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Intrauterine xenotransplantation of human Wharton jelly-derived mesenchymal stem cells into the liver of rabbit fetuses: A preliminary study for *in vivo* expression of the human liver genes

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ABSTRACT

Objective(s): End-stage hepatic failure is a potentially life-threatening condition for which orthotopic liver transplantation is the only effective treatment. However, a shortage of available donor organs for transplantation each year results in the death of many patients waiting for liver transplantation. Xenotransplantation, or the transplantation of cells, tissues, or organs between different species, was proposed as a possible solution to the worldwide shortage of human organs and tissues for transplantation. The purpose of this preliminary study was to reconstruct human liver tissue by xenotransplantation of human Wharton jelly mesenchymal stem cells (hWJ-MSCs) into fetal rabbit.

Materials and Methods: Isolation and confirmation of hWJ-MSCs from human umbilical cord was performed. Eight rabbits at gestational day 14 were anesthetized. All rabbits carried pregnancies to term yielding 40 rabbit fetuses. Intrauterine injection of hWJ-MSCs was performed in 24 fetuses. Twenty-seven fetuses were born alive. Ten liver samples from injected fetuses were sampled, eight rabbits 3 days after birth and two rabbits 21 days after birth. The non-injected fetuses served as positive control. Fetuses of non-injected rabbits were negative controls. Using real-time polymerase chain reaction (RT-PCR), mRNA expression of albumin (ALB), α-fetoprotein (AFP), hepatic nuclear factor 4 (HNF4), and CYP2B6 (CYP) were detected in liver samples.

Results: The human *ALB, AFP, HNF4,* and *CYP* mRNAs were expressed in the injected sampled fetuses by hWJ-MSCs into fetuses of rabbits *in utero*.

Conclusion: Developing xenotransplantation of hWJ-MSCs into rabbit uterus can introduce an applied approach for producing human liver tissue in rabbits.

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Introduction

Chronic liver disease or viral infections may lead to continual loss of liver function (1). Indeed, the loss of liver function caused more than 38,000 deaths in the USA in 2014 (2). In addition, orthotopic liver transplantation is currently the only clinical treatment for patients with acute and chronic liver. Orthotopic liver transplantation needs long-term immunosuppression (3). Other ways of liver transplantation are hepatocyte-based extracorporeal bioartificial livers (BALs) (4), hepatocyte transplantation (5), and *in vitro* liver tissue engineering (6, 7). These approaches have some limitations. The BALs could provide temporary support for patients who receive a partial hepatectomy due to acute liver failure or are awaiting orthotopic liver transplantation (8). However, weak engraftment of transplanted hepatocytes remains the main barrier to the successful extension of hepatocyte transplantation therapy (5). Liver tissue engineering *in vitro* requires three-dimensional (3D) culture conditions (9).

Xenotransplantation is a potential approach for liver tissue reconstruction. Present interest in xenotransplantation stems from the global deficiency of human organs, tissues, and cells for use in clinical transplantation. As a preliminary study on liver xenotransplantation, liver transplants after recipient immunosuppressive treatment from baboons to adult patients were performed in the 1990s with one

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patient surviving for 70 days (10). On the other hand, xenotransplantation of stem cells in the affected fetuses is an effective treatment for a number of genetic disorders. *In utero* stem cell transplantation is a potential therapy for a number of these disorders and has some potential advantages over postnatal therapy (11). Mesenchymal stem cells (MSCs) of human umbilical cord matrix were used for this study. Human umbilical cord matrix, Wharton jelly (WJ), has recently become the suitable source of stem cells; it is regarded as an extra-embryonic tissue, which is naturally a waste product after birth (12). This tissue can be a ready source of MSCs with little ethical worry and a large amount capacity for use in regenerative medicine (13, 14). Human WJ-MSCs (hWJ-MSCs) can be considered as multipotent stem cells with self-renewal and immune-regulatory potential. They are also able to differentiate towards adipocyte (15) chondrocytes, osteocytes (16), cardiomyocytes (17), skeletal myocytes (18), neuron-like cells (19), insulin-producing cells (20), and hepatocytes (21) under acceptable situations.

The hWJ-MSCs have an extent gene expression profile. They can express the embryonic and adult stem cell markers (22). These cells represent a positive reaction to MSC markers, such as CD10, CD13, CD29, CD44, and CD90 and a negative reaction to CD14, CD33, CD56, CD31, CD34, CD45, and HLA-DR (23). Moreover, they show a number of genes found in undifferentiated embryonic stem cells (ESCs), such as the markers set to pluripotency. Besides, some markers of three germ layers, i.e., endoderm, ectoderm, and mesoderm, were shown to be expressed by the hWJ-MSCs (24). Furthermore, the hWJ-MSCs have shown the ability to make hepatocyte markers and can be regarded as a very interesting cell source for the treatment of liver diseases (25). Therefore, it can be an appropriate candidate for liver tissue reconstruction in xenotransplantation approach.

After stem cells as a source of *in vivo* tissue reconstruction in xenotransplantation, selection of an appropriate recipient is the other step. The reason for selecting rabbits in our research is that the fetal rabbit is an established animal model for *in utero* transplantation of stem cells. The general anatomic, physiologic, phylogenetic and immunologic similarities between rabbit and human fetal development make it a suitable tool for the evaluation of the clinical usage of *in utero* therapy (26). Also, the short period of gestation (28 to 31 days) and the large number of embryos during each pregnancy (5 to 8 embryos) make the rabbit a good candidate of MSCs recipient in *in utero* xenotransplantation approach.

This study has several innovations including a new method for production of human liver tissue using *in utero* xenotransplantation, introducing rabbit as an appropriate recipient for this purpose, introducing an *in-vivo* differentiation method of hWJ-MSCs into human liver cells, and introducing specific genes for real-time detection of human liver cell production. Therefore, the aim of the present study was to assess human liver production in rabbit fetus using an *in utero* injection of hWJ-MSCs.

Materials and Methods

Animals

Ten pregnant time-mated New Zealand white female rabbits weighing from 2.2 to 3.3 kg were purchased from Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences and their pregnancy was confirmed by ultrasonography (Hitachi EUB, Hitachi Medical Corp, Tokyo, Japan) (27). All experiments on the rabbits were done according to the Ethical Instructions for Research on Laboratory Animals of Shiraz University of Medical Sciences. The rabbits have been selected as xenotransplant recipients of human cells because as a halal herbivore species (considering the Islamic rules) it can produce several infants in each pregnancy, has a short pregnancy duration, and intrauterine injection of the large fetus is convenient, which increase chances of success in *in utero* xenotransplantation.

Harvesting and preparation of hWJ-MSCs

Human umbilical cords were obtained from 15 full-term infants born through caesarian delivery at Shafa, Hafez, and Kawsar Hospitals. Samples were collected and prepared after getting informed consent from the parents and human sampling was approved by the Research Ethics Committee of the Islamic Azad University, as a related project that were approved (project# IR.MIAU.REC.1396.603). In this study, we obtained MSCs from human umbilical cords by a rapid and effective method (28). In detail, the umbilical cords were collected in cold phosphate buffered saline (PBS) containing 100 µg/ml penicillin and 100 U/ml streptomycin (Sigma, USA). Then the arteries were discarded and the endothelium of the vein and amniotic epithelium were scraped. Afterward, small explant pieces (about 4×5 mm) were prepared from umbilical cord matrix. The α-minimum essential medium (α-MEM, Sigma, USA) containing 10% fetal bovine serum (FBS, Gibco, Germany), 1% L-glutamine (Sigma, USA), 100 µg/ml penicillin, and 100 U/ml streptomycin were added after 15 min. During this period, the medium was changed twice a week. The MSCs reached 70–80% confluence after about 15 days.

Flow cytometry analysis for cell surface markers

To stain the hWJ-MSCs, at passage 3 the cells were detached from the culture flask. Afterward, 1×10^6 cells/ml were washed in cold PBS containing 10% FBS as blocking solution for 20 min. Then, the cells were incubated with phycoerythrin (PE)-conjugated anti-CD44, CD73 and CD144, Fluorescein isothiocyanate-conjugated anti-CD34, CD90 and CD106, and PerCP-

Table 1. Number of injected fetuses in each rabbit after xenotransplantation of human Wharton jelly-derived mesenchymal stem cells (hWJ-MSCs)

Item	Injected	Non-injected	Total
Rabbit #1	3	2	5
Rabbit #2	3	2	5
Rabbit #3	3	2	5
Rabbit #4	4	2	6
Rabbit #5	3	2	5
Rabbit #6	2	2	4
Rabbit #7	3	2	5
Rabbit #8	3	2	5
Total	24	16	40

conjugated anti-CD105 antibodies (all from Abcam, Cambridge, UK) for 30 min. After the washing phase, the cells were re-suspended in PBS containing 10% FBS. Then, the cells were subjected to flowcytometry (BD, USA) and the data were analyzed using the FlowJo™ software (TreeStar, Ashland, OR, USA).

Adipogenic and osteogenic differentiation of hWJ-MSCs

The cultured hWJ-MSCs were differentiated into osteocytes and adipocytes by exposure to osteogenic (MACS, Germany) and adipogenic media (Stem Cell Technologies Inc., Canada) for 4 and 3 weeks, respectively. The culture media were replaced twice a week. Then, the hWJ-MSCs differentiated toward osteogenic lineage were fixed with 4% paraformaldehyde and stained with alizarin red S (Sigma, USA). To demonstrate the adipogenic differentiation, the cells were stained with oil red O (Sigma, USA).

In utero xenotransplantation

Rabbits generate their antibody repertoire first by *VDJ* gene rearrangement as early as gestation day 14 in fetal liver (29). According to the onset of the immune system developing time in rabbits, *in utero* xenotransplantation was performed at day 14 of pregnancy. Eight rabbits were selected for cell injection (Table 1). The fetuses of those rabbits that were not injected were selected as positive controls. The other 2 pregnant rabbits served as negative controls. Rabbits were anesthetized with intramuscular injection of xylazine (1 mg/kg, Alfasan, Worden, Holland) and ketamine (2 mg/kg, Alfasan, Worden, Holland) and postured in a dorsal position. Using a transabdominal ultrasound with a 3.5-MHz curved array probe, presence, position, and viability of the fetuses were determined, and crown-rump length was measured to confirm the gestational age (Figure 1) (30). Exposing the uterus from an abdominal wall incision, a 24-gauge spinal needle was inserted through the uterine wall into the amniotic cavity and then into the peritoneal cavity and liver of the fetuses under continuous ultrasound guidance by using the freehand technique (Figure 1) (31, 32). After confirmation of the appropriate positioning of the

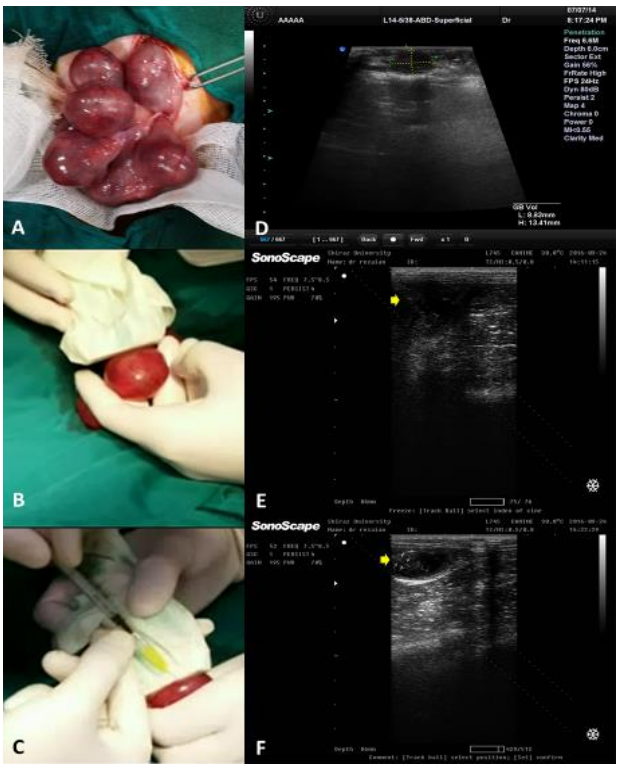

Figure 1. Intrauterine injection of human Wharton jelly-derived mesenchymal stem cells (hWJ-MSCs) into the rabbit fetuses. A) Ventral midline laparotomy to expose rabbit uterine horn. B and D) determining the exact position of the peritoneal cavity by ultrasonography and measurement of the crown-rump length of the fetuses. C, E, and F) needle insertion and injection of hWJ-MSCs in the peritoneal cavity of the fetus (arrows)

needle, the MSCs were slowly injected in a total volume of 3×10^6/200 µl. During infusion, the distribution of a small echogenic fluid in the peritoneal cavity could be observed in ultrasound images (Figure 1). The same procedure was repeated in other fetuses. After transplantation and suturing of the abdominal wall, the fetal heartbeat was monitored by ultrasonography until the dam recovery. The dams were checked daily for health status and signs of abortion. The rabbit fetuses that were delivered normally 13 to 15 days post-transplantation were included in the next step of the study.

Real time-PCR

Three days and 21 days after parturition from live pups (Table 3), liver tissue samples were collected on dry ice. After storage at -70 °C, real time-PCR analysis was done on nitrogen liquid ground tissue. As a negative control, we used RNA from infant rabbit liver tissue samples of a non-injected mother and RNA from human liver cells (HepG2 cell line was purchased from Pasteur Institute, Iran) as a positive control. Biazol isolation reagent (Bioflux, Kenari Harapan Sdn. Bhd. Malaysia) was used to extract total RNA according to the manufacturer's instructions. The quantity of RNA was estimated

Table 2. The primer sequences used for quantitative real-time polymerase chain reaction (RT-PCR)

Primer	Sequence	Size (base pair)
Albumin	5'-ACAGAGACTCAAGTGTGCCAGT-3' 5'-GCAAGGTCCGCCCTGTCATC-3'	198 bp
α-fetoprotein	5'-TTCATATGCCAACAGGAGGC-3' 5'-TGAGAAACTCTTGCTTCATCGT-3'	152 bp
CYP2B6	5'-TTCTTCCGGGGATATGGTGT-3' 5'-TCCCGAAGTCCCTCATAGTG-3'	91 bp
Hepatic nuclear factor 4	5'-AAGAAATGCTTCCGGGCTGG-3' 5'-GACGGGGGAGGTGATCTGTC-3'	156 bp
Human GAPDH	5'-GGCTGTTGTCATACTTCTCATG-3' 5'-CCATCTTCCAGGAGCGAGA-3'	207 bp

Table 3. Number of adult rabbits or fetuses in different stages of the study after xenotransplantation of human Wharton jelly-derived mesenchymal stem cells (hWJ-MSCs)

Item	Injected	Non-injected	Total
Pregnant rabbits	8	0	8
Total detected fetuses during laparotomy	24	16	40
Perinatal dead fetuses	8	0	8
Alive born fetuses	11	16	27
Dead born fetuses	5	0	5
Sampling 3 days after birth	6	0	6
Sampling 21 days after birth	2	0	2

* Absorbed or aborted between 7 to 14 days after injection

at 260/280 nm wavelength by Biophotometer (Eppendorf, Germany). Additionally, cDNA (ABI cDNA kit, USA) was made of 1 µg RNA according to the manufacturer's instructions. The cDNA (2 µl) was used as the template for real-time PCR reactions. Primer-BLAST online program was used to design the primers based on the human DNA sequences found in the GenBank (Table 2) (33). The SYBR green PCR master mix (Applied Biosystems, Rotkreuz, Switzerland) was used for real-time PCR and the cDNA was amplified for 50 cycles. The initial denaturation temperature was 95 °C for 10 min, continued by cycling denaturation at 95 °C for 15 sec and annealing at 60 °C for 1 min. After amplification, the melting curve was analyzed to ensure that no primer dimers were produced in samples and the accuracy of the reactions was confirmed. The data were normalized by comparing them with the housekeeping gene, human *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase).

Results

Culture and characterization of hWJ-MSCs

The morphology of the hWJ-MSCs was a typical characteristic of MSCs; fibroblast-like and spindle shaped (Figures 2 A–C). The hWJ-MSCs were characterized by flowcytometry and showed that they expressed CD44 (89.4%), CD73 (97.7%), CD90 (94.5%), CD105 (43.9%), and CD106 (29.3%). However, the cells were negative for hematopoietic stem cell CD34 (3.20%) and endothelial cell markers (CD144, 3.25%) (Figure 2D). Furthermore, the alizarin red S and oil red O staining revealed the capability of the cells to differentiate toward osteogenic and adipogenic cell lineages, respectively (Figures 2E and F).

In utero xenotransplantation and birth outcomes

The eight rabbits had 40 fetuses (Table 3). We injected 24 fetuses with the hWJ-MSCs. The data of born infants are summarized in Table 3. A few of the fetuses that had died before birth were either absorbed or aborted mostly between 7 to 14 days after *in utero* xenotransplantation.

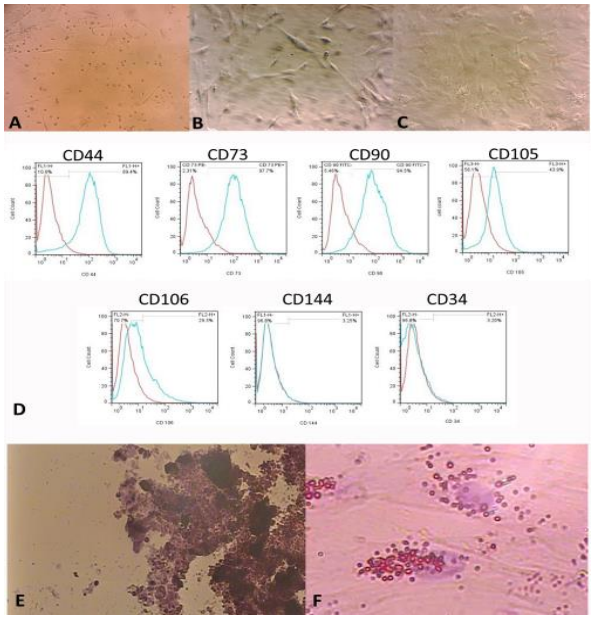

Figure 2. Characterization of human Wharton jelly-derived mesenchymal stem cells (hWJ-MSCs). A to C) Fibroblast-like and spindle shape morphology of the hWJ-MSCs a typical characteristic of MSCs in passage 0 (10x, A), passage 1 (10x, B), and passage 2 (10x, C). D) flowcytometric graphs show the CD marker expression pattern of hWJ-MSCs. hWJ-MSCs were differentiated into osteogenic (E) and adipogenic cell lineages (F)

Table 4. Real-time PCR threshold cycle (CT) of human liver genes in rabbit livers for evaluation of presence of human liver cells after xenotransplantation of human Wharton jelly-derived mesenchymal stem cells (hWJ-MSCs)

Liver tissue samples	Sampling day after birth	CYP2B6	Hepatic nuclear factor 4	α-fetoprotein	Albumin
Sample #1	21 days	38.72	23.99	21.4	19.4
Sample #2	21 days	UD	36.07	33.7	UD
Sample #3	3 days	UD	UD	37.6	38.2
Sample #4	3 days	UD	UD	36.3	UD
Sample #5	3 days	UD	36.8	34.0	UD
Sample #6	3 days	UD	14.4	30.05	27.9
Sample #7	3 days	UD	UD	36.8	UD
Sample #8	3 days	UD	UD	36.6	UD
Negative control	21 days	UD	UD	UD	UD
NTC	-	UD	UD	UD	UD

UD, undetermined; NTC, non-template control

Figure 3. A) Rabbit infants 19 days after intrauterine human Wharton jelly-derived mesenchymal stem cells (hWJ-MSCs) injection. B) Two-week-old rabbits one month after hWJ-MSCs injection. C) Liver tissue sampling. D) Results of gel electrophoresis of HepG2 cells as positive control (CP), xenotransplanted rabbit liver tissue 21 days after birth (XT) and negative control rabbit liver tissue (CN)

Gene expression of human liver cells after xenotransplantation

The expressions of *ALB, AFP, HNF4*, and *CYP2B6* mRNA were evaluated in the samples of liver tissue (Figures 3A to 3C) and compared to the control group (Table 4). According to the results, the samples of rabbit liver tissue could express different levels of some human liver-specific genes. An increase in gene expression was observed when the sampling of rabbit liver tissue was performed after 21 days of birth compared with the tissues sampled after 3 days (Table 4). Gel electrophoresis was used to confirm the real-time PCR results according to the bands formed by the expression of genes (Figure 3D).

Discussion

Xenotransplantation of human MSCs *in utero* through midline laparotomy of the gravid uterus by ultrasonography of fetuses resulted in differentiation and production of human liver cells in the liver of rabbit infants. The analysis of liver cells by real-time PCR showed the existence of the human *ALB*, *AFP*, *HNF4,* and *CYP* mRNA in rabbit infants. Considering the fact that this method is a traumatic surgical technique that causes fetal loss due to the abortions, the number of rabbit offspring decreased.

On the other hand, in the current study, we used hWJ-MSCs for xenotransplantation. WJ-MSCs are now regarded interesting factors not only for autologous but also for allogeneic cell therapy approaches of malignant and non-malignant, hematopoietic and non-hematopoietic, and inherited and gained diseases (12). The ability to regulate immunological responses can grade hWJ-MSCs as a significant suitable stem cell type for therapeutic applications in xenotransplan-tation. Decreased immunogenicity for xenotransplan-tation is suggested by the absence of acute rejection response by the host tracking xenotransplantation into

immune-competent animals (34). Although, the cells were injected in rabbits before immune system development as early as gestation day 14 in fetal liver (29), mechanisms that may let MSCs escape from the immune system in the hosts include the adjustment of host dendritic and T-cell function, progress of regulatory T-cell induction, and limited expression of alloantigen (35). Low MHC-I level and lack of MHC-II expression in hWJ-MSCs keep them from NK-mediated lysis by the host (36). In addition, WJ-MSCs express high levels of leukocyte antigen G6 (HLA-G6), the same that is made by trophoblasts and keeps the embryo from immune-based destruction (37). Thus, prescription of immunosuppressive drugs is not needed, thereby protecting the patient against their side effects.

Furthermore, it has been recommend that hWJ-MSCs is a good candidate for hepatocyte differentiation (38). They have been demonstrated to express some hepatic markers, such as *ALB, AFP,* and *HNF4,* which can be considered as a gene with an essential role in hepatocyte differentiation (39). The hWJ-MSCs population has shown the early hepatic markers, such as albumin, α-fetoprotein (AFP), and a low level of hematopoietic markers, such as c-kit and phosphoenolpyruvate carboxykinase (PEPCK). Nevertheless, expression of the hepatic nuclear factor-4 (HNF4) as an endodermal marker and a key regulator of hepatogenic fate is described (40). There is a low level of *CYP2B6* expression in hWJ-MSCs. Different types of MSCs have been isolated from the bone marrow (41-49), adipose tissue (44, 50-52), endometrium (53, 54), dental pulp (55), umbilical cord (39), and menstrual blood (56). They possess multilineage properties differentiating to osteoblasts (50), adipocytes (54), chondrocytes (50), and neuronal-like cells (41). In the current study, differentiated hWJ-MSCs in rabbit fetus expressed several human liver markers after xenotransplantation. Other types of MSCs also may have liver cell differentiation potential in *in utero* xenotransplantation and need further investigation.

Human liver production by xenotransplantation also needs development of a suitable animal model for *in vivo* research before clinical application. In the current study, we developed a rabbit model for cell xenotransplan-tation. This model has benefits such as a large number of offspring in each delivery and short breeding cycle. The early-stage rabbit embryos are immunologically immature, providing a good tolerance of cell xenotrans-plantation and an allowable model to recognize the biology of stem cells following their transplantation. Therefore, an animal model is definitive to develop improved strategies for clinical protocols aiming to progress engraftment levels to get a clinical advantage (29, 30).

Conclusion

These results indicate the usefulness of ultrasound-guided procedures as less traumatic and invasive methods to inject human cells *in utero* in the rabbit model. So, this technique may increase the efficiency of breeding alive animals with express human genes, therefore, advancing xenotransplantation study and helping to reconstruct human liver tissue in rabbit fetuses. It is also valuable for patients with hepatic impairment in the future.

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