



**Original Article**

# Optimizing Lipofectamine LTX Complex and G-418 Concentration for Improvement of Transfection Efficiency in Human Mesenchymal Stem Cells

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## Abstract

Conventional cancer therapies, including surgery, radiotherapy, and chemotherapy, are not tumor site-specific and have cytotoxic and harmful side effects for normal cells. Mesenchymal stem cells (MSCs), due to their tumor-tropism migration property, are a promising alternative to deliver and produce antitumor agents. However, MSCs are difficult-to-transfect cells, and introducing the exogenous therapeutic gene into MSCs is challenging yet needs improvement. Transfection using chemical reagents, including Lipofectamine, is more convenient and less cytotoxic compared with different methods of introducing exogenous DNA into MSCs. Nonetheless, the major limitation of Lipofectamine is low transfection efficiency in MSCs. Therefore, the purpose of this study was to evaluate and suggest the optimum quantities of lipoplex components to enhance the transfection efficiency of human adipose tissue-derived MSCs (hASCs). Finding the best transgene expression time point and the optimum concentration of G-418 for antibiotic-based selection was another goal of this study. hASCs were transfected in a series of experiments with altering the quantities of Lipofectamine LTX® (Lip-LTX), the related “PLUS” reagent, and a plasmid DNA (pDNA) expressing the enhanced green fluorescent protein (eGFP). After transfection, the percentage of eGFP-expressing cells was evaluated using fluorescence microscopy and ImageJ software in 12-hour intervals for 48 hours. Also, the viability of hASCs exposed to different concentrations of G-418 was measured using an MTT assay. The results demonstrated that a combination of 2 µL Lip-LTX, 0.75 µL of its “PLUS” reagent, and 0.75 µg pDNA (6484 bp) improve the transfection efficiency of hASCs (23.75%), and the best period for evaluation of fluorescence for these cells is 12 to 24h post-transfection. Also, the optimum concentration of G-418 for antibiotic-based selection of hASCs was 0.25mg/mL. In conclusion, this study indicates that the setting up of optimized quantities of lipoplex components and the golden time of evaluation for transgene expression could increase the possibility of transgene expression in hASCs before beginning research and clinical application. Also, the definition of optimal dose of selection antibiotic for purification of transfected hASCs seems to be necessary for maximum transgene expression effects in the cell population.

**Keywords:** Genetic engineering, Mesenchymal stem cells, Transfection, Lipofectamine LTX

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## 1. Introduction

Cancer is one of the top life-threatening diseases worldwide. It is the second leading cause of death in the United States (1) and the third most common cause of death in Iran after cardiovascular diseases and traffic accidents (2). The incidence and mortality rate of various cancers has been severely increased in Iran in the last decade (3). Thus, various therapeutic approaches have been exploited, and novel treatment methods are under development.

Numerous conventional therapeutic approaches for cancer treatment, including surgery, radiotherapy, and chemotherapy (4), are nonspecific and unable to distinguish and eliminate tumor cells exclusively, so they cause harm to the normal tissue cells too. Thus, tissue injuries, cell toxicity, and severe side effects are inevitable in cancer patients. Perhaps the low efficiency of these conventional cancer therapies has been the reason to witness only 13% less mortality related to cancer (5).

Human MSCs (hMSCs) provide a promising alternative cell-based therapy for cancer treatment. These cells have been widely studied for their multiple biological features (6). Several of these features include easy isolation and ethical derivation of hMSCs from bone marrow and adipose tissues, quick *in vitro* expansion, multilineage differentiation potential *in vitro* (7), and immune-privileged in autologous transplantations (8). In particular, MSCs have an inherent tendency to migrate toward the tumor or injured tissue (5).

Based on the promising properties of hASCs, these cells have been extensively used in gene modification studies to generate genetically engineered MSCs as a therapeutic gene delivery tool. Particularly, due to the homing of hASCs following the migration toward wounded tissue or tumors, these cells keep a great promise in targeted delivery of either cytotoxic inducible antitumor transgene or its therapeutic products precisely at the side of tumor or wound, respectively (9). However, the hASCs are known as hard-to-transfect cells, similar to the other primary cells

(10). Therefore, the critical step for using hMSCs in cell-based therapy is introducing the exogenous therapeutic DNA (transgene) into the cells, a process called transfection (11).

Current techniques for gene introduction into hMSCs using viral methods are costly, challenging to perform, and inefficient for delivering large transgenes. There are also numerous biosafety concerns about using viral vectors, including possible immunogenicity, mutagenicity, and even tumorigenic consequences (11). In contrast, the non-viral transfection methods are safer, economical, easy to perform, with no limitation for the transgene size. However, these methods are inefficient for the transfection of hMSCs, which are known as hard-to-transfect cell types (12). For non-viral methods, a low-to-moderate transfection efficiency (less than 15%) has been reported in hMSCs (13).

The transfection of hMSCs has been usually performed using commercial cationic lipids, including Lipofectamine as a non-viral method (14). Cell transfection using Lipofectamine, also known as “liposome/lipid-based transfection” or “Lipofection,” uses a lipid-DNA complex (Lipoplex) to deliver transgene DNA to the cells. The animal/human cell membranes are composed of a bilayer of phospholipids with hydrophilic surfaces facing the cytoplasm and extracellular environment. Lipofection technology uses small vesicular structures called “liposomes” with the same composition as the cell membrane. During lipoplex preparation, a simple reaction causes liposome formation around the transgene DNA sequence to be transfected. Depending on the liposome and cell type, the liposome can be directly fused with the cell membrane or endocytosed to release the transgene DNA construct into the host cells (15). According to the literature and ThermoFisher Scientific® website, Lipofectamine® LTX (Lip-LTX) is a plasmid transfection reagent, which provides the highest level of transfection into primary cells, including MSCs, compared with the other lipid or polymer reagents, if combined with PLUS™ reagents. The manufacturers’

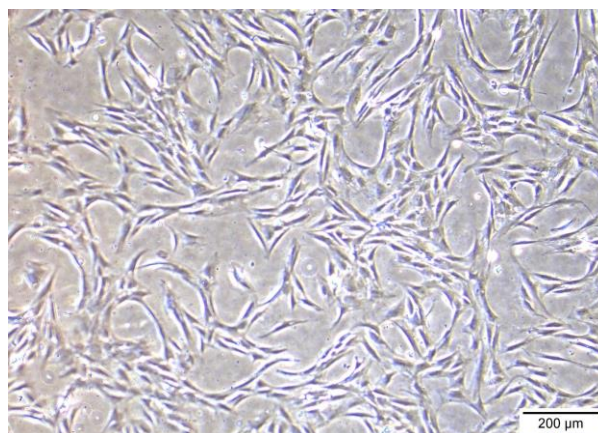
typical instructions usually have not been set up for all cell types, which lead to lower transfection efficiency and higher cytotoxicity for specific cell types, including MSCs (16).

This study investigated whether altering the proportion and quantity of the transfection reagent (Lipo-LTX and its “PLUS” reagent) to the mass of plasmid DNA (pDNA) could improve the transfection efficiency of hASCs using a large-sized transient expressing plasmid. Also, we tried to find the best time window for the study of transgene expression. Furthermore, we aimed to determine the optimum selective concentration of G-418 as an aminoglycoside phosphotransferase antibiotic to kill non-transfected hASCs and select the transfected cells.

## 2. Materials and Methods

### 2.1. Isolation, Culture, and Characterization of hASCs

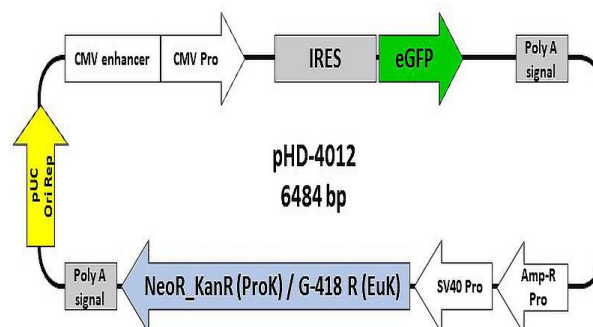
Lipoaspirate wastes were collected from the subcutaneous abdominal and waist fat tissues of healthy women aged 30–50 years who had no underlying disease and underwent aesthetic surgery with informed consent (approved by the local ethics committee, IR.ACECR.JDM.REC.1396.7). hASCs were isolated from the lipoaspirate and were cultured and characterized in consecutive steps, as clarified in our previous study (17). In brief, the lipoaspirate content was first washed with sterile phosphate-buffered saline (PBS). In the next step, lipoaspirate (3 mL) was treated with a tissue digestion solution containing 1 mg collagenase type I (Invitrogen, Carlsbad, CA), 10 mg BSA (Bio west, Nuaille, France), 2mM CaCl<sub>2</sub> in 1 mL PBS. After mild shaking in a water bath for 45 min at 37°C, the dissociated adipose tissue was centrifugated. The infranatant pellet (stromal vascular fraction cells; SVF) was suspended in low-glucose Dulbecco’s modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and subcultured in continuous passages to obtain purified adherent hASCs at the end of passage 3 (Figure 1). After that, hASCs at the 3<sup>rd</sup> passage were used for all subsequent experiments.



**Figure 1.** Morphology of human adipose-derived mesenchymal stem cells (hASCs) at passage 3. Magnification is 200X

### 2.2. Plasmids and Transfection

The transfection efficiency of hASCs was assayed using different quantities of lipoplex components in different post-transfection times. All transfection assessments were performed using a pDNA, pHD-4012 (a generous gift from Prof. Hesam Dehghani, Research Institute of Biotechnology, FUM, Mashhad, Iran), as a transgene. This plasmid encodes the enhanced green fluorescent protein (eGFP), which is under the control of the cytomegalovirus (CMV) major immediate-early (MIE) enhancer-containing promoter (Figure 2). The plasmid was transferred into DH5α *Escherichia coli* using heat shock transformation. The amplified plasmid was purified using the Plasmid Isolation Kit (DENAZist Asia, Iran) and stored in deionized distilled water following the manufacturer’s instruction. The plasmid concentration was determined by a Nanodrop®2000 spectrometer (Thermo Scientific, USA).



**Figure 2.** Map of the pHD-4012 transgene construct.

The transfection efficiency of hASCs was assessed using different quantities and proportions of pDNA, and different volumes of Lip-LTX, and the "PLUS" reagent (Invitrogen, Thermofisher, USA) to form transfection complexes (Lipoplexes) as summarized in table 1. Briefly, the hASCs with a density of  $4 \times 10^4$  per well were seeded in a 24-well plate (SPL, South Korea) to reach 60-70% confluency before transfection. In the first assessment, different transfection complexes were formed by combining different volumes of Lip-LTX (1, 1.5, 2, and 2.5  $\mu$ L) with a fixed volume of the "PLUS" reagent (0.5  $\mu$ L) and pDNA mass (0.5  $\mu$ g). The second assessment was performed by mixing a fixed volume of Lip-LTX (2  $\mu$ L) combined with different volumes of the "PLUS" reagent (0.5, 0.75, 1, or 2  $\mu$ L) and various amounts of pDNA (0.5, 0.75, 1, or 2  $\mu$ g) (Table 1). The

desired lipoplexes were formed in a reduced-serum medium, OptiMEM (Life Technologies), by incubating the pDNA and the "PLUS" reagent diluted in OptiMEM for 10 minutes at room temperature (RT) as the DNA mixture. Then, the DNA mixture was added to the Lip-LTX diluted in Opti-MEM, and the final mixture (lipoplex) was incubated for an additional 30 minutes at RT. After that, the desired lipoplexes were added drop-wise into the wells containing the hASCs monolayer. The preparation was incubated for 4-6 hours at 37°C in an incubator with saturated humidity and 5% CO<sub>2</sub>. Then, the transfection medium was discarded and replaced with 500  $\mu$ L fresh antibiotic-free standard low-glucose DMEM with 10% FBS. After that, the wells were incubated for 48 hours under the same conditions.

**Table 1.** Determination of transfection efficiency of hASCs transfected with different amounts of lipoplex components

Transfection assays	Components of transfection complexes (Quantity and ratio)				Transfection Efficiency (mean %eGFP expressing cells $\pm$ SD)*	
	pDNA ( $\mu$ g)	"PLUS" reagent ( $\mu$ L)	Lip-LTX ( $\mu$ L)	Component's ratio		
1 <sup>st</sup> assessment	I	0.5	0.5	1	1 : 1 : 2	5.58% $\pm$ 0.72
	II	0.5	0.5	1.5	1 : 1 : 3	6.42% $\pm$ 0.95
	III	0.5	0.5	2	1 : 1 : 4	19.42% $\pm$ 1.71
	IV	0.5	0.5	2.5	1 : 1 : 5	3.92% $\pm$ 1.09
2 <sup>nd</sup> assessment	V	0.5	0.5	2	1 : 1 : 4	18.08% $\pm$ 1.19
	VI	0.75	0.75	2	1 : 1 : 2.7	23.75% $\pm$ 1.61
	VII	1	1	2	1 : 1 : 2	8.42% $\pm$ 1.11
	VIII	2	2	2	1 : 1 : 1	5.58% $\pm$ 0.86

\* The results have been presented based on the optimum eGFP expression time point (12 h)

### 2.3. Fluorescence Microscopy

The transfected hASCs were monitored at 12-hour intervals (12, 24, 36, and 48 hours) after lipoplex delivery using a Nikon Ts2RFL fluorescence microscope (Nikon, Japan). The fluorescent images were captured in both bright and fluorescent fields at 12-hour intervals. The number of cells was manually counted using the multi-point option in ImageJ software (National Institutes of Health, NIH) in both fields. Subsequently, the transfection efficiency was calculated by dividing the number of eGFP-expressing cells in fluorescent fields by the total number of cells in

corresponding bright fields multiplied by 100 (% eGFP-expressing cells = transfection efficiency of each image).

Each assessment was done with four replicates (wells) for each lipoplex (lipoplex=groups=4). Then, four images were captured from each well (left, right, up, and down) every 12-hours. After calculation of transfection efficiency in every single image, the average of 4 images in each well was used as a single data for each group (four data from four wells of each group). After that, The microscopic data were statistically analyzed (one-way ANOVA, GraphPad

Prism 8) to determine the best transfection efficiency in hASCs and define the best time point for evaluating the transgene expression.

#### 2.4. Determination of Selection Concentrations of G-418 Using MTT Assay

The quantitative tetrazolium-based colorimetric assay (MTT) determines cytotoxicity, cell viability, and proliferation. In the present study, the MTT assay was used to plot the antibiotic (G-418) kill curve to determine the optimum selection dose of the G-418 in non-transfected hASCs. In other words, the G-418 kill curve is a dose-response experiment in which, non-transfected hASCs are subjected to increasing selection amounts of G-418 to determine the minimum concentration of G-418 that can kill all the non-transfected hASCs (almost 0% cell viability) in a specific period. Also, using this curve, we could determine the half-maximal selection concentrations of G418, known as IC50 value that is the concentration of drug (here G-418), which results in 50% cell viability. The following steps were performed to achieve these goals.

24h before adding the selection antibiotic, G-418 (Capricorn Scientific, Germany), the trypsinized non-transfected hASCs at the end of the third passage were seeded on a 24-well plate (SPL, South Korea) at a density of  $4 \times 10^4$  cells per well. Since the presence of routinely-used antibiotics in the culture medium, other than G-418, may result in cumulative growth inhibitory effects, cells were cultured in standard media containing low-glucose DMEM with 10% FBS without routine antibiotics on the seeding day.

The day after seeding, the standard medium was discarded. Then, non-transfected hASCs were grouped into ten selection treatments with four replicates (4 wells). The groups were treated with 500  $\mu$ L of selection media containing ten dilution series of G-418 diluted in the standard medium at the final concentrations of 0.1, 0.2, ..., 0.9, or 1 mg/mL, respectively (Figure 4-A). The selection media were refreshed every three days till the end of the 14-day antibiotic selection period. Cells with only standard

media (no antibiotic supplementation) were used as the negative control. The blank wells with only standard media (no cells and antibiotics) were used for background correction of colorimetric readings. As previously suggested (18, 19), the cells in three technical replicates were assayed in 14 days at three timepoints (day7, day10, and day 14) exposed to various concentrations of G-418 using MTT assay. Accordingly, the MTT solution (Sigma, Germany) was added to all wells to a final concentration of 5 mg/mL, and the cells were incubated in the dark at 37°C for 3 hours. After removing the media, the resulting formazan crystals were solubilized in 600  $\mu$ L DMSO per well. The absorbance of the purple formazan-DMSO solution was quantified at 590 nm using an Epoch plate reader spectrophotometer (BioTek, US). The cell survival percentage in all ranges of G-418 concentration was calculated using MTT assay data analysis. Absorbances of treated wells were subtracted from those of the blank wells to obtain the corrected absorbance (CA). Then, the percentage of cell survival in G-418-treated wells (for different concentrations at three time points) compared to untreated control wells were calculated using the below equation:

$$\% \text{ Cell survival} = \frac{\text{Mean (CA}_{\text{Treated wells with a definite concentration of G-418}})}{\text{Mean (CA}_{\text{Untreated control wells}})} \times 100$$

The survival curve was created by plotting cell survival (%) values versus the concentration of G-418 (mg/mL) at three time points. After that, the IC50 value for G-418 was determined using this plot, indicating the required concentration of G-418 for 50% inhibition of non-transfected hASCs growth *in vitro*.

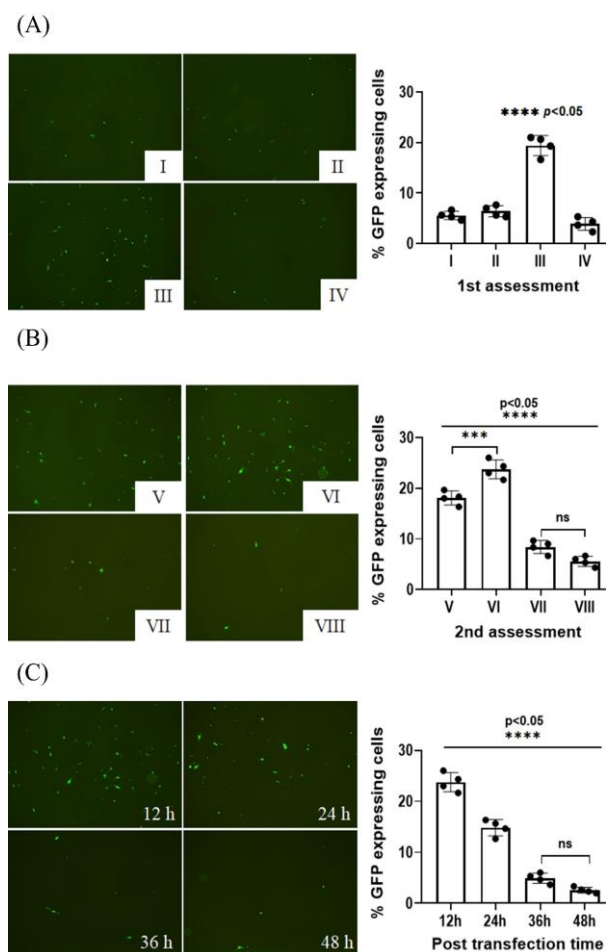
In the next step, this experiment with the same procedure was repeated with more technical replicates of cells exposed to a narrower range of G-418 concentration, including 0.1, 0.25, and 0.5 mg/mL. The MTT assay was repeated at least three times for all replicates exposed to wide and narrow ranges of G-418 concentration. The data were statistically analyzed with two-way ANOVA using GraphPad Prism 8 for both G-418 wide and narrow ranges experiments.

### 3. Results

#### 3.1. Transfection Efficiency in Transfected hASCs

At first, the effects of different volumes of lip-LTX in lipoplexes on the transfection efficiency of hASCs (in the third passage) were compared while the quantities of pDNA mass and the “PLUS” reagent were kept constant (0.5µg:0.5µL). After selecting 2µL Lip-LTX as the efficient and constant volume, the subsequent experiment was performed by altering the quantities of pDNA mass and the “PLUS” reagent with the same numerical values (Table 1). Transfection efficiency was measured as the percentage of eGFP-expressing cells. The first assessment results show that using 2 µL lip-LTX causes a significant improvement in transfection efficiency (19.42% ± 1.71), four folds higher than using lower volumes (Figure 3A). Nonetheless, using a higher volume of Lip-LTX than 2µL resulted in a significant reduction in eGFP-expressing cells and resulted in the lowest transfection efficiency (3.92% ± 1.09). The optimum volume of 2 µL Lip-LTX was used in the subsequent assessment (Table 1 and Figure 3-A). The ratio of pDNA mass to the “PLUS” reagent volume was maintained in a 1:1 ratio. A slight increase in the quantity of the DNA mass and the volume of the “PLUS” reagent, from 0.5µg:0.5µL to 0.75µg:0.75µL respectively, caused a significant increase in transfection efficiency (from 18.85% ± 1.19 to 23.75% ± 1.61). The transfection efficiency was declined by the use of higher amounts of pDNA and the “PLUS” reagent (Table 1 and Figure 3-B).

According to the transfection results, cells were treated with optimum lipoplex components (containing 2 µL Lip-LTX and 0.75µg pDNA:0.75µL “PLUS” reagent) and were monitored in 12-hour intervals for 48 hours after transfection. Image analysis revealed that the highest eGFP expression in hASCs could be recorded 12 hours after transfection (23.75%). After that at different time points post-transfection, the transfection efficiency was significantly reduced to 14.83%, 4.92%, and 2.58% (Figure 3-C). Accordingly, the obtained data in previous double assessments were only analyzed at this optimum time point (12 h) and presented as results.

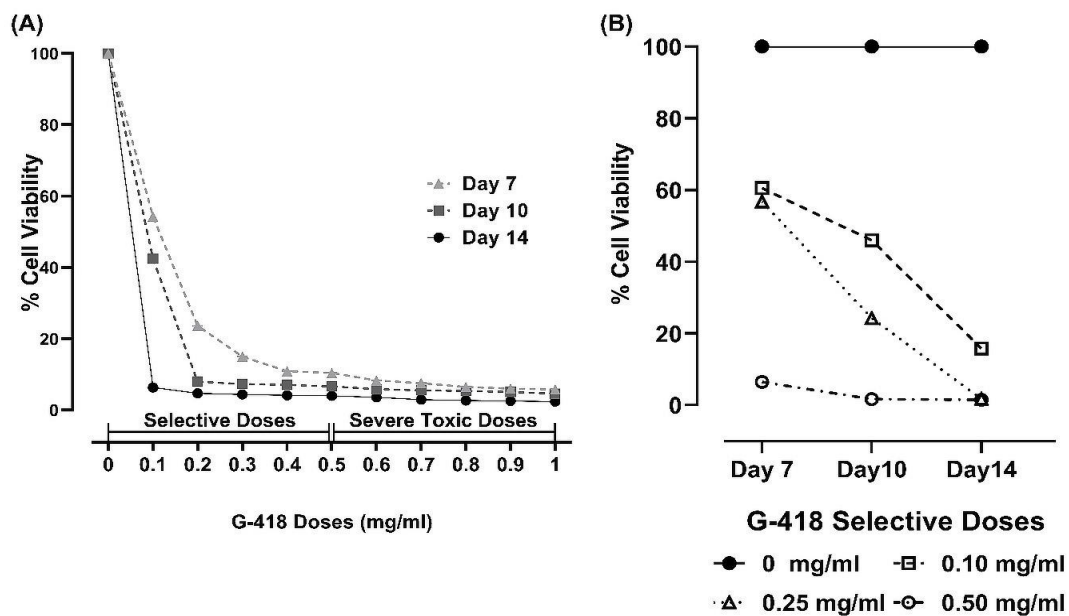


**Figure 3.** Effects of alteration of the quantities of lipoplex components on the percentage of eGFP-expressing cells (transfection efficiency) and transgene expression over time. **(A)** The fluorescent images (I, II, III, IV) and their related graph represent the results of the first assessment to find the highest transfection efficiency by altering Lip-LTX volumes according to Table 1. The most efficient Lip-LTX volume is 2 µL. The results have been presented based on the optimum eGFP expression time point (12 h). **(B)** The fluorescent images (V, VI, VII, VIII) and their related graph represent the second assessment to find the highest transfection efficiency by alteration of the “PLUS” reagent volume and the transgene mass according to Table 1. The most efficient lipoplex for the transfection of hASCs was obtained by a combination of 2 µL Lip-LTX, 0.75µL “PLUS” reagent, and 0.75 µg pDNA (23.75%±1.61). The results have been presented based on the optimum eGFP expression time point (12 h). **(C)** The images and their related graph show the changes in transgene expression in the cells transfected with the highest efficient lipoplex during 12-hour intervals over 48 hours. The results show that the optimum transgene expression time point is 12 h after transfection. Error bars in graphs represent the standard deviation of the mean for replicate wells of assessments (n=4, mean % Transfection Efficiency ± SD). All images were captured with 40X magnification.

### 3.2. Antibiotic-Based Selection of hASCs Using G418

The effect of ten dilution series of G-418 from 0.1 to 1 mg/mL of G-418 on hASCs viability was investigated in 14 days at three timepoints (day7, day10, and day 14) by colorimetric MTT assay (Figure 4). In all triple timepoints, the cell viability generally decreased with an increase in antibiotic concentration, which indicated a dose-dependent manner. Also, the cell viability was reduced by time in every single dose of G-418, which declared the time-dependent manner. Nevertheless, the dose-dependent and time-dependent manners of hASCs viability were not observed significantly in doses higher than 0.60 mg/mL, which indicates the severe toxic doses of G-418 for hASCs. Conversely, the lower doses of G-418, under 0.60 mg/mL, caused a significant gradual reduction in hASCs viability during the selection period that implied selective doses of G-418 for hASCs (Figure 4-A). Therefore, to find a single optimum dose of G-418, the experiment was repeated in the selective range of G-418 concentration, including

0.1, 0.25, and 0.5 mg/mL with eight replicates per dose per day at the same time points. These selective doses of G-418 were compared among all time points based on the IC<sub>50</sub> value (Table 2). Accordingly, 0.50 mg/mL G-418 immediately caused 93.56% cell death at day 7, earlier than the endpoint (day 14), which is not the acceptable selective dose. Although the 0.10 mg/mL of G-418 resulted in a gradual decrease in cell survival (from 60.5% to 45.96%, and 15.47% on days 7, 10, and 14, respectively), it is not an eligible selective dose because of remaining a significant viable population of sensitive non-transfected hASCs at the endpoint (15.47%). In comparison, 0.25 mg/mL not only resulted in a gradual decline in cell viability (from 56.74%, approximately equivalent to IC<sub>50</sub>, to 24.23%, and finally 1.73% on days 7, 10, and 14, respectively), but it also caused the elimination of almost major of the G-418 sensitive non-transfected hASCs (98.23%) at the end of the selection period (day14). Therefore, 0.25 mg/mL of G-418 in standard culture media could be the optimum selective dose for hASCs (Figure 4-B).



**Figure 4.** Evaluation of the viability of hASCs in response to different concentrations of G-418 antibiotic at three-time points by colorimetric MTT assay. **(A)** The viability of hASCs was decreased with an increase in antibiotic concentration and over the selection period. The G-418 toxicity for hASCs occurs at doses over 0.5mg/mL (Severe toxic doses). The significant selection of hASCs occurs at the lower doses of G-418, under 0.50 mg/mL, with a gradual reduction in cell viability during the selection period (Selective doses) (n=4, mean %cell viability  $\pm$  SD). **(B)** Between high (0.5 mg/mL), moderate (0.25 mg/mL) and low (0.1 mg/mL) doses of G-418, the concentration of 0.25 mg/mL is more efficient for selection of hASCs because of gradual and significant decline in hASCs viability over the selection period. (n=8, mean %cell viability  $\pm$  SD).

**Table 2.** Viability of hASCs in response to selective doses of G-418 determined at three timepoints (Mean%  $\pm$  SD.)

Time-points	Selective Doses of G-418			
	0.00 mg/mL	0.10 mg/mL	0.25 mg/mL	0.50 mg/mL
Day 7	100% $\pm$ 0	60.5% $\pm$ 2.01	56.74% $\pm$ 0.83	6.44 % $\pm$ 0.5
Day 10	100% $\pm$ 0	45.96% $\pm$ 0.98	24.23% $\pm$ 2.28	1.62% $\pm$ 0.17
Day 14	100% $\pm$ 0	15.47% $\pm$ 4.41	1.73% $\pm$ 0.10	1.48% $\pm$ 0.15

#### 4. Discussion

A survey in other studies show that various factors influence the transfection efficiency, the duration of transgene expression, and the hASCs resistance against selective antibiotics. These factors briefly include the sex and age of the cell donor, the site of lipoaspiration, the heterogeneity of the cell batch of lipoaspirate even from a similar site, cell passage number, the transgene size, the integrating or nonintegrating transgene, and method of introducing a transgene into the cells (12, 20).

This study indicates that the setting up of optimized quantities of lipoplex components and the golden time of evaluation for transgene expression could increase the possibility of transgene expression in hASCs before beginning research and clinical use. Also, the definition of optimal dose of selective antibiotic for purification of transfected hASCs seems to be necessary for maximum transgene expression effects in the cell population.

Lipofection is one of the most widely used non-viral methods to deliver nucleic acid to a wide variety of cells (21); however, it needs to be optimized based on cell type and sort of Lipofectamine. In this study, we showed an increase in the volume of Lip-LTX, up to 2  $\mu$ L, caused an increase in transfection efficiency; such a result was not observed in higher volume. In addition, we revealed that the efficient amount of "PLUS" reagent and pDNA is an equal ratio of 0.75 $\mu$ L:0.75 $\mu$ g. Accordingly, the optimum lipoplex for highly efficient lipofection of hASCs contained 2  $\mu$ L Lip-LTX, 0.75  $\mu$ L related "PLUS" reagent, and 0.75  $\mu$ g pDNA. Locatelli, Olea (22) found that combining 5  $\mu$ L Lip-LTX and 4  $\mu$ L "PLUS" Reagent with 2  $\mu$ g of DNA caused a high transfection efficiency in ovine marrow

MSCs, While Diaz, Cuevas (23) reported the highest number of eGFP-positive bovine marrow MSCs using a combination of 9  $\mu$ L/mL of Lip-LTX and 750 ng/mL of a transgene. Also, DNA/LTX complex at 1:2 ratio (3  $\mu$ g DNA/6  $\mu$ L LTX) produces the highest expression level in human umbilical cord MSCs (24). The comparison of our evidence with similar studies demonstrated that transfection efficiency in MSCs depends on multiple factors, including the species, the source of MSCs, and exclusively optimized quantities of lipoplex for each species.

In addition to transfection efficiency, eGFP expression duration was evaluated 12, 24, 36, and 48 hours following transfection in hASCs transfected with different lipoplexes as the second goal of this study. The overall eGFP expression obtained from all lipoplexes decreased in hASC with time (Data not shown). Accordingly, the comparison of lipoplexes in eGFP expression was only analyzed at the optimum expression time point. Therefore, the transfected hASCs with the well-formed lipoplex (2  $\mu$ L lip-LTX + 0.75  $\mu$ L "PLUS" reagent + 0.75  $\mu$ g pDNA) were followed in 12-hour intervals for at least 48 hours to achieve the optimum eGFP expression time point. Percentage of eGFP expressing cells from their highest levels at 12 hours decreased by 1.5 orders of magnitude at 24 h, while further decreases were observed in 36 to 48 hours, respectively by 5 to 9 orders of magnitude. Hence, all comparisons were analyzed and presented at this optimum time point (12 h) ( $P \leq 0.05$ ). In this regard Kelly, Plautz (10) followed the transfected human bone marrow MSCs at 24-hour intervals (24, 48, and 72). They observed that the percentage of GFP-expressing cells reduced over time, and the reduction was more pronounced 24 hours after transfection. In addition, several studies have proved the reduction of



transgene expression during the time in MSCs, whether with viral or non-viral methods (10). However, it is reported that the transgene expression in transduced MSCs (viral method) is longer. In contrast, the transfected MSCs (non-viral method) express the transgene for a short time, according to our study results. In general, reducing transgene expression in MSCs over time and the limited duration of expression is a disadvantage for MSCs as candidates for therapeutic genes carrier. Kelly, Plautz (10) represented that priming the MSCs with glucocorticoids before lipofectamine transfection caused more prolonged transgene expression than unprimed cells (25-27). Therefore, glucocorticoid cell priming could be an optimal solution for short-time transgene expression in MSCs (10).

Regardless of transfection efficiency, it is essential to select and purify the transgene expressing hASCs for subsequent research or therapeutic use. The transfected cells are generally selected using an antibiotic. The type of selective antibiotic depends on the antibiotic resistance gene located on transgene, expressed simultaneously with the therapeutic gene. Here, the pHD-4012 was used as a transgene, which expressed the aminoglycoside phosphotransferase resistance gene. The expressed protein saves the transgene-expressing bacteria against the neomycin or kanamycin antibiotics and saves the transgene-expressing eukaryotic cells, hASCs, against the G-418 antibiotic. However, the mammalian cell sensitivity to antibiotics varies from one cell type to another. In order to achieve a population of transgene expressing cells, it is important to determine the minimum concentration of antibiotics required to kill non-transfected or non-transduced cells. Therefore, one of the critical steps for efficiently selecting transfected hASCs is determining the optimal G-418 antibiotic concentration, adequate antibiotic incubation time, and selection period; however, there is no common sense in the literature. For example, different studies have used different concentrations of G-418, ranging 0.3, 0.5, and 0.6 mg/mL, for the

selection of transgene-expressing hASCs (28, 29). Alternatively, in terms of incubation time, Kucerova, Matuskova (30), selected the transduced hASCs in the presence of 0.5 mg/mL of G-418 for ten days, while other studies extended it to 14 21 days with the same concentration of G-418 (29, 31). Therefore, we investigated the effect of G-418 on non-transfected hASCs in a wide range of concentrations from 0.1 to 1 mg/mL to detect the best selective concentration using MTT assay. According to our first MTT analysis, the G-418 had a selective effect in a range of 0.1 to 0.5 mg/mL on hASCs, while the higher concentrations than 0.5 mg/mL caused cell toxicity. Also, the second MTT assay findings in the narrow range of G-418 concentration showed that the optimum selective dose of G-418 was 0.25 mg/mL, which should be treated for 14 days on hASCs. Hence, according to the MTT results of this study and Horizon (19), the 0.25 mg/mL of G-418 is the lowest concentration that kills most non-transfected hASCs in the 14-day selection period with a gradual killing manner from half of the population (56.74%) on day 7 to most of the population on day 14 (1.73%). This optimal dose with a gradual killing manner after transfection of hASCs makes a chance for transfected hASCs to express the G-418 resistance gene over time. Therefore, a pool of viable transgene expressing cells could emerge, and also non-transfected hASCs would be destroyed (32, 33).

## 5. Conclusion

Our findings demonstrated that introducing a transgene into hASCs using Lip-LTX caused a transfection efficiency of 23.75% when the lipoplex was composed of 2  $\mu$ L Lip-LTX, 0.75  $\mu$ L "PLUS" reagent, and 0.75  $\mu$ g of a transgene. Furthermore, the results proved that the transient expression of transgene decreases over time in transfected hASCs. Thereby, the best time for subsequent research or therapeutic use is 12 h after transfection (earlier than 24h). Also, the present study indicated that the optimum condition of G-418 antibiotic for the selection of hASCs is using

0.25 mg/mL for 14 days after transfection. However, the post-transfection events in hASCs depend on multiple factors like transgene uptake by host cells, intracellular stability of the transgene, the access of transgene to the nucleus, and transcription efficiency that remain to be elucidated.

### Authors' Contribution

A. P. and H. D. supervised the study. M. T. performed the experiments, analyzed the data, and drafted the manuscript. A. P., H. D., and H. N. M. critically revised the manuscript. All authors read and approved the final manuscript.

### Ethics

The procedures used in this study to obtain primary stem cells from the human adipose tissue were approved by the Ethics Committee of the ACECR, and all methods were performed under the relevant guidelines and regulations.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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