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Designing a Construct of Chimeric Multi-Epitopes Protein for Contraceptive Vaccine in Mice: An Immunoinformatics and In Silico Study

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ABSTRACT

Background: Contraceptive Vaccines (CVs) can be used as a valuable and alternative method for the prevention of gestation in humans and animals. These vaccines can have several targets, such as superficial sperm proteins. Vaccines based on sperm antigens are quite efficacious to create a contraceptive effect. However, multi-epitope vaccines are more effective in stimulating the immune system and producing more antibodies to reduce the infertility rate.

Materials and Methods: This study aimed to design and evaluate a chimeric fusion protein containing IZUMO, SACA3, and PH-20 epitopes. IZUMO1, SACA3, and PH-20 were assessed, and appropriate regions were selected using various bioinformatics tools, including IEDB, I-TASSER, ProtParam, Asa-View, and Chimera software. Protein epitopes were selected based on various characters, including specificity, solvent accessibility, their weight and length, antigenic intensity, and topology. Epitopes with high antigenic potential were selected and joined together by linkers. The designed fusion protein was simulated using Molecular Dynamic, GROMACS 5, and Chimera 1.14 software.

Results: The results demonstrated that all antigenic plots and availability of epitopes in the new construct remained constant. The spermatic antigens were combined using rigid linkers as a new construct and showed a stable formation with proper solvent accessibility validated by ProSA-web and PROCHECK. Also, comparing the new structure with its original one did not show any structural change.

Conclusion: Based on bioinformatics results, the fusion protein that consists of three spermatic antigens has productive potential to stimulate the immune system and capable of producing more antibodies in circulation and reliable infertility.

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Introduction

Contraceptive Vaccines (CV) may provide an alternative approach to control unintended pregnancies and animal populations [1]. Recent research indicates that 9% to 36% of couples are infertile because of sperm autoimmunity [2, 3]. Sperm antibody is detected in 8% to 21% of the infertile male partners [3, 4] and 6% to 23% of the female partners [2, 4-6]. Several CVs have been studied and introduced to inhibit gamete production (such as LHRH, FSH, and GnRH) [7, 8], sperm and oocyte antigens [9], and gamete outcome (LIF and hCG) [10-12]. The blood-testis barrier separates spermatogonial and immune cells. Sperm antigens releasing to blood lead to autoimmune response against spermatogonial cells [13]. Antigen-antibody complex clots sperms and limits their motility [14]. Immune response to spermatogonial cells results in infertility in both males and females [15].

Various antigens are used to produce contraceptive vaccines. IZUMO1, SACA3, and PH-20 antigens are essential for sperm function and oocyte fertilization [16-18]. Utilizing the full length of these proteins individually with reduced fertility goals can partially interfere with fertility [19-21]. IZUMO1 is a sperm cell surface protein that plays a role in penetrating sperm by interacting with JUNO, which is located on oocyte, and binding of these two proteins together is necessary for oocyte fertilization [22, 23]. Also, studies that focus on gene knockout proves that IZUMO protein has an effective role in sperm penetration [24]. Recently, several variations in the JUNO gene have been found which can be related to female infertility and disrupting sperm-egg binding [25].

SACA3 is a sperm surface membrane protein that is involved in adhesion and fusion of sperm during fertilization. This protein binds to N-acetylglucosamine, which is located on the surface of the oocyte [18, 26]. Reverse transcription-polymerase chain reaction and immunohistochemistry determine the SACA3 expression in the oocyte, corpora lutea, and ovarian follicles [27]. This protein has a critical role in the early phase of embryo development in many species [18, 19, 28].

Hyaluronidase PH-20 protein is encoded by the Spam1 gene and contributes to sperm-oocyte adhesion. This protein is an anchored-GPI protein, which digests hyaluronic acid of cumulus cells to facilitate sperm penetrating oocyte [29, 30]. PH-20 is a multi-functional protein, besides hyaluronidase property, it is a receptor for HA-induced cell signaling and binding to the zona pellucida [31-36].

A series of monoclonal antibodies have been produced using hybridoma and recombinant DNA technology, which interferes in the fertility process in various species of animals [9, 37-40]. The notable ones are SP-10 (sperm protein 10) [41], FA-1 (fertilization antigen 1), FA-2 [42], lactate dehydrogenase (LDH)-C4 [43, 44], ZP3 [45], YLP12 peptide [46], SAMP32 [47, 48], and SAMP14 [49]. The identification of appropriate epitopes with high antigenic and immunogenic potential is the first step in designing a recombinant protein. This procedure can be accomplished by in silico methods and bioinformatics tools. Studies indicate that a combination of epitopes can enhance immunogenicity and create powerful reversible infertility [50].

In a similar study, it was observed that immunization with complete uPA protein might have serious side effects

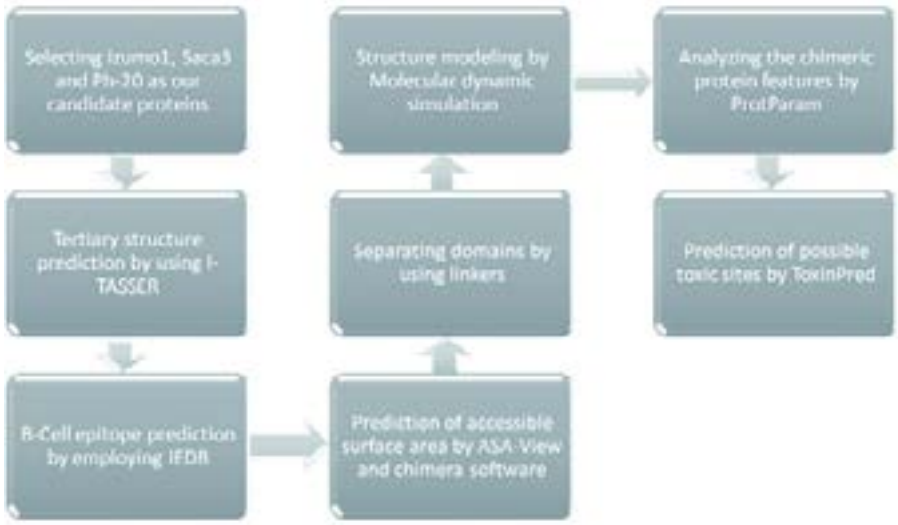

Figure 1. The schematic image of the research stages

and induce immune tolerance at higher doses while using B-cell epitopes reduce adverse effects and enhance the immune specificity [51, 52]. The purpose of this study was to design a new vaccine, consisting of three spermatic proteins (IZUMO1, SACA3, and PH-20), which can induce B cells and results in contraception by the immune response. Multi-epitope vaccines have more potential for stimulating the immune system and producing more antibodies to reduce the infertility rate. This study was done and designed for mice, but the results, process, and methods can be used for other animals, too.

Materials and Methods

The research had several stages, including protein selection, tertiary structure prediction of proteins, epitope prediction, solvent accessibility prediction, protein engineering, structure modeling and simulation, physicochemical parameters, and evaluating toxic fragments. Figure 1 displays the research process steps.

Protein selection

Initially, the selection of proteins for CV production was carried out using UniProt (www.uniprot.org) and Protein Atlas (www.proteinatlas.org) databases. Then IZUMO1 (UniProt: Q9D9J7 and Gene ID: 73456), SACA3 (UniProt: Q9D9X8 and Gene ID: 75622) and PH-20 (UniProt: P48794 and Gene ID: 20690) were selected as desirable candidates. All sequences were obtained from NCBI (www.ncbi.nlm.nih.gov/protein), and the isoforms were analyzed by the BLASTP program and the UniProt database.

Tertiary structure prediction of proteins

Due to the lack of full 3D structures of IZUMO1 and PH-20, the whole structures were simulated using I-TASSER (Iterative Threading ASSEmbly Refinement) [53], which is a hierarchical approach to protein structure and function prediction. I-TASSER server is an integrated platform for automated protein structure and function prediction based on the sequence-structure-function paradigm. The server is free for academic users and available at https://zhanglab.ccmb.med.umich.edu/I-TASSER/. The 5B5K and 2PE4 PDB codes were considered as templates for IZUMO1 and PH-20 simulation, respectively. The structure of the SACA3 with 4yf2 PDB code is fully available in the PDB (https://www.rcsb.org/) database.

Epitope prediction

In this stage, the 3D structure and exposed regions of the proteins are considered. High antigenic areas were determined by employing The Immune Epitope Database (IEDB) [54] (https://www.iedb.org/). IEDB contains an extensive collection of experimentally measured immune epitopes and a suite of tools for predicting and analyzing epitopes. IEDB has antibody, and T cell epitopes for infectious diseases, allergens, autoimmune diseases, and transplant/alloantigens studied in humans, non-human primates, mice, and other animal species. It has two sections: prediction and analysis tools. Prediction tools predict the outcome of experiments, such as MHC class I or class II binding, MHC class I processing and immunogenicity, and for predicting linear and discontinuous (conformational) B cell epitopes. The epitope prediction tools are valuable resources for vaccine researchers when experimentally measured epitopes are not available in IEDB. To assign antigenic potency, epitope regions were analyzed based on sequence (prediction of linear epitopes from protein sequence) and structure in ‘’B cell tool’’.

The antigenicity plots of the extracellular region were obtained according to the seven sequences based methods which are listed in the IEDB software [55-60]. Structural B-cell epitopes were predicted using Ellipro (minimum score of 0.5 and a maximum distance of 6 Å) [61]. Similarity analysis of three epitopes was done using Clustal Omega, which is a multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments between three or more DNA or protein sequences.

Solvent accessibility prediction

The accessible surface area or solvent accessibility of epitopes are predicted by ASA-View [62] (http://ccbb.jnu.ac.in/shandar/servers/asaview/) both quantitatively and qualitatively. This server provides a graphical representation of the solvent accessibility of amino acid residues in proteins with known structures. We also used Chimera 1.13rc software to analyze the available surface of epitopes schematically. Chimera is a program for the interactive visualization and analysis of molecular structures and related data, including density maps, sequence alignments, docking results, and trajectories.

Protein engineering

Non-helical linkers can create multi-functional chimeric proteins, which consist of modules from various proteins.

These linkers create structural rigidity and isolation of linkers from the attached epitopes. Two types of rigid linkers were used for protein engineering to link the epitopes and form a fixed and non-interference structure of fusion protein [63, 64]. Codon optimization and in silico cloning were carried out using EMBOSS and GenSmart servers in Escherichia coli K–12 as a prokaryotic host [65].

Structure modeling and simulation

The fragment structures were combined and modeled by MODELLER 11. It is used for homology or comparative modeling of three-dimensional protein structures. MODELLER implements comparative protein structure modeling by satisfaction of spatial restraints. It can perform many additional tasks, including de novo modeling of loops in protein structures, optimization of various models of protein structure with respect to a flexibly defined objective function, multiple alignments of protein sequences and or structures, clustering, searching of sequence databases, comparison of protein structures, etc. To achieve the final composition of the fusion protein, we performed a Molecular Dynamics (MD) simulation with GROMACS [66].

It is one of the most widely used open-source and free software codes in chemistry and biology used primarily for dynamical simulations of biomolecules. It was developed by Groningen University. GROMACS can work in the operating system Linux. The primary ability of GROMACS is to perform MD simulation and minimization energy [67]. It provides a rich set of calculation types, preparation, and analytic tools. GROMACS 5 was used in the operating system Linux, and the force field was Gromos 54A7 with 50 ns. For simulating the system, sodium (Na+) and chloride (Cl-) ions were used to neutralize, SPC model for filling water, with pressure 1 bar, at the temperature of 300 Kelvin, and neutral pH set.

Root-Mean-Square Deviation (RMSD) and Root-Mean-Square Fluctuation (RMSF) diagrams of simulated protein were obtained from studying the physical movements of atoms and molecules [68]. To recognize the errors in the generated model, coordinates were supplied by uploading 3D structure in PDB format into ProSA-web [69] (https://prosa.services.came.sbg.ac.at/prosa.php) and PROCHECK server [70] (https://servicesn.mbi.ucla.edu/PROCHECK/), which are frequently employed in protein structure validation.

Physiochemical parameters

The protein features such as instability index, molecular weight, aliphatic index, theoretical isoelectric point (pI), half-life in Escherichia coli and mammals, the total number of positive and negative residues, and grand average hydropathy were analyzed in ProtParam [71] (https://web.expasy.org/protparam/).

Evaluating toxic fragments

The existence of potential toxic sites was predicted by ToxinPred [72] (https://webs.iiitd.edu.in/raghava/toxinpred/protein.php) server. ToxinPred is an in silico method, developed to predict and design toxic/non-toxic peptides. The main dataset used in this method consists of 1805 toxic peptides.

Antigenicity and allergenicity assessment of the chimeric protein

IEDB evaluated the antigenicity of the chimeric protein, and the structural database of allergenic proteins (SDAP) and AlgPred assessed the allergenicity of the chimeric protein. SDAP is a server that integrates the database of allergenic proteins with various computational tools and assists structural biology studies related to allergens [73]. AlgPred allows the prediction of allergens based on the similarity of known epitope with any region of the protein [74].

Results

Following the selection of IZUMO1, SACA3, and PH-20, the isoform study by BLASTP demonstrated some similarities in their domains, such as the Ig-like C2 domain in IZUMO1, glycoside hydrolase family 22 in SACA3, and glycoside hydrolase family 56 in PH-20.

The locations of the extracellular region of proteins considered for epitope selection are aa 22-319 in IZUMO1, aa 91-221 in SACA3, and all sequences in PH-20. The IEDB results revealed that some regions of proteins have high antigenicity potential in terms of sequence and structure. In sequence-based methods, each epitope above the threshold is considered. Also, Table 1 lists 8 linear B-cell epitopes represented by analyzing the 3D structure. Table 2 presents the selected epitopes based on the score of sequence and structure methods.

The available position of the IZUMO1 structure is from aa 22-257, while the location of the selected epitope is aa 249-291, so the whole structure by 5B5K pattern code

Table 1. Predicted linear B-cell epitopes for the 3D structure of proteins

Protein	Chain	Start Position	End Position	Peptide	Number of Residues	Score
IZUMO	A	249	256	DVTVLPPK	8	0.844
	A	219	230	TKSMVGPEDAGN	12	0.757
	A	238	241	INQG	4	0.614
SACA3	A	88	129	LASSKAKVFSRCELAKEMHDFGLDGYRGYN-LADWVCLAYYTS	42	0.679
	B	115	128	QGRDLSDWVDGCDF	14	0.733
	C	115	126	QGRDLSDWVDGC	12	0.832
PH-20	A	265	273	KKDLKSNRQ	9	0.688
	A	226	240	NKFQDPKYDGQCPAV	15	0.654

Table 2. Selected epitopes based on top scores in sequences and structure methods

Protein	Chain	Start Position	End Position	Number of Residues	Epitope Sequence
IZUMO1	A	249	291	43	DVTVLPPKHSEENQPPNIITQEEHETPVHVTPQTPPGQEPESE
SACA3	A	119	148	30	ADWVCLAYYTSGFNTNAVDHEADGSTNNGI
PH-20	A	220	247	28	FPDCYNNKFQDPKYDGQCPAVEKKRNDN

was simulated. To simulate the entire PH-20 structure, we used 2PE4 PDB code with a 38% identity between them.

The first used rigid linker for joining IZUMO1 and SACA3 epitopes was AEAAAKA, and the second linker was APAPAP for joining SACA3 and PH-20 epitopes. Rigid linkers can make firm gaps between protein domains, and the distance between them can be controlled by changing their length. For the AEAAAKA linker, we used a single specific repeat, but three AP repeats for the second linker.

Since the protein weight is less than 20 kDa and may not have proper antigenicity and stability, epitopes regions were extended in two sides to reach the ideal weight and size. Table 3 presents the final sequence, which consists of 270 amino acids (epitopes and linkers in the fusion protein).

Analyzing the similarity of three epitopes using Clustal Omega revealed that epitopes have no similarity by themselves. The obtained results by ProtParam showed that protein molecular weight was 30.465 kDa. The theoretical

Table 3. The position of epitopes and linkers in the fusion protein

Construct	Start Position in The Main Protein	End Position in The Main Protein	Start Position in The Fusion Protein	End Position in The Fusion Protein
Epitope region 1 (IZUMO1)	210	300	1	91
Linker 1	AEAAAKA		92	98
Epitope region 2 (SACA3)	100	184	99	183
Linker 2	APAPAP		184	189
Epitope region 3 (PH-20)	195	275	190	270

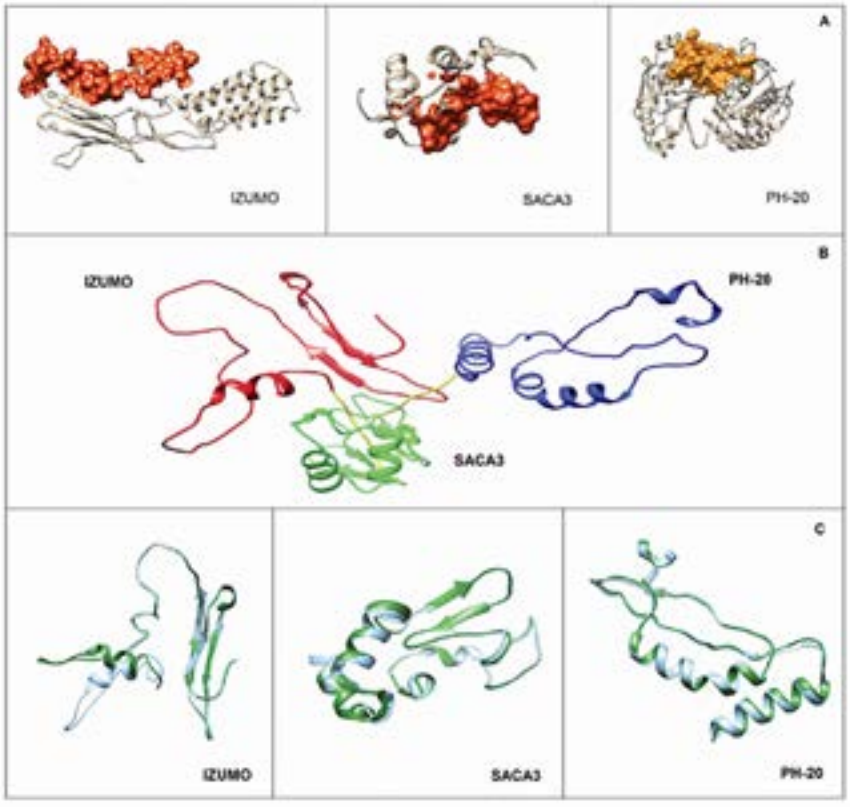

Figure 2. Surface accessibility and analyzing the new construct

A. Localization of potential B-cell epitopes on the protein structure in red; B. The final composition of the fusion protein, using the molecular dynamic output. The fusion protein consists of three epitopes, which colored in red (IZUMO), green (SACA3), blue (PH-20), and yellow (linkers); C. Examining the structural changes in the fusion protein.

isoelectric point (pI) was 5.93. The total number of Asp and Glu amino acids was 34, and Arg and Lys were 29, so fusion protein has a negative charge. The biocomputed half-life in mammals was 20 hours and in E. coli greater than 10 hours. Based on the instability index, Expasy's ProtParam classified the optimized chimeric protein as stable (instability index: 39.93). Aliphatic index of chimeric protein was 68.33. The extinction coefficient of optimized chimeric protein at 280 nm was 50225 M-1 cm-1.

The solvent accessibility scores of epitope regions obtained by Asa-View in IZUMO1, SACA3, and PH-20 were 0.54, 0.37, and 0.33, respectively. After obtaining PH-20 and IZUMO1 structure from I-TASSER, and by possessing the SACA3 structure, we used Chimera software to determine the available epitope surface regions, as shown in Figure 2. The findings showed that epitope regions are located at the surface of the fusion protein (Figure 2a). The final structure of the fusion protein obtained using MD is shown in Figure 2b. To examine the structural changes in the fusion protein, we aligned the structures derived from MD with previous structures obtained by I-TASSER and PDB web server. The SACA3 and PH-20 epitope regions are perfectly matched, but there is a slight change in IZUMO1 epitope region alignment that could not have a significant impact on the main structure (Figure 2c). Next, a Ramachandran plot analysis of the model was obtained from PROCHECK. Analysis of the simulated fusion protein revealed that 98.7% of the residues are placed in the favored region and 1.3% in the disallowed part of the Ramachandran plot (Figure 3a). The z-score of the model simulated by MD for overall model quality was -4.99, and within the range of scores typically found in native proteins of similar size (Figure 3b).

The results of IEDB to examine the remaining constant of antigenicity plots in fusion protein demonstrated that all antigenicity plots in all seven methods did not change. Figure 4 shows two examples of diagrams for BepiPred linear epitope prediction and Chou and Fasman beta-turn prediction.

The RMSD diagram declared that fusion protein reaches a stable structure after 50 ns, and the RMSF diagram

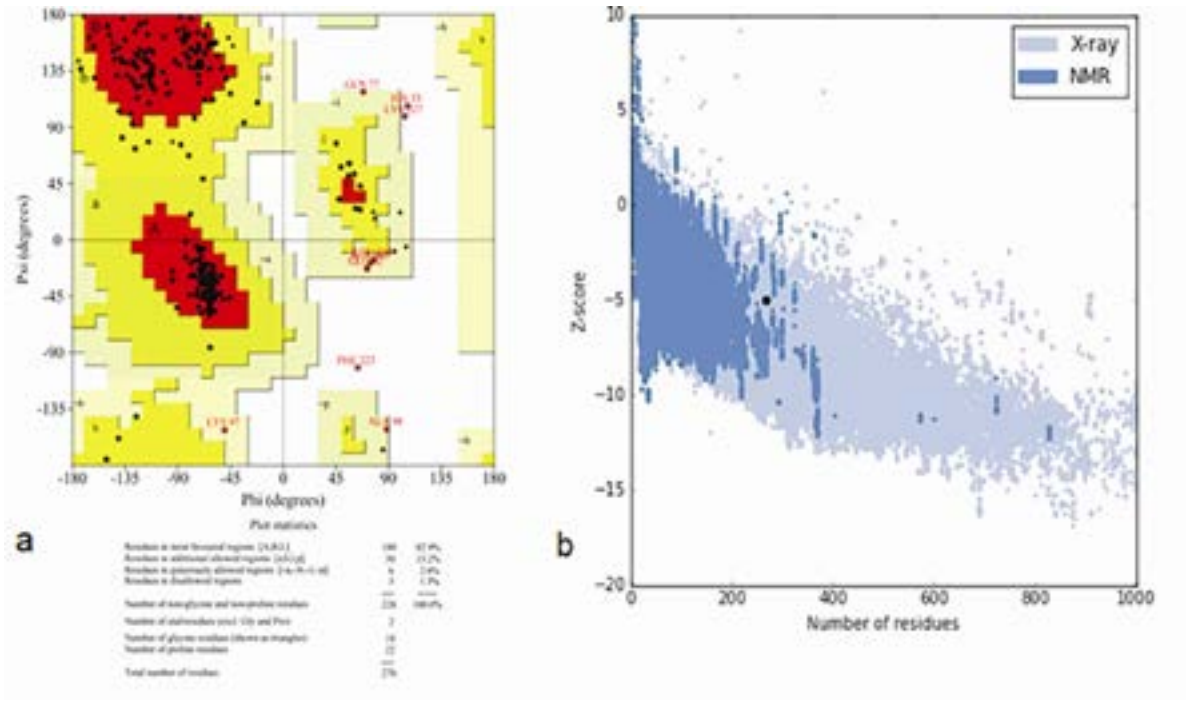

Figure 3. Ramachandran plots and z-score analysis

A. Ramachandran plot analysis for the fusion protein: 189 residues in most favored regions (82.9%), 30 residues in the additional allowed regions (13.2%), 6 residues in the generously allowed regions (2.6%), 3 residues in the disallowed regions (1.3%). B. Z-score plot for the tertiary structure of constructing: The z-score checks the input structure within the range of scores generally found in native proteins from different sources (X-ray, NMR) with a similar size (69)

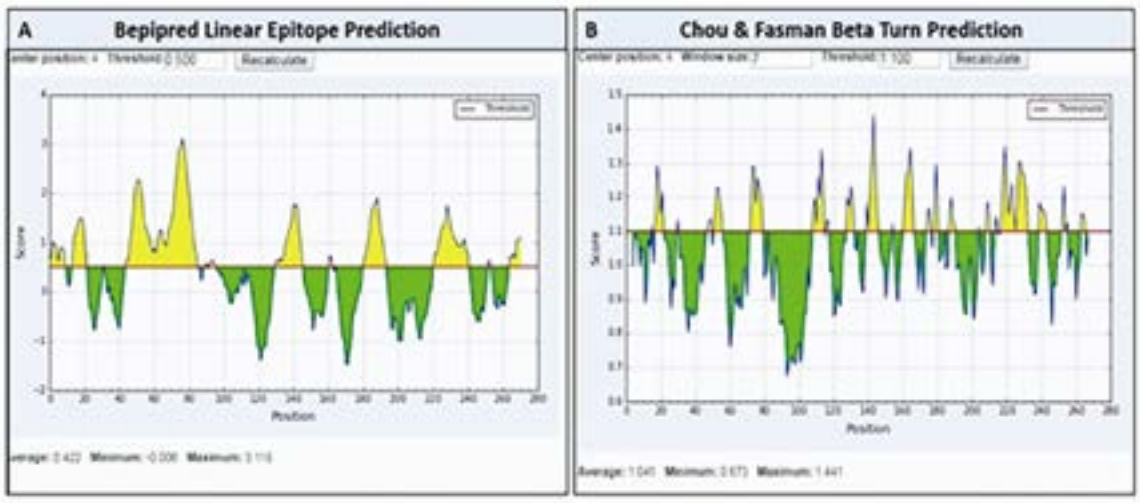

Figure 4. Two examples of antigenicity plots of the fusion protein obtained by IEDB: Yellow peaks exhibit high antigenic areas, and green peaks denote low antigenic regions of the fusion protein.

A. BepiPred linear epitope prediction (threshold 0.500). The peaks above the threshold are recognized by antibodies. B. Chou and Fasman beta-turn prediction (threshold 1.100).

The regions above the threshold have beta turns in the fusion protein.

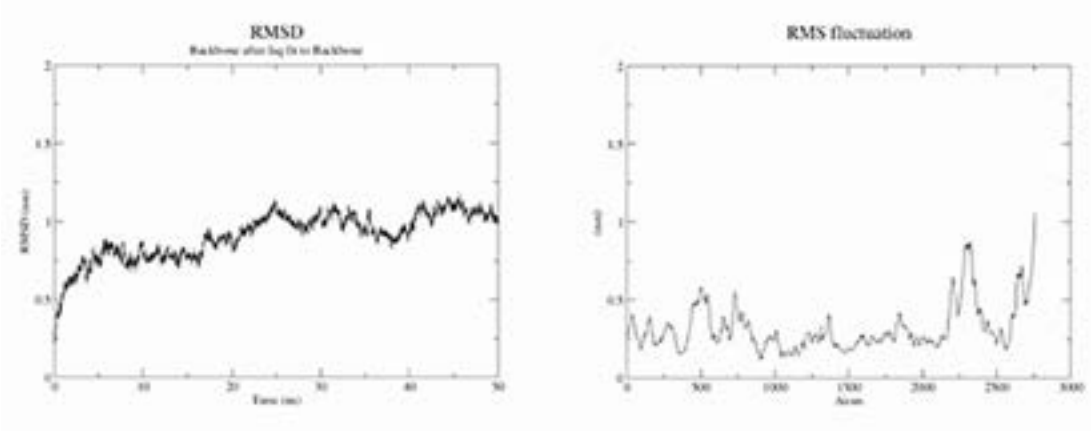

Figure 5. Root-mean-square deviation (RMSD) and root-mean-square fluctuation (RMSF) diagrams

shows the activity of atoms and their movements in a biological environment in which they are acceptable in all epitopes (Figure 5). RMSD can be employed to recognize large changes in protein structure as compared with the starting point. In molecular dynamics, we are interested in how structures and parts of structures change over time as compared with the starting point. RMSF is a calculation of individual residue flexibility, or how much a particular residue moves during a simulation [73].

ToxinPred data results of the chimeric protein did not show significant toxicity. Only two segments (LFPDCYNNKF and FPDCYNNKFQ), which is related to PH-20 protein with 0.1 E-value, indicated mild toxicity, which is not noticeable. Allergenicity assessment of the chimeric protein did not reveal any similarity and cross-reactivity with the known allergens.

Discussion

Several proteins have been introduced as Contraceptive Vaccine (CV) candidates so far. These vaccines must not have side effects. Hence, sperm-specific molecules are the best choice for antisperm CV development. Similar to the other vaccine production procedures, the progress in CV development has been lingering owing to the variability of immune response and assorted antibody titers after vaccination [75, 76]. However, we suggested that multi-epitope vaccines can enhance the efficacy of the variability of the response.

Also, due to the variable duration of antibodies circulation and its effect on fertility, new explorations have focused on enhancing the immunogenicity of the CV based on sperm-specific proteins [77]. The reason for the selection of three proteins is to reach more potent and prolonged immunogenicity [78].

Various antigens are used to produce contraceptive vaccines. In this regard, IZUMO1, SACA3, and PH-20 antigens that are essential proteins for sperm function and oocyte fertilization can be the promising candidates. Utilizing the full length or partial length of these proteins individually with reduced fertility goals can partly interfere with fertility [19-21]. Since IZUMO1, SACA3, and PH-20 play a critical role in sperm function, deactivation of these proteins might have a significant impact on fertility [50]. The combination of various peptides involved in different steps of the fertilization, such as sperm-oocyte binding and penetrating sperm into the oocyte, can enhance the efficacy compared to using these peptides alone [79]. Up to now, no one has reported 100% infertility after immunization with sperm antigens. So the combination of these epitopes may enhance the rate of infertility in animal models.

Similar observations have also been reported with the combination of IZUMO and ZP3 epitopes to elicit a robust immune response compared with using IZUMO or ZP3 alone, thereby immunization of female mice with a recombinant fusion protein versus its individual fragments resulted in higher antibody titer [80].

RMSD plots indicate that the chimeric protein is stable during the simulation. MD simulation showed that the predicted structure is stable, and the selected rigid linkers can separate the domains of designed fusion constructs effectively. Toxicity and allergenicity assessment of the chimeric protein is significant and noticeable. The various components of the vaccines (i.e., protein or polysaccharide antigen, adjuvants, and excipients) should be assessed for their direct effects, and be safe without similarity or cross-reactivity with the known allergens and toxins [81].

Our results also demonstrated that this fusion protein has a suitable half-life in mammals like mice, which can stimulate the immune system for a longer time and provide a stronger response. The in silico data revealed that the fusion protein structure has not changed compared with the original structure and makes us use it instead of one protein to enhance the immunogenicity. Since the previous experiments that used surface sperm antigens for infertility did not yield reliable results, so the combination of these proteins is a good option, which requires more clinical trials.

Conclusion

The results of the present study can help us to select an appropriate fusion vaccine for contraception. In summary, all computational analyses showed that the fusion vaccine has structural stability and suitable distance between domains. Also, the flexibility and the solvent accessibility of the construct has an adequate antigen presentation for the immune system. The combination of epitopes as a fusion vaccine can be employed instead of using specific spermatic protein for CV production. Besides, it is expected to have high antibody titer and prolonged sterilization by injecting into animal models.

Ethical Considerations

Compliance with ethical guidelines

Compliance with ethical guideline: All ethical principles were considered in this article.

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Authors contribution's

All authors contributed in preparing this article.

Conflict of interest

The authors declared no conflict of interests.

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