Research in Molecular Medicine

The Modified Recombinant Proinsulin: A Simple and Efficient Way to Produce Insulin Glargine in *E. coli*

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ABSTRACT

Background: Recombinant insulin glargine, a long-acting analog of insulin, is expressed as proinsulin in the host cell and, after purification and refolding steps, is processed to mature insulin by using trypsin and carboxypeptidase B. Because of several internal residues of arginine and lysine in the proinsulin B and C chains, several unwanted products are formed after treatment with these enzymes. To overcome this problem, we introduced three thrombin recognition sites into the proinsulin encoding sequence.

Materials and Methods: After the design, the modified proinsulin encoding sequence containing the 5′ His-Tag tail and three thrombin recognition sites located between the His-Tag and B chain, B and C chains, and C and A chains, respectively, was synthesized by overlap extension Polymerase Chain Reaction (PCR) using seven specific primers in multiple sequential PCR reactions. The final amplified fragment was cloned in the pGEM-5zf vector by the EcoRV enzyme. After sequencing, the modified proinsulin encoding sequence was subcloned into the pET-26b(+) vector using NdeI and XhoI enzymes. Finally, the modified proinsulin was expressed in *E. coli* BL-21(DE3) by induction with Isopropyl β-d-1-thiogalactopyranoside (IPTG).

Results: The accuracy of the synthesized modified proinsulin sequence was confirmed by DNA sequencing. The modified proinsulin cloning was evaluated by PCR with specific primers and digestion with specific restriction enzymes. In this study, the modified proinsulin protein was expressed up to 40%. The modified proinsulin protein expression was assessed using sodium dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blotting.

Conclusion: This modified proinsulin can be used to easily and efficiently produce insulin glargine without any impurities after processing with thrombin in one step in a nickel chromatographic column.

Introduction

Recombinant Deoxyribonucleic acid (DNA) technology has created an excellent opportunity to produce different pharmaceutical and industrial proteins [1, 2]. Insulin was one of the first proteins produced by this method [3]. In 1979, Goeddel et al. cloned and expressed the insulin in the *E. coli* expression system [4]. They cloned and expressed the A and B chains separately. Since then, many attempts have been made to clone insulin in various expression systems, and differ-

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ent strategies have been used to express this protein as proinsulin and then process it into adult insulin [5-20].

There are two different forms of insulin, one with an immediate effect and the other with a prolonged effect. Rapid-acting insulin such as lispro, aspart, and glulisine, begins to lower blood sugar within 15-30 min after injection and continues to work up to 5 h. Long-lasting insulin as insulin glargine, insulin detemir, and insulin degludec reach the bloodstream and begin to lower blood glucose within 1-2 h and continue to work up to 40 h. As a long-acting basal human insulin analog, insulin glargine (Lantus) has a smooth time-action profile and no significant peak [21]. Compared to other long-acting insulins, this analog closely mimics normal physiologic concentrations of insulin [22]. Insulin glargine is composed of A and B chains connected through C-peptide. There are two main cleavage sites in insulin glargine: one between A-chain and C-peptide and the other between B-chain and C-peptide. However, some alternative trypsin cleavage sites in the sequence reduce the efficiency of the active insulin production.

In this study, we investigated whether the human proinsulin encoding sequence containing thrombin sites could be synthesized with Polymerase Chain Reaction (PCR) and if the synthesized fragment could be properly expressed in *Escherichia coli*. For this purpose, we designed the human proinsulin sequence with three thrombin recognition sites. The first recognition site separates the histidine tag from the proinsulin protein. The second site separates B-chain from C-peptide, and the last one separates the C-peptide from A-chain.

Materials and Methods

Chemicals, enzymes, and media

Luria-Bertani agar and Luria-Bertani broth were purchased from Sisco research laboratories. Agarose, sodium dodecyl sulfate (SDS), glycine, glycerol, acrylamide, and bis-acrylamide were purchased from Sigma Company (Germany). Bromophenol blue, 2-mercaptoethanol, Coomassie Brilliant Blue G-250, Ethylenediaminetetraacetic Acid (EDTA), Ammonium Persulfate (APS), Tetramethylethylenediamine (TEMED), Bovine Serum Albumin (BSA), diaminobenzidine (DAB), and other chemicals were purchased from Merck Company (Germany). Isopropyl β-D-1-thiogalactopyranoside (IPTG), anti-His tag monoclonal antibody, and anti-mouse HRP were purchased from Roche Company (Germany). PCR master mix, GeneRuler DNA Ladders, and restriction enzymes (EcoRV, NdeI, and XhoI) were purchased from Thermo Fisher Scientific Company (USA). DNA and plasmid extraction kits were purchased from GeneAll Company (South Korea). *E. coli* DH5α and BL-21(DE3) strains and pET-26b(+) expression vector were purchased from Novagen Company (USA). PGEM-5zf cloning vector was purchased from Promega Company (USA).

Designing the modified proinsulin sequence

A DNA sequence encoding the entire length of modified proinsulin, containing three thrombin cleavage sites and the 6His-Tag tail, was designed using GenBank sequences for proinsulin gene (NC_000011.10), a synthetic human proinsulin sequence that had been cloned into pBHA plasmid vector, and thrombin cleaving sites (GSLQPRF, RGHRP, and GSLQPRG). The sequence was flanked by NdeI and XhoI recognition sites for the following cloning step. To reach the high-rate expression, the sequence was optimized based on *E. coli* codon usage and analyzed by the RNA Draw program (http://www.rnadraw.com).

Synthesis of the modified proinsulin

A synthetic proinsulin sequence, cloned into pBHA plasmid in a previous study by Feizi et al. [12], was used as a template to synthesize the modified proinsulin sequence by overlap extension polymerase chain reaction

Table 1. Primers used in this study

Primer Name	Sequence (5'→3')	Length, bp
F11	GGTCATATGCACCATCACCACCATCACGGATCACTGCAACCACGTTTTGTAATCAGCACCTTTGTGG	68
F1	GGTCATATGCACCATCACCACCATCACGGATC	32
F2	CTCGCCGTGGTCATCGTCCTGAAGCGGAAGATCTCCAG	38
F3	GAAGGCTCGTTACAACCGCGTGGCATTGTTGAGCAGTG	38
R1	GGACTCGAGATTAGCCGCAGTAGTTCTCCAGCTG	34
R2	CTTCCGCTTCAGGACGATGACCACGGCGAGTTTTCGGCGT	40
R3	CTCAACAATGCCACGCGGTTGTAACGAGCCTTCCAACG	38

(SOEing PCR) (Figure 1). To do overlap extension PCR, specific primers were designed using Gene Runner software v 6.5.52 Beta (Table 1). The PCR reactions were prepared and performed according to kit instructions.

In PCR1, F11 and R1 primers were used to insert the 6His-Tag sequence and the first thrombin recognition site at the 5' end of the proinsulin. In the next step, the first overlap extension PCR was used to introduce the second thrombin recognition site, which separates the B-chain from C-peptide. Therefore, F_1/R_2 and F_2/R_1 primers were separately used to amplify two fragments from the PCR1 product. Then, the PCR2 and PCR3 products were gel-purified using Expin™ Gel SV (GeneAll, South Korea) and according to the manufacturer's instructions. The purified fragments were exploited as the templates for PCR4 reaction using F_1 and R_1 primers. The PCR4 product was the 328-bp full-length fragment of proinsulin containing the first and second thrombin site sequences. The second overlap extension PCR was performed to introduce the third thrombin recognition site, which separates the C-peptide from A-chain. Thus, F_1/R_3 and F_3/R_1 primers were separately used to amplify two fragments from the PCR4 product. Then, the PCR5 and PCR6 products were gel purified, and the purified fragments were applied as the template for the PCR7 reaction using F_1 and R_1 primers. The PCR product of the final step (PCR7) was the 328-bp full-length fragment of modified proinsulin containing the three thrombin recognition sites sequences.

Cloning the modified proinsulin coding sequence

First, the pGEM-5zf vector was digested by the EcoRV restriction enzyme. Both digested vector and modified proinsulin coding sequence were recovered from agarose gel with GeneAll Expin Gel SV kit. After ligation, the transformation was performed in *E. coli* DH5α competent cells, and transformants were cultured on LB-agar plates containing ampicillin (100 µg/mL) and incubated at 37°C overnight. To verify the accuracy of the recombinant vector (pGEM-proInsG), 5 colonies were selected, and plasmid extraction was performed by GeneAll Exprep Plasmid SV kit according to the manufacturer's instruction. The modified proinsulin coding sequence was verified by colony PCR amplification using F_1 and R_1 primers and restriction enzyme mapping. The modified proinsulin fragment was sequenced to show the expected mutation in the sequence. Then, the sequence was aligned with the expected sequence using Clustal Omega online software v. 1.2.2[1]. Afterward, the modified coding sequence was subcloned in pET-26b(+)using NdeI and XhoI enzymes. Restriction enzyme mapping and PCR amplification were performed to confirm the encoding sequence in the vector.

1. https://www.ebi.ac.uk/Tools/msa/clustalo/

Expression of the recombinant modified proinsulin protein

The recombinant plasmid (pET-proInsG) was transformed into the competent *E. coli* BL-21(DE3) cells, and expression of proinsulin protein was induced by adding IPTG at a final concentration of 1 mM. The 1 mL-cell samples were harvested before induction and every hour from 1 to 4 (h) after induction for 1 min centrifuging at 10000×g. Then, the cell samples were lysed by adding lysis buffer (urea 8 M, NaH2PO4 100 mM, Tris-Cl 10 mM) for 2 h at 37°C. The mixture was centrifuged for 30 (min) at 13000×g and supernatant electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed to confirm the expression of proinsulin using an anti-His tag monoclonal antibody.

Results

Modified proinsulin designing

In this research, the proinsulin coding sequence was designed and synthesized as [His-tag]-[Gly Ser Leu Gln Pro Arg]-[B-chain]-[Arg Arg Gly His Arg Pro Glu Ala]-[C-chain, without two Arg residues at the end of 5']-[Gly Ser Leu Gln Pro Arg]-[A-chain] wherein the His-Tag tail was considered for 1-step purification using the Ni^{+2}-NTA column.

Synthesis of the modified proinsulin sequence

First, using F_{11} and R_1 primers, the 6His-Tag and first thrombin recognition site were introduced in the proinsulin coding sequence. The PCR product of this step (PCR1) was a 316-bp full-length fragment of proinsulin containing 6His-tag and the first thrombin recognition site without two other sites (Figure 2, lane 1). In the next step, two overlap extension PCRs were used to introduce the second and third thrombin recognition sites in the proinsulin coding sequence. F_1 and R_2 primers were used to amplify the first fragment of the first gene SOE. It was expected that the PCR product of this amplification reaction (PCR2) would be a 163-bp fragment (Figure 2, lane 2). Also, F_2 and R_1 primers were used to amplify the second fragment of the first gene SOE. The PCR product of this amplification reaction (PCR3) was a 195-bp fragment (Figure 2, lane 3). Then, gel-purified fragments of the PCR2 and PCR3 were used to construct and amplify

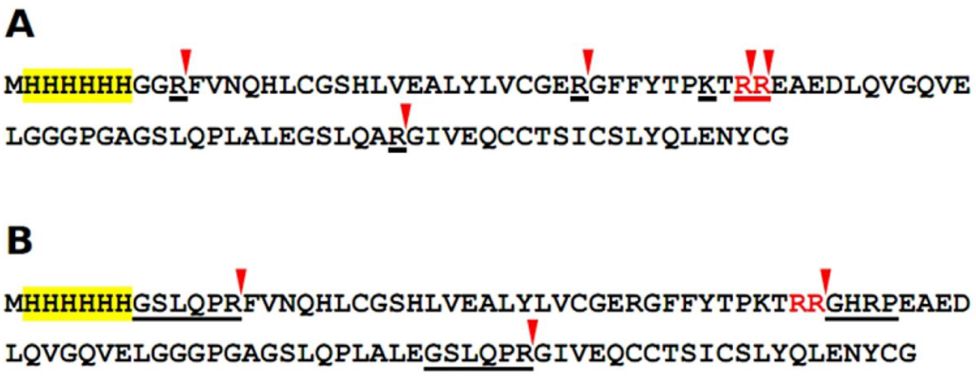

Figure 1. Primary amino acid sequences of template (A) and modified (B) proinsulin proteins

In both sequences, the His-Tag tail has been highlighted, and the trypsin (A) and thrombin (B) recognition sites have been presented as underlined. The trypsin and thrombin cleavage sites have been shown with red arrows.

the first gene SOE using F_1 and R_1 primers. The PCR product of this step (PCR4) was a 328-bp full-length fragment of proinsulin containing the 6His-tag, first and second thrombin recognition sites (Figure 2, lane 4). Then, using the first SOE PCR product as the template and F_1 and R_3 primers, a 264-bp fragment was amplified (PCR5) (Figure 2, lane 5). The same reaction was performed using F_3 and R_1 primers, which resulted in a 97-bp fragment (PCR6) (Figure 2, lane 6). Further amplification using F_1 and R_1 primers and the products of the two previous reactions (PCR5 and PCR6) as templates resulted in the amplification of a 328-bp fragment which was the modified proinsulin coding sequence containing 6His-Tag and 3 thrombin recognition sites (Figure 2, lane 7).

Cloning the modified proinsulin coding sequence into pGEM-5zf vector

After cloning the modified proinsulin into the pGEM-5zf vector, the accuracy of the encoding sequence in the vector was evaluated using PCR and restriction enzyme digestion (Figure 3). As seen in Figure 3A, the 328-bp fragment was amplified using F_1 and R_1 primers. The cloning process was confirmed by digestion of extract-

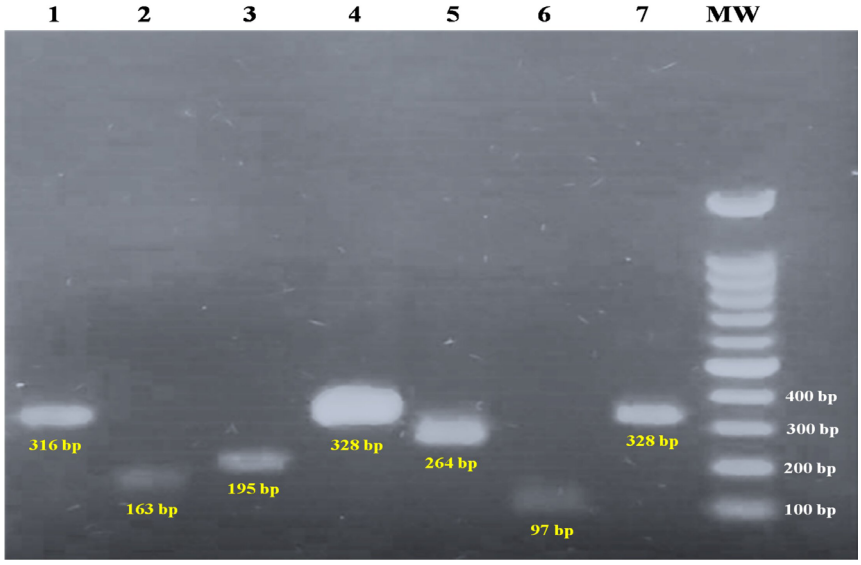

Figure 2. Construction of modified proinsulin fragment using overlap extension PCR

Lane 1, Amplification using F_{11} and R_1 primers to introduce the first thrombin recognition site (316 bp); Lane 2, Amplification of the first fragment of the first gene SOE using F_1 and R_2 primers (163 bp); Lane 3, Amplifying the second fragment of the first gene SOE using F_2 and R_1 primers (195 bp); Lane 4, Amplifying the first gene SOE by using F_1 and R_1 primers to introduce the second thrombin recognition site (328 bp); Lane 5, Amplification of the first fragment of the second gene SOE using F_1 and R_3 primers (264 bp); Lane 6, Amplification of the second fragment of the second gene SOE using F_3 and R1 as primers (97 bp); Lane 7, Amplifying the second gene SOE by using F_1 and R_1 primers to introduce the third thrombin recognition site (328 bp); MW1, 50-bp DNA ladder, and MW2, 100 bp DNA ladder.

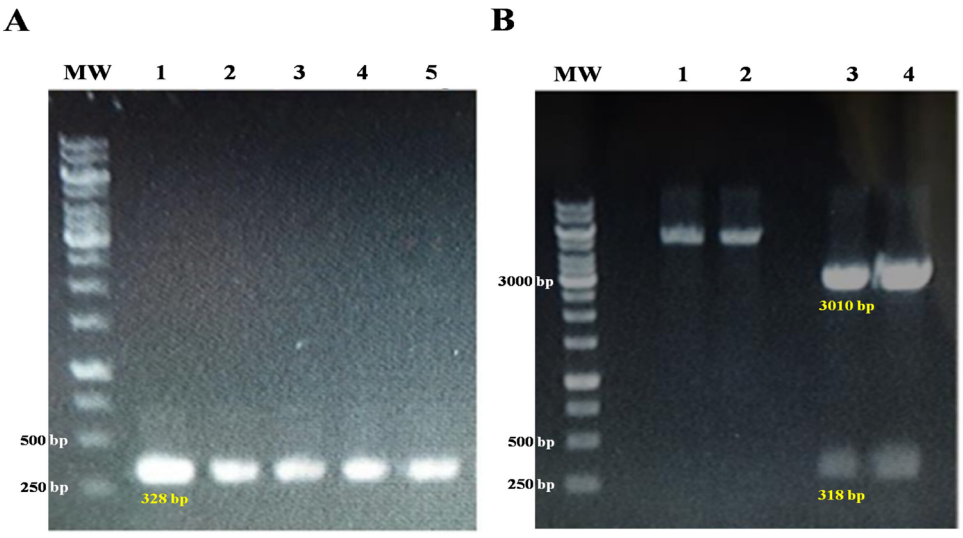

Figure 3. Evaluation of The Modified Proinsulin Cloning in the pGEM-5zf

A: Confirmation of cloning by PCR using F_1 and R_1 primers. Lanes 1-5, PCR product from 5 different colonies; MW, 1 kb DNA ladder; B: Evaluation of the insertion of the modified proinsulin fragment into the pGEM-5zf cloning vector by using NdeI and XhoI digestion. Lane 1, 1 kb DNA ladder; Lanes 1-2, undigested plasmids from two different colonies; Lanes 3-4, digestion product from the same plasmids.

ed plasmids using NdeI and XhoI restriction enzymes (Figure 3B). As seen, the 318-bp and 3010-bp fragments have been obtained from the slit of the recombinant plasmid (lanes 3 and 4). Then, the modified proinsulin fragment was sequenced, and the result confirmed the accuracy of the cloned fragment. As seen, the sequence has been modified successfully. After sequencing, the modified proinsulin encoding sequence was recorded with accession number MF927971 in the Gene Bank.

Modified proinsulin expression

To express the modified proinsulin coding sequence, it was first subcloned into pET-26b and confirmed by digestion with NdeI and XhoI (data not shown). Then, the proinsulin gene was expressed in *E. coli* BL-21(DE3) through induction by IPTG, and the result was analyzed by SDS-PAGE (Figure 4A). The Figure shows the presence of an 11-KD protein on the gel. Western blot confirmed the proinsulin protein expression using an anti-His-Tag antibody (Figure 4B).

Discussion

The human insulin encoding gene was first chemically synthesized in 1978 [3] and cloned and expressed in *E. coli* in 1979 [4]. Since then, this protein and its several analogs have been produced in different expression systems, including *E. coli* [5-12], mammalian cells [13-15], yeast [16], transgenic plants [17], etc. Glargine, one

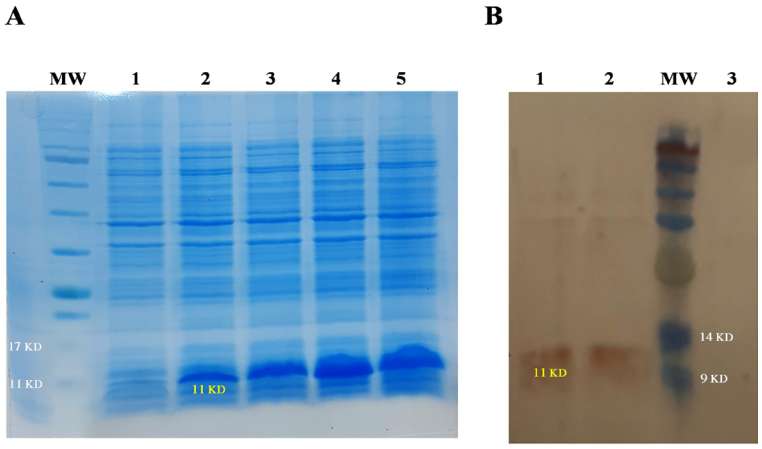

Figure 4. Analysis of the modified proinsulin expression

A: SDS-PAGE analysis. Lane 1, uninduced sample; Lanes 2-5, induced samples at 1 to 4 hours after induction; B: Western blotting analysis using an antibody against His-tag. Lanes 1-2, induced samples; Lane 3, uninduced sample; MW, protein molecular weight marker.

of the insulin analogs, due to its pharmacodynamics and pharmacokinetic properties, and basal long activity, is preferred to fast-acting analogs of insulin, such as lispro and glulisine (Apidra) and has been developed by Aventis Pharmaceuticals and approved by US regulatory authorities in 2004.

Glargine is expressed as proinsulin, and then the trypsin enzyme is used to cut the C-peptide out and form mature insulin. However, because of the alternative recognition sites of trypsin in the proinsulin protein sequence, one drawback of this method is the production of unwanted insulin derivatives, such as des-threonine, which in turn reduces the efficiency of the insulin production. Many strategies have been exploited to solve this problem and reduce the production of unwanted insulin products. Hilgenfeld et al. reviewed several studies performed to produce and use insulin glargine in the care of diabetes [18]. Son et al. used citraconic anhydride to decrease the accumulation of unwanted insulin derivatives [19]. The process consists of two steps. In the first step, lysine residue in position 29 of the B-chain (B29) is reversibly blocked through citraconylation. In the next step, the precursor insulin is cut in specific sites by using trypsin and carboxypeptidase B. Based on this method, some patents are also invented [20]. Saeedinia et al. designed and expressed synthetically modified proinsulin to produce insulin glargine using this method [22]. They used a pET-21b(+) expression vector to express synthetic human proinsulin in *E. coli* BL21(DE3), which expressed approximately 40% of the total protein and was 80% pure after purification. Hwang et al. have cloned proinsulin sequence in a pPT-GI vector expression and transformed it into *E. coli* JM109 cells [23]. They also used this method to produce recombinant glargine insulin. In their study, the dry cell mass was 18 g/L, and the produced recombinant protein was 38.52% of the total protein. In addition to these methods, Abd El-Aziz et al. used in vitro design and synthesis of a modified proinsulin gene by computational methods [24]. Despite these achievements in the production of insulin glargine, the proinsulin protein still had limitations due to the presence of Arg and Lys residues when cut with trypsin. To solve this problem, Oka et al. designed the modified proinsulin containing three thrombin recognition sites to produce the regular insulin in Bacillus subtilis cells [25].

In this study, we have employed the mentioned method to produce insulin glargine in *E. coli*. In the present study, a new construct for the proinsulin expression was designed and constructed via site-directed mutagenesis using overlap extension PCR, in which three thrombin recognition sites are introduced into the proinsulin encoding sequence. This study indicates the proper and precise design of primers to the desired mutations in the initial sequence containing the trypsin cleavage site and constructs the correct sequence of the proinsulin glargine encoding sequence containing the thrombin cleavage site. SDS-PAGE and western blotting displays the suitability of the pET26-ProInsG expression system to produce the recombinant proinsulin protein up to 40% of the total protein in inclusion body form in *E. coli*. We aim to use an appropriate strain such as *E. coli* Rosetta-gami B (DE3) to express the soluble and proper recombinant proinsulin protein.

Conclusion

In this study, a new modified proinsulin coding sequence was designed and synthesized. The modified proinsulin can be used to simply and efficiently produce the insulin glargine without any impurity following thrombin treatment within the chromatography column containing nickel-agarose beads.

Ethical Considerations

Compliance with ethical guidelines

There were no ethical considerations to be considered in this research.

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Authors contribution's

Writing the original draft, preparation, and investigation: Seyyedeh Khadijah Hosseini Oula; Writing, reviewing and editing, supervision, and investigation: Ali Reza Saeedinia; Reviewing and editing: Mehdi Zeinoddini.

Conflict of interest

The authors declared no conflict of interest.

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