



Research Paper

Evaluation of the Effect of Newcastle Disease Virus on
Mouse Spleen Cells and Induction of Mouse T-helper
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ABSTRACT

Introduction: Newcastle disease (ND) is a highly lethal and contagious disease affecting a diverse range of avian species, caused by the Newcastle disease virus (NDV). This study aims to evaluate the cytotoxic effects of NDV on mouse spleen cells and to assess the activation of specific immune responses against NDV mediated by helper T cells.**Materials & Methods:** NDV isolates evaluated in this study were collected from broiler farms with disease symptoms in Alborz Province. NDV isolates were propagated by inoculation into the allantoic cavity of ten-day-old embryonated eggs. Varying concentrations of the virus were introduced to the leukocytes in the first and second groups, while the third group served as a control, receiving no NDV. Following incubation, the gene expression levels were quantified using reverse transcription polymerase chain reaction (RT-PCR), and the cytotoxicity of the virus toward the cells was assessed through the MTT assay.**Results:** The expression levels of the genes *GATA3*, *T-bet*, *IL-4*, and *IFN-γ* did not exhibit significant changes in the group of spleen cells exposed to lower viral concentrations. The evaluation of the cytotoxic effects of NDV on spleen cells revealed no cytotoxic impact in the first passage of the NDV treatment group; however, cytotoxic effects were noted during the second passage, although these results were not statistically significant ($P > 0.05$).**Conclusion:** NDV is capable of activating both cellular and humoral immune responses by stimulating the activation of type 1 and type 2 helper T lymphocytes. Moreover, it influences the survival and proliferation of leukocytes.

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1. Introduction

Newcastle disease (ND) is a highly contagious avian disease transmitted through the respiratory tract, causing substantial economic losses in the global poultry industry. Newcastle disease virus (NDV) strains are classified as velogenic, lentogenic, or mesogenic. In addition, NDV has beneficial clinical effects in cancer patients [1]. Studies suggest that NDV disrupts the innate immune response by degrading phospho-STAT1, MDA5, and MAVS via the viral V protein [2]. NDV has been shown to impact dendritic cells (DCs) during the early stage of infection, facilitating DC activation and the cross-priming of naïve T cells into tumor-specific T cells [3, 4]. A recent study has shown that infection with a velogenic strain of NDV activates the extrinsic apoptosis pathway in DCs, inhibiting CD4⁺T cell proliferation. As a result, the overall effects of viral infection on adaptive immunity remain insufficiently explored [5].

In a number of previous studies, mice have been used as an experimental model to investigate the effects of NDV on the immune system. Given that the spleen is the largest lymphoid organ in the body and plays a crucial role in mounting specific immune responses, and that NDV is capable of targeting lymphoid organs, including the spleen, the present study utilized mouse splenic leukocytes to examine the impact of NDV on immune responses. Infection with NDV elicits a robust innate immune response through DCs, yet the immunostimulatory capabilities of infected DCs that prime naïve T cells remain inadequately characterized [6]. The successful generation of an efficient adaptive immune response by T cells via DCs necessitates three signals: the antigen-specific T cell receptor (TCR)-peptide-major histocompatibility complex (MHC), co-stimulatory molecules, and cytokines that promote the polarization of cluster of differentiation 4-positive (CD4⁺) helper T (Th) cells [7]. NDV has the ability to activate a synergistic response involving mucosal, cellular, and humoral immunity while simultaneously neutralizing host innate immunity through the viral V protein, leading to apoptosis of DCs which impedes antigen presentation. Recent investigations have demonstrated that NDV infection results in the generation of phenotypically mature DCs; however, these cells exhibit dysfunction in stimulating T cell responses. Furthermore, the specific mechanisms underlying the interaction between NDV and DCs remain largely unexplored [8].

This study aimed to evaluate the cytotoxic effects of NDV on spleen cells and the induction of T-helper cell responses.

2. Material and Methods

NDV was isolated from an infected poultry farm in Alborz Province (Iran) in 2022 to evaluate the cytotoxic effects of the virus on spleen cells and the induction of T-helper cell responses.

2.1. Isolation of Newcastle disease virus

NDV was isolated from broiler farms in Alborz Province, and its pathotype was evaluated using the mean death time (MDT) and pathogenicity indices. During the fall of 2022, four-week-old broiler chickens from an industrial poultry farm exhibiting high mortality and suspected acute NDV infection were sampled. The Ross breed chickens were identified based on clinical signs, including lethargy, eye swelling, and severe greenish diarrhea, along with necropsy findings associated with NDV. The farms maintained high management standards, featuring adequate ventilation and the provision of necessary equipment, feed, and water. All birds were offered food and water ad libitum.

Samples were collected from the trachea and lungs of ten freshly euthanized four-week-old chickens under aseptic conditions and immediately transferred to the virology laboratory at the Faculty of Veterinary Medicine. The samples were dissected into approximately 2-mm fragments using sterile tools and ground in a sterile mortar with sterile sand and 10 mL of phosphate-buffered saline (PBS) at pH 7.4. This suspension was placed in sterile 15-mL falcon tubes, and antibiotics, penicillin (1,000 IU/mL) and streptomycin (10 mg/mL) were added. After centrifugation at 2,000 rpm for 10 minutes at 4 °C, the supernatants were filtered through a 0.22-μm syringe filter and transferred to sterile 2-mL microtubes. The samples were either used immediately or stored at -80 °C. One hundred microliters of the samples were inoculated into the allantoic cavity of ten 7- to 9-day-old embryonated eggs, with three control eggs injected with 100 μL of sterile PBS. The puncture in the eggshell was sealed with wax, and the eggs were incubated for five days in an incubator at 37 °C and 50-60% humidity. Daily viability assessments were conducted; embryos that died within 24 hours post-inoculation were removed due to non-specific causes. Eggs with embryo mortality after 24 hours were transferred to a refrigerator at 4 °C. Eggs with surviving embryos were also refrigerated overnight. Subsequently, allantoic fluid was collected from the refrigerated eggs, and the hemagglutination (HA) test was performed on the samples.

2.2. Detection of viral particles in the allantoic fluid of eggs by hemagglutination test

All strains of NDV possess the capability to agglutinate red blood cells of avian species. This phenomenon, known as HA, results from the binding of specific antigenic proteins, namely hemagglutinin and neuraminidase, located on the viral surface to their corresponding receptors on red blood cells. The interaction between the virus and the red blood cells leads to the aggregation or agglutination of the cells, which is referred to as HA.

The HA test was conducted to assess the presence of NDV in the allantoic fluid derived from embryonated eggs, following these procedural steps:

1. Fifty microliters of phosphate buffer were added to the wells of a 96-well plate with a U-shaped bottom.
2. Fifty microliters of allantoic fluid from the embryonated eggs were dispensed into the first well, and subsequent dilutions were prepared in a series, specifically at ratios of 1:2, 1:4, 1:8, and so forth.
3. Fifty microliters of 1% chicken red blood cells were introduced into each well.
4. The contents of the plate were mixed thoroughly, and the plate was subsequently incubated at 20 °C for a duration of 40 minutes.
5. The last dilution of the virus that elicited HA was recorded as one HA unit (1 HA).

2.3. Detection of Newcastle disease virus in allantoic fluid by reverse transcription polymerase chain reaction

A total of 100 µL of allantoic fluid was mixed with 1,000 µL of Trizol and centrifuged at 12,000×g for 15 minutes at 4 °C. The RNA-containing supernatant was transferred to an RNase-free microtube, followed by the addition of 1,000 µL of isopropanol. After a second centrifugation at 12,000×g for 10 minutes at 4 °C, 500 µL of 75% ethanol was added to the pellet, which was then centrifuged at 7,500×g for 5 minutes at 4 °C. The pellet was rehydrated with DEPC-treated water, and the extracted RNA was dissolved in 20 µL of RNase-free water and stored at -70 °C.

Complementary DNA (cDNA) synthesis using m-MULV reverse transcriptase, enzyme buffer, nucleotides, and oligo(dT) primers in a 0.2-mL DNase/RNase-free microtube. The reaction was thermocycled at 25 °C

for 5 minutes, 37 °C for 2 hours, and 85 °C for 5 minutes, followed by storage at -20 °C [9, 10].

Polymerase chain reaction (PCR) was performed with a master mix containing Taq DNA polymerase, buffer, nucleotides, MgCl₂, and primers specific to the *F* gene of NDV in a 25 µL volume. The PCR consisted of 40 cycles with three stages: denaturation at 95 °C for 1 minute, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute, during which the DNA polymerase synthesized a new DNA strand from the template [9, 10].

2.4. Assessment of NDV pathogenicity

Numerous strains of NDV exist, each exhibiting varying levels of pathogenicity. This disparity in pathogenicity is evident in the severity of the disease manifested in infected chickens and is utilized to classify the pathotypes of the virus. To evaluate the pathogenicity of NDV isolates, we employed the methodologies of assessing MDT and conducting the intracerebral pathogenicity index (ICPI) test.

2.5. Newcastle disease virus intracerebral pathogenicity test

For the intracerebral pathogenicity test (ICPI), fresh allantoic fluid was diluted at a ratio of 1:10 in sterile isotonic saline, without the addition of antibiotics. Using a sterile tuberculin syringe, 0.05 mL of the freshly diluted allantoic fluid was inoculated intracerebrally into the caudal brain regions of ten chicks, 24 to 40 hours old, obtained from a specific pathogen-free (SPF) flock. The chicks were monitored for clinical signs every 24 hours over a period of eight days. Control chicks, which did not receive inoculation with allantoic fluid, were housed separately from the experimental group.

2.6. Maintenance of laboratory mice

Ten female BALB/c mice, aged 6 to 8 weeks, were maintained in an animal facility with a 12-hour light-dark cycle and a temperature of 22 to 27 °C for three months. They had unrestricted access to a commercial pellet diet and water, with efforts made to minimize stress.

2.7. Measurement of gene expression levels and cytotoxic effect in leukocytes cultured in the presence of Newcastle disease virus

BALB/c mice were euthanized, and spleen cells were collected. Red blood cells were lysed, and the remaining cells were washed and cultured in RPMI1640 medium supplemented with 25 mM HEPES and streptomycin. Leukocytes were cultured in RPMI1640 medium con-

taining 10% fetal bovine serum, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. The cultures were divided into three groups: the first group received 2 HAU of virus, the second group received 8 HAU of virus, and the third group served as the control group with no viral addition. All cells were incubated at 37 °C in 90-95% humidity with 5% CO₂ for 48 hours. Following incubation, RNA was extracted using Trizol buffer with chloroform and isopropanol, and the quality was assessed via absorbance measurements at 230, 260, and 280 nm using a NanoDrop spectrophotometer. Residual DNA was removed using RNase-free DNase.

cDNA synthesis was conducted with M-MuLV reverse transcriptase, random hexamer primers, dNTPs, and RNA guard under specified temperature conditions. For real-time RT-PCR, a PCR master mix was prepared with Taq DNA polymerase, SYBR Green I, and specific primers for target genes, including *T-bet*, *GATA3*, interferon-gamma (*IFN-γ*) and interleukin-4 (*IL-4*), followed by a temperature cycling program in an Applied Biosystems StepOnePlus device. Melt curve analysis was performed post-PCR, and the data were analyzed using the ΔΔC method.

To evaluate the cytotoxic effects of NDV, BALB/c mice were euthanized, and spleen leukocytes were cultured in RPMI1640 medium supplemented with 25 mM HEPES and streptomycin. The treatment group was exposed to NDV, while the control group remained uninfected. The cytotoxicity was assessed via the MTT assay after 96 hours of incubation. The percentage of cytotoxicity was calculated using the Equation 1:

$$1. \text{Cytotoxicity (\%)} = [1 - (\text{Sample optical absorption} / \text{Control optical absorption})] \times 100$$

2.8. Data analysis

The SPSS statistical program version 22 (IBM Corp., Armonk, NY, USA) was used to perform statistical calculations. After entering the data into the SPSS software, they were analyzed using the t-test and ANOVA. The experiments were repeated at least three times, and the average of the results was used in the statistical calculations. The difference was considered significant at the P<0.05 level.

3. Results

Allantoic fluid was harvested from embryonated eggs five days post-inoculation. The results of the HA assay indicated HA of red blood cells in seven samples. A sum-

mary of the findings from the HA assay is presented in Table 1. Specifically, two samples exhibited an HA titer of 8, one sample displayed an HA titer of 16, and one sample showed an HA titer of 32.

We measured gene expression levels in T-helper cells cultured in the presence of NDV. Mouse spleen leukocytes were divided into three groups: one group exposed to NDV at a dose of 2 HA units (HAU), another group exposed to 8 HAU, and a control group cultured in the absence of NDV. All cultures were incubated for 48 hours at 37 °C, with 90-95% humidity and 5% CO₂. Gene expression levels of *T-bet*, *GATA3*, *IL-4*, and *IFN-γ* were quantified using real-time RT-PCR and analyzed with the ΔΔCT method. Results showed no significant changes in the 2 HAU-exposed group. In contrast, the 8 HAU-exposed group exhibited increases: *T-bet* by 2.49-fold, *GATA3* by 3.7-fold, *IFN-γ* by 2.74-fold, and *IL-4* by 4.53-fold (Figures 1 and 2).

The cytotoxic effects of NDV on mouse spleen cells were assessed following a defined culturing protocol. Spleen cells were incubated with 8 HAU of NDV at 37 °C, with 90-95% humidity and 5% CO₂, and subjected to two passages at four-day intervals. The MTT assay, a colorimetric method for evaluating cellular metabolic activity, was employed to measure cytotoxicity. Cells from both treatment and control groups were incubated for 96 hours, followed by the addition of 10 µL of MTT solution (5 mg/mL) and a subsequent 4-hour incubation. After adding 100 µL of solubilizing solution, absorbance was measured at 570 nm. In the first passage of the NDV treatment group, no significant cytotoxic effects were observed. In the second passage, cytotoxic effects were noted, but they were not statistically significant (P>0.05) (Figure 3).

4. Discussion

In this study, we investigated the effects of NDV on splenic leukocytes harvested from BALB/c mice. The leukocytes were cultured with the NDV strain to assess any potential cytotoxic effects over two passages. In the first passage, which lasted four days, we observed no significant cytotoxicity affecting the spleen cells. However, during the second passage, an increase in cytotoxic effects became apparent; although they were noted, these effects did not reach statistical significance. This observation prompts further investigation to clarify whether the noted changes in cell viability are a direct outcome of NDV-induced cell death or a result of NDV's impact on the proliferation of lymphocytes. Our findings suggest that NDV possesses the capability to engage both cel-

Table 1. HI test results

Sample	HI Titre
1	2 HA
2	-
3	-
4	4 HA
5	16 HA
6	32 HA
7	8 HA
8	8 HA
9	2 HA
10	-

lular and humoral immune responses, primarily through the priming of type 1 helper T (Th1) and type 2 helper T (Th2) lymphocytes. This versatility underscores the importance of understanding NDV's mechanisms of action within the immune system.

Cellular immunity represents a crucial component of specific or acquired immunity, primarily mediated by T lymphocytes. It plays a significant role in providing protection against natural NDV infections and contributes to viral clearance. T lymphocyte subsets, which include cytokine-secreting CD4⁺ T helper cells and CD8⁺ cy-

totoxic T lymphocytes, constitute the principal cells involved in the cellular immune response [9-11].

In contrast to humoral immunoassays, such as enzyme-linked immunosorbent assay (ELISA) or HI inhibition assays, cell-mediated immunoassays are more labor-intensive and necessitate more advanced methodologies. These assays encompass tests for interferon- γ induction by stimulated lymphocytes, as well as evaluations of cellular responses to antigen or mitogen challenges through proliferation, flow cytometry analysis of lymphocytes, and levels of cytotoxicity displayed by NDV-specific CD8⁺ T lymphocytes against virus-infected target cells.

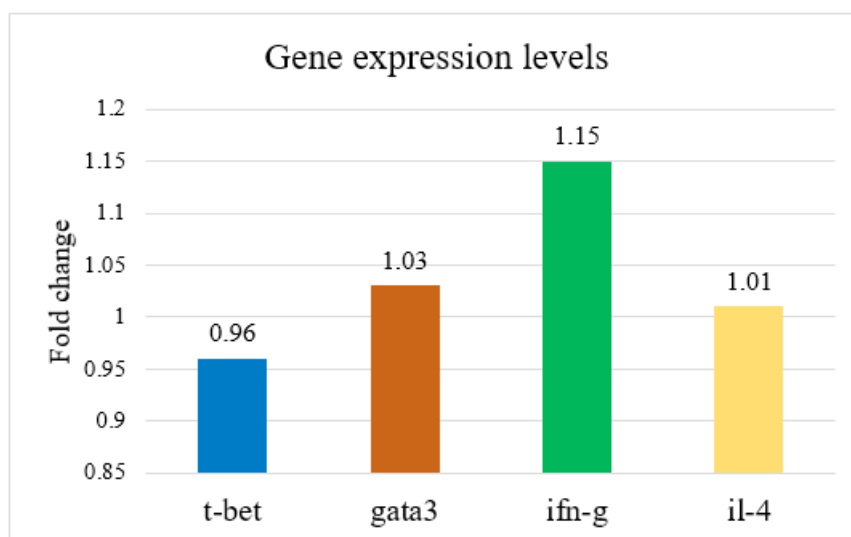


Figure 1. Expression levels of *T-bet*, *GATA3*, *IFN- γ* , and *IL-4* genes in spleen cells cultured in the presence of 2 HAU of NDV

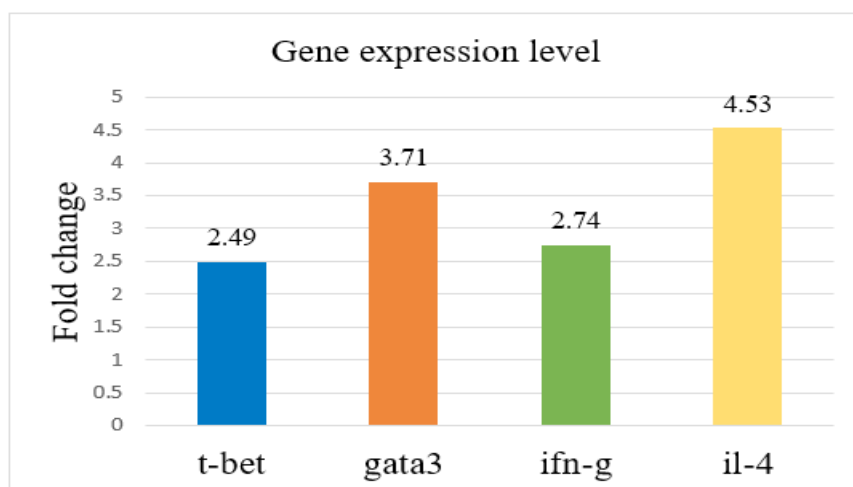


Figure 2. Expression levels of *T-bet*, *GATA3*, *IFN-γ*, and *IL-4* genes in spleen cells cultured in the presence of 8 HAU of NDV

Evidence of cellular immunity priming is detectable 2 to 3 days post-infection with NDV, particularly in turkeys [12]. Recent studies have further confirmed that cellular immune responses to NDV can be identified shortly after vaccination with a live NDV vaccine [13].

In these investigations, chickens demonstrating specific cellular immunity to NDV, as assessed by a blastocyst microassay with inactivated NDV, were not protected from lethal NDV challenges in the absence of HI-inhibiting antibodies. Conversely, birds possessing NDV-specific antibodies were observed to be protected. The findings indicate that antibodies generated against NDV serve as critical modulators of protection; however, cellular immunity likely aids in reducing viral shedding by selectively targeting and eliminating NDV-infected cells [14, 15].

Despite the increasing capability to measure cellular immune responses over recent years, there remains a scarcity of studies investigating the induction of cellular immunity by NDV. Type 1 helper T lymphocytes are responsible for producing and secreting various cytokines, particularly interferon- γ , which is essential for cellular immunity. In contrast, type 2 helper T lymphocytes produce and secrete interleukin-4, among other cytokines, which is pivotal for establishing humoral immunity mediated by antibodies [16-19].

In the study by Ginting et al. (2019), it was demonstrated that NDV strongly induces the production of antiviral type I interferons (IFN- α and IFN- β) and type III interferon (IFN- λ) in cell lines, whereas in normal cells, it predominantly induces type III interferon [21]. In the study by Xiang et al. (2018), NDV was capable of infecting and replicating in mature DCs derived from chick-

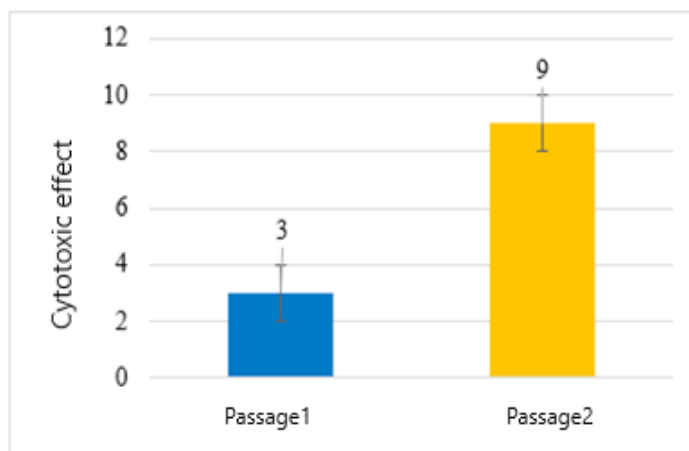


Figure 3. Results of evaluating the cytotoxic effect of NDV on mouse spleen cells

en bone marrow. Infection of chicken DCs with NDV was associated with alterations in the gene expression profile of these cells [6]. In the study by Zhang et al. (2019), higher NDV replication in chicken tissues correlated with a stronger immune response, characterized by increased expression of most Toll-like receptors, as well as certain avian defensins and cytokines [22]. Sun et al. (2019) showed that the NDV V protein inhibits host type I interferon production via the E3 ubiquitin ligase RNF5 [2]. In the study by Xu et al. (2020), recombinant NDV induced the maturation of immature DCs derived from human monocytes *ex vivo*, as evidenced by the upregulation of costimulatory molecules and antigen-presenting molecules, as well as the production of type I interferons [23].

NDV infection induces apoptosis in both tumor and normal cells, as demonstrated by the activation of caspase-3 and Annexin-V staining [3]. In our previous study, NDV increased the expression of *BAX*, *caspase-9*, and *caspase-3* genes in human cancer cells and enhanced the rate of cell death in these cells [8]. In the study by Yurchenko et al. (2019), passaging NDV in a human cell line led to increased viral cytotoxicity against human cells [24]. In NDV genotype VII (NDV-GVII), an emerging velogenic NDV strain, viral tropism has shifted from respiratory–enteric tropism to lymphotropism. This virus targets lymphoid organs, including the spleen and the bursa of Fabricius in birds, leading to severe lymphocytopenia. In the study by Rabie et al. (2021), gene expression profiling of the spleen in chickens inoculated with this virus revealed that activation of synaptogenesis, lymphotropism, and autophagy-mediated cell death pathways may contribute to the pathogenesis of this emerging virus [25].

In the study by Heicappell et al. (1986), postoperative injection of NDV-modified tumor cells in mice resulted in prevention of metastatic spread and increased long-term survival of mice [26]. In another study, recombinant NDV strains derived from the velogenic strain Italian induced syncytium formation and cell death as well as prolonged survival of tumor-bearing mice [2].

The results from this study revealed that NDV is capable of activating both cellular and humoral immunity by stimulating type 1 helper T and type 2 helper T lymphocytes. These results contrast with those reported by Tan et al., who observed that NDV infection in mouse DCs elicited stronger type 1 T helper responses (IFN- γ and IL-12), while type 2 T helper responses (IL-4 and IL-10) remained unchanged [5]. Nevertheless, our results align somewhat with those of Nan et al., who documented that

NDV-infected mouse DCs induced type 2 T helper responses. Importantly, our findings also demonstrate that type 1 T helper responses are elicited by NDV [20].

5. Conclusion

Our findings indicate that NDV can activate both cellular and humoral immunity by activating type 1 helper T and type 2 helper T lymphocytes. Additionally, we found that the NDV treatment group did not demonstrate any cytotoxicity during the two passages.

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Compliance with ethical guidelines

The study did not use any animals or biological samples. All analyses were performed with publicly available sequence data from online databases.

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Authors' contributions

Conceptualization, investigation, formal analysis, and writing the original draft: Kia Gharari; Supervision review and editing: Hadi Keyvanfar, Alireza Monadi Sefidan, and Samad Farashi Bonab.

Conflict of interest

The authors declared no conflict of interest.

Data availability

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

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