell nuclear antigen (PCNA, 1/3200) antibody, according to the method of Greenwell et al. (23). Proliferative index (PI) was expressed as the number of proliferating cells (in G1, S, G2, and M phases) per 100 hepatocytes, in ten high-power fields (9).

Enzymatic activities and percentage of prothrombin time determinations

Blood samples were obtained via cardiac puncture under anesthesia at seven and 60 days post-transplantation to determinate alkaline phosphatase (ALP) and alanine and aspartate aminotransferases (alanine aminotransferase [ALT] and aspartate aminotransferase [AST], respectively) activities. The percentage of prothrombin time (PT) was determined using spectrophotometry at 25 °C using fresh serum. For PT measurement, 2.5 ml blood was drawn into a test tube containing 0.25 ml of sodium citrate and expressed as a unit known as the Quick value. In this case, the measured PT was expressed in relation to the coagulation time of a healthy individual. The value obtained was the "percentage of the standard value" (24,25).

Statistical analysis

Results were expressed as the mean ± SEM. The number of FAH per $cm^2$ of hepatic tissue and the percentage of liver occupied by foci were analyzed using the independent sample t-tests. The results of PI were evaluated by two-way analysis of variance (ANOVA). The comparison of means was performed using the Tukey's test. ALP, AST and ALT activities and PT data were analyzed by two-way parametric or non-parametric ANOVA, as appropriate. An aligned-rank transformation of the data was used when the assumptions of normality and homoscedasticity (which are requirements for a classical analysis) were not met. Data were analyzed using the ARTool packages and lsmeans of the R 3.2 software. A p-value lower than 0.05 was regarded as significantly different.

## RESULTS

**Histological studies**

Liver architecture was conserved in the Sham and R groups. Endothelial cells were attached to the perisinusoidal extracellular matrix, hepatocyte cords were preserved and sinu-

soids and Kupffer cells appeared normal at seven and 60 days post- transplantation (Fig. 1).

### Immunohistochemical studies

All rats from group D had abundant FAH in the liver parenchyma at the end of the treatment to induce hepatic preneoplasia. As expected, animals that received only vehicles of the drugs did not have FAH. There were no signs of cirrhosis in group D (Fig. 2). Whereas, all animals in group R had very few, small and scattered FAH, as expected. The sham rats did not develop any FAH (Fig. 3A).

### FAH quantification

The number of FAH per $cm^2$ of hepatic tissue and the percentage of liver occupied by foci were analyzed. The number of FAH reflects the amount of initiated cells capable of developing into clones of FAH, whereas the volume percentage generally reflected the growth rate and total cellular population of the FAH (26). There were no statistical differences between the number and percentage volume occupied by

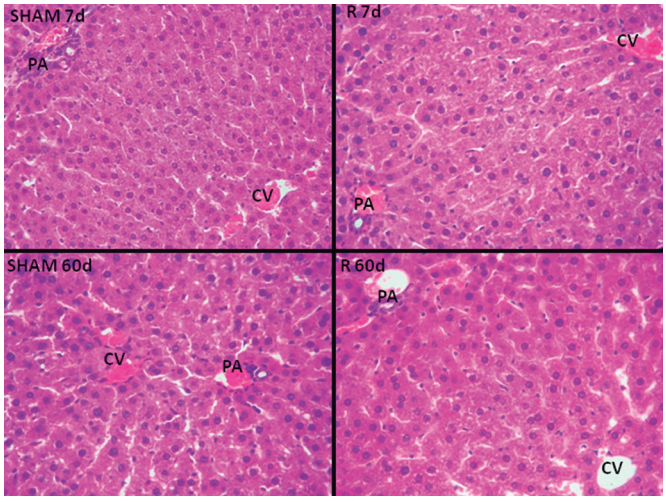


**Fig. 1.** *Histological analysis. The sinusoidal and hepatocyte architectures (H&E staining) were conserved. No morphological alterations were seen upon transplantation in the Sham and R groups at both times of euthanasia. Portal areas (PA) and central vein (CV). Magnification: 200X.*

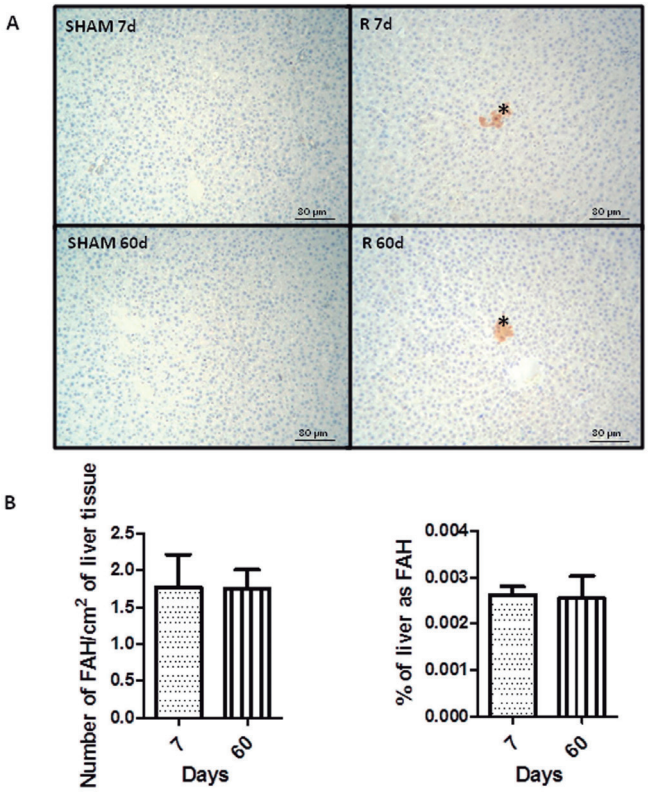


**Fig. 3.** *Immunohistochemical studies of rGSTPi expression and FAH quantification performed in group R. A. Very few and scattered FAH were found in group R at seven (R7d) and 60 (R60d) days after transplantation (asterisks, right panel). The Sham group did not develop FAH, either seven days (Sham7d) or 60 days (Sham60d) post-transplant (left panel). Magnification: 100X. B. Number of FAH/cm2 of liver tissue and the percentage of liver as FAH. Each bar represents the mean ± SEM. No statistically significant differences were found between these parameters at seven and 60 days post-transplantation.*

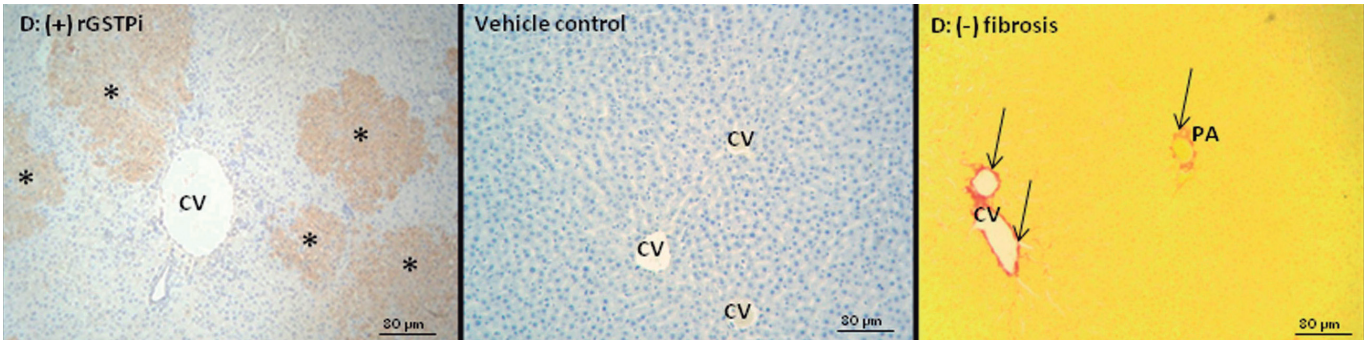


**Fig. 2.** *Immunohistochemical and fibrosis studies performed in livers of group D animals before hepatocyte isolation and vehicle controls. D: (+) GSTPi: abundant FAH (asterisks) were present in the hepatic parenchyma of group D at the end of preneoplastic induction. Vehicle controls: representative area of the hepatic parenchyma from animals that received the vehicles of the drugs. No FAH developed. D: (-) fibrosis: direct red 80 stain showed normal red collagen fibers deposition (arrows) in group D livers. CV: central vein; PA: portal area. Magnification: 100X.*

FAH at seven and 60 days post-transplantation. The number of FAH in group R at seven days was 1.76 ± 0.45 and 1.75 ± 0.26 at 60 days. The volume percentages of FAH in group R at seven days was 2.63 x 10-3 ± 1.84 x 10-4 and 2.55 x 10-3 ± 4.80 x 10-4 at 60 days. These results are summarized in figure 3B.

### Determination of PI

PI values are summarized in table 1. Figure 4 shows representative images of PCNA staining, with a marked decrease in the number of positive hepatocytes after 60 days with respect to seven days in both groups. Furthermore, immunohistochemistry showed an increment in PI in group R with respect to the Sham group at seven days.

### Enzymes activities and PT determination

ALT activity of group R was significantly higher than the Sham group seven days after transplantation and was comparable to the value after 60 days. ALT in the Sham group after seven days was 17.2 (UI/l) ± 1.4 and 17.1 (UI/l) ± 1.8 after 60 days, and 28.9 ± 3.0 in group R after seven days and 16.8 ± 2.1 in group R after 60 days. There were no statistically significant differences between the groups 60 days after transplant (Fig. 5A). There were no statistically significant differences between AST, ALP and PT in the Sham group at seven and 60 days post-transplant. AST (UI/l) was 47 ± 8.9 after seven days and 53.3 ± 4.4 after 60 days in the Sham group and 70.3 ± 8.1 after seven days and 54.3 ± 2.9 after 60 days in group R. ALP (UI/l) was 280.3 ± 16.3 after seven days and 285.7 ± 8.9 after 60 days in the Sham group and 384.0 ± 40.5 after seven days and 269.0 ± 14.6 after 60 days in group R. PT (%) was 114.0 ± 3.9 after seven days and 111.7 ± 1.7 after 60 days in the Sham group and 105.2 ± 3.3 after seven days and 104.3 ± 2.5 after 60 days in group R (Figs. 5B-D).

**Table 1. PCNA proliferative index (PI)**

| Days | Group | PI |
|---|---|---|
| 7 | SHAM | 13.8 ± 0.27 |
| 60 | SHAM | 2.9 ± 0.24* |
| 7 | R | 19.3 ± 1.6† |
| 60 | R | 3.1 ± 0.11* |

PI was expressed as proliferating cells per 100 hepatocytes. All values are shown as the mean ± SEM. *p < 0.05 *vs* corresponding to seven days. †p < 0.05 *vs* respective Sham.

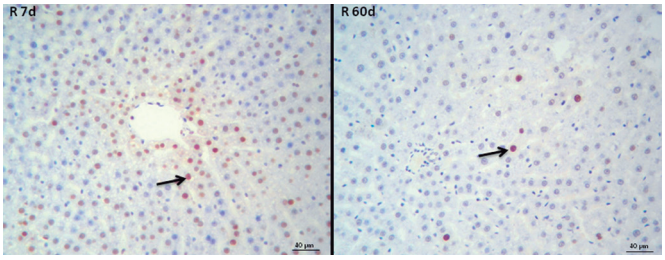


**Fig. 4.** *PCNA immunohistochemistry of rat liver from group R. Representative images showing positive PCNA cells (arrows) seven days (R7D) and 60 days (R60D) post-transplantation. Magnification 200X.*

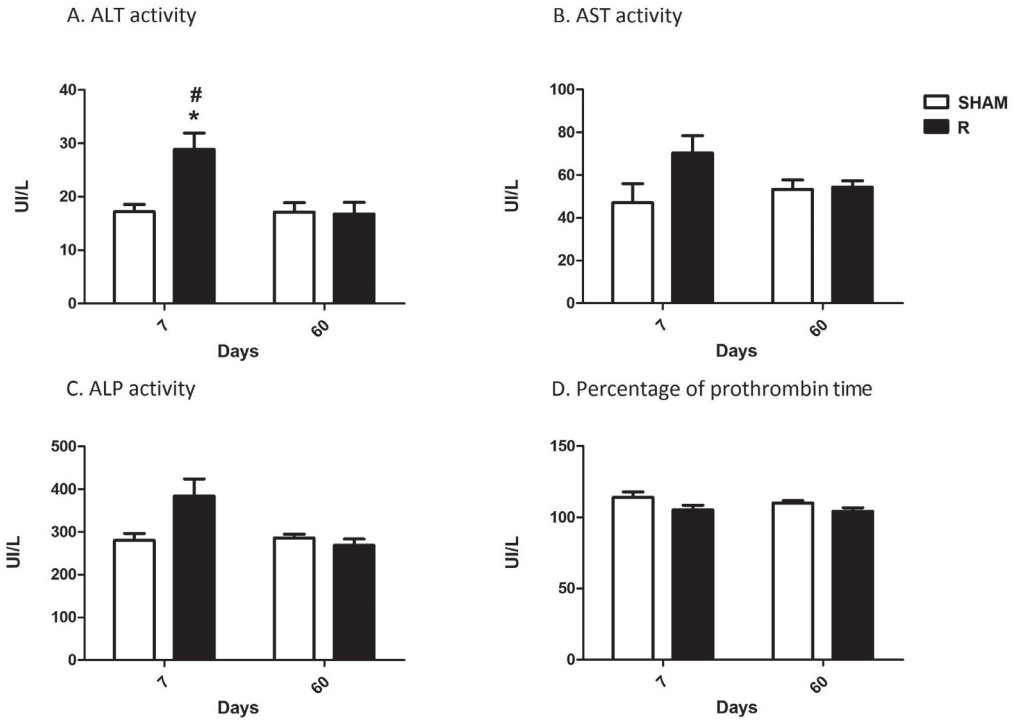


**Fig. 5.** *Hepatic enzymatic activities and percentage of prothrombin time. Each bar represents the mean ± SEM. A. ALT activity. *p < 0.05* vs *Sham at day 7. #p < 0.05* vs *Group R at day 60. B. AST activity. C. ALP activity. D. Percentage of prothrombin time.*

## DISCUSSION

HT performed with hepatocytes obtained from preneoplastic rat livers and the impact of this procedure on healthy recipients was analyzed in this study. We proved that all recipients from group R developed small and very isolated FAH. We also showed that these FAH did not progress to PHDT and persisted in the receptor livers during the study period.

The enzymatic and PT activities were normal and there were no statistical differences between the number of FAH per $cm^2$ of hepatic tissue and the percentage of liver occupied by foci from day seven and 60 after HT. These results suggest that liver biochemical feature and liver protein biosynthetic capability were intact after two months of HT. Furthermore, even though there were a few and scattered FAH in recipient livers, they did not increase in numbers or size. Moreover, no foci progressed to PHDT; this data was obtained when the hepatic morphology was analyzed with hematoxylin-eosin and rGST-Pi immunostaining 60 days after transplantation. The PI indicated that replicating cells were scarce at day 60 compared to day 7 after partial hepatectomy when livers were still in the regenerating process. This was true for recipients who received 150,000 hepatocytes or the vehicle alone. HT did not interfere in the hepatic regeneration process, as there were no statistically significant differences in PI at day 7.

This is the first study to demonstrate a preliminary assessment of the risks of HT in rats when using marginal donors. Thus, this study could be useful to estimate how long the preneoplastic-transplanted hepatocytes remain in the recipient, in cases where HT is used as a temporal bridge and the whole liver is transplanted within few days. Further studies on FAH development in rat recipient livers are needed to assess if this constitutes a severe risk for the recipients' health.

There are some limitations associated to this procedure. Even though rGST-Pi is a frequently used and reliable marker for FAH in rat livers, it does not mean that persistent FAH could change their cellular phenotype during progression, as demonstrated in many studies by cytomorphological, cytochemical, microbiochemical and molecular methods (27). Thus, it is necessary to analyze FAH phenotype evolution over time in our experimental study. Furthermore, there is a chance of uneven transplanted cell distribution, which may cause overestimation or underestimation of engrafted cells with the stereological method used. Finally, other tissues must be monitored as transplanted cells have the potential to induce the development of HCC. The organs that should be studied include the spleen, since many injected hepatocytes remain in the splenic parenchyma, and also the lungs, as transplanted hepatocytes can migrate to these organs *via* the inferior cava vein (28,29). Lymph nodes should also be studied as some preneoplastic cells can be trapped in the parenchyma arriving *via* lymphatic circulation. If the amount of cells transplanted is controlled, the formation of micro emboli of hepatocytes could be avoided and the HT can be performed directly by injection in the portal vein, thus avoiding the permanence of these cells in the spleen (29). Functional studies in recipients should also be performed together with a complete histological analysis of the organs mentioned above.

We conclude that all rat recipients developed FAH and they persisted until 60 days post-transplant. FAH number and size did not increase during the study period and PI confirmed normal hepatic regeneration. Furthermore, the enzymatic profile and the percentage of PT activity was normal 60 days post-HT and finally and most importantly, FAH did not progress to PHDT under our experimental conditions.

## ACKNOWLEDGEMENTS

The authors would like to acknowledge the histotechnologist Alejandra Inés Martinez for her excellent technical assistance and Wiener Laboratories S.A.I.C. (Rosario, Argentina) for providing the reagents for enzymatic determinations. This study was supported by the Secretary of Science, Technology and Innovation of Santa Fe, Scientific Project No. 2010-093-11, Argentina.

## REFERENCES

1. Bartlett DC, Newsome PN. Hepatocyte cell therapy in liver disease. Expert Rev Gastroenterol Hepatol 2015;9:1261-72. DOI: 10.1586/ 17474124.2015.1073106
2. Gewartowska M, Olszewski WL. Hepatocyte transplantation-biology and application. Ann Transplant 2007;12:27-36.
3. Pareja E, Cortés M, Martínez A, et al. Trasplante celular hepático: un nuevo tratamiento en las enfermedades hepáticas. Cir Esp 2010;88:3-11. DOI: 10.1016/j.ciresp.2010.03.013
4. Fondevila C, Jiménez-Galanes S, García-Valdecasas JC. ¿Cómo incrementar el número de trasplantes hepáticos? Gastroenterol Hepatol 2009; 32:519-30.
5. Russo FP, Ferrarese A, Zanetto A. Recent advances in understanding and managing liver transplantation. F1000Res 2016;5:2895. DOI: 10.12688/f1000research.8768.1
6. Cholankeril G, Patel R, Khurana S, et al. Hepatocellular carcinoma in non-alcoholic steatohepatitis: current knowledge and implications for management. World J Hepatol 2017;9:533-43. DOI: 10.4254/wjh.v9.i11.533
7. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington, D.C.: National Academies Press; 2011.
8. Solt D, Farber E. New principle for the analysis of chemical carcinogenesis. Nature 1976;263:701-3. DOI: 10.1038/263701a0
9. De Luján Álvarez M, Cerliani JP, Monti J, et al. The in vivo apoptotic effect of interferon alfa-2b on rat preneoplastic liver involves Bax protein. Hepatology 2002;35:824-33. DOI: 10.1053/jhep.2002.32099
10. Borbath I, Leclercq I, Abarca-Quinones J, et al. Inhibition of early preneoplastic events in the rat liver by the somatostatin analog lanreotide. Cancer Sci 2007;98:1831-9. DOI: 10.1111/j.1349-7006.2007.00626.x
11. Gerlyng P, Seglen PO. Effects of the tumor promoter 2-acetylaminofluorene and of 4-acetylaminofluorene on rat liver growth. Bull Cancer 1990;77:461-6.
12. Pisani GB, Valenti JL, Quintana AB. Hepatic preneoplasia induction in male Wistar rats: histological studies up to five months post treatment. Rev Esp Enferm Dig 2016;108(8):457-63. DOI: 10.17235/reed.2016.4183/2015
13. Seglen PO. Preparation of rat liver cells. III. Enzymatic requirements for tissue dispersion. Exp Cell Res 1973;82:391-8.
14. Gkretsi V, Zhang Y, Tu Y, et al. Physical and functional association of migfilin with cell-cell adhesions. J Cell Sci 2005;118:697-710. DOI: 10.1242/jcs.01638

15. Baur H, Kasperek S, Pfaff E. Criteria of viability of isolated liver cells. Hoppe Seylers Z Physiol Chem 1975;356:827-38. DOI: 10.1515/bchm2.1975.356.s1.827

16. Higgins G, Anderson R. Experimental pathology of liver. I. Restoration of liver white rat following partial surgical removal. Arch Pathol 1931;12:186-202.

17. Ascher NL. Hepatocyte transplantation. Liver Transplant Surg 1995;1:139-42. DOI: 10.1002/lt.500010212

18. Strom SC, Fisher RA, Rubinstein WS, et al. Transplantation of human hepatocytes. Transplant Proc 1997;29:2103-6. DOI: 10.1016/S0041-1345(97)00252-2

19. Nishie M, Tateno C, Utoh R, et al. Hepatocytes from fibrotic liver possess high growth potential in vivo. Cell Transplant 2009;18:665-75. DOI: 10.1177/096368970901805-623

20. Cascales Angosto M. El extraordinario fenómeno de la regeneración hepática. In: Bases celulares y moleculares de la regeneración hepática. Madrid, Spain: Instituto de España; 2008. pp. 35-70.

21. Imai T, Masui T, Ichinose M, et al. Reduction of glutathione S-transferase P-form mRNA expression in remodeling nodules in rat liver revealed by in situ hybridization. Carcinogenesis 1997;18:545-51. DOI: 10.1093/carcin/18.3.545

22. Saltikov SA. The determination of the size distribution of particles in an opaque material from a measurement of the size distribution of their sections. In: Elias H (ed.). Stereology Springer; 1967. pp.163-73.

23. Greenwell A, Foley JF, Maronpot RR. An enhancement method for immunohistochemical staining of proliferating cell nuclear antigen in archival rodent tissues. Cancer Lett 1991;59:251-6. DOI: 10.1016/0304-3835(91)90149-C

24. Greer J, Foerster J, Rogers G. In: Hemostasis. Wintrobe's Clin Hematol, Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2009. pp. 460-630.

25. CoaguChek. A brief discussion of prothrombin time. Available from: http://www.coaguchek.com/coaguchek_hcp/en/home/coagulation_monitoring/medical_and_scientific_information/prothrombin_time_INR.html

26. Dragan YP, Hully JR, Nakamura J, et al. Biochemical events during initiation of rat hepatocarcinogenesis. Carcinogenesis 1994;15:1451-8. DOI: 10.1093/carcin/15.7.1451

27. Bannasch P, Klimek F, Mayer D. Early bioenergetic changes in hepatocarcinogenesis: preneoplastic phenotypes mimic responses to insulin and thyroid hormone. J Bioenerg Biomembr 1997;29:303-13. DOI: 10.1023/A:1022438528634

28. Gupta S, Aragona E, Vemuru RP, et al. Permanent engraftment and function of hepatocytes delivered to the liver: implications for gene therapy and liver repopulation. Hepatology 1991;14:144-9. DOI: 10.1002/hep.1840140124

29. Ho C-M, Chen Y-H, Chien C-S, et al. Transplantation speed offers early hepatocyte engraftment in acute liver injured rats: a translational study with clinical implications. Liver Transplant 2015;21:652-61. DOI: 10.1002/lt.24106