LncRNA XR_596701 protects H9c2 cells against intermittent hypoxia-induced injury through regulation of the miR-344b-5p/FAIM3 axis

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INTRODUCTION
Obstructive sleep apnea (OSA), a common sleep disorder, is characterized by recurrent episodes of upper airway collapse during sleep, leading to recurrent events of nocturnal hypoxemia, hypercapnia and transient awakening [1]. Until now, several studies have focused on the relationship between OSA and cardiovascular (CV) diseases. Clinical studies have found that the prevalence of OSA in patients with coronary heart disease ranged from 30 to 58% and approximately 70% male hospitalized patients with myocardial infarction (MI) also have OSA [2, 3]. Furthermore, OSA has been recognized a major risk factor for CV diseases [4]. Although it is likely that OSA negatively affects the pathological process of heart diseases through multiple complex mechanisms such as endothelial dysfunction and inflammation [5, 6], intermittent hypoxia (IH) in particular is the important player in OSA-associated CV diseases [7]. However, the potential mechanism between IH and CV disease is largely unknown.

Recent year, long noncoding RNAs (lncRNAs) are considered as the critical noncoding RNAs that transcript longer than 200 nucleotides [8]. Emerging evidence supports that lncRNAs can exert vital roles in cardiac remodeling including MI, proliferation, and apoptosis of cardiomyocyte and myocardial fibrosis [9–11]. For example, overexpression of anti-apoptotic lncRNA Sarrah showed a better recovery of cardiac contractile function in an acute MI model mice compared to normal mice [10]. Down-regulation of lncRNA myocardial infarction-associated transcript (MIAT) significantly alleviated atrial fibrillation (AF) and AF-induced myocardial fibrosis by targeting miR-133a-3p [12]. Likewise, miRNAs as short noncoding RNA molecules also exert crucial effects in abnormal cardiac physiology, particularly concentrating on cardiac hypertrophy, cardiac fibrosis and ischemic heart disease [13, 14]. To date, a numbers of studies focused on the roles of IncRNAs or miRNAs in the potential association between hypoxia/reperfusion (H/R) and cardiac injury [15]. However, the biological role and regulatory mechanism of IncRNAs in IH-mediated myocardial damage have not been studied.

Our previous study has found that several abnormally expressed IncRNAs in a rat myocardial infarction model of IH were identified by using IncRNA microarray experiments and qRT-PCR.
Results demonstrated that lncRNA XR_596701 was markedly elevated in the IH-induced myocardial tissue [16]. However, the expression level, biological function and molecular mechanism of XR_596701 in vitro model of myocardial injury are still unclear. Therefore, this study aimed to explore the effect of XR_596701 in IH-induced H9c2 cells and underlying molecular mechanism of XR_596701.

RESULTS
Molecular characteristics and cellular localization of Rat-derived XR_596701
To explore the biologic characteristics of XR_596701, 938 bp of the full-length nucleotide sequence of XR_596701 was obtained by 5′ and 3′ RACE (Table S2). The full length of XR_596701 was then used in a BLAST search with the genomic sequences of rat for homology analysis. For the rat alignment, the sequence at genomic location 17:23774793–23775588 scored 1573 with an e-val of 0, which suggests that XR_596701 is located in 17:23774793–23775588 (Fig. 1A). Furthermore, analysis based on high-scoring segment pair (HSP) distribution of genome (Fig. 1B) and HSP distribution of query sequence (Fig. 1C) demonstrated that XR_596701 is located on chromosome 17 in rat. The ORF of XR_596701 was predicted by NCBI ORF Finder and further validated the coding capacity by SmartBLAST. The results indicated that the ORF has no coding capacity in rat genome (Table S3). In addition, we also assessed the protein-coding potential of XR_596701 by using CPC, the results indicated that the gene XR_596701 has a very low coding potential (Table S4).

To determine the cellular localization of the XR_596701 transcript, the expression of XR_596701 in subcellular locations was measured. The FISH analysis of XR_596701 in H9c2 cells. XR_596701 is shown in red, and DAPI is shown in blue to indicate the cell nucleus, scale bar 25 µm. XR_596701 predominantly is distributed in the cytoplasmic region in H9c2 cells.

Fig. 1 Characterization of the lncRNA XR_596701 sequence. A Detailed overview of the genome region. The gene colors are as follows: blue, and purple genes: non-coding; red genes: protein coding; the red squares indicate the location of XR_596701. B High-scoring segment pair (HSP) is a subsegment of a pair of sequences that share high level of similarity. Fragments of the query sequence that hit other places in the genome are shown as red boxes. C The level of similarity between the sequences depends on the sequences, the alignment algorithm, and the settings used, one can find numerous HSPs within a given pair of inputted sequences. D The FISH analysis of XR_596701 in H9c2 cells. XR_596701 is shown in red, and DAPI is shown in blue to indicate the cell nucleus, scale bar 25 µm. XR_596701 predominantly is distributed in the cytoplasmic region in H9c2 cells.
XR_596701 might serve as an endogenous RNA (ceRNA) to affect the expression of its target miRNA or mRNA in cytoplasm.

**XR_596701 was upregulated and played a protective role in IH-induced H9c2 cells injury**

To determine the potential functional effects of XR_596701, H9c2 cells were treated with IH for six cycles, and the total RNA of H9c2 cells was extracted for qRT-PCR. The result showed that XR_596701 was upregulated under IH condition compared to the non-treated cells ($P < 0.01$; Fig. 2A). Simultaneously, we performed the loss-of-function experiment by knocking down XR_596701 with si-XR_596701 (Fig. 2B). First of all, our data showed that IH exposure significantly decreased cells proliferation and increased cells apoptosis when compared to the non-treated group, which is consistent with the result of our previous study [18]. After down-regulation of XR_596701, we found that si-

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**Fig. 2** The expression level of XR_596701 and its protective role in IH-induced H9c2 cells injury. A The expression of XR_596701 was detected by qRT-PCR after IH stimulation, Actin mRNA served as an internal control. B H9c2 cells were transfected with siRNAs of XR_596701 or control siRNA (si-NC). Relative XR_596701 expression was measure by qRT-PCR. C, D H9c2 cells were transfected with si- XR_596701 or si-NC and were treated with IH for six cycles. EdU assay was performed to assess the impact of XR_596701 on the proliferation of H9c2 cells. Red (EdU) H9c2 cells indicated proliferating the cell nucleus and blue (DAPI) represented the cell nucleus, scale bar 50 µm. E H9c2 cells were transfected with si- XR_596701 or si-NC and were treated with IH for six cycles. Hoechst 33342/PI staining was performed to assess the effect of XR_596701 on the apoptosis of H9c2 cells. Red (PI) indicated apoptotic H9c2 cells and blue (Hoechst 33342) represented the cell nucleus, scale bar 50 µm. F H9c2 cells were transfected with si- XR_596701 or si-NC and were treated with IH for six cycles. Caspase-3 immunofluorescence was measured to show the apoptosis of H9c2 cells. Caspase-3 is shown in green, and DAPI is shown in blue to indicate the cell nucleus, scale bar 25 µm. G, H H9c2 cells were transfected with si- XR_596701 or si-NC and were treated with IH for six cycles. Expression levels of apoptosis-related proteins (Bcl-2, Bax and Cleaved caspase-3/Caspase-3) by western blot analysis. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$, ****$P < 0.00001$. Data were shown as Mean ± SD based on three independent experiments.
XR_596701 not only significantly decreased cells proliferation, but also promoted cells apoptosis in IH-treated H9c2 cells model when compared to si-NC group. As shown in the Fig. 2C, D, an Edu-S94 staining assay was utilized to show the cells proliferation, the results showed that the proliferation rate of H9c2 cells in si-XR_596701 group is significantly lower than si-NC group under IH exposure. In the Fig. 2E, Hoechst/PI staining assay was conducted to evaluate the cells apoptosis, the results demonstrated that the apoptosis of H9c2 cells in si-XR_596701 group is significantly higher than si-NC group under IH condition. Meanwhile, we also performed the immunofluorescence to investigate the expression level of caspase-3, which serves as a critical executor in the intrinsic and extrinsic apoptotic pathways [19]. As shown Fig. 2F, si-XR_596701 markedly increased caspase-3 immunofluorescence expression in IH-treated H9c2 cells when compared to si-NC group after IH stimulation. Additionally, flow cytometry results also indicated that the cell apoptotic rate in the si-XR_596701 group was markedly upregulated compared to control group (Fig. S2). To further assess the cells apoptosis, we tested the apoptosis-related proteins Bax, Caspase-3, and Bcl-2 by western blot. We found that si-XR_596701 group has lower Bax and Cleaved caspase-3/Bcl-2 ratio with a higher Bcl-2 protein expression level when compared to si-NC group after IH stimulation (Fig. 2G, H).

Moreover, we performed the gain-of-function experiment by overexpressing XR_596701 with oe-XR_596701 plasmid (Fig. S3A). The results indicated that overexpression of XR_596701 could significantly increase the proliferation of H9c2 cells (as detected by Edu-S94 assay in Fig. S3B, C) and reduce the H9c2 cells from IH-mediated apoptosis (as detected by western blot for apoptosis-related protein in Fig. S3D, E) when compared to empty vector group. Together, these outcomes suggest that XR_596701 exerts a protective effect in IH-induced H9c2 cells injury.

miR-344b-5p was downregulated and mediates IH-induced H9c2 cells injury

To further assess the effect of miR-344b-5p in H9c2 cells, we treated H9c2 cells with IH stimulation. Firstly, we examined the expression of miR-344b-5p by qRT-PCR, we found that the expression of miR-344b-5p was down-regulated under IH condition compared to the non-treated group (P < 0.001; Fig. 3A). Next, we applied gain-of-function strategy to examine the biological significance of miR-344b-5p on IH-induced cells injury (Fig. 3B). After transfecting miR-344b-5p mimics or mimics NC, we found that miR-344b-5p mimics could significantly reduce the proliferation of H9c2 cells (as detected by Edu-S94 assay in Fig. 3C, D) and increase the H9c2 cells from IH-induced apoptosis (as detected by Hoechst/PI assay in Fig. 3E, caspase-3 immunofluorescence in Fig. 3F and western blot for apoptosis-related protein in Fig. 3G, H) when compared to mimics NC group after IH stimulation.

miR-344b-5p inhibitor reversed the protective effects of XR_596701 Accumulating evidence indicated that IncRNAs sponges as miRNA sponges in several diseases including hypoxia-mediated injury [20]. The opposite regulatory effects of XR_596701 and miR-344b-5p in IH-induced cells injury suggested that XR_596701 may function as a sponge of miR-344b-5p. To explore the relationship between XR_596701 and miR-344b-5p, we performed qRT-PCR and the luciferase reporter assay. The results of qRT-PCR demonstrated miR-344b-5p expression was markedly increased by knocking down XR_596701 when compared to si-NC group (Fig. 4A). Analogously, and the expression level of XR_596701 significantly changed after transfecting miR-344b-5p mimics or inhibitor when compared to corresponding negative control (Fig. 4B). Additionally, the binding regions between XR_596701 and miR-344b-5p were showed in Fig. 4C. The dual-luciferase reporter indicated that the luciferase activity of wt-XR_596701 rather than mut-XR_596701 can be significantly decreased by miR-344b-5p mimics but not mimics NC (Fig. 4D). These findings supported the direct interaction between XR_596701 and miR-344b-5p.

Furthermore, we examined the effect of miR-344b-5p mimics on XR_596701-associated IH-induced cells damage. H9c2 cells were transfected with si-XR_596701, miR-344b-5p inhibitor and corresponding scramble. As shown in the Fig. 4E-I, miR-344b-5p inhibitor was able to rescue the effect of XR_596701 silence on the obvious reduction of cells proliferation, the significant increase of cells apoptosis and apoptosis-related proteins expression under IH condition. Collectively, these investigations demonstrated that directly targeting miR-344b-5p is an essential mechanism by which XR_596701 mediates H9c2 cells injury under IH stimulation.

Fas apoptotic inhibitory molecule 3 (FAIM3) was confirmed as a direct target of miR-344b-5p and exerted antagonistic effects in miR-344b-5p mediated IH-induced cells damage

We predicted the potential target of miR-344b-5p by bioinformatics analysis. Using TargetScan and miRBase database, FAIM3 was predicted as a new target gene for miR-344b-5p. Binding sites between them were showed in Fig. S1. To verify the interaction between miR-344b-5p and FAIM3, we next conducted qRT-PCR and the dual-luciferase reporter assay. As shown in the Fig. 5A, B, miR-344b-5p expression was markedly decreased by pcDNA3.1-FAIM3. Similarly, the expressions of FAIM3 at mRNA and protein level were dramatically reduced by miR-344b-5p mimics (Fig. 5C–E). Moreover, the results of dual-luciferase reporter assay demonstrated that the luciferase activity of wt-FAIM3 was significantly decreased by miR-344b-5p mimics, whereas almost unchanged in the luciferase activity of mut-FAIM3 (Fig. 5F). These indicated that FAIM3 was a target of miR-344b-5p.

Then, we assessed the functional roles of FAIM3 in miR-344b-5p-inhibited cells damage after IH exposure. As demonstrated in Fig. 5G–K, after co-transfecting miR-344b-5p mimics, pcDNA3.1-FAIM3 and corresponding negative control, overexpression of FAIM3 was able to markedly increase the suppressed cells proliferation while reducing promoted cells apoptosis caused by co-transfection of miR-344b-5p mimics when compared to negative control group. Taken together, these data indicated that FAIM3 could antagonize the effects of miR-344b-5p in IH-induced H9c2 cells injury.

FAIM3 mediated XR_596701-associated cells damage in IH-induced H9c2 cells

Considering that miR-344b-5p reversed the protective roles of XR_596701 and FAIM3 directly targeted miR-344b-5p expression in IH-induced cells injury, we assessed the potential relationship between FAIM3 and XR_596701 under IH stimulation. As shown in the Fig. 6A–D, the expressions of FAIM3 at mRNA and protein level were markedly decreased by si-XR_596701, while XR_596701 mRNA expression was significantly increased after transfecting pcDNA3.1-FAIM3. In addition, we found that FAIM3 expression was markedly upregulated after overexpression of XR_596701 (Fig. S4). Furthermore, by co-transfecting si-XR_596701, pcDNA3.1-FAIM3 and corresponding negative control before IH treatment of H9c2 cells, we found that overexpression of FAIM3 completely abolished the roles of XR_596701 on IH-mediated cells damage (Fig. 6E–I). These findings might explain that XR_596701 functions as a ceRNA to regulate the expression of FAIM3 by sponging miR-344b-5p.

DISCUSSION
Cumulative evidence supports that OSA is an important risk factor for CV diseases, including myocardial infarction, hypertension and heart failure. IH is the common pathophysiological basis of OSA, which may exert critical role in OSA-associated CV diseases. Our previous study has showed that IncRNAs aberrantly expressed in a rat model of IH and IncRNA XR_596701 was markedly increased in the IH-induced rat myocardial tissue. In this study, we
performed a vitro model of IH-mediated cardiomyocytes injury to evaluate biological function and molecular mechanism of XR_596701. The results demonstrated that XR_596701 was upregulated and played a critical role in IH-induced H9c2 cells injury. In addition, we found that miR-344b-5p was verified to be a direct target of XR_596701 and miR-344b-5p inhibitor reversed the protective effects of XR_596701. Furthermore, FAIM3, a confirmed target of miR-344b-5p, exerted antagonistic effects in miR-344b-5p mediated IH-induced cells damage and affected protective effects of XR_596701 (Fig. 7).

IncRNAs are a class of non-coding RNA with the length of over 200 nucleotides. They can be divided into several groups and exert critical roles in modulation of gene expressions and biological processes at chromosome, transcription, and post-transcription, including chromosomal activation, cell proliferation, apoptosis, and death according to their length, localization, and genetic location [21, 22]. Recent studies are getting more and more involved relationship between IncRNAs and hypoxia. IncRNAs may regulate cancer proliferation, apoptosis, angiogenesis, metabolism and metastasis in hypoxic microenvironment.
For instance, Dr. Mole et al. showed that down-regulation of NEAT1 markedly suppressed the cell proliferation under hypoxic conditions in breast cancer [23]. Yang et al. demonstrated that histone deacetylase 3 may repressed the expression of lncRNA-LET via decreasing regulation of the lncRNA-LET promoter region [24]. Additionally, lncRNAs also exert an important role in hypoxia/reoxygenation (H/R) model of myocardial, hepatic, and cerebral injury [25–27]. Li et al. found that the expression of IncRNA XIST was markedly up-regulated and suppression of XIST could improve myocardial H/R injury through regulation of the miR-133a/SOCS2 axis [25]. In our study, IncRNA XR_596701 was significantly upregulated after IH stimulation, which was similar with the model of H/R, and played a protective role in IH-induced H9c2 cells injury. Nevertheless, how it functioned and what the underlying regulatory mechanism was is not clear.

MicroRNAs (miRNAs) are a class of important short strand RNA molecules that were first discovered in 1993 [28]. Previous review showed that a number of hypoxia-mediated miRNAs that have been
The putative binding sites between XR_596701 and miR-344b-5p. Expression was measured by qRT-PCR after transfecting miR-344b-5p mimics, miR-344b-5p inhibitor, and corresponding scrambled control. The putative binding sites between XR_596701 and miR-344b-5p. Luciferase reporter assay. Luciferase construct containing either wild-type XR_596701 (XR_596701-wt) or mutant XR_596701 (XR_596701-mut) sequence was co-transfected into H9c2 cells with miR-344b-5p mimic or inhibitor. The luciferase activity was measured and a dual-luciferase reporter assay was performed to evaluate the regulation of H9c2 cells. 

MATERIALS AND METHODS

Cell culture and IH injury model

Rat cardiomyocyte-derived H9c2 cells (ATCC, Shanghai, China; The STR profiling of cell line was showed in the supplementary file) were cultured at 37 °C in 5% CO2 atmosphere with Dulbecco's modified Eagle's medium (HyClone), which contained 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin. IH model of H9c2 cells was established as described previously [18]. Briefly, H9c2 cells were transferred to hypoxic chamber with 1% O2 at 37 °C for 35 min; then, the cells were incubated with normoxic chamber with 21% O2 at 37 °C for 25 min. Cell maintained repeated IH stimulation for six cycles.

LncRNA identification

Full-length XR_596701 cDNA was obtained by 5'- and 3' rapid amplification of cDNA ends (RACE). Subsequently, to identify the characteristic of XR_596701, genome location and protein-coding potential were analyzed by NCBI Genome, ORF Finder (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Coding Potential Calculator (CPC) [40], respectively. ORF finder is a graphical analysis tool, which can search newly sequenced DNA for potential protein encoding fragment and then validate predicted protein by Smart BLAST. Eventually, we combined above results to assess the molecular characteristics of LncRNA.
RNA fluorescent in situ hybridization (RNA-FISH)

DIG-labeled IncRNA XR_596701 probes were obtained from Servicebio (Wuhan, Chian). H9c2 cells were fixed in 4% paraformaldehyde for 20 min at room temperature (RT). After three time washed in PBS, cells were permeabilized with 0.5% Triton-100 for 5 min. Then, the cells were blocked by prehybridization solution for 30 min at 37 °C. After removal of the prehybridization solution, the cells were cultured with the probe hybridization solution containing specific probe overnight in the dark. Finally, the cells were incubated with DAPI for 15 min, and observed using a laser confocal microscope (Leica SP5, Heidelberg, Germany). The sequence for XR_596701 probe is: 5′-DIG-GAGGTCATAAGGAAGTTTGGA-CACCGCAGAA-DIG-3′.
Fig. 5 FAIM3 was confirmed as a direct target of miR-344b-5p and exerted antagonistic effects in miR-344b-5p mediated IH-induced cells damage. A Relative FAIM3 expression was measure by qRT-PCR after transfecting pcDNA3.1 or pcDNA3.1-FAIM3. B Relative miR-344b-5p expression was measure by qRT-PCR after transfecting pcDNA3.1 or pcDNA3.1-FAIM3. C Relative FAIM3 expression was measure by qRT-PCR after transfecting miR-344b-5p mimic or mimics NC. D, E Relative FAIM3 protein expression was measure by western blot after transfecting miR-344b-5p mimic or mimics NC. F Luciferase reporter assay. Cells were co-transfected with FAIM3-wt or FAIM3-mut 3′-UTR reporters and miR-344b-5p mimic or mimics NC and pcDNA3.1-FAIM3 (or pcDNA3.1) were co-transfected into H9c2 cells. Cells were treated with IH for six cycles. EdU staining assay was performed to evaluate the proliferation of H9c2 cells, scale bar 50 µm. G Hoechst 33342/PI staining was performed to evaluate the apoptosis of H9c2 cells. miR-344b-5p mimic (or mimics NC) and pcDNA3.1-FAIM3 (or pcDNA3.1) were co-transfected into H9c2 cells, scale bar 50 µm. Cells were treated with IH for six cycles. J, K miR-344b-5p mimic (or mimics NC) and pcDNA3.1-FAIM3 (or pcDNA3.1) were co-transfected into H9c2 cells. Cells were treated with IH for six cycles. Expression levels of FAIM3 and apoptosis-related proteins (Bcl-2, Bax, and Cleaved caspase-3/Caspase-3) by western blot. *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.00001. Data were shown as Mean ± SD based on three independent experiments.

Fig. 6 FAIM3 mediated XR_596701-associated cells damage in IH-induced H9c2 cells. A Relative FAIM3 expression was measure by qRT-PCR after transfecting si-XR_596701 or si-NC. B, C Relative FAIM3 protein expression was measure by western blot after transfecting si-XR_596701 or si-NC. D Relative XR_596701 expression was measure by qRT-PCR after transfecting pcDNA3.1 or pcDNA3.1-FAIM3. E, F si-XR_596701 (or si-NC) and pcDNA3.1-FAIM3 (or pcDNA3.1) were co-transfected into H9c2 cells. Cells were treated with IH for six cycles. EdU staining assay was performed to evaluate the proliferation of H9c2 cells, scale bar 50 µm. G Hoechst 33342/PI staining was performed to evaluate the apoptosis of H9c2 cells, scale bar 50 µm. H, I si-XR_596701 (or si-NC) and pcDNA3.1-FAIM3 (or pcDNA3.1) were co-transfected into H9c2 cells. Cells were treated with IH for six cycles. Expression levels of FAIM3 and apoptosis-related proteins (Bcl-2, Bax, and Cleaved caspase-3/Caspase-3) by western blot. *P < 0.05, **P < 0.001, ***P < 0.0001, ****P < 0.00001. Data were shown as Mean ± SD based on three independent experiments.
H9c2 cells were transfected with miR-344b-5p mimics using Lipofectamine 3000. The luciferase activity was measured after transfection by utilizing the Dual-Luciferase Reporter Assay kit (Promega) and analyzed with a luciferase reporter assay system (Promega). Luciferase activity was normalized to renilla luciferase activities.

Western blot assay
H9c2 cells were lysed using Mammalian Protein Extraction Reagent (CWBio, Beijing, China) and removed the cells debris through centrifuging for 15 min (15,000 rpm, 4°C). Total protein was harvested and quantified using BCA assay kit (DINGGUO, Beijing, China). For each sample, approximately 20 µg proteins was separated by SDS-PAGE and transferred to a PVDF membrane. Then PVDF membrane was blocked in 5% non-fat dry milk for 2 h, followed by incubation with primary antibodies against Caspase-3 (1:1000 dilution, #14220, Cell Signaling Technology), Bcl-2 (1:1000 dilution, #2785, Cell Signaling Technology), Bax (1:5000 dilution, #2743, Cell Signaling Technology), and β-actin (1:5000 dilution, #4967, Cell Signaling Technology). The bands were quantified by measuring the band intensity for each group.

Statistical analysis
The SPSS 19 software and GraphPad Prism 8 software were used to carry out statistical analysis. All quantitative data are expressed as mean ± SD from at least three independent experiments. The t-test was performed for comparisons between two groups and one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was used for comparisons among multiple groups. The P value less than 0.05 was considered statistically significant.

DATA AVAILABILITY
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS
QC, GL, LL: study design, performing experiments, data analysis, writing, and revising the manuscript. LC, JH: study design and data analysis. NL: study guidance and supervision. MC: study design and revising the manuscript. AZ: study design and obtaining fundings. QL: study design and supervision, obtaining fundings, revising the manuscript. All authors approve the final version of the manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

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