

Effect of Photodynamic Therapy With Two Different Photosensitizers on the Viability of Human Dental Pulp Stem Cells



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Abstract

Introduction: Photodynamic therapy (PDT) is a minimally invasive technique increasingly used in dentistry for its antimicrobial properties. This research aimed to evaluate the influence of photodynamic therapy (PDT) on the viability of dental pulp stem cells (DPSCs).

Methods: In this laboratory-based, experimental study, DPSCs were cultured in Dulbecco's modified Eagle's medium and maintained at 37 °C. The cells were separated into five groups: Toluidine blue (TBO) at concentration of 0.1 mg/mL and 0.5 mg/mL, as well as methylene blue (MB) at concentrations of 0.01 mg/mL and 0.05 mg/mL were added to the wells in groups 1 to 4. The fifth group served as the control group. After 5 minutes of incubation, the experimental groups were irradiated with Fotosan® light-emitting diode (LED) for one minute. Cell viability was assessed after 8, 24, 48, and 72 hours using the methyl thiazolyl tetrazolium (MTT) assay.

Results: Time ($P < 0.000$), photosensitizer type/concentration ($P < 0.0001$), and their interaction effect ($P < 0.000$) on cell viability were all significant. Viability in both MB groups was considerably higher than that in the control group at 8 hours ($P < 0.001$). At 24 hours, no significant difference was observed between the experimental groups and the control ($P > 0.05$). At 48 and 72 hours, cell viability in the TBO groups was markedly lower compared to the control group ($P < 0.01$).

Conclusion: PDT with MB at the tested concentrations had no adverse effect on DPSCs even in the long-term (48 and 72 hours).

Keywords: Photochemotherapy; Methylene blue; Tolonium chloride; Cell survival.

Introduction

Efficient chemo-mechanical preparation of the root canal system and its hermetic three-dimensional seal are imperative for successful endodontic treatment.^{1,2} Also, elimination or minimization of bacterial load in the root canal system is the ultimate goal of treatment to prevent reinfection.^{3,4} Several strategies have been proposed for eradicating pathogenic microorganisms from the root canal system, and assessment of their efficacy and biocompatibility for the adjacent tissues are among the important research topics.⁵

Photodynamic therapy (PDT) or light-activated therapy is a relatively novel treatment strategy that involves the interactions of a non-toxic photosensitizer such as methylene blue (MB) or toluidine blue (TBO) with a safe light source in the presence of oxygen. This process produces reactive oxygen species that trigger a chain of biological events that eventually result in apoptosis and death of microorganisms.^{6,7} In PDT, the stimulated photosensitizer binds to molecular oxygen

to produce reactive species responsible for cell death.⁶ Fast effect, reproducibility, selectivity, access to hard-to-reach areas such as furcation areas and root surface depressions and porosities, low risk of associated bacteremia in immunocompromised patients or those with systemic underlying conditions, reduction of dentin hypersensitivity following root planing, alleviation of pain and edema following surgery, low cost, short treatment duration, and no interference with the adjacent healthy tissues are among the main advantages of PDT.⁸ PDT has been introduced as an effective adjunct to endodontic therapy for further reduction of bacterial load. However, further studies are required on its efficacy and safety for different tissues.⁹

Stem cells are the origin of all cell types in the human body. They have high potential for cell division and can differentiate into different cell types such as hematopoietic, cardiac, and neural cells as well as chondrocytes and osteocytes. The preservation of stem cells and the induction of their proliferation are imperative to achieve

optimal clinical results. In order to achieve this goal, the root canal system should be well disinfected and optimized.^{10,11} Dental pulp stem cells (DPSCs) and stem cells from the apical papilla can be effectively used for tissue regeneration.¹⁰

Although PDT has shown optimal efficacy as an adjunct to the conventional chemomechanical instrumentation of the root canal system,^{12,13} its effects on DPSCs have not been adequately investigated. Low-level lasers do not usually cause any morphological changes in teeth.¹⁴ However, some parameters in PDT, such as penetration of photosensitizer into the cells, and the effects of light irradiation of photosensitizer and its possible impact on the viability of host cells, which need to be further scrutinized may damage the pulp and periapical cells.^{15,16} Any damage to the host cells can impair healing and lead to treatment failure.⁵ The assessment of the possible cytotoxicity of PDT in the use of different types and concentrations of photosensitizers can help in finding the safest protocol for tissue regeneration and healing.^{15,16}

Considering the significance of the viability of stem cells, this study sought to assess the impact of PDT on the viability of DPSCs.

Methods and Materials

The study protocol received ethical approval from Shahid Beheshti University of Medical Sciences Ethics Committee (IR.SBMU.DRC.REC.1398.171). Conducted as an in vitro, experimental study, it utilized human DPSCs sourced from the Iranian Cell Bank. The cells were cultured in Dulbecco's modified Eagle's medium, maintained at 37 °C with 5% CO₂. The cells were kept as semi-confluent to prevent their differentiation. The culture medium was renewed every two days and 20% fetal bovine serum was added to enrich the medium. After the completion of cell culture, the cells were separated into five groups including one control group with no intervention and four experimental groups subjected to PDT using either 0.1 mg/mL or 0.5 mg/mL concentrations of TBO and 0.01 mg/mL or 0.05 mg/mL concentrations of MB.¹⁶ Five wells were allocated to each group. Each group contained 1.5×10^5 cells in 0.1 mL of the culture medium in a microtiter plate. After 24 hours, photosensitizers were added to the wells. Group 1 received 0.1 mg/mL TBO, group 2 received 0.5 mg/mL TBO, group 3 received 0.01 mg/mL MB, group 4 received 0.05 mg/mL MB, and group 5 served as the control group. After the addition of photosensitizers to the wells in the four experimental groups, the samples were incubated for 5 minutes and were then irradiated with Fotosan® light-emitting diode (LED; CMS Dental, Denmark) at 630 nm wavelength and 200 mW/cm² power for 1 minute. Several empty wells were considered between the experimental and control wells in order to prevent unwanted irradiation of wells by the scattered radiation. Cell viability was assessed at 8, 24,

48, and 72 hours using the methyl thiazolyl tetrazolium (MTT) assay.

The data were analyzed by GraphPad Prism V8 software, and cell viability was reported using measures of central tendency and dispersion for five groups across various time points. The effects of type and concentration of photosensitizer and assessment time point on cell viability were evaluated using a two-way ANOVA. In case of a significant effect, pairwise comparisons were conducted using the Bonferroni multiple comparisons test. The level of significance was set at 0.05.

Results

The analysis showed significant effects of time ($P < 0.000$), the type and concentration of photosensitizer ($P < 0.000$), and their interaction ($P < 0.000$) on cell viability.

Table 1 (& Figure 1) presents the mean viability of DPSCs in the 0.1% TBO group in comparison with the control group across different time intervals. As shown, no significant difference was observed between the 0.1% TBO group and the control group in cell viability at 8 and 24 hours (both $P > 0.05$). However, at 48 ($P < 0.001$) and 72 ($P < 0.01$) hours, cell viability in the 0.1 mg/mL TBO group was significantly lower than that of the control group.

Table 2 (& Figure 2) presents the mean viability of DPSCs in the 0.5% TBO group relative to the control group at different time points. At 8 and 24 hours, no significant difference was noted in cell viability between the 0.5% TBO group and the control group ($P > 0.05$). However, at 48 ($P < 0.01$) and 72 ($P < 0.01$) hours, the viability of the 0.5% TBO group was significantly lower than that of the control group.

Table 3 (& Figure 3) compares the mean viability of DPSCs in the 0.01% MB group in comparison with the control group at different time points. At 8 hours, the mean viability of cells in the 0.01% MB group showed significantly higher cell viability compared to the control group. However no significant difference was found at 24 ($P > 0.05$), 48 ($P > 0.05$), or 72 ($P > 0.05$) hours.

Table 4 presents the mean viability of DPSCs in the 0.05% MB group compared to the control group. At 8 hours, the mean cell viability in the 0.05% MB group was significantly greater than that in the control group. No significant differences were found at 24 ($P > 0.05$), 48 ($P > 0.05$), or 72 ($P > 0.05$) hours.

ANOVA showed a significant difference in cell viability between the five groups at 8 hours ($P < 0.000$). Tukey's post-hoc test revealed significant differences between the control and 0.01 mg/mL MB ($P < 0.006$), control and 0.05 mg/mL MB ($P < 0.000$), 0.1 mg/mL TBO and 0.05 mg/mL MB ($P < 0.000$), and 0.5 mg/mL TBO and 0.05 mg/mL MB ($P < 0.005$) groups. No other significant differences were observed ($P > 0.05$).

At 24 hours, ANOVA did not detect any significant

Table 1. Mean Viability of DPSCs in 0.1 mg/mL TBO Group in Comparison With the Control Group at Different Time Points Using the Bonferroni Test

Time	Mean Viability in the Control Group	Mean Viability in 0.1 mg/mL TBO	Difference	95% CI	t	P Value
8 hours	100%	106.2%	6.175%	17.85% 5.49%	1.602	>0.05
24 hours	100%	91.4%	8.599%	3.075% 20.27%	2.231	>0.05
48 hours	100%	83.79%	16.21%	4.53% 27.88%	4.206	<0.001
72 hours	100%	85.06%	14.94%	3.27% 26.62%	3.877	<0.01

Table 2. Mean Viability of DPSCs in 0.5 mg/mL TBO Group in Comparison With the Control Group at Different Time Points Using the Bonferroni Test

Time	Mean Viability in the Control Group	Mean Viability in 0.5 mg/mL TBO	Difference	95% CI	t	P Value
8 hours	100%	110.70%	10.70%	-0.972%-22.38%	2.77	>0.05
24 hours	100%	94.85%	5.151%	-16.68%-6.52%	1.34	>0.05
48 hours	100%	85.73%	14.27%	-25.95%-(-2.6%)	3.7	<0.01
72 hours	100%	85.9%	14.1%	-25.77%-(-2.43%)	3.66	<0.01

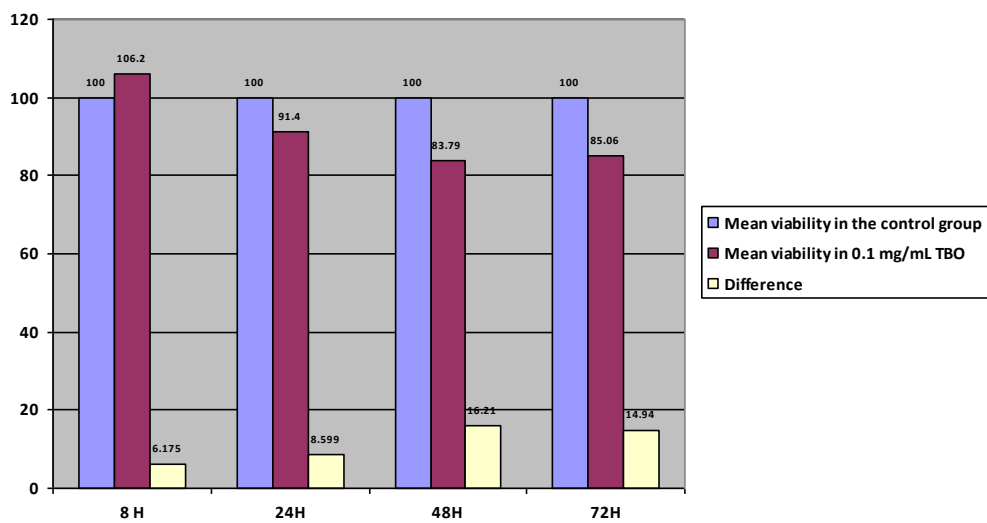


Figure 1. Mean Viability of DPSCs in 0.1 mg/mL TBO Group in Comparison With the Control Group at Different Time Points

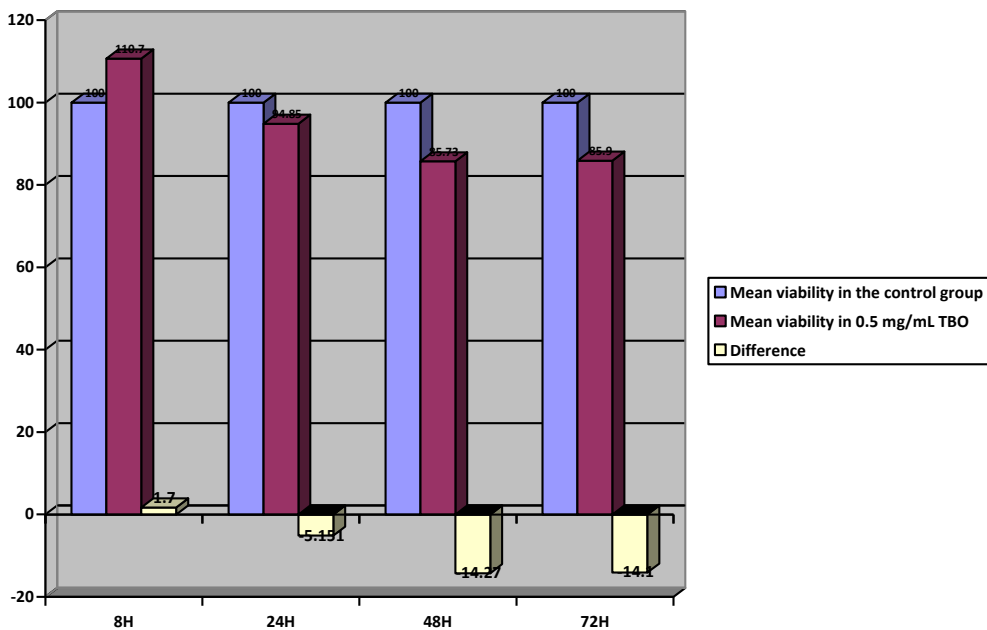


Figure 2. Mean Viability of DPSCs in 0.5 mg/mL TBO Group in Comparison With the Control Group at Different Time Points

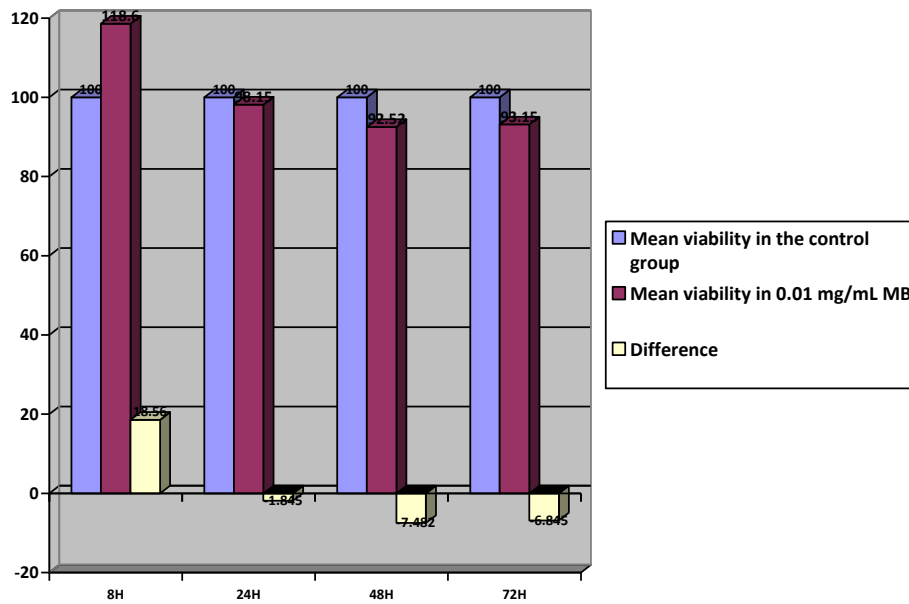


Figure 3. Mean Viability of DPSCs in 0.01 mg/mL MB Group in Comparison With the Control Group at Different Time Points

Table 3. Mean Viability of DPSCs in 0.01 mg/mL MB Group in Comparison With the Control Group At Different Time Points Using the Bonferroni Test

Time	Mean Viability in the Control Group	Mean Viability in 0.01 mg/mL MB	Difference	95% CI	t	P Value
8 hours	100%	118.6%	18.56%	6.882%-30.23%	4.81	<0.001
24 hours	100%	98.15%	1.845%	-13.52%-9.828%	0.478	>0.05
48 hours	100%	92.52	7.482%	-19.16%-4.192%	1.941	>0.05
72 hours	100%	93.15%	6.845%	-18.52%-(-4.82%)	1.776	>0.05

Table 4. Mean Viability of DPSCs in 0.05 mg/mL MB Group in Comparison With the Control group at Different Time Points Using the Bonferroni Test

Time	Mean Viability in the Control Group	Mean Viability in 0.05 mg/mL TBO	Difference	95% CI	t	P Value
8 hours	100%	129.7%	29.68%	-18.0%-41.35%	7.7	<0.05
24 hours	100%	101.4%	1.399%	-10.27%-13.07%	0.363	>0.05
48 hours	100%	101.9%	1.887%	-9.787%-3.56%	0.489	>0.05
72 hours	100%	94.41%	-5.592%	-17.27%-6.08%	1.451	>0.05

differences in cell viability between the groups ($P=0.07$). At 48 hours, a significant difference was found in cell viability between the groups ($P<0.000$). Pairwise comparisons using Tukey's test indicated significant differences between the control and 0.1 mg/mL TBO ($P<0.000$), control and 0.5 mg/mL TBO ($P<0.000$), 0.1 mg/mL TBO and 0.05 mg/mL MB ($P<0.000$), 0.5 mg/mL TBO and 0.05 mg/mL MB ($P<0.000$), and 0.01 mg/mL TBO and 0.05 mg/mL MB ($P=0.04$) groups. No other significant differences were noted ($P>0.05$).

At 72 hours, the results of ANOVA again revealed a significant difference in cell viability between the groups ($P<0.001$). Tukey's test showed significant differences between the control group and the 0.1 mg/mL TBO group ($P<0.003$), and between the control group and the 0.5 mg/mL TBO group ($P<0.005$). There were no other differences ($P>0.05$).

Discussion

PDT can eliminate the residual bacteria from the root canal system.^{9,13,17-19} However, it should not have any detrimental effect on the host cells. This study aimed to assess the effects of PDT on the viability of DPSCs.

The results showed higher cell viability in all experimental groups compared with the control group at 8 hours, which is due to the fact that at 8 hours, the effects of TBO and MB have yet to be exerted, and it was the pure effect of LED irradiation that increased cell proliferation in comparison to the control group. This increase in viability was significant in both MB groups. The increase in TBO groups did not reach statistical significance. Over time (from 8 to 72 hours), cell viability decreased in all experimental groups. At 24 hours, cell viability was comparable between the experimental groups. However, at 48 and 72 hours, cell viability in TBO groups was lower than that in the control group, while the MB

groups showed no significant difference from the control. This finding may be related to the low concentration of photosensitizers used in this study.

Kofler et al¹⁹ studied the impact of PDT with MB and diode laser on head and neck cancer cell line. They showed that the combined use of MB and diode laser decreased cell viability by 5% compared to controls. When MB was applied alone for 4 minutes, it yielded 46% cell viability compared with the control group. Their results were different from the present findings since in the current study, MB significantly increased the cell viability at 8 hours compared with the control group. Cell viability decreased over time in MB groups compared with the control group but with no significant difference. Variations in the results of the two studies may be due to variations in the cell types and concentrations of photosensitizers. Frame et al²⁰ reported that PDT decreased the viability of stem-like cells and more differentiated cells in the culture of primary prostate epithelial cells, and it induced their necrosis and autophagy. Paschoal et al²¹ compared PDT with the diode laser and LED and photosensitizer alone and found no reduction in the number of viable bacteria in any group. Their results were somehow in line with the present findings despite different methodologies. Diniz et al¹⁵ explored the effect of antimicrobial PDT on human DPSCs and reported a reduction in cell viability in the use of 0.05 mg/mL and 0.025 mg/mL concentrations of MB. They concluded that MB in 0.0125 mg/mL concentration is probably safe for application in dental restorative treatments; however, PDT with concentrations of MB > 0.025 mg/mL along with the irradiation of red laser light may adversely affect the viability of DPSCs. Xu et al evaluated the synergistic effects of MB and red light on human gingival fibroblasts and human osteoblasts and showed that PDT had insignificant effects on cells at 24 hours. They concluded that PDT is a safe modality and can inactivate endodontic pathogens with no adverse effect on the host cells.¹⁶ Antimicrobial agents may affect the stem cells in two ways: leakage into the apical papilla and affecting the stem cells at this region, and exposure of residual stem cells in the canal to the antimicrobial agents.^{22,23}

Some previous researchers defined optimal conditions for PDT in terms of safety and efficacy.²⁴ A previous study showed that incubation time of up to 10 minutes, low concentration of the photosensitizer, and energy density and power of light < 50 mW/cm² and 5 J/cm² resulted in the eradication of microorganisms with no adverse effect on human fibroblasts and keratinocytes.²⁴ Another study showed that PDT with water-soluble MB at 10-100 µM concentration eliminated 36% of fibroblasts and 100% of *Enterococcus faecalis* (after 20 minutes of incubation following exposure to red light with 36 J power).²⁵ The same results were reported for human periodontal ligament cells.²⁶

PDT can also induce oxidative stress and apoptosis.²⁷ Xu et al¹⁶ reported apoptosis in mammal cells after PDT. However, another study used the Comet kit and found no DNA damage in keratinocytes following incubation with MB and exposure to visible light.²⁸ However, the same kit used in another study showed DNA damage in human myeloid leukemia cells after PDT with MB.²⁹ Some others reported mitochondrial-dependent apoptosis after PDT with MB.³⁰ Sturmeijer et al³¹ showed that use of MB along with white light caused dose- and time-dependent DNA damage in vitro, and 30% of the cell population underwent apoptosis in response to treatment.

Zafari et al³² investigated the impact of MB, LED, TAP and DAP on DPSCs. Their Study found that an LED alone did not significantly affect cell viability. However, MB-induced cell Death occurred in a concentration-dependent manner. The concentration of 1 µg /mL and 10 µg /mL MB showed no significant effect on cell viability, and thus, the researchers selected 10 µg /mL MB for further experiments as the highest noncytotoxic concentration. They also observed that all tested concentrations of TAP and DAP (1, 1.5, 2, 2.5 and 5 mg/mL) led to the death of DPSCs ($P < 0.001$).

Several factors such as the light parameters and type and concentration of photosensitizers are involved in the efficacy and safety of PDT.^{33,34} The maximum absorption wavelength of a photosensitizer should match the light wavelength to generate reactive oxygen species, which are responsible for the eradication of bacteria.^{35,36} The maximum absorption wavelengths of MB and TBO are 656 and 625 nm, respectively.³⁶ The selection of MB and TBO and their respective concentrations in this study was based on the findings of a previous study that showed no cytotoxicity of these concentrations for the host cells.¹⁶

The cytotoxicity of MB and its derivatives against tumor cells in mammals have been previously confirmed in such a way that a cytotoxicity of 7.9% has been reported for MB at a concentration of 18.7 M/L.³⁷ The cytotoxic effects of MB on human brain tumor cells have also been documented, indicating its potential for therapeutic applications.³⁸ Similarly, Kofler et al¹⁹ demonstrated the cytotoxic effects of MB alone and combined use of 660 nm laser and MB and MB alone on head and neck cancer cells.

The diode laser is commonly used for PDT due to its cost-effectiveness, easy handling, and simple application.³⁹ Also, it can be used in body cavities.⁴⁰ Evidence shows the higher antibacterial efficacy of lower laser powers compared with higher powers.^{40,41} Thus, a minimal radiation dose is often used for photo-activation to benefit from both the antimicrobial and photobiological effects of lasers (such as pain relief and acceleration of wound healing).^{40,42}

It should be noted that this study had an in vitro design, and since a number of confounders are present in the

clinical setting which cannot be simulated in vitro, the results should be interpreted with caution. Clinical studies are required to further scrutinize this topic.

Conclusion

PDT with MB at the tested concentrations increased the viability of DPSCs in the short-term (8 hours) and had no adverse effect on the viability of DPSCs in the long-term (48 and 72 hours).

PDT with TBO at the tested concentrations had no significant difference in cell viability between the TBO group and the control group at 8 and 24 hours. However, at 48 and 72 hours, the viability of TBO group was significantly lower than that of the control group.

Authors' Contribution

Conceptualization: Shiva Shojaeian.

Data curation: Arash Heidari.

Formal analysis: Arash Heidari.

Funding acquisition: Arash Heidari.

Investigation: Arash Heidari.

Methodology: Shiva Shojaeian.

Project administration: Shiva Shojaeian.

Resources: Mohamad Asnaashari.

Software: Mahsa Sadeghi.

Supervision: Mohamad Asnaashari.

Validation: Mohamad Asnaashari.

Visualization: Mohamad Asnaashari.

Writing—original draft: Mahsa Sadeghi, Pegah Mehrabinia.

Writing—review & editing: Mahsa Sadeghi.

Competing Interests

None declared.

Ethical Approval

Ethics approval code has been obtained from the university's ethics committee (IR.SBMU.DRC.REC.1398.171).

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