Polarization of T-helper 2 to 1 phenotype has arisen in rat asthmatic pulmonary tissue after intra-tracheal administration of bone marrow-derived c-Kit+ cells

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Abstract

Introduction: This study investigated the paracrine therapeutic effects of intra-tracheal administration of bone marrow-derived c-Kit+ and c-Kit- cells on the T helper (Th)1/Th2 balance in ovalbumin-induced acute asthma in male rats.

Methods: Forty male Wistar rats were randomly allocated into four experimental groups; healthy (group C) and sensitized (group S) rats received PBS (Phosphate-buffered saline); sensitized rats received PBS containing c-Kit (group S + c-Kit-) and c-Kit+ cells (group S + c-Kit+). Total and percentages of differential leukocytes were calculated in bronchoalveolar Lavage. The lung cellular contents of interleukin (IL)-4, IL-10, and interferon gamma (IFN-γ) mRNAs were measured quantitatively. Moreover, the existence of excessive collagen deposition in pulmonary interstitial space was evaluated through Masson's trichrome staining.

Results: The results showed the successful homing of c-Kit+ cells into the asthmatic niche. The significantly increased total number of leukocyte, eosinophil, neutrophil, and IL-4 mRNA levels, as well as decreased lymphocyte count, IL-10, IFN-γ, and IL-4/IFN-γ ratio, were observed in asthmatic rats compared to C group (P<0.001). C-Kit+ cells, but not c-Kit- cells, had the potential to participate in these changes (P<0.001 to P<0.05). The deposition of collagen fibers in the asthmatic pulmonary tissue decreased after administration of both c-Kit+ and c-Kit- cells, which were more prominent in the S + c-Kit+ group.

Conclusions: The results of the current experiment highlighted the therapeutic capacity of c-Kit+ cells in the alleviation of asthmatic changes at the cellular level.

Introduction

Asthma is a progressive and destructive condition of the pulmonary system, accounting for a high rate of human deaths annually with a heavy socio-economic burden.1,2 It is thought that asthma per se is related to type2 T helpers (Th2) malfunction in response to airborne pollutants, leading to airway hypersensitivity, reversible bronchoalveolar constriction, and aberrant tissue remodeling.3,4 The current treatment for asthma, including inhaled β2-agonists and glucocorticoids, are not definite therapeutic choice and symptoms return when the treatment discontinued.5

Given the apparent activity of Th2 cells in the promotion of asthmatic pathologies, novel approaches are highly required to switch the dynamic of these cells toward inflammation suppression by targeting the decrease of Th2/Th1 levels, which is in accordance with any changes of IL-4/IFN-γ ratio.3,5 Within the therapeutic modalities, cell-based therapy is touted as one of the most promising alternatives to restore partially and/or complete damaged tissues to a state with normally function.6 In recent years, therapies based on stem cells originating from different tissues have received guarantee in terms of various pathologies treatment, particularly in the resistance form.7 However, the most efficacious subset of stem cells is still unknown for lung repair.7

The among the systems used for stem cells isolation, it seems that enrichment of stem cells based on cell surface 

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c-Kit marker show significant regenerative potential even under various insults in different in vivo settings and contributes to achieve the therapeutic outcomes, especially in ischemic cardiac disease. Loffredo and colleagues in a study found that enriched BM-derived c-kit+ cells, but not mesenchymal stem cells (MSCs), improved cardiac function following the regeneration of infarct regions and the increase of endogenous cardiac progenitors. C-kit is known as a type III tyrosine kinase receptor commonly utilized as a preeminent identifier of BM-derived and tissue stem cells. This receptor is expressed in several populations of BM-derived cells, including MSCs, hematopoietic stem cells, and endothelial progenitor cells.

To our knowledge, there are few experiments related to the application of c-Kit-positive cells in asthma. In line with this issue, a pressing need exists to unveil the therapeutic effect of c-kit-expressing BM-derived stem cells in asthmatic rats. It is well-established that there is a large number of c-Kit+ cells isolated from bone marrow due to the existence of a unique microenvironment, while these cells are accounted for approximately 1–4% of total marrow cells. An inherent potency to dictate the inflammatory responses via engaging both paracrine and juxtacrine interaction make these cells eligible as a novel therapeutic cell source. Commensurate with these findings, we aimed to investigate the paracrine therapeutic effects of intra-tracheal administration of bone marrow-derived c-Kit+ cells on the Th1/Th2 balance in ovalbumin-induced acute asthma in male rats.

**Materials and Methods**

**Experimental groups**

Fifty male Wistar rats (weighing 200-250 g, approximately 8–10 weeks old) were enrolled in this study. The rats were maintained at 20–22°C, with relative humidity of 50 ± 10% in a regular light-dark interval. Rats were allowed to access a standard chow diet and tap water ad libitum. After a two-week inhabitation period, 10 rats were blindly sacrificed for bone marrow content collection to enrich c-Kit+ cells. The remaining animals were randomly allocated into four experimental groups (each in 10 rats) as follows: healthy rats received intratracheally 50 µL PBS (group C); sensitized rats received intratracheally 50 µL PBS (group S); sensitized rats received intratracheally 50 µL PBS containing $3 \times 10^5$ c-kit negative cells (group S + c-kit-); and sensitized rats received intratracheally 50 µL PBS containing $3 \times 10^5$ c-kit positive cells (group S + c-kit+).

**Induction of acute asthma in rats**

According to our previously published protocol, the sensitization procedure was fulfilled over a period of 32 ± 1 days. Briefly, to induce sensitization, each animal was received intraperitoneally 1 mg ovalbumin (OVA, Sigma-Aldrich, USA) and 200 mg aluminum hydroxide dissolved in 1 mL normal saline on days 1 and 8. During the distinct time, from days 14 to 32 ± 1 (for 18 ± 1 consecutive days), rats were challenged with 4% aerosolized OVA forming by a nebulizer (CX3, Omron Co., Netherland) for 5 min daily in a whole-body inhalation exposure chamber with dimensions $30 \times 20 \times 20 \text{ cm}^3$. In non-treated rats, normal saline solution was administrated using the same protocol. One day after the last OVA challenge (on day 33), either PBS or c-kit negative and positive cells were directly given into the trachea via a small ventral neck incision. All animals were euthanized two weeks after treatment for the assessment of therapeutic impact (Figure 1).

**c-Kit+ cells enrichment by magnetic-activated cell sorting (MACS) technique**

In order to achieve high c-Kit+ cell purity, normal rats were sacrificed humanely by standard protocols (n = 10). In this study, we aimed to obtain bone marrow c-kit+ cells from the femur. The bone marrow content was collected after cutting the extremities by pushing via PBS containing 2% fetal bovine serum (FBS; Gibco, USA). Thereafter, mononuclear cells (MNCs) were isolated from each sample using Ficoll (Sigma-Aldrich, USA) density gradient method. Cells were centrifuged at 400 g for 20 minutes and gently collected at the interphase followed by PBS wash. MNCs from all rats were pooled and incubated with 1% FBS at 4 °C for 30 min to inhibit the non-specific binding of antibodies. Then, a mouse-anti human c-Kit microbead (Miltenyi Biotec, Germany) was added to cell suspension according to the manufacturer’s recommendation. After the completion of antibody incubation, cells were passed through the LS column (Miltenyi Biotec, Germany) system. Both c-Kit positive and negative cells were separated and used in different analyses.

**C-kit+ cells characterization**

To affirm the efficiency of the MACS procedure, the isolated cells were analyzed via flow cytometry to determine the percentage of the c-kit-positive cells. After MACS enrichment, positively and negatively selected cells were suspended in solution containing mouse-anti human CD117 (c-kit+) and kept at 4 °C for 30 minutes. The samples were computerized by BD FACSCalibur and raw data were analyzed with the Flow jo software (version 7.6.1).

**Cells tracking after transplantation**

To determine the exact location of transplant cells after administration, both c-kit+ and c-kit- cells were exposed to 20µM CellTracker™ CM-Dil at 37 °C for 40 minutes.
followed by PBS wash. Aliquots of 50 µL PBS containing 3 × 10⁵ c-Kit+ and c-Kit− cells were prepared. To visualize the labeled cells, either c-Kit+ or c-Kit− cells, pulmonary samples were taken at the end of the experimental period, and snap-frozen in OCT solution (Cat no: 4583; Scigen; USA). Then, we prepared a 5-µm thick section of samples and stained them with DAPI solution (dilution: 1 µg/mL; Sigma-Aldrich).

### Bronchoalveolar lavage fluid (BALF) preparation

Following tracheal removal, BALF was carefully collected using five consecutive 1 mL instillations of normal saline through a catheter connected to the rest of each trachea. An equal volume of BAL fluid and Turk solution (1:1 ratio) was used to stain leukocytes, and total numbers were calculated by a Neubauer slide. The rest of the lung fluid was centrifuged at 2500 g for 10 minutes at 4 °C and then the supernatant was removed. A thin smear of collected cells was prepared and stained with Wright-Giemsa stain. Finally, the percentage of differential leukocytes was determined using a light microscope according to the cellular morphological features.

### Quantitative real-time PCR assay

The cellular contents of IL-4, IL-10, and IFN-γ were measured quantitatively by assessing the real-time PCR method. The lobes of left lung were also collected and stored in liquid nitrogen. Total RNA was extracted using a total RNA extraction mini kit (YTA, Iran) according to the instructions of the supplier and then quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, 19810 USA). Then, the total extracted RNA was transformed into cDNA using a SYBR Green master mix (YTA, Iran). Real-time PCR was also performed on a Corbett Rotor-Gene 3000 instrument (Corbett Life Science, Australia). Validated housekeeping gene, GAPDH, was used to normalize the mRNA values. Finally, the results were analyzed with the 2^−ΔΔCt method. All primers designed for the current experiments have been outlined in Table 1.

### Histopathological examination

Right pulmonary lobes were sampled and placed in a 10% neutral buffered formalin solution for one week. Then, specimens were paraffin-embedded and slides were prepared by cutting into 4-µm thick longitudinal sections. We performed Masson’s trichrome staining to monitor the existence of excessive deposition of collagen type I in pulmonary interstitial space.

### Data analysis

All quantitative results were presented as mean ± SEM and analyzed using a one-way ANOVA with the Tukey–Kramer post hoc test. Statistical significance was set at P<0.05.

### Results

#### Immunophenotyping and characterization

We performed flow cytometry analysis to show the purity of enriched cells by MACS. Data showed a high rate (over 90%) of cells expressing the c-kit marker after MACS, indicating appropriate cell isolation prior to transplantation to the asthmatic niche (Figure 2).

#### Successful homing of transplanted cells into the asthmatic niche

IF staining revealed the red-colored Dil+ cells, either from c-kit negative or positive groups, in the pulmonary niche. Data confirmed that cells were successfully recruited to the pulmonary tissue after local intra-tracheal administration (Figure 3). Based on the data, the numbers of c-Kit negative cells in lung tissue were lower than c-Kit positive cells. We also found that c-Kit-positive cells were able to reside in the bronchial epithelium. In the asthmatic group, the intensity of DAPI-positive cells was high, indicating the recruitment of immune cells toward pulmonary tissue.

#### Local transplantation of c-kit+ cells diminished the elevated leukocyte count in asthmatic rats

To monitor whether the administration of c-kit positive cells could decrease the number of infiltrated immune cells to bronchoalveolar fluid, we calculated the number and type of total leukocytes. Based on the results, the

### Table 1. Forward and reverse primer sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Primer Names</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10:F</td>
<td>TGAGAATAAAAGCAAGCTGGG</td>
</tr>
<tr>
<td>IL-10:R</td>
<td>GTAGGCTTCTATGCAGTTGCA</td>
</tr>
<tr>
<td>IL-4:F</td>
<td>CTGTCACCCTGGTCTGCTTTCT</td>
</tr>
<tr>
<td>IL-4:R</td>
<td>CTGGTACAAACATCTCGGTGCA</td>
</tr>
<tr>
<td>IFN-G:F</td>
<td>ATGCATATGGAGGAAGAGGCC</td>
</tr>
<tr>
<td>IFN-G:R</td>
<td>TGTGGITACGTGCTGAAGAG</td>
</tr>
</tbody>
</table>

![Figure 2. Flow cytometry analysis of c-Kit cells after MACS enrichment. Data showed 91.45 ± 4.6% of cells were positive against the c-Kit marker, showing a high degree of purification after MACS](image-url)
mean total leukocyte counts were significantly increased in asthmatic pulmonary fluid compared to the rats of the group C, indicating a stimulated immune response in the asthmatic niche ($P<0.001$; Table 2). We found that intratracheal administration of c-kit positive cells had the potential to decrease the number of immune cells recalled to the asthmatic lungs as compared with asthmatic rats without any manipulations or received c-kit negative cells ($P<0.001$, Table 2). Cell differentiation analysis revealed an increased eosinophil and neutrophil number in lung lavage fluid during 32-day exposure to OVA ($P<0.001$ to $P<0.01$; Table 2).

Following local c-kit transplantation, the percentage of specific leukocytes number, either eosinophils or neutrophils, was closer to the normal values rather than other asthmatic groups ($P<0.01$ to $P<0.05$). Also, the administration of cells devoid of the c-kit marker was unable to exert modulatory effects on the types of leukocytes. Concomitant with the reduction of total leukocyte count in asthmatic rats ($P<0.001$ to $P<0.01$), we found an increased lymphocyte number in rats that received c-kit+cells ($P<0.01$; Table 2). Non-significant

Table 2. Total and differential percentages of leukocyte values in different groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>C</th>
<th>S</th>
<th>S + c-kit</th>
<th>S + c-kit*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC (number/ mm$^3$)</td>
<td>5745 ± 207</td>
<td>11650 ± 220***</td>
<td>11284 ± 192***</td>
<td>7875 ± 165******</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>3.5 ± 0.42</td>
<td>17.34 ± 1.1***</td>
<td>16.34 ± 1***</td>
<td>12.34 ± 4.4***</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>17.34 ± 0.6</td>
<td>25.5 ± 1.2***</td>
<td>25.17 ± 1.1***</td>
<td>21 ± 0.6***</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>69.5 ± 1.6</td>
<td>48.5 ± 1.7***</td>
<td>50 ± 1.4***</td>
<td>58 ± 1.9***</td>
</tr>
</tbody>
</table>

Note: Total and differential percentage of leukocyte counts in lung lavage fluid of C (control group), S (sensitized animals with ovalbumin), S + c-kit (sensitized animals received c-kit- cells), S + c-kit+ (sensitized animals received c-kit+ cells). Statistical differences between control and different groups: ***$P<0.001$ and ****$P<0.001$. Statistical differences between S + c-kit- and S + c-kit+ vs. S group: *$P<0.05$ and **$P<0.01$. Statistical differences between S + c-kit- and S + c-kit+ groups: $^+$*P<0.05, $^+$+$P<0.01 and $^+$+$+$P<0.001. Bars represent the mean ± SEM.

Figure 3. IF imaging of pulmonary tissue. IF imaging revealed the existence of c-Kit labeled cells inside the pulmonary niche shown by red colored appearance. In the c-Kit positive group, the cells showed the potency to locate on the bronchial epithelium and through the alveolar niche. The number of labeled c-Kit negative cells was low compared to the c-Kit positive cells. C (control group), S (sensitized animals with ovalbumin), S + c-kit (sensitized animals received c-kit- cells), S + c-kit+ (sensitized animals received c-kit+ cells)
differences were found in the number of lymphocytes between the asthmatic rats with c-kit. Commensurate with these descriptions, c-kit+ cells could be touted as an alternative cell source to govern excessive immunological reactions in the experimental model of acute asthma.

Levels of cytokines were changed in asthmatic lungs post-c-kit+ administration

According to data from a real-time PCR panel, transcription of IL-4 cytokine was increased after the OVA challenge followed by the down-regulation of IL-10 and IFN-γ (P<0.001; Figure 4a, b,d). In addition, the IFN-γ/IL-4 ratio was reduced (P<0.001; Figure 4c). For corroborating these data, the activity of type 1 T helper and T suppressor was also diminished following the onset of asthmatic modifications. In line with the changes in total leukocyte number, mRNA expression levels of IL-10, IFN-γ, and IFN-γ/IL-4 ratio were also modified after c-kit+ cell administration (P<0.001 to P<0.01; Figure 4a and 4d). As expected, these cells were able to suppress the transcription of IL-4 in the asthmatic milieu compared to the non-treated asthmatic rats (P<0.001; Figure 4b). No modulatory impact of c-kit- cells was observed in asthmatic rats (Figure 4a-d). These data demonstrated that the changes in the dynamic of inflammatory cytokines along with a shift from T lymphocyte subtypes could result in the orientation of inflammatory status after transplantation of c-kit+ cells.

Pathological findings were attenuated in the presence of c-Kit+ cells

Histopathological analysis revealed the substantial deposition of blue collagen fibers in the periphery of bronchioles after induction of asthma compared to the control samples (Figure 5). Based on these data, the deposition of collagen fibers in the asthmatic pulmonary tissue was decreased after intratracheal administration of c-Kit+ positive and negative cells. These changes were more prominent in asthmatic rats received c-Kit+ positive cells compared to the S + c-Kit- group. These data demonstrated that the administration of c-Kit+ positive cells could ameliorate the asthmatic damage by reducing the collagen level intensity.

Discussion

Considering several therapeutic strategies applied for asthmatic subjects, this chronic state is not completely curable. Despite of favorable outcomes in patients who undergone cell therapy, statistics reported some pitfalls and a lack of complete reconstitution in injured asthmatic tissues, mainly lungs and bronchoalveolar conduits. Therefore, an appropriate selection of cell types, cell numbers, and the route of administration is highly recommended to elevate therapeutic efficiency in asthmatic candidates. As previously mentioned in scientific literature, MSCs from different sources have been extensively applied in animal and human medicine to accelerate the regeneration of target tissues. Notably, the possibility of multiple side effects cannot be neglected in candidates receiving MSCs; for instance, uncontrolled aging, possible heterogeneity, and undesirable genetic alterations are common in MSCs isolated from the bone marrow and other tissues. To circumvent these limitations, many attempts have been exploited to introduce novel cell types with abilities to obviate these pitfalls. Among multiple progenitor cells identified in pulmonary niches, the critical role of c-kit+ cells is well-established in the pulmonary homeostasis and reconstruction either pathological or physiological conditions. To the best of our knowledge, there are

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Figure 4. Expression levels of IFN-γ (a), IL-4 (b), IFN-γ/IL-4 (c) and IL-10 (d) mRNAs in the lung tissues of C (control group), S (sensitized animals with ovalbumin), S + c-kit (sensitized animals received c-kit cells), S + c-kit+ (sensitized animals received c-kit+ cells). Statistical differences between control and different groups: ***P<0.001. Statistical differences between S + c-kit- and S + c-kit+ vs S group: ***P<0.001. Statistical differences between S + c-kit- and S + c-kit+ groups: $P<0.01$ and $$$P<0.001. Bars represent the mean ± SEM
numerous experiments either preclinical or clinical studies conducted to evaluate the c-kit+ cells in cardiovascular diseases. Of note, the number of cells harboring the c-kit marker is trivial in pulmonary tissue, while documents noted a large population of c-kit+ cells inside the bone marrow microenvironment. In addition, the isolation and expansion of c-kit+ cells from pulmonary sources encounter many ethical issues and invasive surgical manipulations. In line with these statements, the present study was conducted to assess the therapeutic benefits of bone marrow-derived c-kit positive cells administrated by the intra-tracheal route in alleviating asthmatic changes in the rat model.

Based on the results, we noted the successful recruitment of transplanted c-kit+ cells into the asthmatic niche. It was previously demonstrated that cells harboring surface c-kit markers could appropriately respond to chemokines and pro-inflammatory factors in a concentration-gradient manner. A similar function was also shown for the c-kit- cells. These data showed that cells from different lineages could appropriately respond to the inflammatory condition and be recalled to the injured sites. An increased number of total leukocytes in BAL simultaneously with promoted Th2/1 and eosinophil/lymphocyte ratio showing the efficiency of our protocol in the induction of acute asthma in rats exposed to OVA. Compared to c-kit- cells, we noted superior effects of c-kit+ cells in alleviating pulmonary inflammation by the reduction of immune cells migration into asthmatic niche. Moreover, the total number of eosinophils was diminished and the Th2/1 ratio was close to the control levels. To find the dynamic of the pre-and post-cell treatment, we also measured the local expression of cytokines such as IL-4, IL-10, and IFN-γ in the pulmonary milieu using real-time PCR assay. As previously described, these cytokines are associated with the regulation of asthma. The elevation of pulmonary IL-4 occurred in response to an increased Th2/1 ratio, contributing to excessive mucus secretion and allergic features. Based on our data, c-kit+ cells had the potential to increase the expression levels of IFN-γ which are correlated with the induction of T helper type 1. Although, we found an increase in transcription of IFN-γ in the c-kit+ group, these effects were slight when compared to the group received the c-kit- cells. Along with the dynamic changes of IFN-γ, the IL-10 expression increased concomitant with the decreased expression of IL-4. Besides, the therapeutic effects of c-kit+ cells likelihood be associated with the immuno-modulatory properties.

Non-significant results emphasized the alleviation of immune-related response in rats that received c-kit- cells. One reason would be that the c-kit- fraction consisted of both mature immune cell types and the direct subjecting of these cells to the asthmatic niche not only could not quench the asthmatic injury, but also exacerbate the immunological response. In addition, it was revealed that mature immune cells could be activated after exposure to the asthmatic niche. In line with these data, we also performed pathological examination of pulmonary tissue from different groups. The injection of c-kit+ cells into asthmatic lungs decreased chronic changes which coincided with the decrease of collagen deposition and

Figure 5. Masson's trichrome staining of pulmonary tissue. Masson's trichrome staining revealed the existence of blue collagen fiber around the bronchioles after the induction of asthmatic condition. The injection of c-Kit cells, mainly c-Kit-positive cells, decreased the collagen fiber deposition in asthmatic rats. The levels of collagen fibers were also decreased in asthmatic rats received c-Kit negative cells but these effects were less compared to the c-Kit positive group.
aberrant remodeling. It seems that the ability of c-kit+ cells to decrease pathological changes could be related to paracrine, juxtacrine, and trans-differentiating into epithelial-like cells.

Conclusion
In conclusion, the results from the current experiment highlighted the successful homing of c-kit+ cells into the asthmatic niche and their therapeutic capacity in the alleviation of asthmatic changes. It seems that their ability could be related to paracrine, juxtacrine, and trans-differentiating into epithelial-like cells.

Study limitation
The current experiment has some limitations. At first, we did not evaluate the involved intracellular singling pathways associated with the release and synthesis of anti-inflammatory factors. Therefore, further research is highly recommended, considering these properties to address the therapeutic potential of c-kit+ cells in the face of asthmatic injury.

Study Highlights

What is current knowledge?
- There are few experiments related to application of c-Kit positive cells in asthma. In line with this issue, a pressing need exists to unveil the therapeutic effect of c-kit-expressing BM-derived stem cells in asthmatic rats. An inherent potency to dictate the inflammatory responses via engaging both paracrine and juxtacrine interaction, make these cells eligible as a novel therapeutic cell source. Commensurate with these comments, we aimed to investigate the paracrine therapeutic effects of intra-tracheal administration of bone marrow derived c-Kit+ cells on the dynamic of Th1/Th2 balance in ovalbumin induced acute asthma in male rats.

What is new here?
- Based on the results, we demonstrated successful recruitment of transplanted c-kit cells into asthmatic niche. Compared to c-kit- cells, we noted superior effects of c-kit+ cells in diminishing pulmonary inflammation by the reduction of immune cells migration into asthmatic niche. Moreover, total number of eosinophils was diminished and Th2/1 ratio closed to the control levels. Based on our data, c-kit+ cells had potential to increase the expression of IFN-γ which is correlated with the induction of Th helper type 1. Along with the dynamic of IFN-γ, the IL-10 expression increased coincided with decreased expression of IL-4.

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Funding acquisition: Rana Keyhanmanesh.
Writing—original draft: Fatemeh Mir-ershadi.
Writing—review & editing: Mahdi Ahmadi, Reza Rabarghazri, Rana Keyhanmanesh.

Competing Interests
None declared.

Ethical Approval
All of the experiments were designed by the previously delivered guidelines (NIH Publication No. 85-23, revised 1996) and confirmed by the ethics committee of Tabriz University of Medical Sciences (No: IR.TBZMED.VCR.REC.1397.404).

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