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Engineering a DYRK1B R102C mutation: insights into metabolic syndrome pathogenesis through lentiviral gene delivery

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Abstract

Background: A rare heterozygous DYRK1B mutation (R102C) recently linked to a familial form of metabolic syndrome prompted this study to introduce the R102C mutation into the mouse *DYRK1B gene*, utilizing recombinant lentiviruses for long-term gene expression.

Methods: In the present fundamental study, the DYRK1B R102C mutation was generated via Overlap Extension-PCR (OE-PCR) and inserted into the LeGO-iG2 transfer vector with a GFP marker. Recombinant lentiviruses were produced by co-transfection of the transfer vector carrying DYRK1B R102C, psPAX2 (Packaging vector), and pMD2 (Envelope vector) into HEK-293T cells.

Results: The accuracy of the intended mutation was confirmed through OE-PCR and sequencing. Expression of DYRK1B and successful gene transfer were visualized using a fluorescence microscope to detect the GFP marker. Lentiviral titer was quantified using flow cytometry, with an infection efficiency of 108 TU/ml in HEK-293T cells.

Conclusion: DYRK1B plays a crucial role in the pathogenesis of metabolic syndrome, central obesity, early-onset coronary artery disease, hypertension, type 2 diabetes, and adipogenesis, suggesting its potential as a target for therapeutic interventions. Lentiviruses carrying the DYRK1B R102C mutation offer significant advantages for both *in vitro* and *in vivo* research on metabolic syndrome. This study showcases the successful application of recombinant lentiviral vectors for gene transfer into eukaryotic cells.

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Introduction

Metabolic syndrome (MetS) is determined by a set of multiple physical diseases that concertedly increase diabetes mellitus, cardiovascular disease, and vascular and neurological complications such as cerebrovascular accidents (1-4). According to the World Health Organization (WHO), blood pressure, obesity, and lipid disorders are essential components of MetS (5-7). This syndrome is accompanied by a decrease in the life quality of patients and causes serious damage to the healthcare system (8,9). It should be noted that MetS has become a global health problem. The prevalence of MetS worldwide reaches 20% to 30% according to increases with age and by gender (10-12).

The *DYRK1B gene* is involved in some diseases, such as MetS. Missense mutations in the *DYRK1B* are associated with MetS. An arginine to cysteine substitution at position 102 (R102C) in the highly conserved domain alters the protein's function (13-15). MetS is a rare autosomal dominant disorder caused by two missense mutations, H90P and R102C, in DYRK1B located on chromosome q13.219. MetS is characterized by abdominal obesity, type 2 diabetes, hypertension, and premature coronary artery disease (16-18). According to the results, the mutant alleles increase the DYRK1B function. DYRK1B-H90P or DYRK1B-R102C overexpression in HepG2 hepatocytes results in greater induction of the gluconeogenesis enzyme (Glucose-6-phosphatase) than normal *DYRK1B*. Additionally, R102C mutation affects the effect of DYRK1B on Hedgehog and Wnt signaling pathways and promotes the fat differentiation in 3T3-L1 preadipocyte cells (19-22).

Site-directed mutagenesis, one of the essential techniques in molecular biology, has been widely used to investigate the structure and function of nucleic acids and proteins, the mechanisms of genetic diseases, and the effect of genome modification (23-25). PCR-mediated site-directed mutagenesis is one of the most powerful methods for creating gene mutations *in vitro* (26-29). This method consists of two sequential steps of PCR. The primary PCR reactions produce two mutated DNA fragments with overlapping ends and a secondary reaction that creates a single piece by joining two components (30,31).

Lentiviral vectors are handy for investigating mutant gene expression. Lentiviruses are of interest considering their ability to infect dividing and non-dividing cells, enter the cell genome, explore gene and protein function, low level of cellular toxicity, and their application in gene therapy and cell therapy (32-35). Therefore, their unique characteristics make them suitable for investigating the expression of the desired mutant gene in the *in vitro/in vivo* environment (36-38).

Currently, gene therapy using lentivirus is being performed in a wide range of diseases. The purpose of this study is to describe the scientific steps of engineering recombinant lentivirus carrying *DYRK1B-R102C* and placing it in the transgenic pathway. The production of such a lentivirus can help in creating MetS models and more comprehensive investigations of this disease.

Methods

Study design

The Shiraz University of Medical Sciences, which follows the National Institutes of Health guidelines for the care and use of animals, approved the protocols and experiments in this study. In the present fundamental study, RNA samples were extracted from the fat tissues of 3-month-old mice free of the pathogen to create mutations in the desired gene. Considering the higher expression of *DYRK1B* in adipose tissues, samples were prepared from the adipose tissues around the mice's testicles. Then, RNA was extracted from the samples and used for cDNA synthesis. To isolate adipose tissues, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (100/10 mg/kg). A small incision was made in the abdomen with a sterile scissor, and the fat mass around the testicles was removed with the help of sterile forceps. The steps for separating adipose tissue from mice are shown in Figure 1.

RNA isolation and cDNA synthesis

TriPure Isolation Reagent (Roche, Germany) accomplished the RNA extraction from mouse fat tissue. Optical density ratios were examined using 260/280 nm; the extraction quality was assessed using a Nanodrop™ spectrophotometer (Nanodrop; Thermo Fisher Scientific, Wilmington, DE, USA). The cDNA was synthesized using 3.0 µg total RNA by RevertAid™ F first S and cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). In brief, the reaction was performed with 3.0 µg total RNA, 4 µl 5x reaction buffer, 1 µl oligo-dT or random hexamer primers, 2µl dNTP mix, 1 µl reverse transcriptase, and 1ml Ribolock inhibitor. The reaction conditions were as follows: 25 °C for 5 minutes, 42 °C for 60 minutes, 70 °C for 5 minutes, and the cDNA was stored at -80 °C.

Primer design

The gene-specific primers (GSP), including forward (FGSP) and reverse (RGSP), were designed to contain restriction sites NotIand EcoRI by AllelID v.7.5 software (PREMIER Biosoft, Palo Alto, CA, USA). The forward mutagenic

primers (FMP) and reverse mutagenic primers (RMP) were complementary to each other, with the mutagenic bases (CT) in the center of each primer. The primer sequence is shown in Table 1.

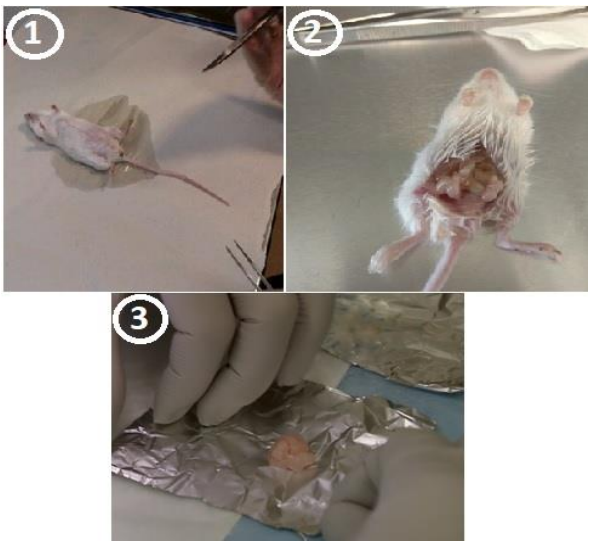

Figure 1. The steps for adipose tissue isolation from mice

Table 1. The list of primers used in this study. The enzyme cleavage position and site-directed mutagenesis for primers are shown as well

Primer name	Sequence (5′ → 3′)	Description
FGSP	GGTCGGAATTCTGCTGGTTGCATTACTGGGTA	Primer for EcoRI restriction enzyme
RGSP	AGCGGCCGCGGGTAGCAGCAAGTCCAGTC	Primer for NotI restriction enzyme
FMP	TACATTGTGtGCAGTGGCGAG	Primer for site-directed mutagenesis
RMP	CTCGCCACTGCaCACAATGTA	Primer for site-directed mutagenesis

Mutagenesis by overlap extension-PCR

Mutagenesis by OE-PCR consists of two primary PCR reactions and a secondary PCR reaction. The direct PCR reaction for each primer pair was carried out in tubes of separate, using 10 µl 2x Master Mix Red (Ampliqon A/S, Odense, Denmark), 1 µl FGSP, 1 µl RMP, 1 µl cDNA, 7 µl water, and another tube consist of 10 µl Mastermix, 1 µl RGSP,1 µl FMP,1 µl cDNA, 7 µl water. The PCR profile was used as follows: initial denaturation for 5 minutes at 95 °C, followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at 60 °C, and 1 minute at 72 °C. A final elongation of 72 °C followed this profile for 5 minutes. PCR reactions were performed using the Applied Biosystems Veriti 96-Well Thermal Cycler Instrument (ABI, Veriti, USA). At 60 °C for all primers, set the annealing temperature (Ta). Subsequently, 1% agarose gel stained with a safe stain visualized the primary PCR products. Gel excise and purify the PCR products, then for the overlap PCR, using a 1:1 ratio of both excised fragments (~75ng each) in the master mix without the primers for the first 10-13 cycles. Later the forward primer of the first fragment and the reverse primer of the last piece were added to the cocktail and ran for another 25-30 cycles, which produced the full-length mutagenic DNA and then excised gel-purified to be used for cloning.

Cloning and transformation

The mutagenic DNA and LeGO-iG2 plasmid were digested with EcoRI and NotI (Fermentas, Germany). These enzymes have restriction sites in LeGO-iG2 and the prepared mutagenic DNA. The digestion reaction was performed using 5 µl of DYRK1B DNA (212 ng/µl), 1µl of tango buffer, 1 µl of EcoRI (10 U/µl), 1 µl of NotI (10 U/µl), and 2 µl of DDW. In another reaction LeGO-iG2 2.5 µl (487 ng/µl), tango buffer 1 µl, EcoRI 1µl (10 U/µl), NotI 1 µl (10 U/µl), and DDW 2 µl.

The digested products were purified from the gel using the gel extraction kit (Qiagen, USA). The ligation reaction was carried out as follows: 20µl of DYRK1B, 7µl of LeGO-iG2, 3.3µl of T4 DNA ligase buffer, 1.7 µl of T4 DNA ligase (5 U/µl) and incubate at 16 °C for 20 hours. 10 µl of ligation reaction in 70 µL of competent Escherichia coli DH5 α were transformed using the heat shock method. The transformed bacteria were cultured on LB agar containing ampicillin 100 µg/mL and incubated overnight at 37 °C. Colony-PCR was carried out to approve colonies containing the recombinant vector. In the next step, the recombinant vector was extracted by a QIAprep Miniprep Kit (Qiagen, USA). The cloning accuracy was confirmed by enzyme digestion with EcoRI (10 U/µl) and NotI (10 U/µl), followed by sequencing (DNAMAN software). Enzymatic digestion was carried out using recombinant vector 5 µl (238 ng/µl), 1 µl of Buffer, 1 µl of NotI (10 U/µl), 1µl of EcoRI (10 U/µl), and DDW 3 µl.

Virus production and titration

A lentivirus-based vector expressing GFP was produced using transient co-transfection of Hek-293T cells with a three-plasmid combination. In brief, (i) transfer lentiviral vector 15µg (LeGO-iG2), (ii) envelope plasmid VSV-G 10µg (psPAX2), and (iii) packaging plasmid 10µg (pMD2) were used in a calcium phosphate transfection mix and transfected into human embryonic kidney cells (HEK 293T cells) (Pasteur Institute, Iran) and culture medium was replaced to fresh medium 18 hours after the transfection and viral supernatants were harvested 36-, 48- and 72-hours post-transfection. We centrifuged at low speed (300 g for 5 minutes) and filtered through a 0.45 m filter. The lentiviruses supernatant was concentrated by ultra-centrifugation at 70,000 g in a swinging bucket rotor for 1 hour at 4 °C. The viral pellet was then re-suspended in 200 µl of PBS 1X. Titration of the eGFP-expressing lentivirus vector was carried out by HEK 293T cell transduction, and the eGFP-expressing cell was examined by flow cytometry (BD Biosciences). The lentivirus stocks were diluted in four concentrations (10X, 100X, 1,000X, and 10,000X), and then these dilutions were added to a four-well plate with 2×10^5 293T cells per well. GFP-positive cells were estimated by flow cytometry. The titration results by flow cytometry are shown in Figure 2. Using the formula: TU/ml = seeding cell number × fluorescent cell number percentage × dilution factor/transduction volume in ml, Transducing Units (TU) were calculated.

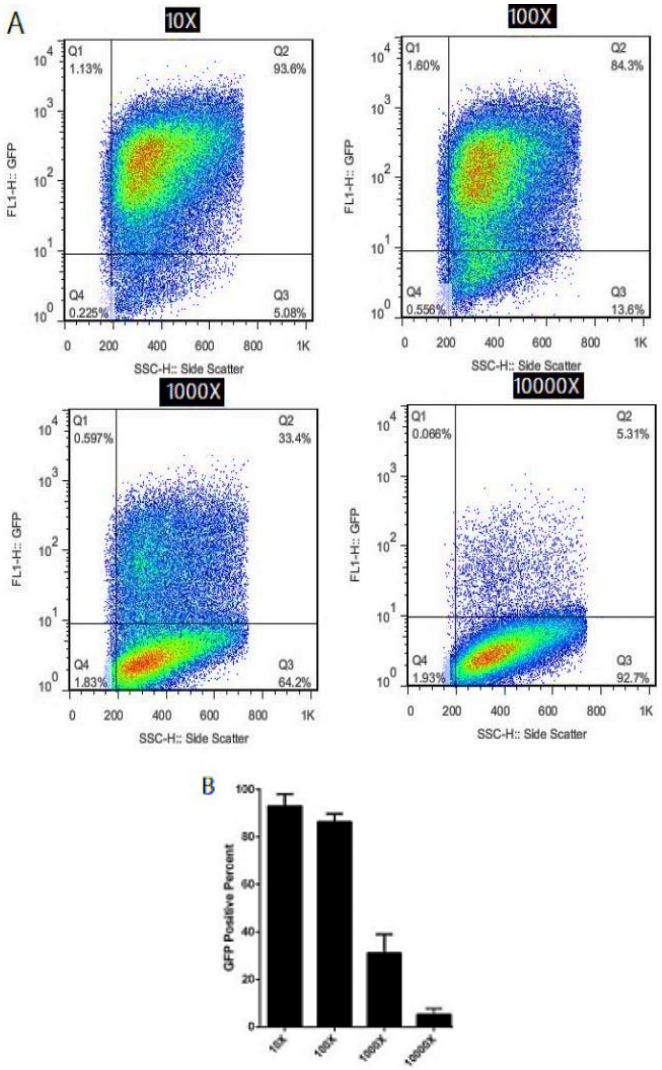

Figure 2. The lentirivus titration by flow cytometry. A) GFP analysis on HEK293T cells after the transduction with different concentrations of lentirivus (10X, 100X, 1,000X, and 10,000X). B) The GFP positive percentage.

Results

Mutagenesis by overlap extention PCR

The results showed that the desired mutation was performed correctly using the OE-PCR method. Following the primary PCR reactions, the secondary PCR reactions were produced in multiple bands, including the expected band, which was gel-excised and sent for sequencing. These reaction results are shown in Figure 3. DNA sequencing indicated that the point mutation was correctly performed.

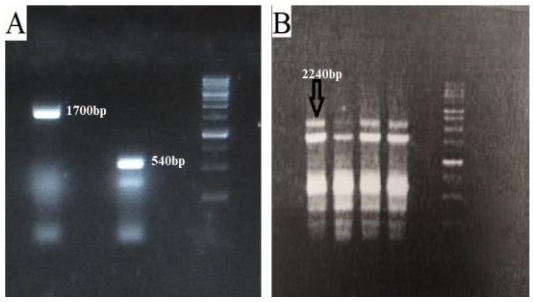

Figure 3. The mutagenesis overlaps extension PCR product. A) Both primary PCR reactions produced single bands estimated at 540bp and 1,700bp, which matched the expected size. Since single bands were obtained, gel purification was unnecessary. B) The secondary PCR reaction produced multiple bands, including the expected band, at 2,240 bp (Indicated by black arrow). The gel purification had to be done because the single band was not created. DNA ladder, 1 Kb.

Recombinant vector production

The mutagenic DNA was electrophoresed after digestion with EcoRI and NotI. A fragment without any considerable size change was observed. The LeGO iG2 vector was also digested with the same enzymes, and a 7829-bp band was observed. In the next step, the target gene was inserted into the LeGO-iG2 using T4 DNA ligase. The ligation product was used to transform Escherichia coli DH5, and colony-PCR was carried out using the forward and reverse primers for DYRK1B to confirm gene insertion into LeGO-iG2. The colony-PCR product was electrophoresed in gel, and a fragment of 2240 bp was observed. The recombinant vector was purified using the QIAprep Miniprep Kit and subjected to digestion with EcoRI and NotI (i), the 7829 bp band was identical to the linearized vector, and (ii) observed two pieces were. Another band was consistent with DYRK1B (2240 bp band). The DYRK1B 2240 bp band is shown in Figure 4. The extracted vectors were sent for sequencing, and the results were checked with BLAST. Successful cloning was finally confirmed by sequencing the recombinant vector, and the sequencing output is shown in Figure 5.

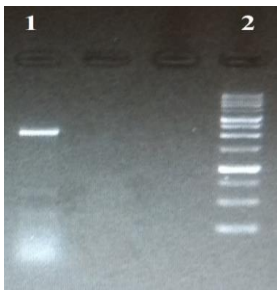

Figure 4. The colony-PCR was performed to confirm colonies. After DNA extraction from the bacterial colonies, the PCR reaction was performed to confirm the presence of the *DYRK1B R102C* gene, sample 1 colony-PCR (2240 bp band). Sample 2, 1 Kb DNA ladder.

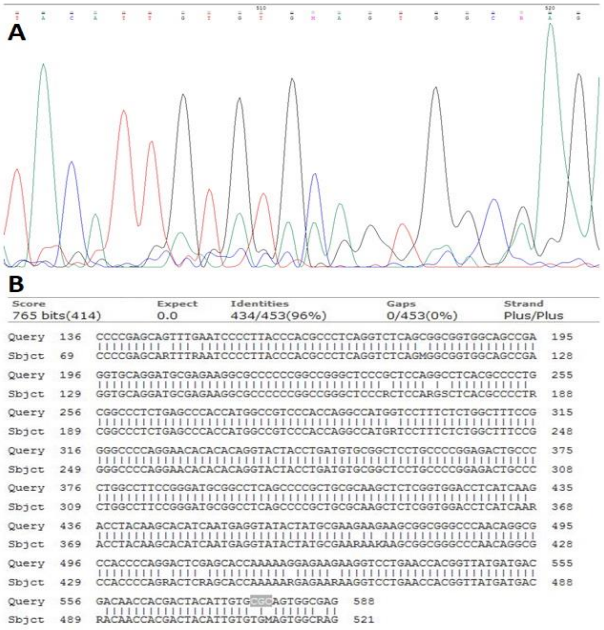

Figure 5. Sequencing to confirm cloning. A) Sequencing chromatogram with forward primer to confirm the accuracy of cloned gene fragment in LeGO-iG2 vector. B) Sequencing chromatogram showing the mutations as dots and highlighted sections in the aligned sequence.

Virus production and titration

HEK 293T cell line was co-transfected with the plasmids leGO-iG2-DYRK1B, pMD2, and psPAX2 to produce a GFP-DYRK1B expressing lentiviral. Fluorescent microscopy showed that GFP was expressed 48 hours post-transfection in more than 90% of these cells Figure 6. Lentiviral-containing supernatant was concentrated using the ultracentrifuge-based method. The flow cytometry for viral titer measuring was performed 72- hours post-transduction of HEK 293T cells. Calculate virus titration using GFP positive cell percentage in 10,000X concentration (5%). The viral titer was 10^8 TU/ml on HEK293T cells.

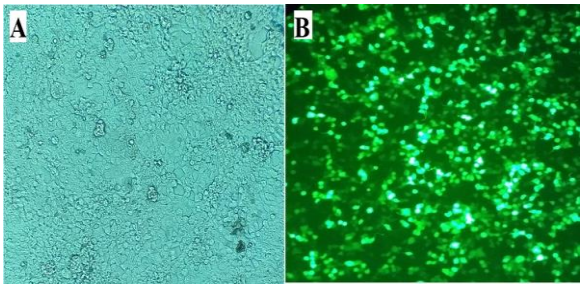

Figure 6. Lentivirus production and *GFP-DYRK1B* gene expression under fluorescent microscopy. A) HEK293T cells before transfection, B) HEK293T cells 48 hours post-transfection

Discussion

Today, gene transfer is one of the essential techniques in diagnosing, preventing and treating diseases under gene therapy. Different vectors are used for gene transfer. Among the viral vectors, lentiviruses have gained attention in recent years, considering their outstanding characteristics and important implications for clinical trials. As essential tools to investigate the molecular pathways involved in the disease, they are being developed and can be helpful in the design of the disease model (39-41). These viruses belong to the retroviridae family and, like retroviruses, can insert their genome into the host's genome after entering the host cell (37,41-43). Can do this during the cell division phase. Nevertheless, lentiviruses can enter the host cell's nucleus and insert their genome into the host's genome without requiring cell division (37,42,44,45). Many unnecessary and pathogenic genes of the virus have been removed, and DNA replication defects increase biological safety in these vectors (24,43).

Second-generation lentiviral vectors are composed of three separate lentiviral vectors. One of which in this study is the leGO-iG2 transfer vector, and the rest are lentiviral packaging vectors (pMD2 and psPAX2). In the present study, after being mutated in the mouse *DYRK1B*, it was cloned in the leGO-iG2 transfer vector containing the GFP marker and then transfected with the pMD2 and psPAX2 vectors in HEK 293T cells to produce lentivirus in these cells. After observing the green light resulting from the GFP expression in HEK 293T cells under a fluorescent microscope, recombinant lentivirus was confirmed, and virus titration was performed by flow cytometry as well.

This study is based on the production of recombinant lentivirus, which is achieved by inserting a gene into its genome, and we described the technique of producing recombinant lentivirus carrying the mutated gene. The use of recombinant lentivirus carrying DYRK1B R102C to produce disease models in animals, including mice, can be very useful in discovering the molecular mechanisms and pathways involved in metabolic syndrome.

DYRK1B overexpression has been found to promote cell cycle progression in certain cancers (46-49), and it has been suggested that DYRK1B protein plays a vital role in pathways that are disrupted in MetS (19,50-53). The molecular mechanisms of this event are not very clear. However, it seems that Dyrk1b protein inhibits the rate of glucose uptake and glycolysis by inhibiting the Ras-RAF-MEK pathway. Further, it has been found that the R102C mutation augments p27Kip turnover and enhances the adipogenic effects of DYRK1B (54). We acknowledge that the limitation of this study is that several other options for stages of lentivirus production were not investigated in this study. However, the primary purpose of this study was to identify steps that can be easily replicated in most laboratories when working with hard-to-transfect cells without having to purchase any additional equipment or incur extra costs. It would have been better to investigate other mutations involved in the function of the *DYRK1B*, such as the H90P missense mutation. For this, more cost and time were required, which we hope will be done by us or others in future research. Considering the advantages of lentiviruses in vivo use, one of our limitations is the lack of in vivo use of these lentiviruses. In the future, our focus is on using these lentiviruses to create transgenic eukaryotic cells and MetS disease models. It is necessary to mention that, according to the goals of lentivirus production containing the DYRK1B R102C mutation, which is transferred to eukaryotic cells, and finally, production of the disease model, we did not evaluate immune responses to lentivirus or lentivirus components in this study, but it should be noted that cell turnover *in vitro* is not representative of an *in vivo* setting.

This lentivirus was used in the present study to express the *DYRK1B-R102C* stably. There are no reports on lentiviral production for genes involved in MetS. Lentiviral vector-based cell therapy is safe in human and animal experiments. Therefore, this method is essential for treating patients with MetS.

Conclusion

We have described a method that can be successfully used for lentiviral gene transfer into eukaryotic cells. Lentiviruses carrying the *DYRK1B R102C* mutation offer significant advantages for *in vitro* and *in vivo* research on metabolic syndrome. The use of lentiviruses can be a strong approach to developing gene therapy and treating rare and incurable diseases.

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Ethical statement

Not applicable.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

All authors have contributed equally.

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