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Expression of Recombinant Coagulation Factor IX in Human Amniotic Membrane-derived Mesenchymal Stem Cells: A New Strategy to Gene Therapy of Hemophilia B

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Abstract

Background: Hemophilia B is an X-linked hereditary disorder of blood coagulation system which is caused by factor IX (FIX) deficiency. Factor IX is a plasma glycoprotein that participates in the coagulation process leading to the generation of fibrin. Replacement of factor IX with plasma-derived or recombinant factor IX is the conventional treatment for hemophilia B to raise the factor IX level to therapeutic range. Recently, gene therapy has been regarded as a promising approach to treat hemophilia B. This study was aimed to express the factor IX in human amniotic membrane-derived mesenchymal stem cells (hAM-MSCs).

Materials and Methods: Human amniotic membrane-derived mesenchymal stem cells were isolated and characterized from amnion membrane. Factor IX from commercially available plasmid was sub-cloned into pcDNA3.1 vector. Recombinant pcDNA3.1-FIX construct was confirmed by PCR, enzymatic digestion and DNA sequencing. Mesenchymal stem cells were transfected with the recombinant vector. Expression of factor IX was determined by RT-PCR, ELISA and its biological activity assay was performed using aPTT.

Results: Isolated hAM-MSCs expressed specific mesenchymal stem cells markers and were able to differentiate to osteocytes and adipocytes lineages. hAM-MSCs expressed hrFIX at mRNA and protein level. The maximum amount of hrFIX was 120 ng/ml at 72 hrs after hAM-MSCs transfection. This hrFIX was biologically active (11% activity), formed fibrin clot in aPTT test and caused more than two fold decrease in clotting time.

Conclusion: The hAM-MSCs expressing factor IX would be useful for gene therapy of hemophilia B. However further studies are required to prove these finding.

Key words: Hemophilia B, amnion membrane, mesenchymal stem cell, factor IX, gene therapy.

Introduction

Hemophilia B is an X-linked hereditary disorder of blood coagulation system caused by factor IX (FIX) deficiency or dysfunction. The prevalence of the disease varies in different countries at a rate of 1 case per 30,000 male (approximately 12% of hemophiliacs) [1-4]. FIX is a plasma glycoprotein that participates in the coagulation process leading to the generation of fibrin. Its molecular weight is about kDa56 and it is synthesized by liver. FIX plays an important role in the process of blood coagulation. Following activation by activated factor VII (aFVII) or activated factor XI (aXI) it helps in clot formation by factor X activation. FIX is a vitamin K-dependent coagulation factor [5-7]. Replacement of factor IX with plasma-derived or recombinant factor IX (rFIX) is the conventional treatment for hemophilia B. The therapeutic dose of factor IX varies in different patients and it depends on the patient's condition such as; disease severity, previous treatment and inhibitory antibodies production. However, factor replacement therapy is not curative and patients should receive factor IX continually. Moreover, it is

too expensive [1,8].

Recently, gene therapy has been considered as a promising approach to treatment of hemophilia B. The gene therapy is mainly based on two methods; first, in vivo transduction of liver or muscle cells with recombinant vector caring the target gene and second; ex vivo transduction of suitable cells (such as: fibroblasts, keratinocytes and stem cells) with the recombinant vector, expansion and then injection of modified cells to patients[9-11]. Gene transfer can be performed using any of the biological, chemical or physical techniques [12]. However, immune reaction to vectors (especially viral vectors) and low expression level of target gene are major challenges. Therefore, choosing proper cells and methods for gene therapy is very critical [13-15]. Over the past two decades, the ability to transfer genes into stem cells has improved and several gene therapy-based approaches have been developed [16,17].

Mesenchymal stem cells (MSCs) are multipotent stem cells which have the capacity of differentiation to various cell types. MSCs are attractive for therapeutic applications because they have immunomodulatory properties. These cells can be isolated from several sources such as adipose tissue, dermis, peripheral blood and bone marrow. Other important sources of MSCs are umbilical cord (UC), umbilical cord blood (UCB), placenta and amnion membrane [18-20]. Amniotic membrane is an appropriate source for collection of the MSCs. Being easily accessible without any

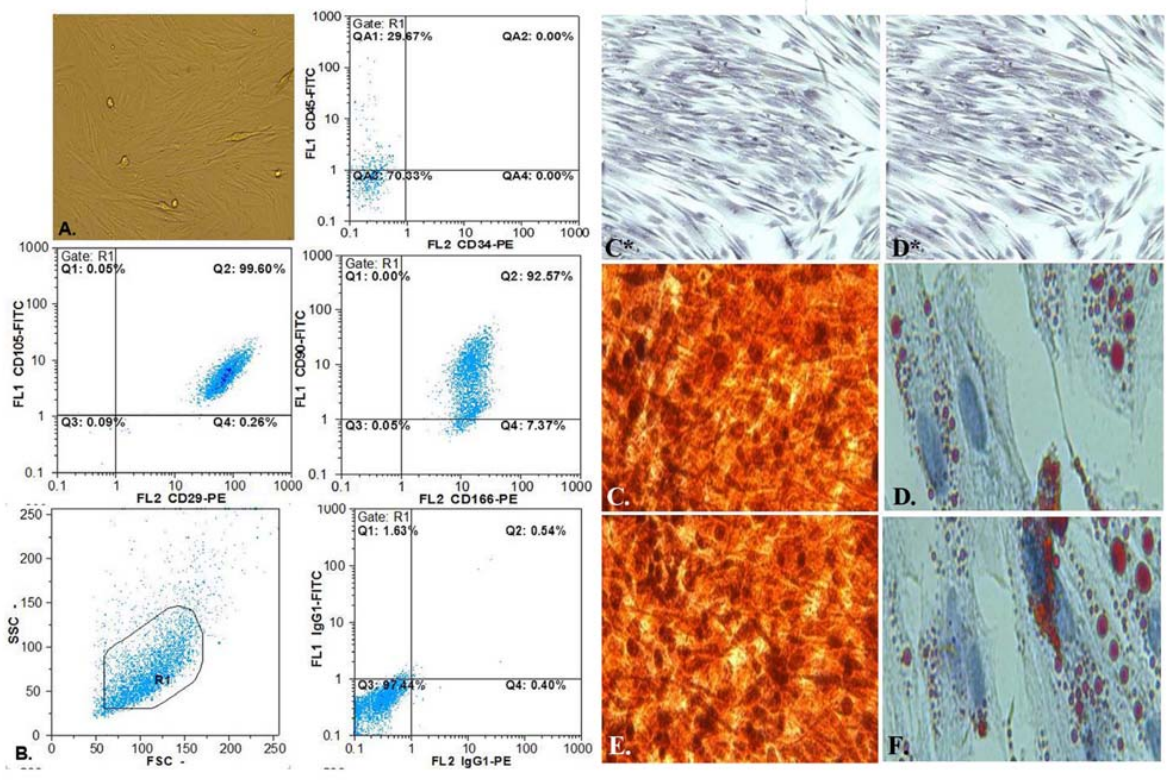

Figure1: **Characterization of human amnion membrane-derived mesenchymal stem cells (hAM-MSCs). (A)** Fibroblastic-like morphology of AM-MSCs after 8 days from third passage. **(B)** Flow cytometric analysis showed that AM-MSCs expressed CD105, CD90, CD29, CD166 and did not express CD34, CD45. **(C)** After cultivation of hAM-MSCs in osteogenic differentiation medium and staining with Alizarin red S 2%, osteocytes were identified by detection of calcium mineralization. **(D)** Accumulated neutral lipids stained with oil red O indicated adipogenic differentiation of AM-MSCs. Negative controls for **(C*)** Osteogenic and **(D*)** Adipogenic lineages. The differentiation potential of hAM-MSCs was tested after transduction with rpcDNA3.1-FIX and the results showed that transfection of rpcDNA3.1-FIX into hAM-MSCs did not affect on hAM-MSCs **(E)** osteogenesis and **(F)** adipogenesis capacity.

invasive procedure needed to obtain them is the most important advantage of these cells [21-23]. In the past few years, the use of MSCs in both cell-based and gene-based therapies has gained much interest.

In this study, we genetically manipulated human amniotic membrane-derived mesenchymal stem cells (hAM-MSCs) with hFIX gene and in vitro expansion of the engineered hAM-MSCs. Our results suggested that hAM-MSCs expressing hFIX would be a promising strategy to treat hemophilia B.

Materials and Methods

Isolation of MSCs from amnion membrane

After filling out the consent form by parents, human amnion membrane tissue was obtained immediately after delivery. Samples were washed several times and transferred in Hank's Balanced Salt solution (Invitrogen, Germany) supplemented with 100 U penicillin / streptomycin (Gibco, Germany). Following removal of blood clots, the samples were cut into smaller pieces , washed with Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG) (Gibco, Germany) and treated with collagenase type IV (2 mg/ml) for 2 hours at 37°C. Then, the samples were washed and incubated with 2.5% trypsin (Invitrogen, Germany) for 30 min at 37°C. Isolated cells were suspended in DMEM-LG with 10% FBS (Gibco, Germany) and antibiotic then seeded in appropriate flasks. The cells were incubated in a 37°C incubator containing 5% CO_2 and 90% humidity. Non-adherent cells were removed after 24 hrs. The culture medium was changed every 2-3 days.

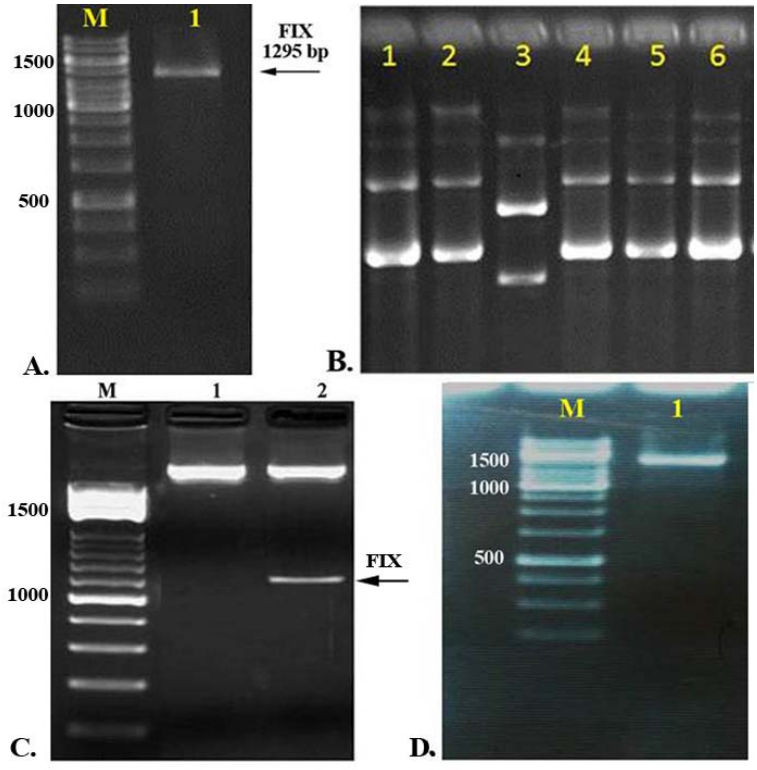

Figure 2: Cloning of FIX into pcDNA3.1 plasmid. (A) PCR reaction to amplify full length of hFIX. **(B)** Electrophoresis of recombinant plasmids. Plasmids of No. 1, 2, 4, 5 and 6 contained hFIX sequence **(C)** Digestion of plasmid with restriction enzymes. M: 100bp DNA marker. Lan1: empty vector, Lan2: recombinant vector. DNA fragment with 1295 bp confirmed insertion of FIX. **(D)** PCR reaction to confirm existence of FIX in the ligated vector. M: 100bp DNA marker. Lan1: 1295 bp amplicon of FIX .

Surface immunophenotyping of hAM-MSCs by flow cytometry

For flow cytometry, hAM-MSCs were trypsinized with 0.05% trypsin/EDTA, washed and re-suspended in PBS. 1×10^5 cells were incubated with specific PE or FITC conjugated anti CD34, CD45, CD166, CD105, CD90 and CD29 monoclonal antibodies for 30 minutes at 4ºC and in a dark place. The hAM-MSCs were analyzed using flow cytometry. Immunoglobulin isotype control was also evaluated in each run.

Differentiation studies

The differentiation potential of cells was examined on third passage of the hAM-MSCs. For induction of osteogenic differentiation, the hAM-MSCs were plated in six-well plates at a density of 10000 cells/cm^2. After 24 hrs, osteogenic differentiation medium containing dexamethasone, L-glutamine, ascorbate, D3 vitamin and β- glycerophosphate was added to the test cells, and DMEM-LG was added to the control cells. Medium refreshment was performed every 3-4 days for 21 days. Finally, the cells were washed with PBS, fixed with 4% paraformaldehyde and stained with alizarin red S 2% to detect the presence of calcium deposition in osteocytes.

To differentiate hAM-MSCs to adipocytes, the hAM-MSCs were plated in six-well plates at a density of 10000cells/cm^2. After 24 hrs, adipocyte differentiation medium containing recombinant human insulin, L-glutamine, dexamethasone, indomethacin, and IBMX (3-isobutylmethyl-xanthine) was added to the cells. In case of controls, DMEM-LG was added to the cells. After 2-3 weeks, the cells were stained with oil red O 0.5% to detect the presence of neutral lipid vacuoles in adipocytes.

Plasmid and bacteria

pcDNA3.1(+) plasmid vector (Invitrogen, Carlsbad, CA, USA) was used for cloning and expression of rFIX. The bacterial strain Escherichia Coli DH5 (Cinagen, Tehran, Iran) was utilized as a host for cloning procedure.

Construction of recombinant expression vector carrying FIX

FIX was sub-cloned into pcDNA3.1(+) plasmid from commercially available plasmid. Briefly, at first FIX was PCR amplified using specific primers containing NheI and EcoRI restriction enzymes sites. Then, the PCR product was cleaned up by high pure PCR product purification kit (Roche, Germany), and cut with NheI, EcoRI restriction enzymes to make compatible sticky ends. pcDNA3.1(+) plasmid vector was also digested by Nhe I and EcoR I

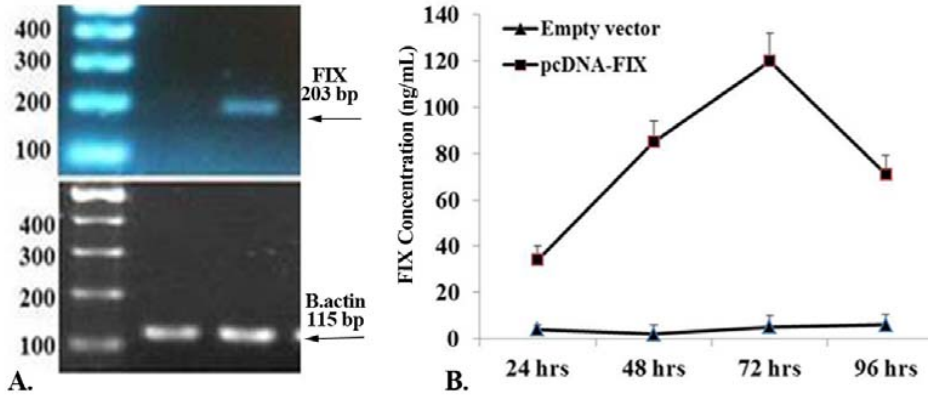

Figure 3: Expression of rFIX in the transfected hAM-MSCs. (A) RT-PCR analysis of transfected hAM-MSCs with rpcDNA3.1-FIX and empty pcDNA3.1vectors. M: 100bp DNA marker lane 1: transfected hAM-MSCs with rpcDNA3.1-FIX, lane 2: transfected hAM-MSCs with empty pcDNA3.1 vector. After RNA extraction, RT-PCR was performed to detect hrFIX in mRNA level. Transfection of hAM-MSCs with rpcDNA3.1-FIX resulted in expression of hrFIX. **(B)** ELISA quantification of hrFIX in the supernatant medium. ELISA was performed at 24, 48, 72 and 96 hrs after transfection. Maximum amount of hrFIX was 120 ng/ml at 72 hrs.

restriction enzymes followed by cleaning up. Then, FIX was ligated to pcDNA3.1(+) plasmid vector using T4 ligase to produce expression recombinant pcDNA3.1-FIX vector (rpcDNA3.1-FIX). Then, rpcDNA3.1-FIX was transformed to competent E.coli. The recombinant construct was screened by PCR, enzymatic digestion and finally confirmed by DNA sequencing.

Transfection of pcDNA3.1-FIX into hAM-MSCs

Transfusion was performed by HD transfection reagent (Roche, Germany) according to manufacture instructions. Stable DNA integration was selected in the presence of G418 (200µg/ml). Expression of hrFIX in hAM-MSCs was detected by RT-PCR and Enzyme-Linked Immunosorbent (ELISA) assay techniques.

PCR and RT-PCR

PCR was performed to isolate full length FIX. It was performed using Pfu and Taq DNA polymerase (Cinnagene, Iran) in a GeneAmp PCR system 9600 (PerkinElmer Life And Analytical Sciences, Inc., Wellesley, MA, USA). The forward primer contained Kozak sequences and the NheI restriction enzyme site was 5'-CGCTAGCACCATGGTGCAGCGCGTGAACATGATCATG -3' and the reverse primer containing EcoR1 restriction enzyme site was 5'-CAGAATTCTTAAGTGAGCTTTGTTTTTTCC- 3. The PCR condition for full length FIX was initial denaturation at 94°C for 5 min followed by 30 amplification cycles consisting denaturation at 94°C for 30 sec., annealing at 60 °C for 30 sec. and extension at 72°C for 30 sec. RT-PCR was performed to evaluate expression of FIX in mRNA level following the transfection. Primer set for a 203 bp fragment of FIX was, forward 5'- ACCCGTGCTGAGACTGTTTT -3' and reverse 5'- GGGCAGCAGTTACAATCCAT -3'. For normalization, expression of β- actin was examined and the primer set was; forward 5'-TTCTACAATGAGCTGCGTGTGG -3' and reverse 5'-GTGTTGAAGGTCTCAAACATGAT- 3'. PCR annealing temperature was 59 °C for internal FIX and beta-actin. PCR products were separated in agarose gel.

ELISA assay to quantify hrFIX

The amount of hrFIX in the supernatant of transfected cells was determined by sandwich ELISA kit (Affinity Biologicals™) at 24, 48, 72 and 96 hrs after transfection. Human pooled normal plasma (PNP) was used as a standard reference.

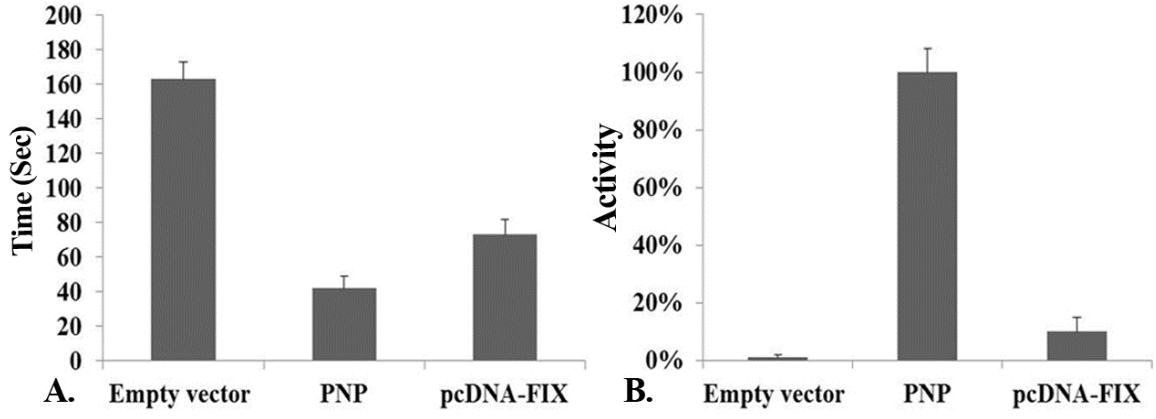

Figure 4: Biological activity of hrFIX. (A) Biological activity of secreted hrFIX was assayed by activated partial thromboplastin time (aPTT) test. hrFIX decreased clotting time more than two fold compared with pooled normal plasma (PNP) that decreased it four fold. Medium of hAM-MSCs transfected with empty vector did not decrease clotting time. **(B)** Activity percentage of hrFIX: hrFIX had biological activity about 11%. While biological activity of medium of hAM-MSCs transfected with empty vector was less than 1% compared with PNP biological activity (100%).

One hundred µl of samples and standard were applied to individual anti-hFIX-coated wells, incubated at room temperature for 30 min. After 3 times washing, diluted HRP-conjugated secondary antibody was added to each well and incubated at room temperature for 30 min. All wells were washed; 100µL of substrate (TMB) was added to each well and incubated for 10 min at room temperature. Then reactions were stopped using 50µL of H_2SO_4 and the optical density (OD) of the samples were taken at 490 nm using an ELISA plate reader.

aPTT to assay biological activity of hrFIX

Activated partial thromboplastin time (aPTT) was used to quantify the functional FIX (FIX:C levels) of hrFIX. One hundred µl of diluted supernatant media and standards (increasing dilutions of pooled normal plasma) were mixed with 100µl FIX-deficient plasma (STAGO, France) and incubated at 37°C. Following by addition of aPTT reagent to the diluted samples and 2-3 min incubation at 37°C, 100µl of 20 mM calcium chloride (Sigma, USA) was added. The clotting times were measured. The standard curve was drawn by plotting the clot times against activity of diluted standards. aPTT was performed on supernatant media harvested from transfected hAM-MSCs with rpcDNA3.1-FIX and empty pcDNA3.1 vector 72 hrs after transfection.

Results

hAM-MSCs were fibroblast-like and expressed MSCs surface markers

Following disruption of amnion tissue and isolation of hAM-MSCs, their morphological characters were studied under invert microscope. Fibroblastic-like appearance, a special character of MSCs, was observed (Figure 1A). Flow cytometric analysis of the hAM-MSCs confirmed the presence of MSCs markers i.e. CD105, CD90, CD166 and CD29 and the absence of hematopoietic stem cell markers i.e. CD34 and CD45 (Figure 1B). Taken together, the results confirmed that the isolated cells were MSCs.

hAM-MSCs differentiated to osteocytes and adipocytes

After isolation of the hAM-MSCs, osteogenic differentiation was induced. Twenty one days after induction, the Alizarin red S staining of the cells confirmed their osteogenic differentiation (Figure 1C). In addition, the adipogenic differentiation capacity of the isolated cells was confirmed following staining of lipid droplets by oil red O (Figure 1D). However cultivation of the hAM-MSCs in the presences of DMEM-LG culture medium (as negative controls) did not show any sign of differentiation (C*-D*). Overall, these findings further confirmed the characters of MSCs. Osteogenic (Figure 1E) and adipogenic (Figure 1E) differentiation of hAM-MSCs was analyzed after genetic manipulation. hAM-MSCs saved their differentiation capacity after transfection.

Cloning and construction of recombinant plasmid carrying the hFIX

hFIX was sub cloned in pcDNA3.1 plasmid. To perform sub-cloning, at first full-length hFIX was isolated from commercially available vector by PCR (Figure 2A) and ligated into pcDNA3.1 vector digested with appropriate restriction enzymes followed by transformation to E.coli DH5 bacteria. Plasmid electrophoresis was performed to select bacteria colonies containing rpcDNA3.1-FIX vector (Figure 2B). Existence of FIX insert in the recombinant plasmid was confirmed by restriction enzymes digestion (Figure 2C) and PCR (Figure 2D). Finally DNA sequencing results confirmed accuracy of the hFIX sequence and its frame in the recombinant vector.

Expression of recombinant hFIX in hAM-MSCs vector

After total RNA extraction from hAM-MSCs transfected with rpcDNA3.1-FIX and empty pcDNA3.1 (as a control) RT-PCR was performed to detect hFIX mRNA using specific internal primers for hFIX to amplify a fragment with 203 bp length. RT-PCR evaluation indicated that the engineered hAM-MSCs expressed hFIX mRNA but no PCR product was observed in hAM-MSCs transfected with the empty vector (Figure 3A).

Next, to detect expression of hrFIX in protein level, ELISA was performed. The supernatant medium was collected at 24, 48, 72 and 96 hrs after transfection. The hAM-MSCs transfected with pcDNA3.1-FIX expressed hrFIX, while cells transfected with empty pcDNA3.1 vector did not. Seventy two hours after transfection, the highest level of hrFIX expression (120 ng/ml) was observed

in the condition medium and declined thereafter (Figure 3B).

hrFIX decreased clotting time and was biologically active

aPTT assay was performed to find out whether the expressed hrFIX is biologically active. More than two fold decrease in clotting time was observed when supernatant medium containing hrFIX was used in combination with human FIX-deficient plasma for aPTT assay. Pooled normal plasma was used as control and about four fold decrease in clotting time by using it was also observed. Clotting time was too long when the supernatant medium of hAM-MSCs transfected with empty vector was used (Figure 4A). Also activity percentage of hrFIX was calculated using aPTT and plotting standard curve. hAM-MSCs transfected with rpcDNA3.1-FIX vector produced biologically active hrFIX with 11% activity in 72 hrs supernatant medium. While in the supernatant medium of hAM-MSCs transfected with empty pcDNA3.1 vector; activity was less than 1% compared with pooled normal plasma (Figure 4B).

Discussion

Replacement of factor IX with plasma-derived or recombinant factor IX (rFIX) is a conventional treatment for hemophilia B. However, this is not a real and curative treatment.

Recently, gene therapy or genome editing has been introduced as a very promising method for hemophilia B treatment and it is under intensive research. Appropriate cells and methods for gene therapy are very critical [13-15].

In this study, we genetically manipulated hAM-MSCs to over-express rFIX. We suggested a new source of cell i.e. hAM-MSCs for gene therapy of hemophilia B. Due to long lifespan and homing ability of MSCs, MSCs have recently been regarded as an attractive cell gen-based therapy purpose [24, 25].

Amniotic membrane is an appropriate source to isolate MSCs. It is easily accessible, without any invasive procedure, and has high multipotency capacity and immunomodulatory effects Moreover; hAM-MSCs are more primitive cells [21-23].

In this study, we used cationic liposome transfection reagent to transfect the recombinant vector to hAM-MSCs. This reagent is very safe and there are no risks of contaminations, infections or immune reactions. The amount of hrFIX protein was 120 ng/ml in the maximum level. Secreted hrFIX was able to form fibrin clot and decreased clotting time. Biological activity of hrFIX was about 11%. We quantified the secreted hrFIX with ELISA technique. ELISA is a very sensitive method to determine protein concentration in different samples.

Recently Sayyar et al.[28] (2012), transfected umbilical cord-derived mesenchymal stem cells (UC-MSCs) with lentivirus vectors then encapsulated UC-MSCs with fibrinogen–alginate microcapsules to produce rFX. Interestingly in their study, the expression level of rFIX was about 4000 ng/ml as determined by ELISA. Difference in the expression system, lentiviral expression system in their study versus plasmid in our study, might account for this discrepancy. However, they produced rFIX by extensive laboratory works. Moreover, concern about safety and immune reaction due to viral vector are other limitations of their study.

Amit et al.[29] (2011), produced expression construct FIX using adenovirus vectors (scAAV2/8-LP1-hFIXco). Fallowing by intravenous injection of vectors in 6 patients, they estimated that FIX level in plasma raised about 4-12%. Similar to Sayyar et al. study, concern about safety and immune reaction are limitations of Amit et al. study as well. Intravenous injection of vectors may also lead to fast clearance of vector from plasma and limit expression of FIX.

Chen et al.[30] (2009), used retrovirus-transfected human umbilical cord tissue derived mesenchymal stem cells to produce hrFIX. They detected hrFIX by western blot and ELISA techniques. They tested biological activity of secreted hrFIX using aPTT assay and showed that the biological activity of hrFIX was 100-130% compared with pooled normal plasma. Again safety is a major concern in their study.

Conclusion

In this study we genetically engineered hAM-MSCs to express recombinant hFIX. The secreted hrFIX protein was biologically active and participated in fibrin clot formation. These hAM-MSCs may be useful for MSCs-based gene therapy of hemophilia B in future. However, further and complementary studies are required. Improvement of expression level to increase the amount of secreted hrFIX and in vivo studies are recommended as future

investigations.

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