Atorvastatin Inhibits Viability and Migration of MCF7 Breast Cancer Cells

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Abstract

Objective: Atorvastatin is commonly used as a lipid lowering drug. The emerging interest in statins as anticancer agents is based on their pleiotropic effects on cancer cells. Among the statins, atorvastatin, and in cancers, breast malignancies have received less attention in preclinical investigations. In order to enhance the efficacy of cancer treatment, adjuvant, less expensive therapeutic strategies have been recently noticed. In this case, we investigated the in-vitro effect of atorvastatin on viability and migration of MCF7 breast cancer cell line. Methods: We tested the cytotoxicity of atorvastatin on breast cancer cells survival by MTT assay. Annexin-V / PI staining and then flow cytometry of cancer cells in addition to quantitative real-time PCR tests quantified the apoptosis and necrosis of cancer cells. We figured out the impact of atorvastatin on cancer cell migration capability through scratch-wound healing assay and transwell migration examination. Inverted light microscope and fluorescent imaging displayed the morphological changes following treatment of MCF7 cells with atorvastatin. Result: We resulted that atorvastatin can trigger MCF7 cancer cells to undergo necrosis and caspase-dependent apoptosis based on the viable/dead cell number, mitotic cell cycle, gene expression, and morphological assays. The results were dose- and time-dependent and the half-maximal inhibitory concentration of atorvastatin for cancer cells’ viability inhibition was 9.1 μM/L(nM/mL). Moreover, the migration of MCF7 cells were inhibited in the treated group as we figured out in two- and three-dimensional migration methods. Conclusion: In-vitro inspection of drug-cancer cell interactions paves the way for future in-vivo research studies. These in-vitro results revealed that atorvastatin has anti-viability and anti-migration effects on breast cancer cells.

Keywords: Statin- MCF7Cell line- migration- apoptosis
Accordingly, we investigated the efficacy of atorvastatin on MCF7 cell line. We hypothesize that atorvastatin benefits breast cancer cells through inhibition of cellular viability, and suppression of migration.

**Materials and Methods**

**Cell line and intervention preparation**

The MCF7 cell line (DSMZ collection) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), High Glucose (Sigma Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS)(Invitrogen), 100 μg/ml streptomycin and 100 U/ml penicillin (Gibco). Cancer cells were incubated at 37°C, 5% CO\(_2\) in a sterile biosafety hood environment, and medium was changed every 48-h. Freeze, thaw, passage, and counting protocols were accomplished based on ATCC method (ATCC Home, 2021). Cancer cells grew up to 80-90% confluence and remained in the serum-free culture medium overnight. On the next day, senescence detached cells were removed and medium exchanged with DMEM High Glucose and FBS0.5% for three hrs (Hours). Therefore, growing cells are unified and synchronized in a similar logarithmic phase (Langan and Chou, 2011). Atorvastatin Calcium (C33H34 FN2O5) 2Ca. 3H2O, Molecular weight:1155.363 g/m\(^3\) (Sigma Aldrich) is a hydrophobic substance, which is dissolved in organic solvents such as Dimethyl sulfoxide (DMSO), Dimethyl formamide (DMF), and ethanol (National Library of Medicine, 2021; Product information, 2021). DMSO (Beyotime, Shanghai, China) has a lower final concentration (less than 0.1% in DMEM medium), thus we used it as an atorvastatin solvent. After making the solution up to a stock concentration of 86.55 μM/L(nM/mL) in Phosphate Buffer Solution (PBS) (Gibco) (1 mg Atorvastatin + 100 μl DMSO + 9,900 μl PBS), atorvastatin diluted with DMEM medium based on the C1×V1 = C2×V2 equation to the final concentrations of 5, 10, 15, 20, 30, 40, 50 μM/L. Three replicates of each concentration and media were employed for treated and untreated groups respectively.

**Cell viability assay**

**MTT test**

We utilized the MTT colorimetric protocol to assay the mitochondrial respiration rate of viable cells with and without treatment (Riss, 2016; MTT Cell Proliferation Assay, 2021). The MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich) was dissolved in PBS at a final concentration of 0.5 mg/ml. Exponentially growing cells were seeded in 96 well plates (1×10\(^4\) cells/ml in each well). The cells were attached after overnight incubation. Culture medium containing cells without atorvastatin were considered as control. Blank wells had medium and MTT without cells. Samples were treated with various concentrations of atorvastatin. After one-day-incubation, we removed the medium, and 40 μl MTT reagents were added to each well and left for three hrs at 37°C in a CO\(_2\) incubator. After removing the MTT reagent, the colored Formazan product was dissolved in 200 μl DMSO and plates were agitated for 20 min at room temperature for fully dissolving the MTT product.

We measured the optical density (OD) of products at a maximum absorbance wavelength of 570 nm with an ELISA micro-plate reader (Bio Rad Laboratories, Inc., Hercules, CA, USA). The relative decrease in cell viability (% inhibition) was measured as follows:

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100-[(\text{mean O.D of samples} - \text{mean O.D of blanks})/\text{mean O.D of controls-mean O.D of blanks}] \times 100.
\]

The half-maximal inhibitory concentration (IC\(_{50}\)); The concentration of atorvastatin which reduces the cell viability by 50% of the upper asymptote) calculated in a dose-response x-y plot equation. The result was employed for subsequent viability and migration tests.

**Flow cytometry**

Equal numbers of MCF7 (1×10\(^6\) cells /ml) were seeded in each well of a six-well plate, allowed to attach, synchronized, and treated with atorvastatin for 24 and 48 hrs. Untreated cells were considered as the control group. Annexin-V/PI double staining can discriminate necrotic/late apoptotic, necrotic, early apoptotic, and viable cells. The cells harvested by trypsinization (Gibco, UK), were washed twice with pre-cooled (4°C) PBS, incubated with 5 μl Annexin V Fluorescein isothiocyanate (FITC) for 15 min, followed by incubation with 10 μl Propidium Iodide (PI) for 5 min in dark at room temperature (Sigma-Aldrich kit). The number of viable/dead cells and DNA content (during a mitotic cell cycle) were measured by the BD Accuri™ C6 flow cytometer and analyzed with Flow-Jo(version7) software.

**Quantitative Real-time PCR**

After 24 and 48 hrs of incubation with atorvastatin, the mRNA expressions of apoptosis genes (caspase 3,8,9), anti-apoptosis gene (bcl2), and internal control (beta2microglobulin gene (B2M)) were measured by ABI PRISM 7500 fast real-time sequence detection system (Applied Biosystems, Foster City, CA, USA). The total mRNA extraction with Trizol (Invitrogen, USA) and CDNA synthesis with cDNA synthesis kit (Takara, Japan) was performed according to the manufacturer’s protocol. The primers were designed through the Primer3 software and synthesized by Takara Biotechnology (Japan)(Table1).

The PCR parameters were denaturation at 93°C for one min, annealing at 59°C for 1 min, and extension at 72°C for 1 min, for 40 cycles. Relative mRNA quantification was calculated through fold-change equation (2^(-ΔΔCt)).

**Characterization of Cell Morphology**

Following 24 and 48 hrs of incubation in 24-well plates, morphological characterization of treated and untreated MCF7 cells was photographed at 20 and 40 magnifications with a reverse light microscope (TCM400-LABOMED).

**Migration evaluation**

**Scratch-wound healing assay**

The scratch-wound assay is a simple, two-dimensional (2D), reproducible, common assay. This
in-vitro healing process surveys the migration speed in a sheet of cells responding to crude wound formation. In order to photograph from the same fixed point at different times, we innovatively marked the cross lines behind the bottom of wells before cell seeding. The intersection of two lines was compared as a fixed criterion (Figure 3A). In each well, 5×10⁴ cells/ml were seeded into a 24-well plate and cultured in a serum-free medium. Once the cells grew up to 90% confluence, the medium and cell debris were carefully aspirated. Then, we scratched a thin vertical wound line through the center on a monolayer cell culture, which gets straight firmly and swiftly from top to bottom of the floor with a sterile yellow 200 μl pipette tip. Then detached cells were slowly washed with PBS once. One ml of atorvastatin and culture medium was added to treated and control groups, respectively. The first picture was immediately acquired with an inverted light microscope and the plate was incubated in the incubator. The speed of cell migration and wound closure was quantified by taking snapshot pictures after 3, 24, 48, 72 hrs. Pictures were analysed with the freely available ImageJ software and the rate of closure [(width of the wound in 0 hr – width of the wound after 3, 24, 48, 72 hrs/width of the wound in 0 hr) × 100%] was calculated (Justus et al., 2014).

Trans-well migration examination
The transwell assay explores the directional cell migration toward the chemo-attractant medium. In the upper compartment of 24-chamber trans-well plate (8.0 μm pore size insert, Corning, USA), 5× 10⁴ cells/ml were seeded. Serum-free medium with and without atorvastatin (100μl) was included to cells and 600μl medium+20% FBS was added into the lower chamber as a chemottractant. The bottom of upper filter membrane contacted with a lower medium for migration of cells through the pores. Incubated cells passed through the filter. After three and nine days, the migrated cells on the lower chamber were fixed with 4%paraformaldehyde, stained with 1μg/ml DAPI, and photographed by fluorescence microscope. We computed cells with ImageJ-Fiji software.

Statistical Analysis
Data from control and experimental groups were analyzed employing IBM SPSS statistics (Version 22) software. The mean values were analyzed. Statistical comparisons between continuous, not categorical groups were measured with the Pearson Chi-Square test. We used the Fisher Exact test for nominal variables and Linear regression measurement to predict the value of a variable based on another variable. We measured the association between exposure and an outcome with the Odds Ratio based on another variable. We measured the association employing IBM SPSS statistics (Version 22) software. The mean values were analyzed. Statistical comparisons between continuous, not categorical groups were measured with the Pearson Chi-Square test. We used the Fisher Exact test for nominal variables and Linear regression measurement to predict the value of a variable based on another variable. We measured the association between exposure and an outcome with the Odds Ratio test. The P-value less than 0.05 was considered significant.

Table 1. Primer Sequences Used for Real Time-PCR

| Genes   | Size (bp) | Forward primer sequence (5’à3’) | Reverse primer sequence |
|---------|-----------|---------------------------------|-------------------------|
| Caspase3| 143       | CAGTGAGGCCGGACTCTTG             | TGGCACAAACGACTGGAT      |
| Caspase8| 161       | GGATGGGACTGTAATAACTG            | TCGAGGATCTCCCTTCCTCA   |
| Caspase9| 150       | TGTCTACTCTACTTCCCCAGGGTT       | GTGAGGGACAATGCCTGAAAGAT | |
| BCL2    | 156       | CTT TTG CTG TGG GTT TTT GT     | GTC ATT CTG GCC TCT CT GC |
| B2M     | 147       | TACATGTCTCGATCCCCACTIAACTAT    | AGCGTACTCCAAAGATTCAAGGT |

Results
Cytotoxic effects of atorvastatin
The concentration range of 5 to 50(5,10,15,20,30,40,50) μM/L of atorvastatin inhibits cancer cell viability about 9.5% to 43.2 %. The more concentration of atorvastatin, the less MCF7 cells viability(Figure 1A). Based on the equation of the straight linear regression fitted line (y=4.5054x+8.9714), IC50 accounts 9.1 μM/L. The relation between atorvastatin dosage and cell toxicity is linear based on linear regression analysis (R Square: 0.854, P=0.003).

Based on the flow cytometry assay (Figure 1C), following 24 hr exposure, early apoptosis (Q3 quadrant) in the control group are less than 2% but after 24 hrs of incubation with 9.1 μm atorvastatin, inflates to 17.1% and after 48 hrs, rises to more than 41%. Increment of percentages after 24 and 48 hrs is significant between the treated groups and control group as they were compared with each other (Pearson Chi-Square test P<0.05). Necrotic cells (Q2 quadrant) in the control group are 5.44%. After 24 and 48 hrs of incubation, necrosis rise to 7.12% and 12.7 % respectively. Only the difference between 48-h treated and untreated groups is significant. The highest Odds Ratio is in the percentage of early apoptosis after 24 hrs of atorvastatin exposure compared with the control group (OR =41.911, 95% CI =9.700-181.082). Necrotic/late apoptotic cells (Q1 quadrant) increase from 0.357% in control to 6.25% after 24 hrs, and to 0.947% after 48 hrs exposure to the atorvastatin. Only the elevation after 24 hrs is significant compared to the control group (Fisher’s Exact Test P=0.008) (Figure 1D). Atorvastatin modifies the mitotic cell cycle phases in malignant cells. After 24 hrs, the sub-G0/G1 (apoptosis) phase in the control group is negative and increases up to 3.28 in atorvastatin treated group.%. After 48 hrs, about 1/3(29.42%) of the cells belong to sub-G0/G1 part. Differences between cell frequencies at different stages of the cell cycle is significant (Pearson Chi-Square P<0.05) (Figure 1E).

Fold change mRNA expressions of apoptosis and anti-apoptosis genes represent apoptosis enhancement. The up-regulation of caspases 3, 8, 9, and down-regulation of Bcl-2 after 48 hrs are more than 24 hrs exposure to the atorvastatin compared to control (Figure 1B).

We photographed control and treated cells after 24 and 48 hrs exposure to the atorvastatin. Morphological signs of apoptosis under the inverted microscope include increasing in color and density, cell shrinkage, membrane blebbing, and pyknotic nucleus. These findings clearly
increase after 48 hrs (Figure 2).

The cholesterol biosynthesis mevalonate pathway is strictly regulated in normal cells, while the pathway deregulation promotes transformation (Pandyra et al., 2015). Highly proliferating cancer cells generate lipid bi-layer membranes rapidly, increasing cholesterol biosynthesis (Mullen et al., 2016). The mevalonate pathway rate-limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) development is higher in estrogen-dependent mammary tumors than healthy glands (El-Sohemy and Archer, 2000). Likewise, this molecule has been considered as a new and special anti-cancer target. Statins inhibit the HMG-CoA reductase enzyme, representing direct anti-cancer activity and multidrug resistance reversal ability in diverse cancers (Steven, 2004), and hinder cancer cell proliferation and survival (Campbell et al., 2006). In this investigation, results confirm that atorvastatin decreases breast cancer MCF7 cell viability and movement in a dose- and time-dependent manner. Within the concentration range of 5 to 50 µm, we found 9.5-43.2% viability inhibition, but Seeger et al. (2003) obtained wider resultants (9-62%) with similar concentrations. The IC$_{50}$ value in our estimation was 9.1 µm/l following 24 hrs exposure similar to the study of Al-khafaji and Arif (12.3 µm) after 72 hrs (Al-khafaji and Arif, 2018). However, others measured
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Further (Seeger et al., 2003; Alkhaitb and Al-Saedi, 2017; Beckwitt, 2018; Sánchez et al., 2008) or lesser (Kimbung et al., 2016) values of atorvastatin. Six serial dilutions of atorvastatin (1.25 μm to 40 μm) showed significant inhibition of MCF7 cell line in dose-dependent manner after 72 hrs, comparing to the negative control rather

Figure 2. Morphological Changes Following 9.1 μm/l Treating with Atorvastatin Captured by Inverted Microscope: A: Control after 24 hrs. B-C: Treated after 24 hrs. D: Control after 48 hrs. E-F: Treated after 48 hrs. Magnification in: A-B-D-E pictures: ×20 objective lens. C-F: ×40 objective lens. Red arrows in the C and F pictures: A=Membrane blebbing, B=Cell shrinkage, C= marginal and pyknotic nucleus, D=Apoptotic bodies, E=Intracellular spaces. Scale bar:100μm

Figure 3. Migration Evaluation of MCF7 Cancer Cells in Control (Untreated) and Treated Groups with 9.1 μm/l Atorvastatin with Scratch-Wound Healing Assay. A: Vertical and horizontal lines crossing under wells. B: The pictures of MCF7 cells migrated in control (Line1) and treated cells(Line3) are captured by an inverted light microscope. Wound area adjusted with ImageJ software (Line2 and4): C-Line chart of scratch area surface (%) in control and treated cells. Scale bar: 100 μm.
than other incubation periods (24, 48 hrs) (Al-khafaji and Arif, 2018). The atorvastatin was examined in the concentration range of 1.6 μm to 50 μm. The inhibitory values were between 10% and 90%, using an adenosine triphosphate-chemo sensitivity test (Mueck et al., 2003). In Martinez et al.’s investigation inhibition effect of 5μm to 80 μm concentrations of atorvastatin were statistically significant at 48 hrs, but after 24 hrs only 40 and 80 μm of atorvastatin exhibited significant effects (Martinez et al., 2018). In other research (Kimbung et al., 2016) MCF7 cells were less sensitive to atorvastatin requiring doses more than 20 μm to carry out the proliferation rates under 30% at 72 hrs. Nevertheless, in 10 μl concentration of atorvastatin, a significant decrease was observed in the survival of MCF7 cells after 24, 48, and 72 hrs compared to control cells by Shahrokhabadi et al.’s study (Shahrokhabadi et al., 2014). The survival of MCF7 cells did not change by 5μm atorvastatin at 72 hrs of exposure (Beckwitt et al., 2018). Clearly, Shahrokhabadi et al. (2014) did not prove statistical significance in the cytotoxicity effect of lower concentrations (0.1 to 1 μm). These diversities could be the result of different atorvastatin manufacturers, the solvent, cell culture medium, viability examination, and the time of test after incubation.

Based on the viable/dead cell number, mitotic cycle, and gene expression, we suggest that atorvastatin significantly induces apoptosis and increases necrosis in the MCF7 cell line, specifically after 48 hrs. The caspases 8 and 9 augmentation demonstrate that the effect of atorvastatin on breast cancer cells is through an external and internal cascade of apoptosis. After 48 hours of exposure to atorvastatin, more than 54% of Annexin-V / PI-stained cells were in early and late apoptotic and necrotic stages. Fold change mRNA expressions of apoptosis and anti-apoptosis genes represent apoptosis enhancement. The up-regulation of caspases 3, 8, 9, and down-regulation of Bcl-2 after 48 hrs were more than 24 hrs exposure to the atorvastatin compared to the control. Qing et al. propounded that the number of early and late apoptosis of breast cancer cells was enhanced significantly after 48 hrs of treatment with 2 μm atorvastatin, detected by annexin V-FITC and PI staining (the percentages were not stated) (Ma et al., 2019). Likewise, Mück et al. reported down-regulation of the anti-apoptotic protein Bcl2 (Mück et al., 2004). Besides, atorvastatin increases necrosed as staining by trypsin blue and [3H]-thymidine release, indicating the damage to the cytoplasmic membrane (S´anchez et al., 2008). Atorvastatin caused a concentration-dependent enhancement in the number of MCF7 apoptotic cells at 24 and 48 hrs after 10, 20, and 80 μm treatment, assessing with TUNEL assay (Martinez et al., 2018). However, Mayson et al. reported only 5.67% early apoptotic and 4.23% late apoptotic cells (Alkhaitb
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migration and Al-Saedi (2017). This low quantity could be due to lower concentration of atorvastatin (5µm) compared to our study. Zalatain et al. (2005) concluded that apoptosis is more frequent in arrested cells in the G1 phase. Whereas necrosis is more frequent in cells arrested in the G2/M phase. Flow cytometry analysis of cell cycle phases in our research and Sanchez et al.’s (2008) also represent a reduction in the G2/M and G1 phases. In the Ding et al.’s research (Ding et al., 2016), the percentage of cell cycle phases in the cells treating with 10 µm atorvastatin for 48 hrs was similar to ours (G0/G1=53.87%, S=34.08%, G2/ M=12.05%). However, Ding et al. did not report the sub-G1 phase percentage. Atorvastatin at 40 µm concentration (50% inhibitory activity) after 48 hrs, significantly increased the number of cells in the G2/M and G1 phases of the mitotic cell cycle compared to the non-statin treated cells (Sanchez et al., 2008). None of the obtained data in Sanchez et al.’s study represented the presence of sub-G1 cells. Nevertheless, flow cytometry analysis of cells marked with two different methods (FLICA/ PI and Annexin-V-fluos/PI) represented an increase in the percentage of apoptotic and necrotic cell death (Sanchez et al., 2008). Even 2 µm atorvastatin induces early and late apoptosis of breast cancer cells discovered by annexin V-FITC / PI staining (Ma et al., 2019).

The electron microscopy revealed apoptotic (vacuolization in the cytoplasm, condensed nuclei, and apoptotic bodies) necrotic (completely lost the cellular membrane integrity and degenerated cytoplasmic content), and autophagic (enlarged pleomorphic vacuoles containing moderate electron-dense material in the cytoplasm) degenerative changes in MCF7 cells treated with atorvastatin (Martinez, 2018).

Migration inhibition

Encounter with vertical and horizontal lines crossing under wells (Fig. 3A), we photographed the cellular movements of the wound margins immediately and at the intervals of 3, 24, 48, and 72 hrs post scratching. By adding MRI wound healing tool to ImageJ software, the wound surface area was adjusted and analyzed (Fig. 3B). In the control group, the wound closes after one day, but in the treated groups, the wound is left open even after three days, because many cells do not move and turn into necrotic or apoptotic (Figure 3C). The Pearson Chi-Square computation between time points estimates that cell movements significantly diminish after 72 hrs in atorvastatin treated groups than control groups (P value<0.05). The relation between rate of wound closure (%) and time points is linear based on linear regression analysis (R Square: 0.812, P=0.003).

The fluorescence imaging illustrates the MCF7 cells in control and treated groups, passing the trans-well membrane after three and nine days incubation (Figure 4A, B, C). Atorvastatin detracts the number, area, and average size of migrant cells in both fixing time. Nevertheless, these parameters increase in each group after nine days rather than three days. As shown in Figure 4, there are 3.38 and 2.05-fold decreasing in the cell count, which migrates toward the chemo-attractant culture medium in treated group in comparison to the control after three and nine days, respectively. Total cellular area decrease in treated cells, 0.199% after three days, and 0.447% after nine days related to controls(Figure 4D, E, F). Differences between un-treated and treated cells in cell counts and areas after nine days incomparrison to three days are not significant (P=0.091 and P=1.000, respectively) (Figure 4D and E). However, atorvastatin significantly reduces the average cell sizes; about 19 pixels after three days and 97 pixels after nine days (Pearson Chi-Square P=0.025) (Figure 4F).

We figured out the inhibitory effect of atorvastatin on the migration of MCF7 cells by two-dimensional scratch-wound healing and three-dimensional(3D) trans-well migration examination tests. Ma et al., (2019) again used the wound healing and transwell assays and showed that 2 µm atorvastatin significantly inhibited cell invasion and migration. Although in Mi et al.’s research (Mi et al., 2015), 10 µm atorvastatin treatment did not affect the migration of MCF7 cells through wound healing assay.

Discussion

Hyperlipidemia is prevalent among women (Phan and Toth, 2014) and cholesterol represents a risk factor and impacts clinical outcomes of breast cancer (Laverias et al., 2011). The lipophilic atorvastatin can easily penetrate cell membranes via passive diffusion. In the present study, various methods such as the MTT assay, flow cytometry, quantitative real-time PCR, scratch-wound healing assay, and trans-well migration examination revealed that atorvastatin has anti-viability and anti-motility effects on breast cancer cells. However, cell culture experiments surely do not replace clinical trials. In vitro experiments have to be carefully extrapolated into in vivo conditions.

For cancer patients already receiving statins for other situations, such as hypercholesterolemia, atorvastatin may provide a pleiotropic anti-tumor advantage without adversely affecting their primary indication for statin therapy. In conclusion, several preclinical studies described the efficacy of statins on breast cancer cells. If the therapeutic or preventive effects of statins on breast cancer cells are verified, statins would be available for breast cancer patients as a low-toxicity, cost-effective option for adjuvant therapy.

List of abbreviations

MCF7: Michigan Cancer Foundation-7
HR: Hormone (estrogen or progesterone) receptors
HER2: Human epidermal growth factor receptor 2 gene
IC<sub>50</sub>: The half-maximal inhibitory concentration
nm: Nano mole
µg: Microgram
µm: Micro mole
ml: Milliliter
hrs: Hours
min: Minutes
ATO: Atorvastatin

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Author Contribution Statement

Professor Jafar Ai is the main thesis manager. He created the main conception of the study. Dr. Somayeh Ebrahimi-Barough is the second thesis manager. She revised and approved the manuscript. Dr. Naghmeh Bahrami is primarily responsible for doing the PCR test. Dr.Reyhaneh Abolghasemi is a major contributor in writing the manuscript for Ph.D. thesis of applied cell sciences. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

The experiment was approved by the National Committee of Biological Research Ethics at Tehran University of Medical Sciences, Research and Technology Assistance (Ethical Code: IR. TUMS. VCR. REC.1397.444). The authors confirm that any experimental and research methods reported in this paper were based on mandatory health and safety protocols in the laboratory procedures.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Declaration of interest

The authors have no conflicts of interest to declare.

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