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Original Article

Production of a Novel Multi-Epitope Peptide Vaccine against Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the prevalent cancers in the world with a high recurrence rate. In recent years, different researches have focused on designing efficient multi-epitope peptide vaccines against HCC. In designing these vaccines, over-expressed antigens in HCC patients, such as α- fetoprotein (AFP) and glypican-3 (GPC-3), have been employed. In our previous study, a multi-epitope peptide vaccine for HCC was designed by *in-silico* methods. The designed vaccine construct included the AFP, GPC-3, and aspartyl-β-hydroxylase (ASPH) as CytoLoxic T cell Lymphocytes (CTL), one epitope from Tetanus Toxin Fragment C (TTFrC) as Helper T cell Lymphocytes (HTL), and a segment of microbial heat shock protein (HSP70) peptide$_{407-426}$ as an adjuvant. All the mentioned parts were connected by appropriate linkers. The aim of this study is the production of the designed vaccine.

Methods: This research is experimental and was carried out in Fasa, Iran, in 2017**.** The designed vaccine construct gene was transformed to the *Escherchia coli* BL21 (DE3) strain and expressed in different isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations (0.6 and 1 mM), times (4, 6, 8, 16 hours), and temperatures (25 and 37 °C). Then, the expressed protein was analyzed by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the Western blot methods.

Results: The best conditions for protein expression were obtained in the Super Optimal Broth (SOB) medium at 37 °C after the induction of expression by 1 mM IPTG for six hour.

Conclusion: The recombinant HCC vaccine was produced with a proper concentration.

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Keywords ● Hepatocelluar carcinoma ● Vaccine ● Subunit ● Expression ● Designed

What's Known

- There is a limited number of designed multi-epitope peptide vaccines for hepatocellular carcinoma. In designing these vaccines, over-expressed antigens in hepatocellular carcinoma patients, such as α- fetoprotein, glypican-3, and aspartyl-β-hydroxylase, have been considered.
- Using selected epitopes by *in silico* methods in vaccine construct, instead of the whole antigen, leads to design new vaccines construct which their immunogenesity should be evaluated.

What's New

- In this study, a novel multi-epitope peptide vaccine construct against hepatocellular carcinoma was expressed and purified. The designed vaccine construct is predicted to have high antigenicity.
- A segment of heat shock protein-70 was used as a natural adjuvant in the vaccine construct. The applied adjuvant has not been used in hepatocellular multi-epitope vaccines up to now.

Introduction

Hepatocellular carcinoma (HCC) is the seventh most prevalent cancer in the world and the fourth leading cause of cancer mortality worldwide. Therapeutic choices against HCC are poor, and dependent on the cancer phase. For example, treatments such as tumor resection and liver transplantation are effective for patients in the primary phase of HCC. However, the chance of surgical recurrence is approximately 70% in the first five years.[1,2] Patients in the end stage of HCC are treated with chemotherapy and the drug sorafenib, while only 10% of patients survive more than three years.

Sorafenib is a multi-kinase inhibitor that improves patient rehabilitation.[3] Therefore, an effective therapeutic method against HCC is required. In recent years, researchers have used three main approaches to stimulate the immune response against HCC, including adoptive immunotherapy Appropriates Natural Killer (NK) and Cytokine-Induced Killer (CIK) cells, indirect immunological approaches including vaccines, and indirect non-immunological strategies such as the antigen-coding mRNA system. Vaccines, which belong to the indirect immunological strategies, are an effective strategy against tumor cells and are generally divided into three types, including pulsed dendritic cells (DCs), peptide-based vaccines, and DNA-based vaccines. Peptide-based vaccines might include Tumor-Associated Antigens (TAAs) or Tumor-Specific Antigens (TSAs) and could stimulate the adaptive immune system properly.[4] Multi-epitope peptide vaccines are used against pathogenic bacteria, viruses, and tumor cells. Multi-epitope peptide vaccines, which consist of Cytotoxic T cell Lymphocytes (CTL) and Helper T cell Lymphocytes (HTL) epitopes and provoke humoral and adaptive immune responses, are considered as a beneficial approach for tumor immunotherapy. For designing an effective multi-epitope peptide vaccine against cancer, CTL and HTL epitopes could be selected from TSAs or TAAs.[5] In HCC, several antigens, including α- fetoprotein (AFP),[6] glypican-3 (GPC-3), and aspartyl-β-hydroxylase (ASPH),[7] are known as TAAs. Therefore, they are proper targets for the selection of CTL epitopes. AFP is known as a fetal liver protein, and about 80% of HCC patients have AFP in their blood serum, while the level of AFP is 1–3 ng/mL in healthy people. Thus, serum assessment plays an important function in the diagnosis and therapy of HCC.[8] As mentioned, another TAA in HCC is GPC-3, which pertains to heparan sulfate proteoglycans and plays a critical role in cell growth, survival, and development.[9] GPC-3 is overexpressed in 50% of HCC cases, and not in healthy tissues, and hence, it is a perfect biomarker for the diagnosis of HCC.[10] ASPH is an ideal TAA for HCC immunotherapy and is a transmembrane protein that belongs to the α-ketoglutarate-dependent dioxygenase family.[11] This protein is over-expressed in HCC and infrequently detected in healthy adult tissues.[12] Adjuvants are employed in the vaccine construct to effectively stimulate the immune response in the host and additionally reduce the needed dose of vaccine injection.[13] Heat shock proteins (HSPs) possess diverse functions in biological processes, such as protein folding and degradation, elimination of misfolded proteins, and protein transportation.[14] Moreover, HSPs have a significant role in stimulating the innate and adaptive immunity and support providing of antigens to T $CD8^+$ cells via the Major Histocompatibility Complex class I (MHC-I) pathway.[15] Accordingly, HSP can serve as a powerful immunoadjuvant.[16] In previous studies, two tandem repeats of microbial HSP70 $peptide_{407-426}$ were used as an adjuvant, which improves DC maturation, induction of T $CD8^+$, and T-helper 1 cell responses.[17-19]

In this research paper, a novel multi-epitope peptide vaccine construct against HCC, which was designed by bioinformatics tools in our previous study,[20] was cloned and expressed in *Escherchia coli* host and then purified. The vaccine construct carries epitopes of AFP, GPC-3, and ASPH in the role of CTL epitopes, Tetanus Toxin Fragment C (TTFrC) as HTL epitopes, and two tandem repeats of $HSP70_{407-426}$ in the guise of a natural immune-adjuvant.

Materials and Methods

Host Strain and Culture Media

This experimental research was carried out at Fasa University of Medical Sciences, Iran, in 2017. The ethics committee approval code for this research is IR.FUMS.REC.1397.116.

E. coli BL21 (DE3) strain is a Gram-negative straight rod, and an acceptable organism to represent recombinant proteins. This strain can lead to high-yield protein expression, because it is under the control of the T7 promoter. In this study, *E. coli* BL21 (DE3) strain was applied for transformation. Furthermore, the pET-23d expression vector carries a C-terminal and N-terminal His Tag coding sequence and possesses an ampicillin resistance marker. This vector was utilized for gene expression; and Super Optimal Broth (SOB) medium, including 20 g/L trypton, 5 g/L yeast extract, 0.5 g/L NaCl, 2.5 mL 1 M KCl, and 10 mL 1 M $MgCl_2$ was applied as media culture for protein expression.

Designed Vaccine Construct

Using bioinformatics tools, the design of a novel multi-epitope peptide vaccine against HCC was carried out.[20] Several servers were used to increase the accuracy of the vaccine design, including IEDB at http://tools.immuneepitope.org/analyze/html/mhc_binding.html, that uses some methods for prediction of MHC-I binding peptides, including Stabilized Matrix Method (SMM), Average Relative Binding (ARB), and Artificial Neural Network (ANN).[21] ProPred-I at www.Imtech.res.in/raghava/propred1/, uses quantitative matrix in prediction of the MHC class-I peptide.[22] PAComplex at http://

PAcomplex.life.nctu.edu.tw, it is the initial server which predicts peptide-MHC and peptide-TCR interaction.[23] BCPRED at http://ailab-projects1.ist.psu.edu:8080/bcpred/predict.html, the server selected the linear B cell epitopes based on the new method of the subsequence kernel which was based on a data collection of 701 linear B-cell epitopes and 701 nonepitopes.[24] These servers were used for the prediction of various immunogenic epitopes, including HTL, CTL, and B cell epitopes. The selected epitopes were joined together by proper linkers. Finally, antigenic, allergenic, and solubility properties of the designed vaccine construct were evaluated using appropriate servers. More details were mentioned in our previous work.[20] The vaccine construct, which subtends CTL epitopes (AFP, GPC-3, ASPH), HTL epitopes (TTFrC), and immunoadjuvant (two tandem repeats of microbial $HSP70_{407–426}$), was cloned into the pET- 23d vector (ZistEghtesad Mad, Iran).

Transformation

E. coli BL21 (DE3) competent cells were prepared and plasmid vector pET-23d was transformed into competent cells using the calcium chloride method.[25] The transformed cells were grown in a Laurie-broth **(**LB) (Miller, China) plate containing 100 µg/mL ampicillin (Jaberebn Hayyan, Iran) and incubated at 37 °C for 24 hours.

Expression of the Vaccine Protein

The culture media containing transformed *E. coli* was at 37 °C overnight. After that, 1 mL of the culture media was diluted in 5 mL of fresh culture in SOB media comprising of 100 µg/mL ampicillin (Jaberebn Hayyan, Iran) on the shaker incubator at 37 °C, and was shaken until an Optical Density (OD) of 0.6 was achieved at 600 nm after about five hours. Then, the media was induced via two different concentrations of Isopropyl β-D-1-thiogalactopyranoside (IPTG), including 0.6 and 1 mM, and different times, times, including 4, 6, 8, 16 hours, and two different temperatures (25 and 37 °C) were evaluated. Subsequently, the transformed cells were harvested via centrifugation at 10000 rpm for five minutes at room temperature, and the obtained pellets were stored at -20 °C for further research.

SDS-PAGE Analysis

The expression of the recombinant vaccine protein in several above-mentioned conditions was measured with Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE),[26] where 4 λ pellet (harvested in the previous step) was blended in 10 λ sample buffer and 6 λ DW, and heated at 95 °C for five minutes and centrifuged at 15000 rpm for 10 minutes at 4 °C. In the next step, an amount of 10 λ of the sample was loaded on the SDS-PAGE gel (12%) (Bio-Rad-USA), the protein marker and the power supply was run on 100-120 V for about two hours. Following that, the gel was stained with Coomassie Brilliant Blue (Biobasic-Canada) solution for about 16 hours, and then de-stained.

Purification of the Vaccine Protein

The purification of the recombinant protein was carried out using Ni-NTA affinity column chromatography (QIAGEN Company, Germany) following denaturing conditions. The bacterial pellets were solved in the lysis buffer (pH=8, 7 M urea, 0.1 M NaH_2Po_4, 0.01 M Tris-Cl) and shaken for about 20 minutes. In the next step, the cells were disrupted via ultrasonication (six-time six-second cycles at 4 °C). The suspension was centrifuged at 12000 ×g for 20 minutes at 4 °C, and the supernatant was decanted to the Ni-NTA column and centrifuged (two minutes at 890 ×g). The column was washed repeatedly via a wash buffer (pH=6.3, 8 M urea, 0.1 M NaH_2Po_4, 0.01 M Tris-cl) to remove the waste material. Finally, elution buffer (pH=4.5, 8 M urea, 0.1 M NaH_2Po_4, 0.01 M Tris-Cl) was used, and the purified protein was evaluated by SDS-PAGE.

Western Blot Test

To prove the expression and purification of the recombinant vaccine protein, the Western blot method was employed.[27] In this technique first, proteins were detached and transferred onto a polyvinylidenefluoride membrane (PVDF). In the next step, the PVDF paper was blocked with 5% skim milk for about 16 hours at 4 °C. Anti-his tag antibodies were inserted for two hours with agitation. Then, they were washed three times with tween 20 (0.1%) in phosphate-buffered saline (PBS). Finally, after adding a diaminobenzidine (DAB) solution (0.05%) with 30 µL hydrogen peroxidase (30%) for 20 sec, the purified protein became visible.

Results

Vaccine Construct and Transformation

The designed vaccine containing immunogenic epitopes against HCC was cloned into a pET-23d expression vector. The expression vector was transformed inside the competent *E. coli* BL21 (DE3) strain, and then, the transformed *E. Coli* was grown on LB agar containing 100 µg/mL ampicillin (figure 1).

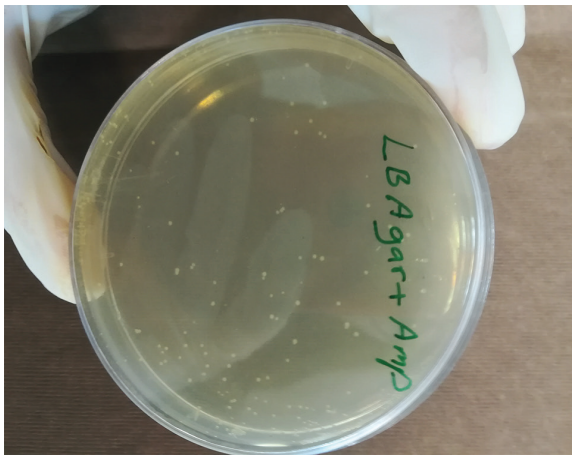

Figure 1: Transformed *E. coli* BL21 (DE3) was grown on an agar plate including 100 µg/ml ampicillin.

Expression of the Vaccine Protein

The recombinant bacteria were cultured in SOB media containing 100 µg/mL ampicillin. Bacteria were grown to reach the OD of 0.6 at 600 nm, the bacterial growth rate over time is shown in table 1. Then, different IPTG concentrations (0.6 and 1 mM) were administrated in different conditions, including several times (4, 6, 8, and 16 hours) and several temperatures (25 and 37 °C). The recombinant protein was verified in all mentioned conditions. The band of the vaccine protein was about 32.3 kDa, which was in accordance with the molecular weight of the designed construct as predicted by the bioinformatics tool. Furthermore, optimal protein expression was noticed in the SOB medium at 37 °C after expression induction by 1 mM IPTG for six hours. The results of the SDS-PAGE analysis are shown in figure 2.

Purification of the Protein

The recombinant protein had His-tag at the C- and N-terminal; therefore, purification was accomplished using Ni-NTA affinity column chromatography. The recombinant protein was purified by denaturing conditions and verified by SDS-PAGE (figure 3).

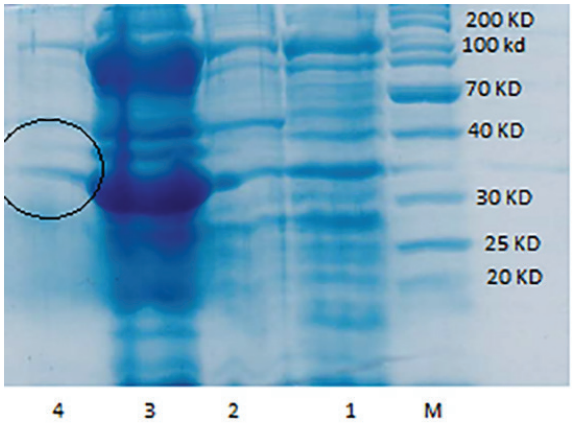

Figure 3: The result of SDS-PAGE analysis of the multi-epitope peptide vaccine purification. Line 1 shows transformed *E. coli* after expression induction for 6 hours using 1M IPTG, lines 2-3 show the unpurified peptide, and line 4 shows the purified peptide vaccine.

Table 1: Bacterial growth rate based on Optical Density at 600 nm

OD	h
0.01	2
0.09	3
0.30	4
0.53	4.30
0.60	4.40

OD: Optical density; h: Hours after bacterial culture

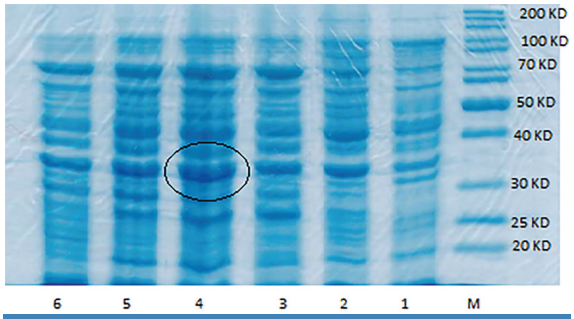

Figure 2: SDS-PAGE analysis of the multi-epitope peptide vaccine expression in 1 mM IPTG on SOB medium at 37 °C. Line1 shows a lysate of *E. coli* BL21 (DE3) as control, line 2 shows the expressed protein before expression induction by IPTG, and lines 3-6 show the protein expression after expression induction by IPTG for 4, 6, 8, and 16 hours, respectively. The black circle represents the optimal protein expression.

Western Blot Test

The expression and purification of the designed multi-epitope peptide vaccine were evaluated by the Western blot analysis. As shown in figure 4, the purified protein was confirmed by the Western blot method.

Discussion

In this research, a novel multi-epitope peptide

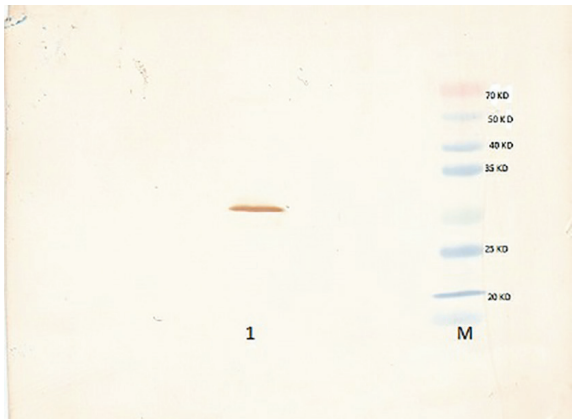

Figure 4: The Western blot analysis results. Line 1 shows the purified protein using the Western blot technique.

vaccine was expressed in a bacterial host (*E. coli* BL21 (DE3) strain) using SOB and LB culture media and purifying it for HCC immunotherapy. The purified peptide vaccine was confirmed by the SDS-PAGE and Western blot techniques, and based on the obtained results, the molecular weight of the purified vaccine was 32.3 KDa.

HCC is a cancer with a high mortality rate. This malignancy is not usually diagnosed in the primary stages, because it is known as a heterogeneous disorder that includes several etiological agents; therefore, it is diagnosed in the advanced stages, when it is usually late for treatment.[19] At the advanced stages, only sorafenib and regorafenib drugs are effective for HCC, and other treatments, such as surgery, are not helpful. Thereby, effective treatment against HCC is needed. Recently, scientists have considered immunotherapeutic methods to treat HCC, and some of the clinical trials on HCC patients include cytokine relying therapies such as interferons,[28] cell-based trials such as dendritic cell vaccines,[29] antibody-based treatments including AFP antibodies,[30] and antigen-specific vaccines such as GPC-3 peptide-based vaccine.[31] In particular, researchers have recently studied multi-epitope peptide vaccines as a new immunotherapeutic approach against HCC. Various investigations have utilized bioinformatics tools to design beneficial multi-epitope peptide vaccines, and bioinformatics makes that process easier.[32] Multi-epitope peptide vaccines possess some advantages over the others. They are detected through several T-cell subsets due to the presence of various MHC-restricted epitopes, which can powerfully stimulate the humoral and cellular immune responses, as they consist of several B-cell, CTL, and HTL epitopes. The presence of adjuvant in their compound increases their immunogenicity.[33] In our previous study, a novel multi-epitope peptide vaccine was designed against HCC via bioinformatics tools.[20] The designed vaccine has several CTL epitopes (AFP, GPC-3, and ASPH), HTL epitopes (TTFrC), and a powerful natural adjuvant (two tandem repeats of microbial $HSP70_{407–426}$). All epitopes were chosen by several bioinformatics servers. Important properties of the designed vaccine construct, such as antigenicity, were checked by ANTIGEN pro at (http://scratch.proteomics.ics.uci.edu) and VaxiJen. (http://www.jenner.ac.uk/VaxiJen), which showed that the designed vaccine possesses high antigenicity. Furthermore, based on the results of the ALgPred server (https://webs.iiitd.edu.in/raghava/algpred2/), the designed vaccine construct was not allergenic, and some physicochemical parameters, such as high half-life and stability, were verified by the ProtParam server (http://web.expasy.org/protparam/). The linear and conformational B-cell epitopes and interferon-gamma-inducing epitopes were recognized in the vaccine construct, because provoking the humoral and cellular immune responses is essential in multi-epitope peptide vaccines.[34] AFP is one of the most important antigens considered in vaccine development against HCC.[35] In 2018, Zhang tested AFP in the development of a dendritic cell-based vaccine for HCC. Adenoviral-encoded synthetic AFP and different forms of AFP were investigated to activate the T $CD8^+$ and T $CD4^+$ cells. These results showed that the secreted form of AFP leads to enhanced T $CD4^+$ and T $CD8^+$ cells responses.[36] For the first time, the Phase I clinical trial of GPC-3 peptide-based vaccine was analyzed in 2012 by Gilboa. Moreover, Yu Sawada and colleagues finished the Phase II clinical trial on 41 patients with HCC in 2016 which demonstrated proper immune response.[37] In recent years, new research has been conducted based on ASPH for the treatment of HCC. Greten and colleagues designed and analyzed the ASPH-DC vaccine *ex vivo* on a mouse model in 2019, and the results showed that the designed vaccine led to increased T-cell response and activated the pre-apoptosis mechanism.[38] Therefore, based on the literature review, we used a combination of the antigens (AFP, GPC-3, and ASPH) that are over-expressed in HCC patients, which are known as suitable targets for the design of peptide-based vaccines. Immunogenic epitopes were used in the vaccine construct instead of the whole antigens. In addition, we used two tandem repeats of $HSP70_{407-426}$ as adjuvans, which showed the potential to stimulate humoral and cellular responses effectively in previous research. HSP70 leads to the production of IFN-γ and IgG2a, which are effective in the Th1 response.[39]

In this study, to join CTL epitopes, the SSL and HEYGAEALERAG motifs were used as linkers. The HEYGAEALERAG linker is a cutting target for the proteasomal machine.[34] Furthermore, HTL epitopes attached via KK linker and EAAAK linker were applied to connect the adjuvant, which is a rigid motif that leads to the increased stability and folding of the vaccine construct.[40] The next step for the development of the multi-epitope peptide vaccine is the prosperous cloning and expression of the vaccine construct in the proper expression vector. In this research, we utilized the expression vector pET-23d, which is an accepted vector for the production of recombinant proteins in bacterial

host *E. coli*.[41] To find the optimal expression of the designed vaccine, the vaccine construct was expressed in different conditions, including various concentrations of IPTG (0.6, 1mM) at two different temperatures (25 and 37 °C) and four different times (4, 6, 8, and 24 hours). According to the SDS-PAGE results, the best conditions for optimal protein expression were in the SOB medium at 37 °C after expression induction by 1 mM IPTG for six hours. The expression and purification of the designed vaccine protein were confirmed by SDS-PAGE and Western blot techniques, which showed a single band with 32.3 KDa molecular weight. Thus, based on the obtained results, we believe that the purified vaccine protein can stimulate humoral and cellular immunity against HCC patients effectively.

Based on the bioinformatics analysis, the produced vaccine has proper characterizations, such as high antigenicity and stability. However, in order to confirm these results, it is necessary to evaluate its immunogenicity in animal models.

Conclusion

In the present research, a novel multi-epitope peptide vaccine against HCC was produced. The multi-epitope construct vaccine contained CTL epitopes selected from AFP, GPC-3, and ASPH antigens, HTL epitopes from TTFrC, and an efficient natural adjuvant (HSP70), which were joined together by appropriate linkers. The best conditions for the vaccine construct expression was found to be the SOB medium at 37 °C after expression induction by 1 mM IPTG for six hours. Hence, the purified peptide vaccine can potentially stimulate the immune system properly, although further *in vitro* and *in vivo* experimental investigations on the designed vaccine are truly necessary.

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Authors' Contribution

F.M.D: Contributed in design of the work, the acquisition, and analysis of data drafting the manuscript; S.H.M: Contributed in interpretation of data for the work and drafting and revising the work critically for important intellectual content. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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