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Inhibition effect of Hsp90 on TLR2, TLR4, and MAPK signaling pathway in melanoma in-vitro

Setayesh Tavakoli [a], Ali Adili [b], Morteza Akbari [a], Rozita Tamjidifar [a], Saeed Tarzi [a], Milad Saadat [a], Leila Sadat Hatamnezhad [c], Babak Sandoghchian Shotorbani [d], Siamak Sandoghchian Shotorbani [a,e,*]

[a] Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[b] Department of Oncology, Tabriz University of Medical Sciences, Tabriz, Iran
[c] Department of Dermatology, Sina Hospital, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, IR, Iran
[d] Department of Oncology, Ardabil University of Medical Sciences, Ardabil, Iran
[e] Department of Immunology, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

Introduction: The Hsp90 is a member of heat shock proteins (HSPs) involved in protein folding and protecting cells from stress. Hsp90 is implicated in melanoma carcinogenesis in which its suppression could have beneficial applicability. This study aimed to investigate the effect of Hsp90 inhibition on TLR2 and TLR4 expression along with the MAPK signaling pathway in melanoma cancer cell line (A-375).

Materials & methods: Melanoma cell line was exposed to Hsp90 inhibitor. After 48 h, the expression of Hsp90 was determined by Real-Time PCR and Western blotting. Then, TLR2 and TLR4 expression levels along with phosphorylated p38, ERK, JNK (MAPK signaling pathway) were assessed by qRT-PCR and Western blotting. MTT assay was used to determine the effect of Hsp90 inhibition on cell proliferation.

Results: The Hsp90 inhibitor decreased the expression of Hsp90 after 48 h. This decrement led to decreased expression of TLR2 and TLR4 through the MAPK signaling pathway in melanoma cells. MTT assay revealed that anti- Hsp90 treatment caused a reduction of cell viability in a time-dependent manner.

Conclusion: The results of this study determine the potential therapeutic ability of Hsp90 inhibitor in melanoma skin cancer cells by regulating TLR2 and TLR4 through MAPK signaling pathway in-vitro.

1. Introduction

A combination of factors, including environmental and genetic factors, are known to cause melanoma. However, it is believed that exposure to ultraviolet (UV) is the main cause of melanoma that leads to melanocytes being mutated and hyperproliferative (Takazawa et al., 2014; Eiro et al., 2013).

Heat shock proteins (HSPs) were first discovered as a group of proteins that are strongly induced by heat shock and stresses in a wide range of species. They were also identified as molecular chaperones or proteins modifying the structures and interactions of other proteins. HSPs overexpression has been known to be contributed to tumor progression and resistance to treatment in different types of cancers (Shomali et al., 2020a; Shotorbani et al., 2017; Shevtsov and Multhoff, 2016). HSPs are classified based on their molecular weights: Hsp10, Hsp40, Hsp60, Hsp70, Hsp90, etc. Hsp90 is a 90-kD chaperone that is overexpressed in a wide range of cancers and performs its role by inducing angiogenesis and metastasis, suppressing apoptosis, impairment of tumor immunity, and bypassing cellular senescence. Hsp90 overexpression has been known as a poor prognosis in cervical, uterine, breast, and bladder carcinomas (Zuehlke et al., 2018; Jhaveri et al., 2014).

Toll-like receptors (TLRs) are involved in the initiation of immune

Abbreviations: HSP, heat shock protein; MAPK, mitogen-activated protein kinase; TLR2, Toll-like receptors 2; TLR4, Toll-like receptors 4; P38, MAP kinase p38; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal Kinase; PAMPs, pathogen-associated molecular patterns; TLR5, Toll-like receptors 5; NF-κB, *nuclear factor-kappa B*; *GA*, gambogic acid; RAS, rat sarcoma; BRAF, serine/threonine-protein kinase B-Raf; NF1, neurofibromatosis type I; PI3K, phosphatidylinositol 3-kinase.

* Corresponding author at: Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
E-mail addresses: siamak1331@gmail.com, sandoghchians@tbzmed.ac.ir (S.S. Shotorbani).

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responses by detecting pathogen-associated molecular patterns (PAMPs). However, it has been revealed that TLRs are overexpressed in a wide range of tumors. TLRs overexpression in the tumor microenvironment not only led to increased proliferative, invasive, and metastatic capacity of tumor cells but also caused tumor cells' resistance to apoptosis through regulating integrin and metalloproteinase. Besides, TLRs' activation leads to pro-inflammatory cytokine and immunosuppressive agent production, leading tumor cells to evade immune responses (Keshavarz et al., 2020; Braunstein et al., 2018; Alizadeh et al., 2020; Shomali et al., 2021).

MAPK signaling pathway is known by its three downstream factors including the p38 MAP kinases (p38), the extracellular signal-regulated protein kinase (ERK), and the c-Jun amino-terminal kinase (JNK), which are functionally active when getting phosphorylated. The relationship between TLR agonist and MAPK signaling pathway has been proven in studies, suggesting the participation of TLRs and MAPK signaling pathway in regulating immune responses (Inamdar et al., 2010; Braicu et al., 2019).

In this study, we aimed to investigate the inhibitory role of Hsp90 on TLR2, TLR4 expression, and the possible regulatory effect of its inhibition on the MAPK signaling pathway in melanoma. Besides, the effect of this inhibition on cell viability was investigated.

2. Material and method

2.1. Cell culture

Melanoma A-375 cells were purchased from Pasteur Cell Bank (Tehran, Iran) and cultured in RPMI1640 medium enriched with FBS 10% and stored in an incubator containing 5% CO_2 at 37 °C.

2.2. Hsp90 blocker

The Hsp90 blocker named CCT018159 was purchased from Millipore Sigma Company located in Singapore. 4.2 μM of this product was added to the cells.

2.3. Real-time PCR

The cells were cultured in a 6-well plate. After treatment with Hsp90 blocker, total RNAs were extracted by the TRIzol reagent based on the related extraction protocol (Takara, Japan). RNAs were then transcribed into cDNAs by reverse transcription enzyme using a cDNA synthesis kit (Takara, Japan). Real-time PCR was performed using a qPCR kit purchased from Takara Company located in Japan via the Corbett Rotor-Gene TM 6000 instrument. The GAPDH gene was considered as the internal control. Primer sequences are summarized in Table 1.

Table 1
Primer sequences.

Name	Sequences	
GAPDH	F	5′-CCCCACACACATGCACTTACC-3′
	R	5′-TTGCCAAGTTGCCTGTCCTT-3′
Hsp90	F	5′-CGATGAATATGCCATGACT-3′
	R	5′-TCCATAGCAGATTCTCCAG-3′
TLR2	F	5′-TCTCCCATTTCCGTCTTTTT-3′
	R	5′-GGTCTTGGTGTTCATTATCTTC-3′
TLR4	F	5′-GAAGCTGGTGGCTGTGGA-3′
	R	5′-GATGTAGAACCCGCAAG-3′
p-P38	F	5′-AGGGCGATGTGACGTTT-3′
	R	5′-CTGGCAGGGTGAAGTTGG-3′
pERK	F	5′-TCAAGCCTTCCAACCTC-3′
	R	5′-GCAGCCCACAGACCAAA-3′
pJNK	F	5′-GCCATTCTGGTAGAGGAAGTTTCTC-3′
	R	5′-CGCCAGTCCAAAATCAAGAATC-3′

2.4. Western blot

In summary, anti-Hsp90 treated cells were lysed by RIPA Lysis Buffer System (Radioimmunoprecipitation) (Sigma Aldrich, 82024 Taufkirchen, Germany). Then, 50 μg extracted protein was added to the gel using vertical electrophoresis. A semidry western blot transfer system, Polyvinylidene difluoride (PVDF) membranes, was used for staining the proteins. Then, cells were tended to be blocked by exposure to Tween 20 (0.5 in PBS) for 2 h. After 2 h, the membrane was incubated with goat monoclonal antibody against aimed target genes (TLR2, TLR4, pP38, pERK, pJNK) and Beta-Actin (Sigma Aldrich, 82024 Taufkirchen, Germany). The last step was to expose the membrane with mouse or rabbit anti-goat secondary antibody conjugated with HRP visualized by an ECL kit (Roche, Germany). The density of proteins was measured using ImageJ software.

2.5. MTT evaluation

Approximately 2000 cells were cultured in 96-well plates and cells were treated with an Hsp90 blocker. The cells were then incubated for 96 h and the MTT test was performed using an MTT assay kit (Abcam, Shanghai, China). Absorbance was measured at OD = 540 nm by an ELISA reader instrument.

2.6. Statistical analysis

Statistical analysis was performed using Graphpad prism 7 software based on ANOVA and Student *t*-test. *P*-values were higher than 0.05 considered significant.

3. Result

3.1. Expression of Hsp90 was attenuated after treating with Hsp90 blocker in the A375 melanoma cell line

qPCR and Western analysis showed that anti-Hsp90 reagent attenuated Hsp90 expression in both mRNA and protein levels (Fig. 1, $^{****}P < 0.0001$).

3.2. The decreased expression of Hsp90 tended to increased expression of TLR2 and TLR4 through the MAPK signaling pathway in melanoma cells

As shown in Figs. 2 and 3, TLR2 and TLR4 expression was attenuated after the suppression of heat shock protein expression. In this regard, the downstream target genes involved in the MAPK signaling pathway including pP38, pERK, pJNK were downregulated in both mRNA and protein levels after inhibition of HSP expression. A collaboration of TLR2 and 4 with MAPK signaling has been proven; in line with studies, our study may propose a new modification approach for attenuation of the TLR signaling pathway via the MAPK signaling route. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$

3.3. Reduction of cell viability by Hsp90 attenuation

To determine the Hsp90 attenuation on cell viability, the optimum concentration of the reagent was added to cells and the cell viability was measured using MTT assay. The results revealed that Hsp90 suppression led to reduced viability of the A-375 melanoma cell line in a time-dependent manner (Fig. 4). $^{**}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

4. Discussion

Melanoma is a malignant skin cancer that is caused by the production of cytokines and activation of TLRs in melanocytes (Ambarus et al., 2018). There are differences in genetics, immunotherapy, and therapeutic effects that could lead to specialized treatment strategies for

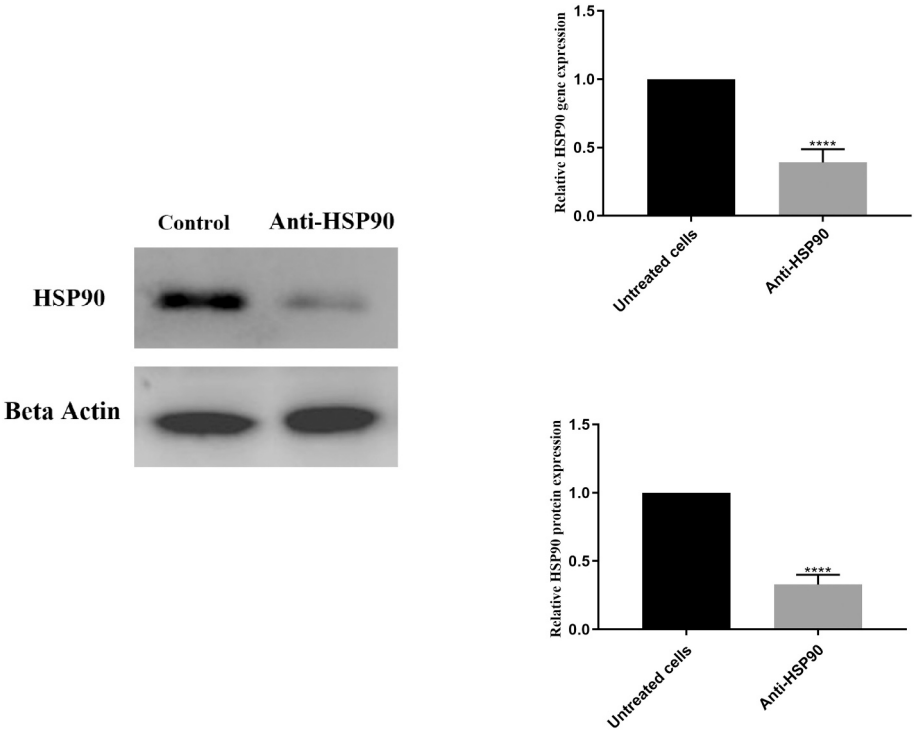

Fig. 1. Effects of anti-Hsp90 reagent on its expression. This figure shows that Hsp90 treatment attenuated Hsp90 expression in both mRNA and protein levels. ****$P < 0.0001$.

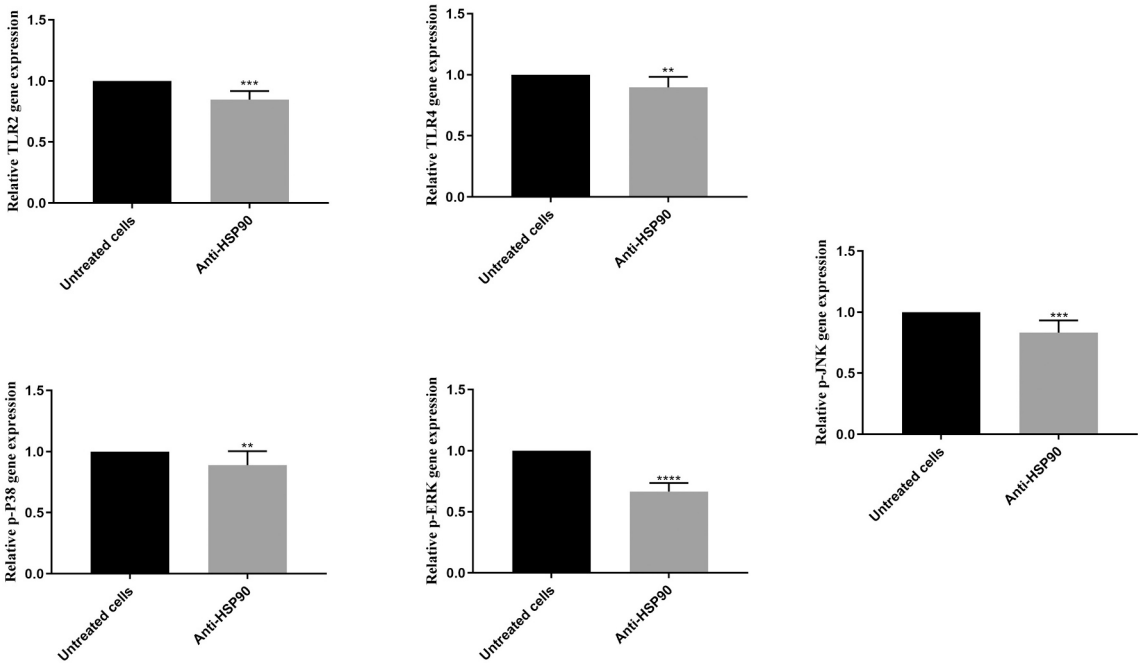

Fig. 2. Effects of Hsp90 attenuation on TLR2, 4 along with MAPK signaling pathway. This graph shows that the decreasing of Hsp90 caused the reduction of TLR2, TLR4, and MAPK signaling pathways (p38, ERK, JNK expression) at mRNA levels compared to untreated cells. **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

melanoma subtypes in the future. By binding to the TLR4 surface, Hsp90 can activate MAPK and trigger inflammatory immune responses (Ambarus et al., 2018; Kastenhuber et al., 2017; Karki et al., 2018).

HSPs are described as intracellular molecules that are released in response to stressful conditions (Mielczarek-Lewandowska et al., 2020a). HSPs play an important role in cancer biology and genetics by regulating a variety of genes. TLR2 and TLR4 may be the major receptors for Hsp60 and Hsp90 which are essential for cancer progression (Shipp et al., 2013). In a study, it was revealed that HSPs are required for TLR folding. In the same vein, similar to our study, Bon Hyang Na et al. showed that anti-Hsp90 therapy reduced TLR5 expression by inhibiting the NF-KB signaling pathway in human myeloid leukemia in-vitro (Mielczarek-Lewandowska et al., 2020b; Na et al., 2018). In line with our study, Vahid et al. revealed that Hsp90 has a regulatory role in TLR4

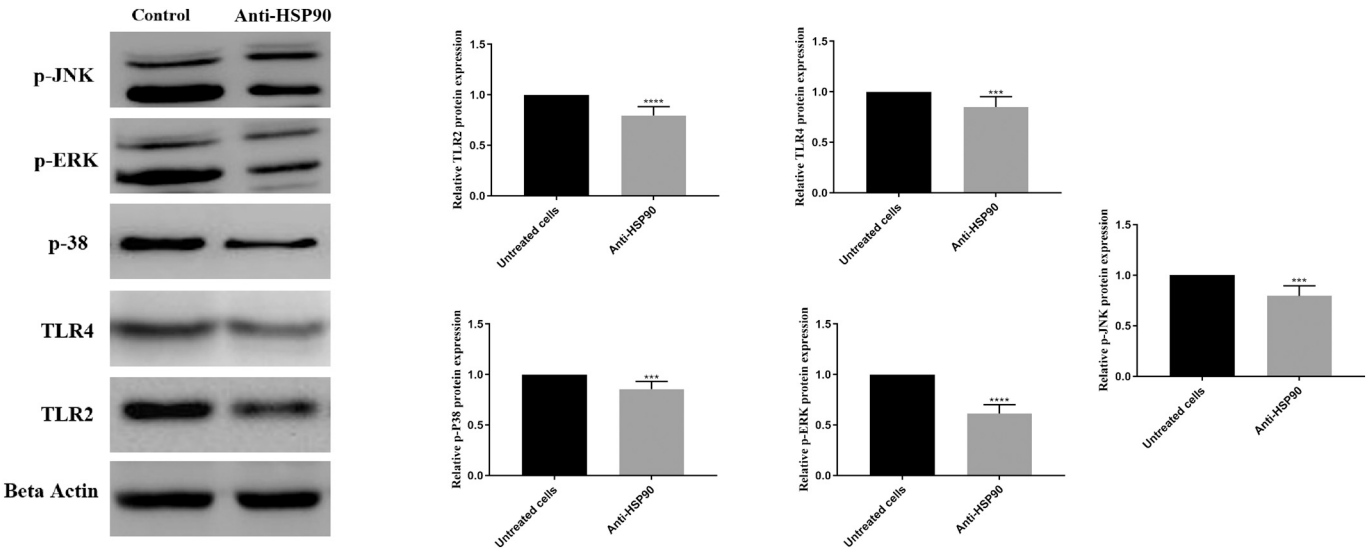

Fig. 3. Effects of Hsp90 attenuation on TLR2, 4 along with MAPK signaling pathway. This graph shows that the decreasing of Hsp90 caused the reduction of TLR2, TLR4, and MAPK signaling pathways (p38, ERK, JNK expression) at protein levels compared to untreated cells. $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

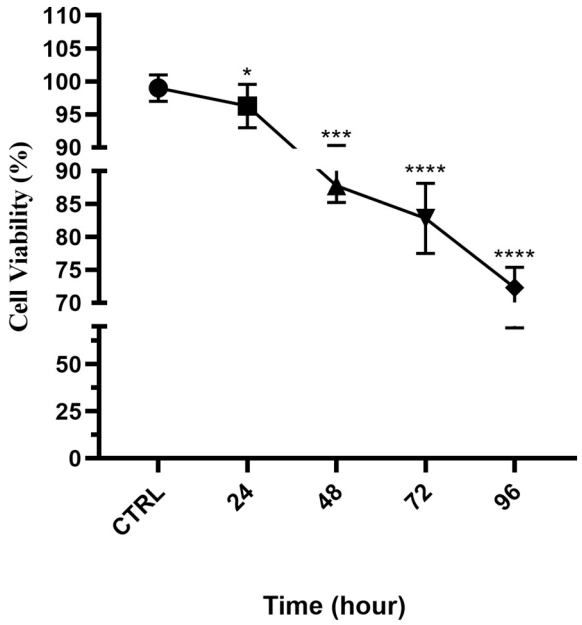

Fig. 4. Hsp90 reduction reduces melanoma cells' viability. This figure reveals that Hsp90 suppression reduced melanoma cells' viability in a time-dependent manner in comparison with untreated cells. $^{*}P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

expression (Vahid et al., 2016).

MAPK signaling pathway has been revealed to be responsible for melanoma cell survival, differentiation, invasion, proliferation, and angiogenesis (Savoia et al., 2019; Cheng et al., 2013). This signaling pathway is activated in melanoma because of genetic alterations in some genes including RAS, BRAF, and NF1 (Hartman et al., 2019; N. Cancer Genome Atlas, 2015).

The role of Hsp90 in the development of melanoma makes this protein a promising therapeutic target. In this regard, it was revealed that gambogic acid (GA) attenuates liver fibrosis by reducing Hsp90 which in turn modulates MAPK signaling pathway (Yu et al., 2019; Wang et al., 2016).

It was reported that DHP1808 (a compound that suppresses melanoma cell proliferation through apoptosis induction) exerted its role via affecting Hsp90/PI3Kα and Hsp90/MAPK in A375 melanoma cells. This compound was found to remarkably inhibited the MAPK signaling pathway by regulating the Hsp90 and suppressing the PI3K-AKT pathway. Thus, Hsp90 and related signaling pathways including PI3K and MAPK could be potential targets of treating melanoma (Zhao et al., 2020).

It has been proven that various tumor-derived antigens are recognized through TLR, so this TLR-mediated recognition of tumors cell leads to activation of the immune system and would be participated counteracting tumor cells (Wang et al., 2016; Kaczanowska et al., 2013). Indeed, there has recently been a great deal of attention to tumor immunotherapy by using TLR agonists to increase the sensitivity immune system especially the innate immune cells to tumor-derived antigens (Vijay, 2018). There are several ongoing preclinical studies and clinical trials evaluating the TLR agonists, some of which have been approved to being used in cancer therapy (Kaczanowska et al., 2013; Anwar et al., 2019). The main problem that reduces the efficacy of such treatments is a potential immunosuppressive microenvironment around the tumors than to activate immune cells (Fu et al., 2015). Besides, TLRs have been expressed on tumor cells which their upregulation causes tumor cells to survive and evade the immune system. Therefore, targeting the TLRs may be an important therapeutic approach for the treatment of tumors (Na et al., 2018; Sato et al., 2009). In this regard, our results suggest that Hsp90 inhibitors suppress TLR2 and TLR4 expression along with the MAPK signaling pathway which has been shown to be involved in oncogenesis, tumor progression, and drug resistance (Braicu et al., 2019; Shomali et al., 2020b), suggesting that Hsp90 inhibitors can be used to treat melanoma.

To sum up, in this study we found that inhibition of Hsp90 could attenuate TLR2 and TLR4 expression. Since it had shown that TLRs and MAPK had a strong association in melanoma, we found that Hsp90 downregulation led to MAPK signaling pathway attenuation. We also revealed that attenuation of Hsp90 could reduce cell viability in a time-dependent manner. Altogether, we determined that Hsp90 has a fundamental role in melanoma carcinogenesis and its suppression can be a promising strategy for patients with melanoma by affecting TLRs and MAPK signaling pathways.

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Ethics approval statement

This study has been approved by the Regional Ethics Committee (IR.TBZMED.VCR.REC.1398.172).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Consent to participate

Not applicable.

Consent to publish

All authors read and approved the final version of the manuscript to be published. Also, they grant the Publisher the sole and exclusive license of the full copyright in the Contribution.

CRediT authorship contribution statement

Setayesh Tavakoli: Conceptualization, Investigation. **Ali Adili:** Conceptualization, Investigation. **Morteza Akbari:** Writing – review & editing. **Rozita Tamjidifar:** Conceptualization, Investigation. **Saeed Tarzi:** Conceptualization. **Milad Saadat:** Investigation. **Leila Sadat Hatamnezhad:** Writing – review & editing. **Babak Sandoghchian Shotorbani:** Formal analysis. **Siamak Sandoghchian Shotorbani:** Conceptualization, Supervision.

Declaration of competing interest

None.

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None.

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