Original Article

Characterization of Serum Concentration of CD8, CD4, Interfron-γ, and Interleukin-4 in 4T1 Tumor Inoculated Mouse Model

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ABSTRACT

Background: Malignancies in domesticated animals, including cats and dogs, present significant health challenges. The 4T1 tumor-inoculated mouse model is pivotal for studying tumor biology and therapeutic strategies.

Objectives: This study aimed to characterize immune system alterations in the 4T1 tumor-inoculated mouse model by assessing serum concentrations of CD4 and CD8 antigens, along with cytokines interferon (IFN)-γ and interleukin (IL)-4.

Methods: BALB/c female mice were subcutaneously inoculated with 4T1 cells. Serum levels of CD4, CD8, IFN-γ, and IL-4 were quantified using ELISA, and histopathological analysis was performed to confirm tumorgenesis.

Results: Tumor-bearing mice exhibited significantly elevated CD8 levels (mean: 7.2613 ng/mL) compared to healthy controls (mean: 1.9749 ng/mL), whereas CD4 levels were lower in tumor-bearing mice (mean: 2.7632 ng/mL) compared to controls (mean: 4.8677 ng/mL). IFN-γ levels were reduced (mean: 10.95238 vs 22.85714 pg/mL), while IL-4 levels were increased (mean: 27.76942 vs 10.85452 pg/mL).

Conclusion: The 4T1 tumor model induces significant immune alterations, highlighting its utility for studying tumor immune evasion mechanisms and advancing immunotherapy strategies.

Keywords: Animals, Cancer, Cytokines, Immune response, Mouse model

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Introduction

Cancer poses a significant health risk to both humans and animals, affecting various species with differing rates of occurrence and mortality. In the animal population, it is one of the primary causes of death, particularly in pets such as dogs and cats. For example, nearly 50% of dogs over the age of 10 are diagnosed with cancer, while roughly 32% of cats are affected (Boddy et al., 2020).

Early efforts in tumor therapy screening predominantly relied on murine tumor models, which played a key role in understanding tumor biology and evaluating treatment effectiveness. These models are typically involved in implanting cancer cells or tissues into mice to study tumor development and responses to therapy in a living organism. While limited by species differences, these pioneering models offered valuable insights and laid the groundwork for more sophisticated preclinical testing methods (Sanmamed et al., 2016).

Immunotherapy introduces a novel strategy in cancer treatment, setting itself apart from traditional therapies like chemotherapy and radiotherapy by using the body's immune system to target and destroy cancer cells through specific pathways. Animal models are vital in the research and testing of new treatments. They provide insight into the biological processes involved, allow for early-stage evaluation of treatment safety and effectiveness, and offer critical data that informs the design of clinical trials for human patients (Tan et al., 2020).

The 4T1 tumor model is well-suited for studying mammary cancer because it can be easily transplanted into the mammary gland, allowing primary tumors to form in anatomically relevant sites. This model closely replicates the spontaneous metastatic progression observed in breast cancer, including metastasis to draining lymph nodes and distant organs. Additionally, the primary tumor can be surgically removed, providing an opportunity to study metastatic disease under conditions that closely resemble clinical situations after tumor resection (Chen et al., 2007; Pulaski & Ostrand-Rosenberg, 2001).

Understanding the complex interplay between tumor microenvironments and therapeutic responses provides a platform for studying novel treatment strategies and evaluating therapeutic efficacy in a clinically relevant context (Snipstad et al., 2023).

In this study, we aim to characterize serum CD4 and CD8 antigens, as well as interferon (IFN)-γ and interleukin (IL)-4, in a 4T1 tumor-inoculated mouse model. This work will help monitor changes in the immune system of mice exposed to 4T1 cells, providing valuable insights into the immunomodulatory profile involving lymphocytes and cytokines. Despite numerous studies on the immunological profile of cancer-inoculated murine models, there remain contrasting opinions about cytokine alterations. Therefore, we designed this study to assess and analyze the data.

Material and Methods

4T1 cell culture

The 4T1 cells were obtained from the cell bank of the National Center of Genetic and Biological Resources of Iran, and cultured in RPMI medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. They were then incubated in an incubator with 5% CO_2 at 37 ˚C (Pulaski & Ostrand-Rosenberg, 2001).

Tumor cell harvesting

The culture medium was removed from the tissue culture plate. Then, 5 mL of serum-free medium was added to the plate, mixed, and the supernatant was discarded. A volume of 1.5 mL of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) solution was added to cover the surface of the plate, and it was incubated at room temperature for 2 minutes. Subsequently, 5 mL of serum-free medium was added to detach the trypsinized cells from the plate, and the cell suspension was transferred to a Falcon tube. The cells were centrifuged at 400 g for 4 minutes at room temperature. The supernatant was discarded, and the first step was repeated (Chen et al., 2007).

Tumor induction in BALB/C mice

In this study, 10 female BALB/c mice, approximately 4 weeks old, were used. The mice were housed in cages in a controlled facility with a room temperature of about 21 ˚C, and they were fed ad libitum with a light/dark cycle of 12 hours. The animal study was conducted in Dr Rastegar's Laboratory at the Faculty of Veterinary Medicine, University of Tehran.

Group 1 (G1) (n=5) consisted of healthy mice that did not receive any tumor inoculation. Group 2 (G2) (n=5), on the other hand, was inoculated with 1×10^6 4T1 cells (Kryzwiec et al., 2021) subcutaneously into the right flank (Zhang et al., 2018) of the mice. Cell counting of

the harvested cells from the culture medium was performed using a Neubauer hemocytometer (Pulaski & Ostrand-Rosenberg, 2001).

Sample collection

After 28 days, the mice were sedated by an intraperitoneal (IP) injection of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine, and then sacrificed through cervical dislocation (Steenbrugge et al., 2018). Blood samples were collected, and the implanted tumors were surgically removed, separated from the surrounding normal tissue, and prepared for histopathology.

Evaluation of serum concentrations of CD4 and CD8 antigens

The standard working solution was added to the first two columns: Each concentration of the solution was added in duplicate, to one well each, side by side (100 µL for each well). The serum samples were added to the other wells (100 µL for each well). The plate was covered with the sealer provided in the kit (CD8: Cat No. NBP2-78729, Novusbio, USA; CD4: Cat No. E2507MO, bt-laboratory, China). Incubation was done for 90 min at 37 ˚C C (Gheysari et al., 2002). Solutions were added to the bottom of the microELISA plate well, and touching the inside wall and causing foaming was avoided as much as possible. The liquid was removed from each well, and washing was not done. Immediately, 100 µL of biotinylated detection Ab working solution was added to each well. The plate sealer was used to cover the plate, and gentle mixing was done. Incubation was done for 1 h at 37 ˚C. The solution was aspirated or decanted from each well, and 350 µL of wash buffer was added to each well. Soaking was done for 1–2 min, and the solution was aspirated or decanted from each well and patted dry against clean absorbent paper. This wash step was repeated 3 times. A microplate washer was used in this step and other wash steps. 100 µL of HRP Conjugate working solution was added to each well. The plate sealer was used to cover the plate and incubated for 30 min at 37 ˚C. The solution was aspirated or decanted from each well, and the wash process was repeated five times as conducted in step 3. 90 µL of substrate reagent was added to each well. A new plate sealer was used to cover the plate and incubated for about 15 min at 37 ˚C. The plate was protected from light. Then, 50 µL of stop solution was added to each well. Adding the stop solution was done in the same order as the substrate solution. The optical density (OD value) of each well was determined at once with a microplate reader set to 450 nm.

IFN-γ evaluation

All reagents, standards, and serum samples were prepared according to the kit instructions and incubated at room temperature for 20 min. Next, 50 µL of the diluent solution from the kit (Cat No. MIF00, R&D, USA) was added to all wells. Then, 50 µL of the sample and standard were added to each well, and incubation was done for 2 h at room temperature. The plate was emptied and washed three times with 400 µL of the wash solution each time. Afterward, 100 µL of the IFN-γ conjugate solution was added to each well, and incubation was done for 2 h at room temperature. The plate was emptied and washed three times with 400 µL of the wash solution from the kit each time. Then, 100 µL of the substrate was added to each well, and incubation was done in the dark for 30 min at room temperature. Finally, 50 µL of the stop solution was added to each well. An ELISA reader (Vira-IRAN) was used to measure the optical density at 450 nm.

IL-4 evaluation

All reagents, standards, and samples were prepared according to the kit (Cat No. M4000B, R&D, USA) instructions. Incubation was done at room temperature for 20 minutes. Fifty microliters of the diluent solution were added to all wells. Then, 50 µL of the sample and standard were added to each well, and incubation was done for 1 h at room temperature. The plate was emptied and washed three times with 400 µL of the wash solution each time (Delirezh et al., 2016). One hundred microliters of the conjugated antibody solution was added to each well, and incubation was done for 2 h at room temperature. The plate was emptied and washed three times with 400 µL of the wash solution from the kit each time. Next, 100 µL of the substrate solution was added to each well, and incubation was done in the dark at 20-25 ˚C for 30 min. One hundred microliters of the stop solution was added to each well. An ELISA reader (Vira-IRAN) was used to measure the optical density at 450 nm.

Pathological assessment

The collected segments were fixed in 10% neutral buffered formalin for at least 24 h. Tissue preparation was then performed using an automatic tissue processor device (DS2080/H; Did Sabz Co.), and paraffin-embedded tissue blocks were prepared using a paraffin dispenser (DS 4 LM; Did Sabz Co.). After that, 4–6 µm thick tissue sections (Rotary Microtome, DS 8402; Did Sabz Co.) were stained with the routine Harris hematoxylin-eosin (H&E) stain. The stained sections were later examined under a light microscope (Olympus CX21), and photos were taken with a camera (KECAM; UCMOS10000KPA).

Data analysis

To analyze the data, following the assessment of normality using the Shapiro-Wilk test, an independent t test was employed. The results were reported at a significance level of 0.05.

Results

Histological evaluation

With tumor progression, ulceration of the skin, and palpable tissue mass formation at the site of 4T1 cell inoculation, significant proliferation of neoplastic cells, accompanied by coagulative necrosis and lymphocytic inflammation, was observed (Figure 1).

CD8+ levels analysis

The study also quantified CD8+ T-cell levels, as these cells are pivotal in mediating antitumor immune responses. The levels of CD8 were assessed in both healthy (G1) and 4T1 tumor-inoculated (G2) mice (Figure 2). The G1 group exhibited the following CD8 levels: 2.174825 ng/mL, 1.65035 ng/mL, 2.066434 ng/mL, 2.178322 ng/mL, and 1.800699 ng/mL, with a mean value of 1.9749 ng/mL. In contrast, the G2 group showed significantly higher CD8 levels: 7.398601 ng/mL, 7.073427 ng/mL, 6.807692 ng/mL, 7.604895 ng/mL, and 7.423077 ng/mL, with a mean value of 7.2613 ng/mL.

CD4+ levels analysis

The levels of CD4 were measured in both healthy (G1) and 4T1 tumor-inoculated (G2) mice. The healthy group (G1) exhibited CD4 levels of 5.281955 ng/mL, 4.137218 ng/mL, 5.281955 ng/mL, 4.701128 ng/mL, and 4.934211 ng/mL, with a mean value of 4.8677 ng/mL. In contrast, the 4T1 tumor group (G2) showed significantly lower CD4 levels of 2.531955 ng/mL, 3.383459 ng/mL, 2.362782 ng/mL, 2.637218 ng/mL, and 2.802632 ng/mL, with a mean value of 2.7632 ng/mL. This reduction in CD4 levels in the tumor-inoculated group suggests an immunosuppressive environment induced by the 4T1 tumor.

INF-γ analysis

The levels of INF-γ were assessed in both healthy (G1) and 4T1 tumor-inoculated (G2) mice. G1 group exhibited the following INF-γ levels: 22.85714 pg/mL, 20.33333 pg/mL, 25.52381 pg/mL, 17.83333 pg/mL, and 11.85714 pg/mL. In contrast, the G2 group showed lower INF-γ levels: 10.95238 pg/mL, 10.80952 pg/mL, 6.47619 pg/mL, 8.547619 pg/mL, and 13.16667 pg/mL. This finding suggests a significant reduction in INF-γ levels in the tumor-inoculated group compared to the healthy controls, indicating an immunosuppressive environment induced by the 4T1 tumor.

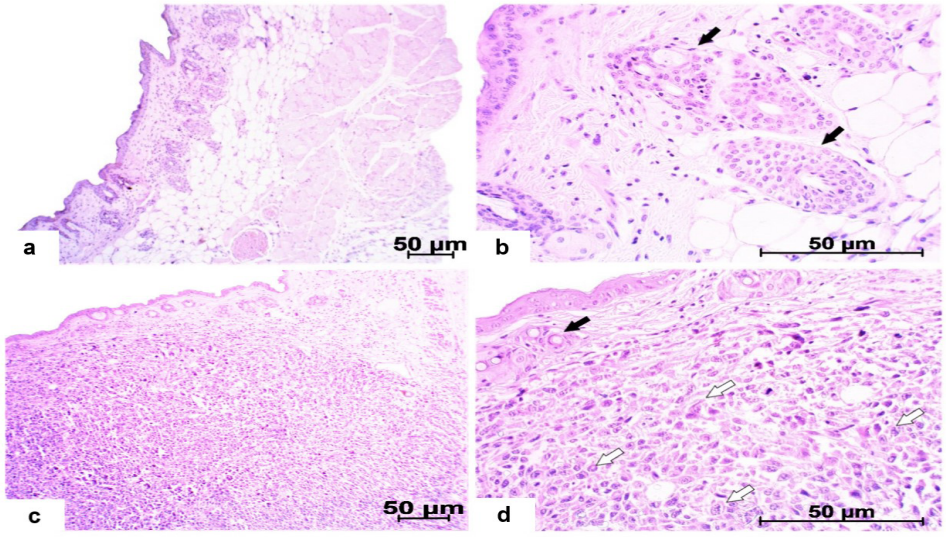

Figure 1. Histologic structure of skin tissue in mouse (H&E staining)

a) Normal histologic structure of skin in group I (×10 magnification), b) Normal histologic structure of skin with dermal adnexa, for instance hair follicles (black arrows) in group I (×40 magnification), c) Neoplastic development within dermis in group 2 (×10 magnification), d) Dermal adnexa atrophy (black arrow) due to neoplastic cell proliferation (white arrows) at the 4T1 inoculation site in group 2 (×40 magnification)

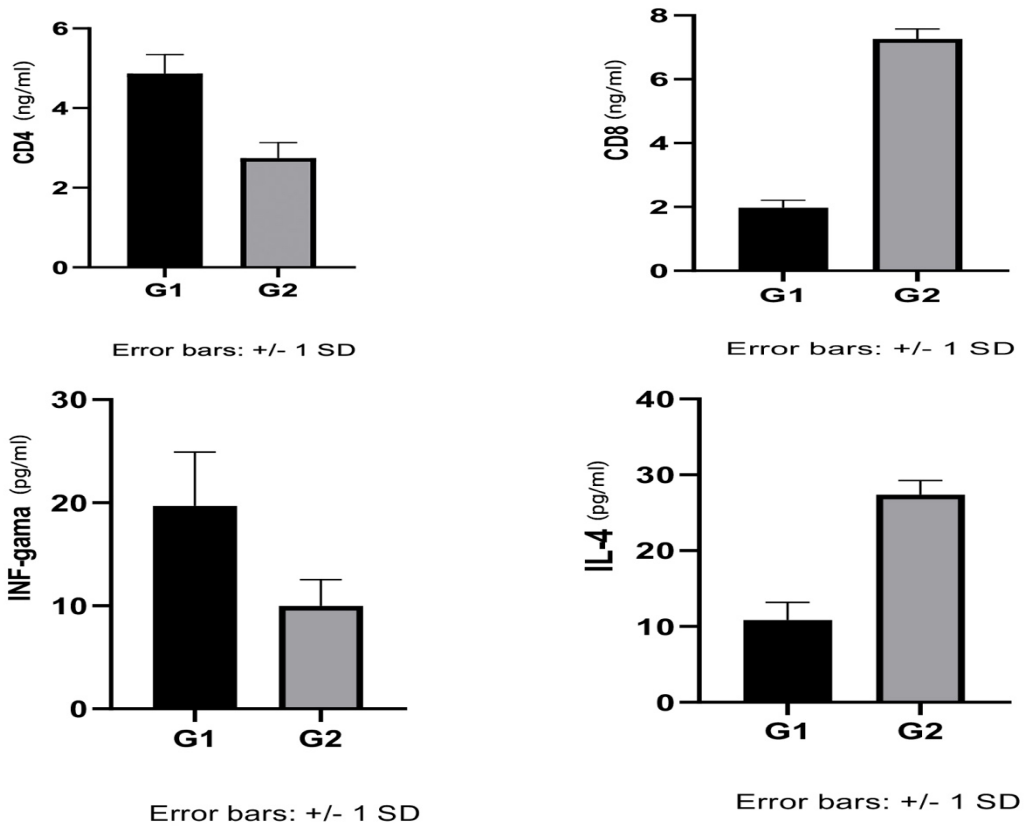

Figure 2. Comparing the mean levels of CD8, IL-4, CD4, INF-γ in rats in group G1 and group G2

IL-4 analysis

IL-4 levels were also measured in both groups. G1 group showed IL-4 levels of 14.1294 pg/mL, 9.91765 pg/mL, 9.41176 pg/mL, 8.43529 pg/mL, and 12.3765 pg/mL. Meanwhile, the G2 group exhibited significantly higher IL-4 levels: 26.8824 pg/mL, 28.2118 pg/mL, 24.3294 pg/mL, 29.0941 pg/mL, and 28.3294 pg/mL. The elevated IL-4 levels in the G2 group suggest an increase in Th2 immune response, which is associated with tumor progression and immune evasion. A comparison of the values revealed a significant difference between G1 and G2, with a $P<0.05$ (Figure 2).

Discussion

Clinical evidence suggests that the immune system plays a crucial role within the tumor microenvironment, a significant factor for the efficacy of targeted therapies such as immunotherapy (Snipstad et al., 2023; Tan et al., 2020). The data highlight the importance of understanding the tumor microenvironment and its influence on immune responses. The findings of this study are vital for the development and enhancement of immunotherapies, emphasizing the need for a detailed understanding of the interactions between tumors and the immune system to improve therapeutic outcomes.

Numerous studies have highlighted the importance of animal models in cancer research, particularly for understanding the tumor microenvironment and immune responses. These models provide valuable insights into how tumors interact with the immune system, including mechanisms of immune evasion and inflammatory responses (Dominguez-oliva et al., 2023; Zhang et al., 2011). In line with this, our study focused on characterizing serum CD4 and CD8 antigens, as well as cytokines like IFN-γ and IL-4, in the 4T1 tumor-inoculated mouse model, offering a more specific perspective on the immune system's alterations in response to mammary cancer. This approach not only enhances our knowledge of immune system dynamics but also lays the groundwork for the development of targeted immunotherapeutic strategies.

In one study, low doses of IFN-γ were shown to boost the effectiveness of cancer therapies and reduce the need for high-dose chemotherapy, which often has significant side effects. In our study, decreased IFN-γ levels were observed in the 4T1 tumor-inoculated mouse model, highlighting the role of IFN-γ in modulating the immune response to cancer. These findings suggest that targeting IFN-γ in cancer treatment can improve therapy outcomes and offer a promising approach to developing better cancer treatments (Jia et al., 2021).

Additionally, in one study, the MC38 colon cancer model demonstrated a robust immune response characterized by increased CD8+ T cell activity. In contrast, the 4T1 breast cancer model exhibited a weaker response with fewer active CD8+ T cells, indicating reduced responsiveness to anti-PD1 therapy. However, in our study, we observed a significant increase in CD8+ T cells within the 4T1 tumor model, highlighting the active involvement of these immune cells in the antitumor response. This finding suggests that, despite the inherent challenges, there is potential for enhancing the immune response in 4T1 tumors through targeted immunomodulatory strategies (Snipstad et al., 2023).

A study demonstrated that blocking IL-4 improves antitumor immunity and slows tumor progression (Eiito et al., 2017). Our findings similarly indicate elevated IL-4 levels in the G2 group, signifying a shift towards a Th2-dominated immune response. Th2 responses, correlated with humoral immunity, can inhibit CTL activity, which is critical for targeting tumor cells, and may potentially aid in tumor growth and metastasis. These results highlight the importance of understanding the tumor microenvironment and its impact on immune responses. Modulating the immune environment to enhance Th1 responses and reduce Th2 dominance could improve immunotherapy efficacy against malignancies.

Moreover, by targeting the specific immune alterations induced by 4T1 tumors, such as boosting INF-γ production and reducing IL-4-mediated Th2 dominance, we can potentially enhance the efficacy of immunotherapies in treating aggressive cancers like 4T1. The data emphasized the importance of selecting appropriate tumor models for preclinical studies and provided valuable insights into the mechanisms of variable responses to immunotherapy.

One study showed that IFN-γ has demonstrated potential as an antitumor agent by inducing apoptosis in cancer cells. Additionally, CD8+ T-cell motility and cytotoxicity were increased against target cells. However, initial clinical trials with recombinant IFN-γ showed limited success and significant side effects. These findings emphasize the need for more targeted approaches to use the therapeutic benefits of IFN-γ while minimizing adverse effects, potentially by focusing on its specific interactions with tumor cells (Jorgovanovic et al., 2020).

In another study, for the 4T1 model, differences in immune cell populations between treated and untreated tumors were only observed for subcutaneous tumors, where the percentage of T cells was significantly lower in the treated tumors. The amount of CD8+ T cells was also lower for the treated group. However, this difference was not significant (Snipstad et al., 2023).

The observed data showed that the presence of 4T1 tumors significantly affects the immune profile in mice. Specifically, the reduced levels of CD4 in the G2 group compared to the G1 group suggest an impaired immune response, as CD4+ T cells are crucial for coordinating the immune response against tumors. This immunosuppressive environment could be a mechanism by which the 4T1 tumor evades immune detection and destruction.

The changes in CD4 levels in the 4T1-inoculated group provide insights into potential therapeutic targets. Strategies to modulate the immune environment to enhance CD4+ T cell responses could improve the efficacy of immunotherapies in treating aggressive tumors like 4T1.

In one study, the 4T1 mouse cancer model treated with immune checkpoint inhibitor (ICI) antibodies showed nonuniform responses, similar to other models. Fine-needle aspiration was used to collect and analyze tumor samples, but unlike the P815 and CT26 models, specific correlations between tumor-infiltrating leukocytes and treatment success were less pronounced for 4T1. This condition highlights the complexity of the 4T1 tumor's immune environment and suggests that additional immunosuppressive factors may contribute to its resistance to ICI therapy (Almonte et al., 2023).

Evidence from the past decade highlights the immune system's dual role in recognizing and eliminating malignant cells while also promoting tumor progression, a process known as cancer immunoediting, which involves elimination, equilibrium, and escape phases. CD8+ T cells play a vital role in this process, particularly in tumors with adaptive immune resistance features, affecting survival outcomes through immune contexture, immunoscore, and tertiary lymphoid structures. Additionally, chronic inflammation fosters an immunosuppressive tumor microenvironment and tumor development, whereas acute inflam-

mation can enhance antitumor immunity. Understanding these dynamics can inform strategies to target immunosuppressive chronic inflammation and improve antitumor responses (Yang, 2015).

The study using the 4T1 mouse mammary carcinoma model found that immunotherapy with MHC class II and B7.1-transfected cells effectively reduced or eliminated spontaneous metastases but did not affect primary tumor growth. These results suggest that cell-based vaccines targeting CD4+ and CD8+ T cells may be effective for treating metastatic breast cancer, which remains resistant to conventional treatments despite the primary tumor being curable (Pulaski & Ostrand-Rosenberg, 2001).

Conclusion

This study was designed to enable the monitoring of immune system alterations in mice inoculated with 4T1 tumor cells by assessing CD4 and CD8 lymphocyte antigen concentrations, as well as cytokines (IL-4, IFN-γ). The observed impairment in Th1 function, along with an increase in Th2 function, affected the overall efficacy of the immune response. Understanding the interplay between cancer and the immune system is essential for addressing cancer immune evasion and developing effective immunotherapies.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the research Ethics Committee of the Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran (Code: IR.UT.VETMED.REC.1403.011).

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Authors' contributions

Conceptualization: Ghazale Vazifehdoust and Shahram Jamshidi; Methodology: Ghazale Vazifehdoust, Gholamreza Nikbakht, and Mahya Lalepoor; Supervision: Shahram Jamshidi; Data collection: Ghazale Vazifehdoust.

Conflict of interest

The authors declared no conflict of interest.

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