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The effect of CRISPR constructs microinjection on the expression of developmental genes in Rag1 knocked-out mice embryo

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Abstract

Despite all the advances in the production of transgenic mice, the production efficiency of these animal models is still low. Given that the expression of developmental genes has a critical role in growth and development of embryo, we determined the expression pattern of pluripotency, trophectoderm and imprinting genes in the Rag1 (recombination-activating gene 1) knocked-out blastocysts resulting from microinjection of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) constructs into the zygote cytoplasm of C57bl6 mice. Following microinjection, the embryos were cultured and the gene expression of developed blastocysts and natural blastocysts (Sham and control groups) were evaluated using real-time PCR. The agarose gel to confirm the deletion in the *Rag1* gene in *Rag1* knocked-out blastocyst. Our results showed that the expression of trophectoderm genes (-*TEAD-4* and *Cdx2*), pluripotency genes (*Nanog* and *Oct-4*) and imprinting gene (*H19*) in the *Rag1* knocked-out group was significantly lower compared with the embryos obtained from Natural fertilization. According to these findings, manipulation, embryo culture and microinjection of CRISPR constructs into the zygote cytoplasm of mice led to reduced expression of imprinting, pluripotency and trophectoderm genes. Therefore, the *Rag1* knocked-out embryos produced by the CRISPR/Cas9 system are of low quality, which reduces the chances of live birth in these animals and may cause various abnormalities in fetuses.

KEYWORDS
CRISPR constructs, developmental genes, microinjection, *Rag1* knocked-out embryos

1 | INTRODUCTION

Modern medicine has made it possible to study a variety of diseases using transgenic animal models. The application of such animals is growing due to need for the reliable models that closely mimic the pathophysiology of a certain disease (Volobueva et al., 2019). Despite favourable results, the production of these animal models is facing with serious challenges that constrain their widespread use (Thornton, 2010). Until now, efforts are being made to find a profitable approach in order to increase their production scale. However, lack of useful information regarding the effect of gene manipulation on the expression of genes involved in embryo development seems to be one of the important issues that must be addressed.

In order for an embryo to develop normally, a set of evolutionary genes including trophectoderm, pluripotency and imprinting genes needs to be expressed in a coordinated manner. The expression of *Oct4* and *Nanog*, as the pluripotency markers in ICM (inner cell mass) and later in epiblast, takes a pivotal role in normal development of embryo. Moreover, *Cdx2* and *Tead4* are the main genes involved in implantation as well as trophectoderm and placenta formation (Nishioka et al., 2008). It should also be noted that, in mammals, imprinting genes including nearly 50 genes that play a critical role in development of embryo and its outer tissues. Impaired expression of such genes is associated with decreased growth of embryo. Furthermore, the relationship between impaired expression of imprinting genes and different genetic disorders such as Prader Willi syndrome, Angelman syndrome, Wilms' tumour and Autism has been well documented (Bartolomei & Ferguson-Smith, 2011). In this context, the role of trophectoderm, pluripotency and imprinting genes is more highlighted, so that any changes in their expression lead to sever fetal growth retardation and various disorders (Piedrahita, 2011; Joy, 2014). Until now, different studies have confirmed the effect of embryo manipulation on the expression of developmental genes (Giritharan et al., 2007; Rathjen, 2014).

It is obvious that any significant changes in the expression of these genes profoundly affect the efficacy of live birth rate in the animal embryos.

Today, newly developed gene-editing technology has been successfully applied to manipulate various cells and organism's genome. Despite the fact that new technologies in the field of gene editing and production of transgenic animal models have been promising in recent years, it seems these methods profoundly affect the expression of genes involved in embryo development including imprinting, pluripotency and trophectoderm genes.

In recent years, CRISPR/Cas9 system as one of the powerful, target-specific, inexpensive and user-friendly techniques (Bosch et al., 2004; Capecchi, 1989; Cibelli et al., 1998) has shown its superiority over the other gene editing methods such as Zink finger nuclease (ZFN) and Transcription activator-like effector nuclease (TALENs) (Solter, 2000). This set of ribonucleoproteins, which is made up of the Cas9 protein and a short crRNA sequence, binds to a target sequence on the basis of conventional base pairing and cuts the double-stranded DNA at a specific sequence. The short length of sgRNA in CRISPR/Cas9 prevents challenges that normally seen in gene transfer by viral vectors. Furthermore, problems with determining DNA sequencing and DNA recombination, as well as the disadvantages associated with long and repetitive TALEN expression vectors (usually 3 Kb or more) are not observed in the CRISPR/Cas9 method (Capecchi, 1989). However, the effect of this method on the expression of genes involved in embryo development is ambiguous. In the present study, we microinjected CRISPR/Cas9 constructs into the zygote cytoplasm of C57bl6 mice in order to produce *Rag1* knocked-out embryo. Later, we evaluated the expression of imprinting (*H19*, *Igf2*), pluripotency (*Oct4*, *Nanog*) and trophectoderm (*Tead4*, *Cdx2*) genes in the transfected embryos.

2 | MATERIALS AND METHOD

2.1 | Materials

The study procedures were confirmed by the Research. Ethics Committee of Shahid Beheshti University Medical of Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1395.5.1). All components of the CRISPR ribonucleoprotein including Cas9 protein and guide RNAs (CrRNA and TracrRNA) were purchased from Integrated DNA Technologies company (Coralville, Iowa, USA). All chemical materials were purchased from Sigma-Aldrich (USA), unless otherwise mentioned. The C57BL/6 mice were obtained from the Pasteur institute of Iran and acclimated in the animal house for few days before the study begun.

2.2 | Experimental groups

In this study, the experimental groups included three groups as follows:

1. –90 Blastocysts obtained from natural mating (the sham group).
2. –86 Blastocysts derived from cultured zygote (zygote obtained from natural mating) as the control group.
3. –93 *Rag1* knocked-out blastocysts derived from microinjection of CRISPR/Cas9 into the cytoplasm of mice zygotes (the test group).

2.3 | Blastocysts preparation procedure

1. **Preparation of sham's** group blastocysts: Initially, 10 units of PMSG (pregnant mare's serum gonadotropin) was injected intraperitoneally into the 12 C57 bl6 female mice (8 weeks) to induce the ovarian follicles growth. After 48 hr, 10 units of hCG (human chorionic gonadotropin) hormone was injected in order to induce ovulation. The female mice then were caged with males for mating. After 14 hr, the mating was confirmed by presence of the vaginal plugs. The female mice with vaginal plugs were selected and sacrificed by cervical dislocation
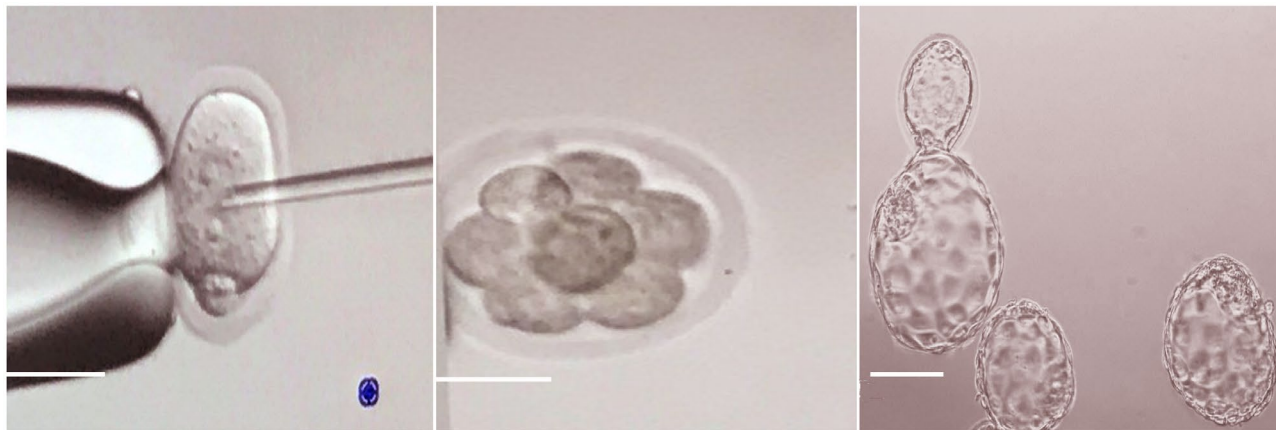
FIGURE 1 Test's group blastocysts, in order from left to right: Injection of buffer containing CRISPR/Cas9 constructs to zygote, 8-Cell (72h) in Blastocysts (96h) formation. Scale bar: 50μm

at 3.5 days post-mating. To obtain blastocysts, the uterine tubes were washed out with M2 medium using insulin syringe and obtained blastocysts were transferred to KSOM^{AA} (Potassium simplex optimization medium Amino Acid) medium (Salimi et al., 2020).

2. **Preparation of control's group blastocysts:** All the hormone therapies were carried out similar to the sham group's procedure. Following mating confirmation, the 12 C57bl6 female mice (8 weeks) with vaginal plugs were sacrificed 12 hr post-mating. Then, the zygotes were removed from the fallopian tubes by a syringe and incubated in KSOM^{AA} medium for 4–5 days, till blastocysts formed (Salimi et al., 2020).

3. **Preparation of Test's group blastocysts:** In vitro transcribed Cas9 mRNA (50–100 ng/μl) and gRNA (10–50 ng/μl) are used for cytoplasmic injection of zygotes. The hormone therapies were performed for 12 C57bl6 female mice (8 weeks) as described in control and sham groups. Several flat M2 drops (10 μl) and then Cas9/gRNA drops (1 μl) were placed on diss. Following zygote collection, 20–30 zygotes was transferred into the M2 and 4–6 pl of injection buffer containing CRISPR/Cas9 constructs was injected into the zygotes by injection pipet (2.5 μm). Injected zygotes were then cultured in KSOM^{AA} medium and incubated for 4–5 days at 37 ℃ to form blastocysts (Horii & Hatada, 2017) (Figure 1).

2.4 | DNA extraction from microinjected zygotes and PCR reaction

Following formation of blastocysts from CRISPR/Cas9 microinjected zygotes, DNA extraction from the developed blastocysts was carried out to confirm the *Rag1* deletion. DNA was isolated from the samples using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The Nested PCR method was used to amplify the gene fragments due to the fact that the concentration of DNA samples extracted from individual blastocytes was very low (DNA extracted from about 60 cells). In

TABLE 1 The sequences of primers used for amplification of the large segment of *Rag1* gene

Primer's name	Sequence (5' to 3')
Rag1-primer F	GAAGAAGCACAGAAGGAGAAG
Rag1-primer R	ATCGGCAAGAGGGACAATAGC

TABLE 2 The sequences of primers used for amplification of the small segment of Rag1 gene

Primer's name	Sequence (5' to 3')
Rag1-Nested-F	GCCGGGAGGCCTGTGGAG
Rag1-Nested-R	CCGTCGGGTGGATGGAGTCAA

the first step, a set of primes (Table 1) was used to amplify the larger segment of *Rag1*. Following conditions were exerted for the first amplification process: 3 min at 95 ℃ followed by 39 cycles including 15 s at 95 ℃, 15 s at 58 ℃, 45 s at 72 ℃ and 5 min at 72. ℃. For the next step, the products obtained from previous step were used as template to amplify the smaller segment of *Rag1* gene. The primers used in the step were shown in the Table 2.

2.5 | Real time PCR

2.5.1 | RNA extraction and cDNA synthesis

The real-time PCR method was carried out to determine the expression rates of *TEAD4*, *Cdx2*, *Nanog*, *Oct-4*, *Igf2* and *H19* genes. To aim this, total RNA was extracted from a single blastocyst using RNA extraction kite (Life Technologies, Gent, Belgium) according to the manufacturer's instructions. The concentration of extracted RNA was determined with spectrophotometer (Pico drop Real-Life) and total RNA suspended in 10 μl of DEPC water was stored at −80 ℃. Later, cDNA was synthesized based on random hexamer method using Prime Script Quanti Tect Kit (Qiagen, Hilden, Germany)

according to the manufacturer's instruction and the following thermocycling condition: 2 min at 42 °C, 15 min at 42°C followed by 3 min at 95 °C. Finally, the synthesized cDNA was stored at 20°C.

2.5.2 | Gene expression evaluation

The real-time PCR was performed on cDNA samples by Rotor-Gene Q instrument (Qiagen, Hilden, Germany) using SYBR Premix Ex TaqII reagent (Takara Bio, Kusatsu, Shiga, Japan) to determine the expression levels of the aforementioned genes. The reactions were carried out under the following conditions: initial denaturation at 95°C for 30 followed by 50 cycle including denaturation at 95°C for 5 s, 60°C for 30 s as annealing/extension and 60 to 95°C with a ramp rate of 0.3°C/s as melting curve. In this study, *H2afz* and *GAPDH* genes were used as the house-keeping genes to normalize the relative expression levels of the target genes. The specific primer sequences used for real-time PCR are listed in Table 3.

2.6 | Statistical analysis

The data obtained from the real-time PCR were analysed by REST software. The $p \leq .05$ was considered to be statistically significant.

3 | RESULTS

3.1 | *Rag1* deletion confirmation

The isolated DNA was PCR amplified using specific primers. The PCR products were visualized on the agarose gel to confirm the deletion in the *Rag1* gene. Our results showed that the RAG1 knocked-out

model was successfully created using CRISPR/Cas9 constructs (Figure 2).

3.2 | The expression level of pluripotency genes

According to the real-time PCR results, the expression levels of *Nanog* and *Oct-4* genes in the *Rag1* knocked-out blastocysts were significantly lower than that in the sham and control groups (Figure 3).

3.3 | The expression level of trophectoderm genes

Our results showed that microinjection of CRISPR/Cas9 constructs into the zygotes profoundly influenced the expression rate of *Cdx2* and *TEAD-4* genes in the microinjected zygotes-derived blastocysts, so that they exhibited lower expression rate compared with the other groups (Figure 4).

3.4 | The expression level of imprinting genes

The expression levels of *H19* gene in the *Rag1* 1 knocked-out blastocysts were remarkably lower than that in the sham and control groups, whereas no significant differences were observed in *Igf2* gene expression in the all experimental groups (Figure 5).

4 | DISCUSSION

Currently, there are several types of animal model with immune system deficiency. Transgenic models with genetic defects are also

TABLE 3 The list of primer sequences

| Genes name	Primer sequences (5′–3′)	Tm
GAPDH	Forward: TTCCAGTATGATTCCACCCAC Reverse: ACTCAGCACCAGCATCACC	55.9 55.7
H2afz	Forward: CTCGTCTCTTCCTCGCTCGT Reverse: CGTCCGTGGCTGGTTGTC	61.3 61.04
Nanog	Forward: CTGAGGAGGAGGAGAACAAGGTC Reverse: CATCTGCTGGAGGCTGAGGTA	58 60
Oct–4	Forward: CGTGTGAGGTGGAGTCTGGA Reverse: GCTGATTGGCGATGTGAGTG	58.7 59.4
Igf2	F: TGTGAGCAAGCGACGGAGT R: GGATTCAGTGGCTGGCAGA	58.3 58.6
H19	F: TGAAGGCGAGGATGACAGGT R: TCCAGAGAGCAGCAGAGAAGTG	58.9 60
TEAD–4	F: CGGAGGAAGGCAAGATGTATG R: ACCTGGATGTGGCTGGAGAC	55.9 55.7
Cdx2	F: GCTGCTGTAGGCGGAATGTAT R: CTCCCGACTTCCCTTCACC	57.9 57
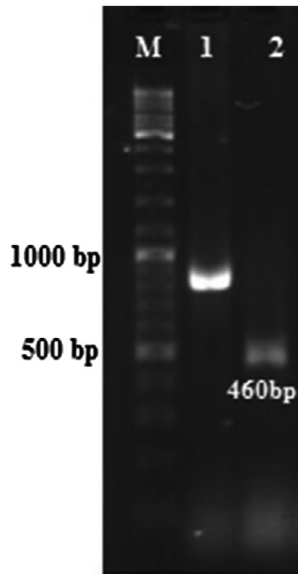
FIGURE 2 The gel electrophoresis results. The line 1 shows the intact *Rag1* amplicon in the control group. The *Rag1* knocked-out blastocysts was confirmed based on observation of a 460 bp fragment on the agarose gel (Line 2)
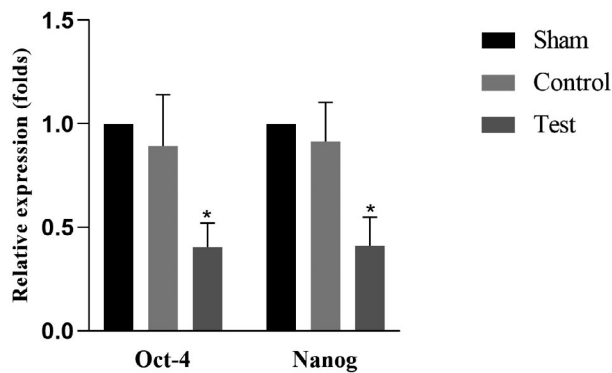

FIGURE 3 The comparison of pluripotency genes expressions in the all experimental groups. The gene expression folds in the sham group were considered as 1. Sham: Blastocysts obtained from natural mating, Control: Blastocysts derived from cultured zygote, Test: blastocysts derived from CRISPR/Cas9 microinjected zygotes, *: $p \leq .05$
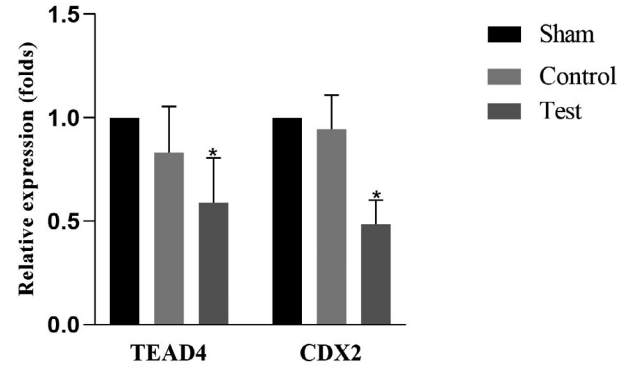

FIGURE 4 The comparison of trophectoderm genes expressions in the all experimental groups. The gene expression folds in the sham group were considered as 1. Sham: Blastocysts obtained from natural mating, Control: Blastocysts derived from cultured zygote, Test: blastocysts derived from CRISPR/Cas9 microinjected zygotes, *: $p \leq .05$
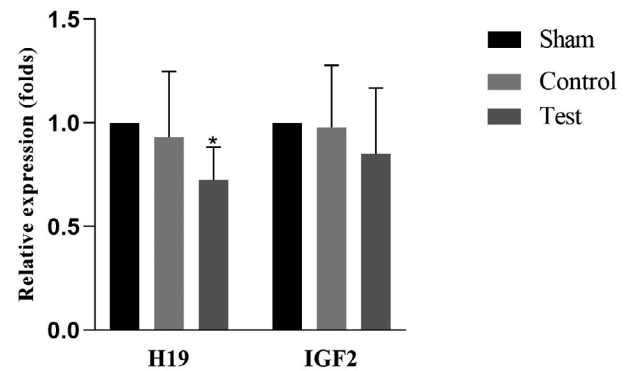

FIGURE 5 The comparison of imprinting genes expressions in the all experimental groups. The gene expression folds in the sham group were considered as 1. Sham: Blastocysts obtained from natural mating, Control: Blastocysts derived from cultured zygote, Test: blastocysts derived from CRISPR/Cas9 microinjected zygotes, *: $p \leq .05$

available for genetic and immunological studies. Mice models with immune deficiency are categorized into three groups: 1 – mice with single mutation such as nude and SCID (severe combined immunodeficiency) mice models; 2 – Rag1 knock-out models and 3 – hybrid mice models with two or three mutations that suffer from innate and adaptive immune system defects (Belizário, 2009). Today, new gene-editing technologies are used to produce such animals. ZFN and TALENs are two of the most widely used techniques, which have been successfully applied to produce a wide range of animal models from Drosophila to pigs (Thomas et al, 2013). Despite promising outcomes, their design is challenging and requires protein engineering for each target sequence.

For many years, CRISPR was only known as a specific repeat element in the genome of prokaryotic organisms (Patrick et al, 2014). Later, with confirming the role of CRISPR in bacterial immunity, the great potential of this system in genome editing was well demonstrated. Need for two '3-GG-'5 nucleotides in the protospacer adjacent motif (PAM) sequence, a sequence located in the downstream of the target region in the genome, makes the Cas9-based genome editing more specific than the other genome-editing technologies (Xuebing et al,2014). Until now, CRISPR/Cas9 system has been used to produce different mice models. Although the rate of transfection and technical efficacy in the CRISPR/Cas9 system is higher than methods such as ZFN and TALEN, some studies reported the low live birth rate in this manner, indicating presence of genetic disorders in animals produced by this technology (Sato et al., 2015).

In the animal models with *Rag1/2* deletions, the gene rearrangement of B- and T-cell receptors does not occur. Moreover,

these cells remain at an undifferentiated stage, and as a result, these animals fail to produce B and T cells. Recently, CRISPR/Cas9 system has been successfully applied to develop *Rag1/2* mice models (Fugmann, 2001). However, there is a need to gather information regarding the effect of this method on the expression of developmental genes in the *Rag1/2* knocked-out mice models. Our results showed that the genome editing with CRISPR/Cas9 system profoundly decreased the expression of genes involved in embryo's growth and development. In line with these findings, the manipulation and embryo culture have been reported to influence the gene expression in the ICM and trophectoderm (TE) cells (Chen et al., 2015; Giritharan et al., 2007). Parameters such as duration of cell manipulation, embryo culture and microinjection of CRISPR/Cas9 constructs lead to abnormalities at the genomic level and reduce the chance of live birth in the microinjected embryos.

Nanog and *Oct-4* are two of the most important transcription factors that play the main role in pluripotency maintenance of embryonic cells (Guilai & Ying 2010). Chen et al. reported that knocking down of these transcription factors significantly promoted the cell growth arrest and apoptosis in embryonic cells (Chen et al., 2012). These findings confirm the critical role of pluripotency genes in embryo development. In our study, the expression of *Nanog* and *Oct-4* genes in the embryos derived from transfected zygotes was significantly lower than those in the sham and control groups. Until now, there is no clear reason why microinjection influence the gene expression in the transfected cells. However, it seems that the effect of laser on zona pellucida during microinjection as well as increased stress following embryo culture alter the epigenetic patterns of developmental genes in the *Rag1* knocked-out mice models.

The attachment of embryo to the endometrial epithelium is a highly complicated and synchronized process that initiate with formation of polar trophectoderm from the outer part of the morula (John et al, 2017). In the light, various transcription factors are involved in differentiation of trophectoderm layer. *Cdx2* and its upstream regulator *TEAD-4* are the key transcription factors in the process. The decreased expression of *Cdx2* has been identified as the main reason of developmental block at 4–8 cell stages in the human IVF embryos (Wuwen et al, 2018). It implies the critical role of trophectoderm genes in embryo development and homing. In the present study, the expression rates of *Cdx2* and *TEAD-4* were lower in the group receiving the CRISPR/Cas9 constructs than the other groups. Our findings showed that the microinjection of CRISPR/Cas9 constructs in zygote cytoplasm may decrease the live birth efficiency in the mice models through hindering the proper formation of trophectoderm layer. However, more investigations are needed to clarify the other possible mechanisms in this context.

Imprinting genes are among the most vital developmental genes that show only maternal or paternal expression pattern. In this light, any disruption, elimination and decrease in the expression of such genes remarkably affect the growth and development

of embryos (Marisa et al, 2011). Dean et al. (1998) found that the embryonic stem cell–derived fetus showed an altered pattern of methylation in *Igf2r*, *H19*, *Igf2* and *U2af1-rs1* genes (Wendy et al, 1998). Our results were in line with those obtained from the Dean's study where the transfected blastocysts exhibited the lower expression rate of *H19* gene compared with the sham and control groups. In contrary, no differences were observed in the expression rate of *Igf2* gene in the all experimental groups. Whether microinjection of CRISPR/Cas9 constructs may selectively alter specific imprinting genes requires further investigations. It is also suggested that the epigenetic status of these embryos be investigated in future studies to assess the reason for the expression pattern of mentioned genes.

5 | CONCLUSION

Our study confirmed the negative effect of CRISPR/Cas9 construct's microinjection on the expression of developmental genes in the mice embryos.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTION
Maryam Salimi: Investigation; Project administration; Writing-original draft; Writing-review & editing. **Abolfazl Shirazi:** Conceptualization; Investigation; Project administration; Supervision; Writing-review & editing. **Koushan Sineh Sepehr:** Data curation; Formal analysis; Methodology; Software. **Mohsen Norouzian:** Funding acquisition; Project administration; Writing-review & editing. **Vahid Ebrahimi:** Formal analysis; Software; Validation. **Maryam Mehravar:** Methodology; Project administration; Visualization. **Mohammad Majidi:** Methodology; Project administration; Software; Writing-review & editing. **Mohammad Mehdi Mehrazar:** Data curation; Formal analysis; Software.

ETHICAL STATEMENTS
The study procedures were confirmed by the Research Ethics Committee of Shahid Beheshti University medical of Sciences, Tehran, Iran (IR.SBMU.MSP.REC.1395.5.1).

PEER REVIEW
The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.380.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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