Effectiveness of Xenotransplantation of Human Fetal Hepatocytes in Spleen of Rats with Acute Liver Failure Induced by CCl4

Human’s fetal hepatocytes (HFH) were intrasplenic transplanted white non-pedigree rats with acute liver failure (ALF) challenged by single per oral administration of hepatotropic toxin diluted in oil CCl4 at a dose 10 ml/kg (volumetric correlation 1:1) (10 mL/kg body weight as a 1:1 mixture of CCl4 and mineral oil). Transplantation had positive effect on all biochemical blood parameters of the studying animals. Morphologic study showed that reparative-restorative processes were arising in hepatic parenchyma after administration of HFH into splenic pulp of rats with model of ALF on days 14-21. Substantial and main factor in restoration of parenchyma was restoration of micro topographic interrelations in acinus as well as polyploidy of hepatic cells expressed in increase of hepatocytes’ nuclei sizes and hypertrophy of cells themselves. It is an indirect confirmation of engraftment of HFH in liver of rats with model of ALF.

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Introduction

According to the World Health Organization data lethality from chronic hepatic insufficiency takes the 5th place among other diseases, and it reaches 70-90% from acute liver failure (ALF) (Zhang, Han, Wu et al., 2010). Hepatic failure may cause complications in not only diseases of hepato-pancreato-duodenal zone but also other organs and systems of human organism. There is description of cases of fulminant liver failure in miliary tuberculosis, pulmonary cancer, Alzheimer disease and other pathology (Laleman, Verbeke, Meersseman et al., 2011).

Treatment of hepatic failure by means of transplantation of mature somatic and fetal hepatocytes is a new stage in development of practice hepatology (Khajibayev, Urazmetova, Madaminov et al., 2011; Touboul, Vallerie and Weber, 2010; Zhou, Lessa, Estrada et al., 2011). Transplantation of hepatocytes provides restoration of the lost hepatic functions of patients and activation of regeneration of the non damaged liver parenchyma. Such an approach explains a fact that a part of parenchymatous cells surrounding necrosis zones in the even quite damaged liver remains viable and may be regenerated in certain conditions (Teng, Wang, Li et al., 2010).

The study examines effectiveness of intrasplenic transplantation of isolated hepatocytes to correct severe form of liver failure in experimental animals.

Materials and methods

Donor materials

Liver from human fetus has been used as donor material. Human’s fetal hepatocytes (HFH) (18-22 weeks of intrauterine development) were obtained as result of legal abortions made in late terms by medical indications in department of pregnancy pathology.
in urban hospitals of Tashkent (Uzbekistan). To obtain hepatocytes from fetal liver were used methods of separation of cells consisting of 4 steps:

1. Non recirculation perfusion of liver by EDTA-containing solution
2. Recirculation perfusion of liver by solution containing 0.025% collagenase
3. Dispersion of liver

Microscopy research of cellular composition determined hepatocytes and their precursors - hepatoblasts up to 60%, hematopoietic cells (including macrophages) - up to 30% and non-parenchymatous cells - up to 10%. Cellular efficiency from 1 g of hepatic tissue was 12.6±0.28 (x108), viability in the first hours after yield was 96.2±3.4%. Viability of cells was estimated by the following method: 300 mcl of trypan blue solution was added to 100 mcl of cellular suspension (final concentration of trypan blue 0.45%). To visualize cellular damage was used ability of nuclear proteins to adsorb dye. Even the weakest coloration of nucleus is an indicator of damage of cellular membrane. Intact parenchymatous cells had yellow color and well outlined convex superficial line reflecting light. They were easy distinguished by this sign from the dark damaged cells even without coloration in conventional light microscope.

**Modeling of ALF**

Model of ALF was reproduced by single per oral administration of the hepatotropic toxin CC14 (Carbon Tetrachloride) diluted in oil at a dose 10 mL/kg (volume correlation 1:1) (10 mL/kg body weigh as a 1:1 mixture of CC14 and mineral oil) sexually mature rats. Animals were divided into two groups containing 20 and 10 health animals. In all in experiment were used 50 sexually mature pedigrees less male rats weighting from 170 to 220 g. The first (control) group formed 20 rats with model of ALF.

The second (comparative) group formed 20 animals which have been treated by means of human’s fetal hepatocytes (HFH) without additive administration of immunosuppressants. For this purpose they were intrasplenic transplanted by the fresh separated HFH on the second day after induction of ALF at a dose 15-20 mln. Cells in volume of 0.15-0.20 ml in nutritive solution RPMI 1640 by laparotomy approach in the lower and median pole of splenic parenchyma in 2-3 points by a slow and careful injection with 1-ml tuberculin syringe during 1.0-1.5 minutes (Figure 1).

**Figure 1. Intraspelic Transplantation of HFH Rats with ALF**

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At a moment of administration spleen was gently moistened with normal saline solution preserving it from desiccation. No bleeding and broken splenic capsule were occurred after completing procedure of administration of cells. Such a method of introduction of HFH has been chosen by us because splenic vein has systemic blood flow with liver that allows the introducing during 20 minutes HFH immediately hit into hepatic tissue. Additionally, by data of different authors, spleen because of generality of phyllogenetic development with liver has ability to keep a part of fetal cells in its parenchyma forming accordingly foci (pools) of reproduction from HFH.

**Keeping of animals**

Animals were kept in accordance with regulations accepted by the European Convention for the protection of pet animals used for experimental aims (Strasbourg, 1986) and endorsed by the National Ethics Committee. Experimental animals were observed during 21 days.

Clinical state has been estimated by means of the unified methods accounting 5-point degree of hepatic encephalopathy (Shimanko and Musselius, 1993). Liver functions have been determined by means of biochemical indicators of animal’s blood serum (total bilirubin, albumin, activity of alanin aminotransferase – AAT and aspartat aminotransferase – AsAT and ammonia) by the unified methods accepted in clinical practice. Biochemical parameters of blood were studied after killing animals on days 0, 7, 14 of observation. Animals were killed by etherization. Evaluation of immune state included determination of relative content of T- and B-lymphocytes (CD3+, CD20+lymphocytes), immune regulatory lymphocytes T-helpers/inductors (CD4) and T-cytotoxic/suppressors (CD8), natural killers (CD16) by method of indirect rosette-formation (Zalyalieva, 2004; Cheredeev, Gorlina and Kozlov, 1999) by using of monoclonal antibodies of “ICO” series (MedBioSpectr, Russia), determination of phagocytosis activity of neutrophils (PhAN) and circulating immune complexes (CIC).

**Morphologic research**

Light microscopy was performed in department of pathomorphology of Republican Research Centre of Emergency Medicine (RRCEM) and clinical biochemical laboratory. For plain light microscopy materials were fixed in 10% neutral solution of formalin. Paraffin microscopic sections dyed by hematoxylin and eosin were studied on light-optic level to estimate cellular morphology, by sudan-III to determine lipids; and by Van Gieson to visualize fibrosis process (Weibel, Kistler and Scherie, 1966).

Histology estimated plethora of central and portal veins, expression of hepatocytes’ fat dystrophy, hepatocytes’ necrosis foci, state of periportal tracts (enlargement of connective tissue, formation of interlobular septum, infiltration) and bile capillaries (changes of endothelium, cholestasis), inflammatory cellular infiltration of parenchyma, availability of visible cells.

**Statistical treatment**

Final statistical treatment of research results was conducted in program MedCalc 10.2 (Belgium). There were calculated M - arithmetical mean of variation range, SD - standard deviation because of minor volume of sampling and distribution of variation data not obeying normal distribution. Reliability of shifts and differences of the comparing indications was estimated by Wilcoxon criterion of range sums. Differences with P<0.05 have considered as reliable. Analysis of survival rate of censored data has been performed by method of Kaplan-Meier; reliability of differences between survival rate curves was confirmed by a means of log rank test.
Results of research

Toxic damage of liver with picture of ALF was observed after administration of carbon tetrachloride. In ALF in blood of rats took place reliable disorders of all biochemical parameters directly related with damage of hepatic tissue. Xenotransplantation of HFH rats with ALF had positive effect on all biochemical parameters of blood of the studying animals (Table 1).

Laboratory sign of hepatic cellular failure is growth of bilirubin concentration. In experiment in rats with model of ALF on the 7th days was detected a reliable 4-fold increase of content of total bilirubin, with peak on the 14th day up to 43.1±13.16 mmol/l (as compared with findings of health individuals 9±3.80). In 5, 12 days after transplantation of HFH was noted a decrease of the increased level of total bilirubin within the limits 20.6 - 31.0%, with reliable reduction to the findings of rats in appropriate terms of research without transplantation on the 7th and 14th days (Wilcoxon test P<0.05).

One of the main indications of activity of pathologic process in the liver is cytolysis. In the studied animals with ALF model activity of indicator enzymes was reliably increased on the 7th and 14th days. So, activity of ALAT was reliably increased 2.0 and 2.2 times, AsAT - 1.6 and 2.0 times. Statistically reliable decrease of activity of enzymes fixed to be in 5-12 days after HFH transplantation: ALAT - 57.4 and 63.8%, AsAT - 54.5 and 59.0%.

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<th>TABLE 1. BIOCHEMICAL INDICATORS OF BLOOD OF RATS AFTER INDUCTION OF ALF AND MEDICAL TREATMENT BY INTRASPLENIC TRANSPLANTATION HFH (±SD)</th>
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Note: * - reliability of differences (Wilcoxon test, P<0.05) to the data of health rats, † - to the data of rats in appropriate terms of study without transplantation.

Main sign of ALF is hepatic encephalopathy increasingly affecting on a course of ALF and prediction of disease. This symptom is developing as a result of penetration of endogen neurotoxins through hematoencephalic barrier and their effect on astroglia as result of incompetence of hepatic cells. Principal role in this mechanism takes ammonia occupying the leading place among endogen neurotoxins. In our studies ammonia concentration in blood in ALF was reliably increased in all the terms of observation 1.9-2.5 times. Transplantation of HFH contributed to reliable decreasing this indicator due to effect of donor HFH on organism of experimental animals that coincided with clinical manifestations of symptomatic reduction of hepatocerebral failure.

Analysis of Kaplan Meier during 504 h of observation exhibited demonstrable and early improvement of survival rate related to using of HFH (Figure 2). In control group consisting of 20 rats during 504 h have been lost 16 animals (80%), and in experimental group - only 9 (45%). The first case of death in control group fixed to be in 12 hours, in experimental one - in 48 h. Thus, animals of experimental group had a more higher,
reliably pronounced duration of life than animals without transplantation of isolated hepatocytes (IH) (Logrank test p = 0.0195). At the same time indicator of relative risk accounted for 0.4287 (95% CI 0.1943 - 0.9457).

Thus, sanogen mechanisms of allotransplantation of HFH include disintoxication and accelerating process of cellular proliferation in acute toxic damage of organ.

Expressed pathology was fixed in macroscopic research of rats died on the 5th-6th days in laparotomy - dense surface of liver of reddish color, granularity and curvature of edges. Microscopy study noted disorders of hepatocytes with necrosis foci (more than 1/3 of shear section) and leucocytes infiltration, areas of incompleteness of beam structures. The most expressed changes were detected on the light optic level in a zone of portal tracts (Figure 3.A).

Changes analogous to the observing ones in rats died on the 5th day were registered in all the rats died on the 7th day. Morphological changes in the liver of rats died on the 8th-9th days were presented by piecemeal necroses, disturbed hepatocytes (up to 1/3 of shear section); neutrophiles’ clusters were observed near the necrotic changed hepatocytes. Infiltration of periportal zones by lymphocytes noted to be in the killed rats on the 10th day. Hepatocytes themselves were polymorphic. As a rule cells were increased in their sizes. Signs of hydropic and granular dystrophy mainly in area of central veins were observed in their cytoplasm. Beam building of the liver was destroyed. There were binuclear hepatocytes. In a number of cases were observed nidus necroses of hepatic cells. Clear spaces of sinusoid capillaries were uneven filled with blood. Disse spaces were somewhat enlarged. Onset of thin fiber bundles of collagen fibers (capillarization of sinusoids) took place in them. Kupffer cells were increased in their volume. Morphologic changes in liver of rats died on the 13th-14th days were presented by fat infiltration of hepatic parenchyma and characterized by gradual reduction of intensity of damage of lobules of liver from periportal zone to center. Thus, were observed damage of total architectonics of liver, microcirculation disturbances, cellular polymorphism, destruction of border plane; incompleteness of hepatocytes, fat, hydropic and balloon dystrophy of hepatocytes, lipofuscinosis of hepatocytes, nidus hepatonecrosis, hyperchromatism and polychromatism, degeneration (vacuolization) of hepatocytes’ nuclei, intralobular lymph-plasmocytic infiltration, pericentral lymphoid infiltration.

**Figure 2. Curves of 504 hours (21 days) survival rate of Kaplan-Meier after induction of ALF (1st group, N=20) and treatment of intrasplenic transplantation of HFH (2nd group, N=20). Statistical value of differences was estimated by means of Log rank test (P= 0.0195)**
Transplantation of HFH contributed to restoration and safe keeping of beam structure with predominance of intact hepatocytes, reduction of fat inclusions, and increase of a number of non-parenchymatous cells of live. HFH occurred to be the most effective correction method of liver failure owing to protective function of transplanted cells (Figure 3.B).

Results of morphologic study of rats’ liver in experimental group after intrasplenic administration of HFH in initial terms (7 days) showed that necrosis degree of hepatic cells and inflammatory cellular infiltration of parenchyma, fibrosis, dystrophy of hepatocytes, and proliferation degree of epithelium of bile ducts were expressed almost to the same degree, that in hepatic changes in rats of control group. Thick connective tissue bands pierced liver parenchyma. Quite great spaces in parenchyma occupied inflammatory infiltrates consisting of lymphocytes, macrophages and fibroblasts. It were revealed edema, nidus hemorrhages and color changes of the liver, sharp dilation of sinusoids, portal vessels and central veins, pronounced stasis of blood elements in them.

To the 14th day edema events of parenchyma were sufficiently decreased, in certain areas up to complete disappearance. Processes of epithelial proliferation of bile ducts were observed. Hepatocytes in hepatic parenchyma located randomly.

**Figure 3. Structure of liver in the CCL4-induced ALF. 7-21 days of experiment, light microscopy. Coloration with hematoxylin and eosin, ×200.**

A - 1st group (control), B - 2nd group (experimental). 1 - central vein; 2 - sinusoids, 3-vacuolating hepatocytes; 4 - foci corresponding to lipid localization by coloration with Sudan III.
Formation of the liver beams was chiefly noted in immediate proximity to enlarged vessels of portal tract and central veins. Great number of hypertrophic hepatocytes with big nuclei was also revealed. Quantity of binuclear hepatocytes was slightly increased. Signs of activation of Kupffer cells especially near to connective-tissue streaks have been determined.

Partial restoration of beam structure (Figure 3.B) noted to be in certain spaces of hepatic parenchyma in the following terms (21 days). Structure and sizes of hepatocytes varied strong. Main mass of parenchyma formed hypertrophic hepatocytes with nuclei of different sizes. Cytoplasm of some hepatic cells was being restoring, they were dyed pink by coloration with hematoxylin and eosin. They were small with dense granular cytoplasm, often binuclear in immediate proximity to portal tracts. Near to periphery of lobules a structure of hepatic beams was not determined; cells were larger, with greater nuclei. Areas with fat dystrophy of hepatocytes were determined in some spaces. Connective-tissue streaks between hepatic lobules became somewhat thin than in previous terms, sometimes they disappeared entirely.

Morphologic studies of liver of rats with transplantation of HFH into splenic pulp exhibited that reparative-restorative processes chiefly expressed in hypertrophy and hyperplasia of hepatocytes were arising in hepatic parenchyma on days 14-21. Connective-tissue cords between hepatic lobules to the end of experiment disappeared not totally that evidenced partial restoration of hepatic structure of animals. Polyploidy of hepatic cells expressed in increase of sizes of hepatocytes’ nuclei and hypertrophy of cells, restoration of micro topographic interrelations in acinus and creation of conditions for hepatocytes’ proliferation is main factor of regeneration in intrasplenic administration of HFH in CC14-induced ALF. These data are an indirect confirmation of engraftment of HFH in liver of rats with modeling acute liver failure.

Discussion

Hepatocytes of mammals present differentiated polyploidy cells that in normal conditions stably are in a phase G0/G1 of cellular cycle (Figure 4). Duration of existence of such mitotic inert hepatocytes corresponds to human’s life duration. Results of scientific studies allow qualify hepatocytes as unipotent committed population of stem cells able to support constancy of structure and functions of the liver when damaging of any etiology (Runovich, Pivovarov, Kurilskaya et al., 2005; Strekalovskiy, Nikiforov, Goldberg et al., 1998). In case of loss of a part of hepatic parenchyma hepatocytes display practically limitless ability to reproduction in extremely high velocity of regeneration - in repeated surgical resection from 70 to 80% of cellular mass of hepatic parenchyma its restoration happens during 5-6 days. Factors produced both by the liver itself and by its extrahepatic tissues interplaying between themselves and specific receptors of cellular membranes, regulate this compensation mechanism.
Sakaida et al., (2004) report that transplanted stem cells of bone marrow decrease hepatic fibrosis on account of expression of matrix metal proteinases and destroy of collagen fibers. That contributes to perfection of survival of mice with CCL4-induced damage of liver. But remains unclear, whether relate mentioned changes with immediate effect of these cells.

It was proved on transgenic mice (ALuPA, Fah-/-) that mature hepatocytes as compared with other somatic cells have great Hayflick limit manifesting their ability to more than 100 replication cycles and completely re-populate liver in animals VI-VIII of transplantation generation (Lu, Gralla, Liu et al., 2008). Therefore, hepatocytes are characterized by their ability to self-support throughout the whole life of organism of one of the main characteristics of stem spaces’ cells and permit consider differentiated parenchymal cell of liver as unipotent stem cell. It is considered that unipotent of hepatocytes related to be with polyploidy set of chromosomes. In postnatal ontogenesis multiplication of hepatocytes is characterized by alternation of acytokinetic and completed mitosis that properly led to uni- and binuclear state. Diploid binuclear cells are not able to self-reproduction; as result single diploid cells of liver in adult animals are remained as precursors of the whole number of polyploidy hepatocytes. After damage of liver mitoses without cytokinesis are excluded and cell division occurs by conventional completed type, with the result that proliferated population of hepatocytes is getting uninuclear. Therefore, binuclear cells are laid in normal slow growth of liver as potential sources of the future clone of uninuclear polyploidy hepatocytes with unlimited number of progenies in conditions of regeneration. Cellular therapy with hepatocytes and other highly differentiated cells allows through culture remove endothelium and admixtures of such over-antigen donor cells that, fore and foremost, are involved in process of immune rejection (Onishenko, Klimenko, Pozdnyakov et al., 2006).

Thus, despite of difference in form characteristics of cellular material, in experiment we have established high effectiveness of their using in treatment of ALF. One of the principal causes is immunologic immaturity of donor fetal cells of human’s fetus due to inferiority of its antigens’ set. Thanks to this fact immune system of rats-recipients, isolated fetal hepatocytes were administered which, are unable to recognize them, and, consequently, reject them. In the second place, fetal cells themselves are still not sufficiently mature to attack a new host. In the third place, transplanted fetal cells mobilize...
and stimulate their own body defenses being, accordingly, a powerful factor stimulating growth of cells. That is characterized by expressed antioxidant and anti-inflammatory features. In spite of implantation of fetal cells is not “gene therapy”, it is quite possible that biological active substances containing in them contribute to expression of “silent gens” genes.

**Conclusion**

Development of toxic hepatitis induced by single administration of tetrachloride carbon was accompanied by fat infiltration of hepatic parenchyma and characterized by gradual reduction of damage intensity of hepatic lobules from periportal zones to center.

Intrasplenic administration of HFH in CCl4-induced ALF contributed to regeneration of liver and creation of conditions for proliferation of hepatocytes of recipient. It was expressed morphologically in polyploidy of hepatic cells, increase of sizes of hepatocytes’ nuclei of their hypertrophy as well as restoration of micro-topographic interrelations in acinus.

Positive effect of xenotransplantation of fetal hepatocytes on damaged liver coincided with regeneration effects of liver that substantiated suitability of transplantation of cells containing separated fetal isolated hepatocytes and created prerequisites to use them in clinical conditions in ALF.

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