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Original Article

Multi-epitope HA Vaccine Confers Cross-protective Immunity to H5N8 and H9N2

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ABSTRACT

Introduction: Avian influenza viruses, notably H5N8 (HPAI) and H9N2 (LPAI), threaten public health due to their zoonotic potential and genetic adaptability. These viruses circulate in poultry and can sometimes directly infect humans after contact with infected birds. Some existing vaccines have proven useful in controlling these infections. Traditional vaccines targeting the variable hemagglutinin (HA) head domains necessitate frequent updates. This study designs a chimeric HA (cHA H9/H5) vaccine targeting conserved epitopes from H5N8 and H9N2 HA proteins to elicit broad immunity.

Materials & Methods: A multi-epitope construct, integrating B-cell and cytotoxic T lymphocyte (CTL) epitopes linked via stabilizing sequences (KK, AAY, GPGPG), was codon-optimized, expressed in *Escherichia coli* BL21(DE3), and purified (>95% purity, 22 mg/L yield). Specific pathogen-free chickens (n=20/group) received two doses of cHA H9/H5 formulated with Alum or Freund's adjuvants, compared to commercial H5N8/H9N2 vaccines. Humoral responses were assessed via hemagglutination inhibition (HI) assays.

Results: The cHA H9/H5 vaccine induced robust HI titers against homologous H9N2 (log_2 GMT 10–12) and hetero-subtypic H5N8 (log_2 GMT 6–8), surpassing commercial vaccines. While H9N2 vaccines lacked cross-reactive H5N8 antibodies, cHA H9/H5 elicited neutralizing titers (20–80) against H5N8. Freund's adjuvant significantly enhanced immunogenicity, with sustained post-boost antibody levels.

Conclusion: These results highlight the cHA H9/H5 vaccine's ability to overcome strain-specific limitations by targeting conserved epitopes, inducing cross-reactive immunity that is critical for pandemic preparedness. Adjuvant selection proved pivotal in optimizing responses, aligning with prior chimeric HA vaccine research. This study advances the development of universal influenza vaccines, offering a promising strategy to mitigate risks posed by evolving avian influenza variants.

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1. Introduction

The influenza virus, a member of the Orthomyxoviridae family, is classified into three subgroups (A, B, and C) based on the antigenic properties of its nucleoprotein and matrix protein [1]. Among these, influenza type A is highly prevalent and exhibits greater pathogenicity compared to the other subgroups. This virus can infect various species of mammals and birds, potentially causing epidemics of varying intensities. Nearly all known subtypes of influenza A viruses have been found in birds. While numerous other animals can also act as hosts for influenza, historically, avian influenza has posed the greatest threat to human health [2, 3].

The influenza virus, particularly subtypes H5N8 (highly pathogenic avian influenza, HPAI) and H9N2 (low pathogenic avian influenza, LPAI), poses significant threats to both poultry and human health due to their potential for genetic reassortment and zoonotic transmission [4, 5]. Existing commercial vaccines, such as inactivated H5N1 and H9N2 vaccines, have been employed to curb poultry outbreaks and reduce human exposure, yet their efficacy is challenged by viral mutations and limited cross-protection [6, 7]. The immune response to influenza vaccines relies on eliciting neutralizing antibodies, primarily against HA, which can be assessed through the hemagglutination inhibition (HI) test—a standard measure of vaccine-induced protection, with HI titers ≥40 commonly indicating sufficient immunity in protection studies [8]. By focusing on conserved HA epitopes, this multi-epitope vaccine seeks to enhance immunogenicity and provide broader, longer-lasting protection against evolving H5N8 and H9N2 strains, addressing gaps in current vaccination strategies [7, 9]. However, mutations in the *HA* gene could potentially enable avian influenza viruses to bind human receptors, enhancing their transmissibility [10].

Simultaneous infections with H5N1 and H9N2 may result in genetic reassortment, producing new subtypes with high pathogenicity in humans [11]. Consequently, the emergence of avian influenza viruses, particularly H5 and H9 subtypes, represents a dual threat to both the poultry industry and public health. The World Health Organization (WHO) lists these subtypes among the most significant pandemic threats. To mitigate risks, some countries have employed various poultry vaccines targeting H5N1 (HPAI) and H9N2 (LPAI) to protect the poultry industry and reduce direct human exposure to infected birds [12]. However, addressing the challenges of increasing viral pressures and controlling outbreaks in poultry requires the timely development of new vaccines. Such vaccines must account for international trade implications and aim to prevent the spread of viral diseases. Based on these insights, this study focuses on designing a multi-epitope influenza vaccine targeting highly conserved regions of the HA protein in H5N8 and H9N2 strains, which are prone to mutations over time.

This study aims to design a multi-epitope vaccine targeting conserved regions of the hemagglutinin (HA) protein in these strains, a key immunogenic component responsible for viral entry by binding to host cell sialic acid receptors [9].

2. Materials and Methods

2.1. Recombinant construct

The recombinant construct was developed in accordance with our previous report [13]. Briefly, the target multi-epitope HA recombinant protein was constructed by selecting antigenic epitopes capable of inducing robust immune responses. B-cell and cytotoxic T lymphocyte (CTL) epitopes were connected linearly using specialized linkers, forming a multi-epitope vaccine construct. The design was verified through various bioinformatics tools to confirm its structural and functional properties.

Key evaluations included allergenicity and antigenicity prediction using AllerTOP v. 2.0 and VaxiJen v. 2.0, respectively, to ensure the construct was non-allergenic and immunogenic. Physicochemical properties, such as molecular weight, isoelectric point, hydropathicity, and stability, were analyzed with ProtParam. Secondary structures, including alpha helices and beta sheets, were modeled with PSIPRED v. 3.3, and tertiary structure prediction was completed using the SwissModel server. The pET-41a+ vector was chosen for expression due to its efficiency and high yield in *Escherichia coli*.

2.2. Expression and purification of recombinant protein

The recombinant pET-41a (+)/HA construct was transformed into *E. coli* BL21 (DE3) cells, optimized for protein expression. Cultures of the transformed *E. coli* were grown in Luria-Bertani (LB) medium supplemented with kanamycin to select for cells harboring the plasmid. Protein expression was induced at the mid-log phase of bacterial growth (OD600 ≈ 0.6) by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final

concentration of 1 mM. The cultures were then incubated for an additional 4 hours at 37 ˚C with constant shaking to optimize protein production.

Following induction, the cells were harvested by centrifugation and lysed using a combination of lysozyme treatment and sonication. The lysate was clarified by centrifugation to remove cell debris, and the supernatant containing the soluble recombinant chimeric HA protein (cHA H9/H5) was collected.

Purification of the cHA H9/H5 protein was performed using nickel-affinity chromatography. The clarified lysate was applied to a nickel-nitrilotriacetic acid (Ni-NTA) agarose column pre-equilibrated with binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). After washing to remove non-specifically bound proteins, the HA protein was eluted using a high concentration of imidazole (250 mM) in the elution buffer.

The purity and identity of the cHA H9/H5 protein were confirmed by SDS-PAGE and Western blotting using anti-HisTag antibodies.

2.3. Immunization and experimental infection of chickens

Seven-day-old SPF White Leghorn chickens (Venky Lab Co., Ltd., Pune, India) were randomly allocated into five experimental groups (n=20 per group) and immunized with antigen-adjuvant formulations as delineated in Table 1. The cHA H9/H5 protein was prepared at a concentration of 1 mg/mL, with each chicken receiving 30 µg of antigen diluted in phosphate-buffered saline (PBS). Freund's adjuvant (complete for the initial dose and incomplete for the subsequent dose) and alum were employed as adjuvants in different groups, prepared in specified ratios with the antigen. Two commercial vaccine groups received inactivated influenza antigens derived from the H9N2 and H5N8 strains, formulated with ISA 71 adjuvant (Seppic, France): Influenza A virus (A/chicken/Iran/B308B/2019(H9N2) ARAK/2009(H9N2)) and influenza A virus (A/poultry/Iran/clade 2344/2017(H5N8)), respectively.

Immunizations were administered subcutaneously in two doses at 14-day intervals. Blood samples were collected weekly from day 0 to day 56 post-immunization, centrifuged to isolate serum, and stored at −20 ˚C for subsequent immunological analysis.

2.4. Evaluation of humoral immune response

2.4.1. HI assay protocol

Antibody responses following immunization were evaluated using HI assays with H9N2 2019,H9N2 ARAK/2009, and H5N8 viral antigens.

For the HI assay, serum samples were subjected to two-fold serial dilutions incubated with 4 HA units of viral antigen at 37 ˚C for 30 minutes, followed by the addition of 1% chicken red blood cells (RBCs). After 30-minute incubation at room temperature, HI titers were determined as the reciprocal of the highest serum dilution that completely inhibited hemagglutination and were converted into log2 values.

2.4.2. Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 9.0 (San Diego, CA, USA). Data were expressed as Mean±SD. Group comparisons were performed using one-way ANOVA followed by Tukey's post hoc test to account for multiple comparisons. A $P<0.05$ was considered statistically significant. All analyses were conducted in accordance with established guidelines to ensure the validity and reproducibility of results.

Table 1. Experimental groups and antigen administration

Groups	Antigen Administered	Adjuvant Type	Dose (µg/chick)	Volume (µL)
cHA H9/H5-Alum	cHA H9/H5	Alum	30	200
cHA H9/H5- Freund's	cHA H9/H5	Complete/incomplete Freund's	30	200
H5N8 vaccine	H5N8 vaccine	None	-	200
H9N2 vaccine	H9N2 vaccine	None	-	200
Mock	PBS	None	-	200

3. Results

The second spherical head of HA in different influenza virus subgroups shows limited homology, while HA2 ectodomains among various subtypes are highly conserved. The amino acid homology level between HA2 regions of H5 or H9 viruses was about 95% within the same subgroup, but only 64-65% identity was observed between H9 and H5 viruses. To develop a multi-subtype vaccine through reverse genetics, we produced a chimeric (cHA) H9/H5 construct, including HA2 regions from A/chicken/Iran/B308B/2004 (H9N2) and A/poultry/Iran/clade 2344/2018 (H5N8)

The retrieved sequences were evaluated using the VaxiJen server to identify the strongest antigenic regions. Prediction scores for HA proteins H9N2 and H5N8 were 0.4333 and 0.5202, respectively —both above the threshold of 0.4. Epitope scores and chemical properties were evaluated, leading to the selection of epitopes E1 to E8 (three from H9N2 and five from H5N8), with two glycines inserted between them to create a linear structure. The final sequence, consisting of 296 amino acids, has an isoelectric point of approximately 7 and an estimated molecular weight of 32.46 kDa, indicating good antigenic potential. The protein is non-allergenic, with an antigenicity score of 0.4828 (Figure 1, Table 2).

The cHA H9/H5 recombinant protein gene was cloned into the pET-41a (+) vector, which includes an N-terminal His-tag sequence, between XhoI and NcoI sites after codon optimization. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells for protein expression. After induction of expression, SDS-PAGE and Western blot analyses using anti-His antibodies confirmed successful expression of the target protein, with an expected molecular weight of approximately 32 kDa (Figure 2). Densitometric analysis confirmed that the purity exceeded 95%. The yield of purified cHA H9/H5 protein was approximately 22 mg per liter of bacterial culture, determined by the Bradford protein assay.

To determine the immunogenicity and protective efficacy of the cHA H9/H5 antigen, we immunized groups of specific pathogen-free (SPF) chickens with the antigen, formulated with different adjuvants. Subsequently, the humoral immune response was measured by the HI assay. It revealed distinct immune responses in chickens immunized with the recombinant chimeric cHA H9/H5 antigen, as well as the H9N2 and H5N8 vaccine antigens (Figure 3).

Chickens receiving the cHA H9/H5 antigen formulated with Alum or Freund's adjuvants exhibited elevated HI geometric mean titers (GMT) against the homologous cHA H9/H5 antigen, indicating robust immunogenicity. Notably, when the cHA H9/H5 antigen was formulated with Freund's adjuvant, it demonstrated elevated cross-reactive responses to the heterologous H9N2 antigen (P<0.05). Commercial vaccine groups

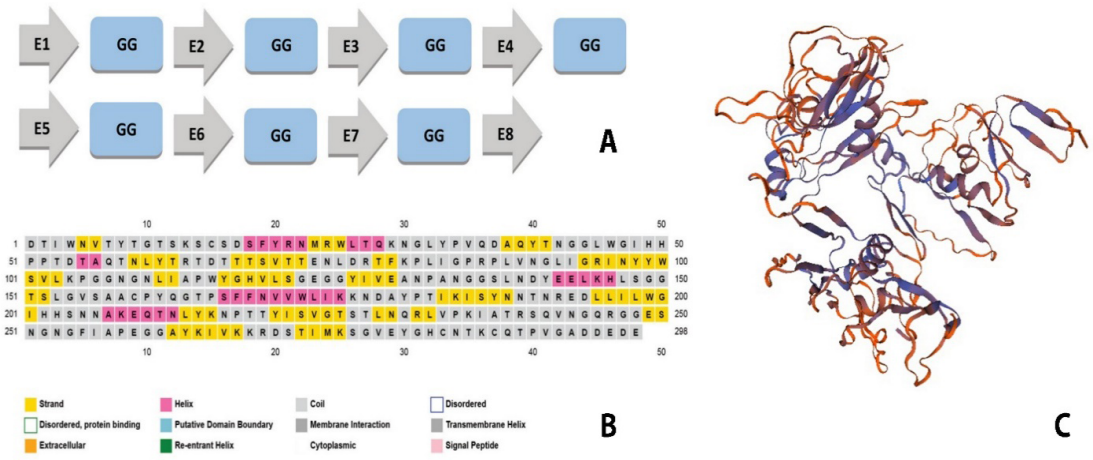

Figure 1. Schematic design and predicted structure of the recombinant HA vaccine antigen

A) The primary structure of the recombinant protein, highlighting the selected epitopes derived from H9N2 and H5N8 strains; B) Predicted secondary and tertiary structures of the designed recombinant protein, illustrating the spatial arrangement of the selected epitopes; C) Three-dimensional model of the recombinant protein, illustrating the overall folding and potential antigenic sites

Table 2. Physicochemical properties of the designed recombinant protein

Molecular Weight	Instability Index	Aliphatic Index	Theoretical pI	No. of Amino acids	Total Number of Atoms	Extinction Co-efficient	Gravy
32465.09	17.93	71.45	7.19	296	4499	64080	-0.568

that were immunized with inactivated H5N8 and H9N2 antigens showed antigen-specific reactivity. The H5N8 vaccine induced strong HI responses to both cHA H9/H5 and H5N8 antigens, while the H9N2 vaccine elicited high titers against cHA H9/H5 and H9N2 antigens but showed lower reactivity to H5N8. These findings underscore the enhanced immunogenicity of adjuvanted cHA H9/H5 and highlight cross-reactivity patterns between the chimeric antigen and conventional H5N8/H9N2 vaccine strains.

4. Discussion

The development of HA protein chimeric vaccines represents a transformative approach to improving influenza immunization strategies. Traditional influenza vaccines target the highly variable HA head domain, necessitating annual updates due to antigenic drift or shift. Chimeric HA vaccines, however, incorporate conserved regions (e.g. the HA stalk), fused with exotic head domains, to elicit broad, cross-reactive immunity against diverse influenza strains, including pandemic variants. Preclinical studies demonstrate that such vaccines induce antibodies targeting conserved epitopes, enhancing protection against heterosubtypic viruses and reducing the need for frequent reformulation [14]. This strategy aligns with the WHO's priority for universal influenza vaccines to mitigate global health burdens. By leveraging conserved antigenic regions, chimeric HA vaccines offer a promising path toward durable, pan-influenza immunity.

In this study, we aimed to elicit a broad and robust immune response by targeting multiple conserved epitopes from different influenza strains. This innovative approach not only enhances cross-protection against diverse influenza subtypes but also paves the way for the development of a universal influenza vaccine, which is crucial for improving global pandemic preparedness and reducing the impact of influenza outbreaks.

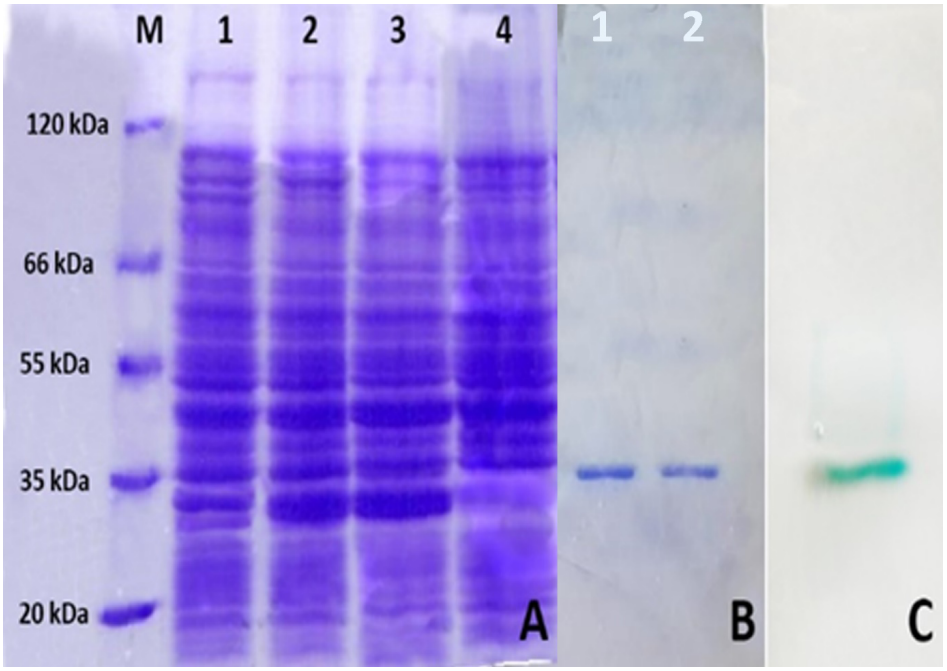

Figure 2. Analysis of protein expression, purification, and Western Blot analysis

A) SDS-PAGE analysis of protein expression, showing a prominent band at approximately 32 kDa: Lane M: Molecular weight markers; Lane 1: Non-induced sample; Lanes 2–4: Recombinant protein expression at 2, 3, and 4 hours post-induction, respectively; B) Purification of the expressed protein using Ni-NTA affinity chromatography, as indicated by the SDS-PAGE analysis (lanes 1-2: Sequential elution of purified recombinant protein); C) Western blot analysis confirming the presence of the target protein at approximately 32 kDa

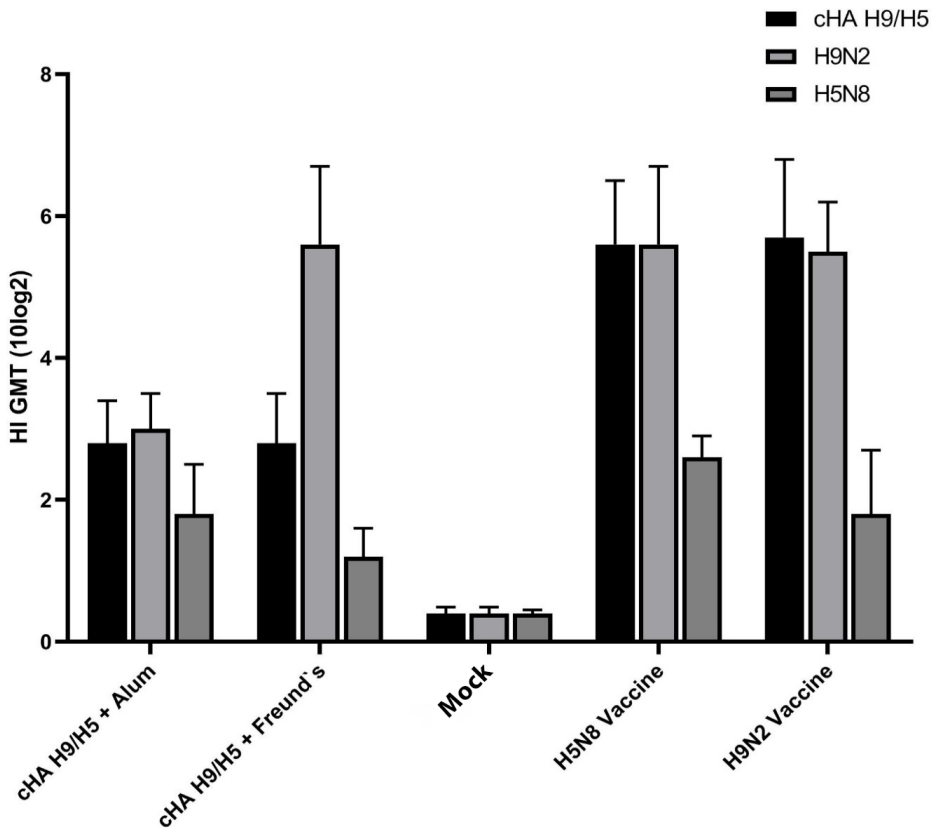

Figure 3. HI GMT against different influenza virus strains

Note: The y-axis represents SN GMT in log base 2 (10 log2) and the x-axis lists different vaccine groups: cHA H9/H5, H9N2, and H5N8.

In comparison to our study, one study utilized an immunoinformatic approach to create a vaccine based on conserved epitopes from HA, neuraminidase, and matrix proteins. This vaccine, designed with specific linkers and evaluated for immunogenicity using computational frameworks, demonstrated promising immune responses in terms of IgG, T helper 1 cells, and interferon gamma levels [15]. Another research effort focused on creating a universal T-cell vaccine using a recombinant vaccinia virus expressing multiple epitopes from influenza virus proteins such as NP, M1, NS1, PB1, and PA, resulting in increased influenza virus-specific-IFN-γ secreting splenocytes and decreased viral load in vaccinated mice [16]. Additionally, a study combining the M2e peptide with stalk HA epitopes of influenza A virus observed enhanced immunogenicity and protective properties, with recombinant proteins providing broad protection against various influenza viruses, including A/H3N2, A/H2N2, and A/H5N1 [17].

It is evident that all studies emphasize the use of conserved epitopes and strategic linkers to enhance stability and immune responses. The use of EAAAK, KK, AAY, and GPGPG linkers for epitope stability and immune activity is consistent with similar approaches in these studies. Additionally, the confirmation of protein expression through Western blotting and SDS-PAGE analysis aligns with common practices in the field.

The study successfully cloned the cHA H9/H5 recombinant protein gene into the pET-41a (+) vector, which was then transformed into *E. coli* BL21 (DE3) cells for expression, confirmed by SDS-PAGE and Western blot analyses. The protein was purified using Ni-NTA agarose affinity chromatography, achieving over 95% purity with a yield of approximately 22 mg per liter of bacterial culture. These results indicate a robust and scalable method for producing cHA H9/H5 recombinant protein for further immunogenicity and functional studies, aligning with other research utilizing *E. coli* for high-yield expression of recombinant influenza proteins, such as those that fused the globular head domain of HA with flagellin to elicit robust antibody responses in mice [18]. Similarly, the study aligns with approaches highlighted in reviews of protein subunit vaccines targeting H1N1/09, emphasizing antigen selection and expression

systems [19]. Additionally, the study's robust method for producing recombinant protein could be further evaluated for immunogenicity and safety, as demonstrated in comparisons of quadrivalent recombinant and inactivated influenza vaccines [20]. Overall, the results provide a scalable method for producing cHA H9/H5 recombinant protein, consistent with studies emphasizing high-yield bacterial expression and purification techniques.

Our study demonstrates that the cHA H9/H5 vaccine formulations with Alum and Freund's adjuvants significantly enhance HI titers against multiple influenza strains, highlighting the superior efficacy of these adjuvants in boosting antibody production. This finding aligns with other studies, such as the one by Kim et al., which showed that a chimeric H9/H5N2 vaccine induced robust HI titers and provided protection against HPAI H5N8 viruses in chickens [21].

5. Conclusion

Overall, our results underscore the potential of the cHA H9/H5 antigen, when combined with adjuvant formulations, to elicit robust immune protection against a broad spectrum of influenza virus strains —consistent with similar research efforts in the field.

Ethical Considerations

Compliance with ethical guidelines

The procedures of this trial were approved by the Ethics Committee of the Islamic Azad University, Karaj Branch, Karaj, Iran (Code: IR.IAU.K.REC.1398.026).

Data availability

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

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Authors' contributions

Conceptualization and study design: Morteza Taghizadeh and Mostafa Ghaderi; Data acquisition: Zahra Bozorgkhoo; Project administration, technical, and material support, data analysis, interpretation and writing: Zahra Bozorgkhoo and Morteza Taghizadeh; Statistical analysis: Behzad Hemmati.

Conflict of interest

The authors declared no conflict of interest.

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