RESEARCH ARTICLE

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Transcriptional Regulation of Epithelial to Mesenchymal Transition Related Genes by Lipopolysaccharide in Human Cervical Cancer Cell Line HeLa

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Abstract

Objective: Cancer is one of the common diseases in the world, and cervical cancer is the fourth one. In this type of cancer, many risk factors, especially infectious diseases, such as human papilloma virus (HPV) and gram-negative bacteria can have important effects on the expression of epithelial to mesenchymal transition related genes like *Snail, E-cadherin*, and *ZEB-1*, responsible for connecting cell tissues. In this study, we have investigated the effect of Escherichia coli O111:B4 Lipopolysaccharide (LPS) on HPV positive cell line (HeLa), the expression level of the (*Snail*, *E-cadherin*, and *ZEB-1*), HPV oncogenes (*E6*, *E7*) and also *microRNA-9*, *192*. **Materials and Methods:** HeLa cell line was treated with LPS to analyze *Snail*, *E-cadherin*, *ZEB-1*, *E6*, *E7* and also *microRNA-9*, *192* expression by quantitative real-time PCR in 24, 48 and 72 hours. **Results:** Quantitative real-time PCR revealed a significant reduction in *E-cadherin mRNA* level at 10ug/L of LPS in three time-points and after 24 hours at 5ug/L of LPS; however, *ZEB-1* at 10ug/L of LPS and Snail at 5, 10ug/L of LPS are up-regulated. *E7* also illustrated a slight increase, but we did not find any relationship between *E7* and LPS treatment. Additionally, there are upward trends in *microRNA-9*, *192* levels. **Conclusion:** The result of this study, LPS is able to reduce *E-cadherin* expression, caused by increase in repressor E-cadherin protein expression and some microRNAs, probably. Since bacterial infection can be in cervical site, it is likely to be effective in reducing the E-cadherin expression in the EMT and enhance cancer process, therefore; removing these infections by using the appropriate antibiotics may result in slowing down this process, which requires more research.

Keywords: HPV- LPS- *Snail- E-cadherin- ZEB-1*

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Introduction

Cancer is one of the most causes of death, and there are multiple types of cancer in the world. Among different types of this disease, cervical cancer causes more than 200,000 deaths, annually (Pisani et al., 2002). This cancer is the fourth common cancer in the word (Lai et al., 2019), and the diagnosis of more than 75,000 new cases of invasive cervical malignancy is estimated in the United States (Louie et al., 2002).

Many risk factors seem to be involved in the progression of cervical cancer, including radiation, environmental pollution, (Scheurer et al., 2014; Eslamizadeh et al., 2018) alcohol consumption, smoking (Kapeu et al., 2009), and even infectious diseases (Salimi et al., 2017; Faghihloo et al., 2014; Vaezjalali et al., 2013). Nowadays, it has been accepted that human papilloma virus (HPV) has a strong relationship with cervical cancer (Franco, 1995; Faghihloo et al., 2014; Mirzaei et al., 2018) HPV is a non-enveloped virus, and has double stranded DNA (Favre, 1995). *E6* and *E7* are the HPV oncoproteins with significant roles in cervical cancer development, and with disrupting tumor suppression like *P53*, *E6* is able to accelerate the progression of cancer (Thomas et al., 1999); in addition, binding E7 to Rb promotes the degradation of tumor suppressor protein proteasome (Syrjänen and Syrjänen, 1999).

Furthermore, many bacteria are able to live in vaginal site, especially gram- negative ones. When the balance of immune system and this flora destroyed, bacteria can replicate; therefore, bacteria tend to produce some inflmatory mediators, probably (Donders et al., 2014). One of the common agents in structure of flora, especially in gram-negative bacteria is cell wall, called Lipopolysaccharide (LPS) (Sharma et al., 2014) LPS can induce *NF-kB* and *TLR-4* expression, which call immune cells to inflmatory site.

Metastasis stage is a hardly curable disease in all kind of cancers, and cervical metastasis is no exception. In invasive cervical cancer, connecting adhesive proteins are reduced between cells; hence, tumor cells are able to spread in all parts of body, replicate, and create

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tumor cell (Granados López and López, 2014; Insinga et al., 2014) This process has been defined as epithelial to mesenchyme transition (*EMT*), related to the low expression of connecting inter cellular proteins (Kalluri and Neilson, 2003) E-cadherin is one of the important *EMT* related protein families, which is involved in the adhesion between cells. Some other *EMT* related proteins like *ZEB-1* and *Snail* may also regulate the expression of *E-cadherin* (Grooteclaes and Frisch, 2000), and reduce the expression of adhesive cell proteins; therefore, metastasis can occur (Vaure and Liu, 2014).

It is supposed that, the level of *EMT* related genes and HPV oncoproteins (*E6*, *E7*) and some *microRNAs* such as *mir-9*, *mir-192* expression can have an effect on cancer progression. Virtually, it has been reported, there is a strong relationship between cervical cancer and HPV; however, the effect of bacterial LPS on cervical cancer, and the relationship between *HPV* and *LPS* on *EMT* related genes expression in cervical cancer is not clear. In this study, human cervical cancer cell line (Hela) was treated with LPS, and the expression of *EMT*-related genes was evaluated.

Materials and Methods

Cell culture and treatment

HeLa cell line (cervical carcinoma), which is HPV positive, cultured in Dulbecco's Modified Eagle Medium (DMEM, gibco) with 2 mM/ L-glutamine (Sigma-Aldrich, MO, USA), supplemented with 10% fetal bovine serum (Life Technologies, Camarillo, CA, USA), and 100 units/ml penicillin/streptomycin (Invitrogen, Life Technologies, Camarillo, CA, USA). Cells were incubated in 37°C and 5% CO_2 (IR.SBMU.MSP.REC.1396.879.).

Lipopolysaccharide preparation

Escherichia coli (E.coli) O111:B4 Lipopolysaccharide (LPS) was prepared from (Sigma-Aldrich, MO, USA). In order to make five different concentrations (1, 2, 5, 10, and 20 ug/l), LPS was dissolved in phosphate buffered saline (PBS).

Proliferation assay

Escherichia coli O111:B4 Lipopolysaccharide (LPS) was evaluated by MTT assay with using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Briefly, HeLa cell line was incubated in 96 well-plates with a condition of 37°C and 5% CO_2 until cells reach sufficient population. Medium containing LPS at concentrations of 0, 1, 2, 5, 10 and 20 ug/l was added into 96-well plates. Then, 20-µl MTT solution (0.25 g/ml) was added in 24, 48 and 72 hours, and incubated for 4 hours in incubator with previous condition. DMSO was added, and the absorbance was measured at 570 nm and 625 nm. HeLa cell line without treatment was employed as control.

RNA extraction

HeLa cell line was seeded in 12 well-plates. After filling the 80 percent of plate , HeLa cell line was treated with medium containing various concentrations of LPS (1 ug/l, 5 ug/l, 10 ug/l) in 24 , 48 and 72 hours . To RNA extraction, RNX-plus solution (Cinnagen, Tehran, Iran) and chloroform were employed to discharge proteins. Then, RNA was precipitated with propanol, and washed with 70% alcohol. Finally, the RNA quality and quantity was evaluated at the wavelength of 260 nm by nanodrop spectrophotometry (Eppendrof, Humburg, Germany). The purity of RNA was also analyzed by running with 1% agarose gel electrophoresis.

cDNA synthesis

cDNA synthesis kit was purchased from BioFACT (Daejeon, South Korea). Cellular gene cDNA was synthesized by reverse transcriptase and random hexamers. We have added 10 µl of RNA, 1 µl of random hexamer and 9 µl reverse transcriptase in a sterile micro centrifuge tube to make a 20 µl final volume.

microRNAcDNA was prepared by 10 µl of RNA, and 10µl of reverse transcriptase, and 0.5 µl of microRNA-9 primer TCCGAGGTATTCGCACTGGATACGACTCATAC-3'), and 0.5 µl of microRNA-192 primer (5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACGGCTGT-3') to make cDNA, respectively.

All samples were incubated in 50°C for 40 minutes, and after that incubated in 95°C for 5 minutes. All cDNAs were diluted (2 times in sterile water), and as template for quantitative real-time PCR analysis.

Quantitative real-time PCR

The expression of cellular genes (Snail, E-cadherin, ZEB-1, and GAPDH) and microRNA (microRNAs -9,192) were analyzed by using the BIOFACT™ 2X real-time PCR master mix (for SYBR Green I; BIOFACT, South Korea); Furthermore, in this study, the expression of *E6* and *E7* genes in HPV positive cell line (HeLa cell line) were evaluated.

SYBR master mix, 1 µl of forward primer, 1 µl of reverse primer, 2 µl of 1/2 diluted cDNA and 6 µl of sterile water were combined. To confirm our results, all samples were run in triplicate at the same time. The primers of all genes were documented in Table 1.

We have two temperature profiles for cellular and microRNA genes: 95°C for 10 minutes; 40 cycles of 95 °C for 30 Seconds, 55°C for 30 Seconds, and 72°C for 30 Seconds for cellular genes, and 95°C for 10 minutes;40 cycles of 95°C for 20 Seconds, 60°C for 20 Seconds, and 72°C for 20 Seconds for microRNA. Melting curve program was between 60 and 95°C.

The threshold cycle (ct) of all genes in treated cell line was compared with the ct of control cell line ,then all cellular and microRNA were normalized with housekeeping gene (GAPDH and u6), respectively, by the $2^{-\Delta\Delta ct}$ method.

Statistical analysis

Graph-Pad Prism software was used to analyze the difference between groups, and ANOVA test shows the comparison between mRNA levels. We consider the P-value less than 0.05 ($P<0.05$) as statistically significant

for the differences.

Results

Optimization of the LPS concentration

The best LPS concentrations to measure the expression of cellular and microRNA are 1, 5 and 10 ug/L of LPS in 24, 48 and 72 hours. The data was not shown.

LPS down-regulates the expression of E-cadherin

After treating with LPS in 24, 48 and 72 hours, RNA was extracted by RNX-plus, and cDNA was synthesized. Quantitative real-time was employed to analyze *EMT* related genes. The E-cadherin has revealed a noticeable down-regulation in all times of 10ug/L of LPS and 24 hours after treating with 5ug/L of LPS (Figure 1).

LPS up-regulates the expression of Snail

In our study, 5 and 10ug/L of LPS can have a strong influence on expression of Snail. We have found in this period of time Snail has an up-ward trend expression, but 1ug/L of LPS ,Snail has not a meaningful increase (Figure 2).

ZEB-1 is over expressed by treating LPS

Analysis of another gene (*ZEB-1*) has demonstrated a meaningful increase in 48 and 72 hours after treating 10ug/L of LPS in HeLa cell line. Generally, LPS induces an increase in ZEB-1 expression at 1 and 5ug/L of LPS,

Table 1. the Primer of Cellular Genes and microRNA, Evaluated with Real-Time PCR

Primer name	Sequence
ZEB-1 (Forward)	5'-GATGATGAATGCGAGTCAGATGC-3'
ZEB-1 (Reverse)	5'-CTGGTCCTCTTCAGGTGCC-3'
Snail 1 (Forward)	5'-CAGACCCACTCAGATGTCAA-3'
Snail 1 (Reverse)	5'-CATAGTTAGTCACACCTCGT-3'
E7 (Forward)	CCGCTCGAGATGCATGGACCTAAGGCAAC
E7 (Reverse)	CGCGGATCCTTACTGCTGGGATGCACACC
E-cadherin (Forward)	AGGGGTTAAGCACAACAGCA
E-cadherin (Reverse)	GGTATTGGGGGCATCAGCAT
GAPDH (Forward)	ATGTTCGTCATGGGTGTGAA
GAPDH (Reverse)	GGTGCTAAGCAGTTGGTGGT
U6 (Forward)	5'-GAGAAGATTAGCATGGCCCCT- 3'
U6 (Reverse)	5'-ATATGGAACGCTTCACGAATTTGC- 3'
miRNA -9-5P (Forward)	5'-CTTTGGTTATCTAGCTGTATGAGTCGT-3'
Forward miRNA -192-5P (Forward)	5'-CTGACCTATGAATTGACAGCCGT-3'
Universal Reverse	5'-ATCCAGTGCAGGGTCCGA-3'

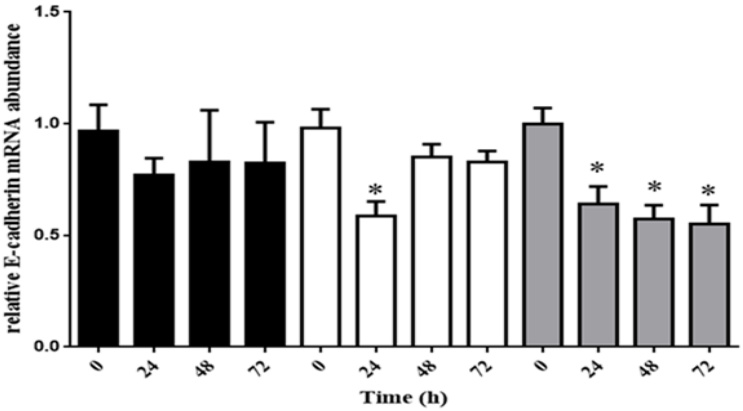

Figure 1. HeLa Cell Line was Treated with LPS by 1, 5 and 10 mmol/L, when the Cells Reached 80% Confluency. In all times of 10 mmol/L of LPS and 24 hours after treating with 5 mmol/L of LPS, E-cadherin expression have been raised.

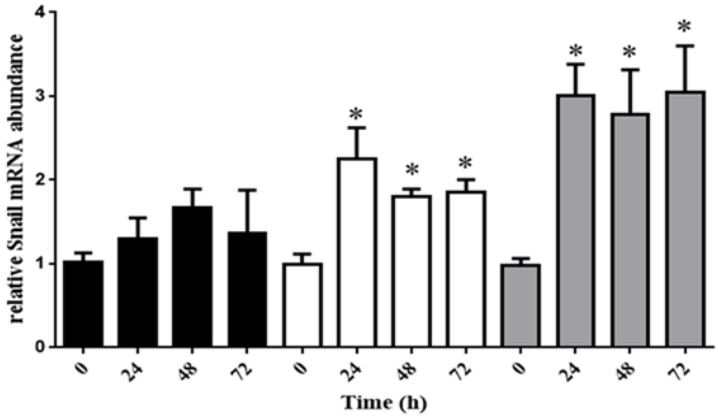

Figure 2. HeLa Cell Line have been Cultured with DMEM Containing LPS at Concentration of 1, 5 and 10 mmol/L. Snail Expression was Revealed a Remarkable Growth in 5 and 10 mmol/L of LPS.

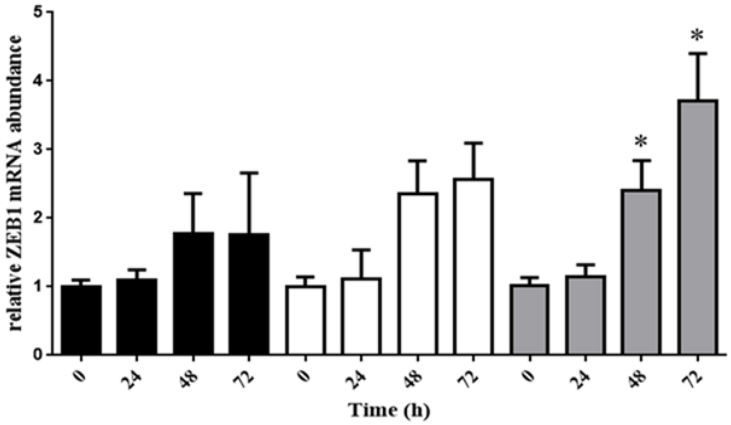

Figure 3. LPS is Able to Increase Expression of *ZEB-1*, in HeLa Cell Line, after 48 and 72 hours in 10 mmol/L.

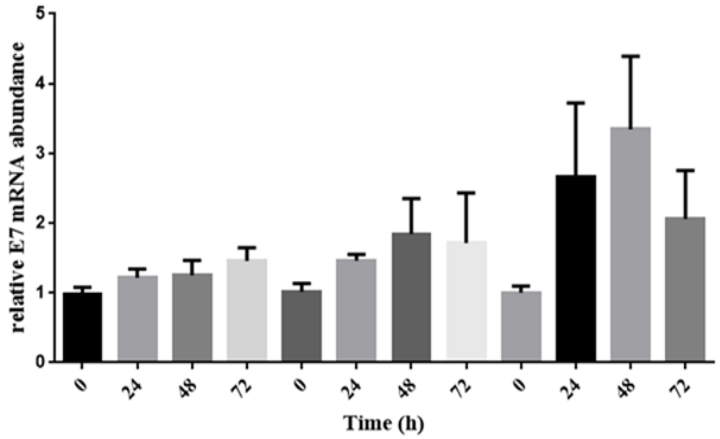

Figure 4. The Level of *E7* HPV Expression after Treating HeLa Cell Line with LPS .The bar graph have demonstrated a meaningful increase in amount of *E7* HPV expression, however it is not meaningful relation.

but it was not meaningful, statistically (Figure 3).

LPS increases E7 HPV expression

LPS is able to increase the level of *E7 HPV* expression, but in our study, we have not found any meaningful relationship between LPS and the expression of *E7 HPV* (Figure 4).

MicroRNA-9 and 192 expression is up-regulated by LPS

We analyzed two microRNAs (*microRNA-9* and *192*) expression .There are an increase in amount of microRNA-9 (almost 12 times) and 192 (almost 8 times) expression (Figure 5).

Discussion

Epigenetic changes may result in changes in gene expression. *EMT* related genes, such as *Snail*, *ZEB-1* are important gene groups help cells in different organs to connect each other, especially *Snail*, *E-cadherin*, and *ZEB-1*. Down, or up-regulation of these genes can have some effects on cancer progression and invasive

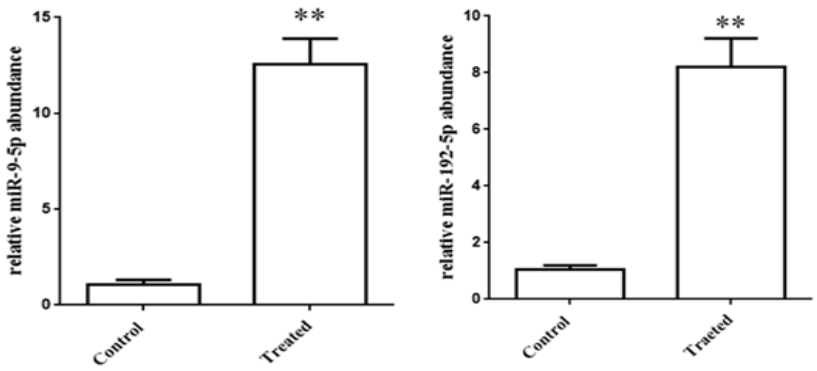

Figure 5. There are a Remarkable Increase in the amount of *microRNA-9* and *192* Expressions after Treating LPS to HeLa Cell Line.

metastasis (Adhya and Basu, 2010).

Cervical cancer is the second most important cancer in women, all around the world (Ferlay et al., 2012), and risk factors, ranging from pollution , radiation, smoking and consumption of alcohol to infectious diseases can affect the *EMT* related genes expression (Scheurer et al., 2014). We studied on the effect of gram- negative bacterial cell wall.

LPS, which is known endotoxin, is a vital structure in all kinds of bacteria, composed of lipid and polysaccharide. This molecule has a complex structure, which can be divided in 3 parts: outer hydrophilic O-antigenic polysaccharide side chain, a hydrophilic core polysaccharide and a hydrophilic lipid, and this hydrophilic lipid acts as an endotoxin (Kilár et al., 2013). We have purchased E.coli O111:B4 LPS to find what might be the relation between bacterial flora and *HPV* is, and how *EMT* related genes expression changes.

Our result shown, 10 ug/L of LPS is able to decrease 1.5 times E-cadherin expression .Other studies have the same results for example, *Porphyromonas gingivalis*-LPS was examined in human gingival epithelial cells, and E-cadherin expression destruct epithelial barrier function (Abe-Yutori et al., 2017) LPS on some cells like macrophages can activate TNF and IL-6 secretion by Raw264.7 pathway, which induces a decrease in *E-cadherin* expression (Van den Bossche et al., 2015). Other studies have used some LPS antagonism, for example it has studied the effects of Shikonin, an antagonism of LPS, and observed that it can prevent down-regulation of E-cadherin from the effect of LPS in breast cancer cell line (Hong et al., 2015).

Another important *EMT* related genes is Snail, after treating HeLa cell line with LPS, considerable increase in amount of Snail is observed. Briefly, LPS at concentration of 5 and 10 ug/l is able to increase the expression of Snail, in HeLa cell line, almost duplicate and triplicate, respectively. This result is similar to some other studies, for example in study of Jing, when human hepatocellular carcinoma cell line (HCC) is treated with LPS. In this case in HCC, LPS is able to activate TLR-4 receptor, promoted NF-κB signaling, and Snail increases, subsequently (Jing et al., 2012), furthermore; activation of TLR-4 by LPS can also induce VEGF, Therefore; VEGF causes increase in *Snail* expression and metastasis progress (Zhu et al., 2016). The study has offered to use Curcumin as an antagonism of LPS. Curcumin can regulate the snail over-expression, induced by LPS (Huang et al., 2013).

We have supposed, LPS with effecting on Snail can lose *E-cadherin* expression. It seems, Snail is a repressor binding to the E-cadherin promoter, and leads to lose of E-cadherin expression (Cano et al., 2000; Takagi et al., 1998). Actually, in order to repress *E-cadherin* promoter, Snail requires histone deacetylase activity (Jiang et al., 2013) Peinado has suggested, trichostatin a can inhibit histone deacetylase activity ,therefore ; snail is not able to reduce E-cadherin expression (Peinado et al., 2014).

In this study, we examined the *ZEB-1* expression by treating HeLa cell line with LPS. 10ug/L of LPS is able to increase in *ZEB-1* expression. It seems, *ZEB-1* with the same mechanism of snail can inhibit the expression of *E-cadherin* (Comijn et al., 2001). We have supposed, LPS by increasing *ZEB-1* level can down-regulate the E-cadherin expression. Recently, it has been suggested that Claudin-1, like LPS can up-regulate *ZEB-1* expression, consequently E-cadherin is repressed (Singh et al., 2011).

HeLa is a HPV positive cell line, and *E7* integrated in HeLa genome. Our result is demonstrated, LPS can cause a slight increase in amount of *E7* expression, but there is no meaningful correlate between *LPS* and *E7*. Probably, *E7* may repress the E-cadherin by *DNA methyltransferase 1 (DNMT1)* expression. Laurson et al., (2010) Caberg also has supposed, decrease in *E-cadherin* expression perhaps result from repressing RB by *E7* (Caberg et al., 2008). One of other mechanisms has reported that *E7* may be over-express cdc6, which is one of key factors to reduce *E-cadherin* expression (Faghihloo et al., 2016). We suppose, LPS by over-expression of *E7*, reduce E-cadherin. It has shown valproic acid has the same effect on *E7*, and *E-cadherin* down-regulate, subsequently. (Faghihloo et al., 2016)

MicroRNA is able to regulate EMT genes (Abba et al., 2016) Observations have shown, different microRNAs can mediate the expression of *E-cadherin* (Wong et al., 2014). Furthermore, in this study, LPS treatment was able to increase in amount of *microRNA-9* and *192* expressions almost 12 and 8 times, respectively. The result of other studies confirm our experiment for example, Flavia Bazzoni has suggested, LPS activate TLR-4, therefore NF-κB signaling initiate, and microRNA-9 increase (Bazzoni et al., 2009). Wu et al., (2012) also found after treating *LPS*, *microRNA-192* over-expressed. Probably, *microRNA-9* is able to suppress *E-cadherin* (Zhou et al., 2017) *LPS* over-expresses *microRNA-9* and *192* , and these microRNAs can down-regulate *E-cadherin* expression, probably.

In conclusion, LPS is able to have effects on EMT related genes, such as *E-cadherin*, *Snail* and *ZEB-1*; therefore, gram-negative bacterial infectious diseases in cervix probably can have the same mechanism, and the cervical cancer progression can occur due to LPS in gram- negative bacteria in this site. To prevent cervical cancer progression in early stages, using appropriate antibiotics, is suggested.

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Ethical Considerations

IR.SBMU.MSP.REC.1396.879.

Conflict of Interest

The authors declare no conflicts of interests.

Authors' Contributions

E.F., SH.T, assisted in the study design and performed the cell culture and molecular experiments. H.G., G.E performed the statistical Analyses. All authors read and approved the final version of the manuscript.

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