Protective effects of dexmedetomidine on hypoxia/reoxygenation injury in cardiomyocytes by regulating the CHOP signaling pathway

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Abstract. Hypoxia/reoxygenation (H/R) injury in myocardial cells occurs frequently during cardiac surgery and affects the prognosis of patients. The present study aimed to investigate the protective effects of dexmedetomidine (Dex) on H/R injury and its association with the C/EBP-homologous protein (CHOP) signaling pathway. An H/R model was constructed in H9C2 cells to investigate the effects of Dex on H/R injury. Cell viability, apoptosis and lactate dehydrogenase (LDH) levels were determined by MTT, flow cytometry and 2,4-dinitrophenylhydrazine colorimetric assays, respectively. The expression levels of inflammatory factors were measured by reverse transcription-quantitative PCR (RT-qPCR), and CHOP and glucose-regulated protein-78 (Grp78) expression levels were detected by RT-qPCR and western blotting. CHOP was overexpressed or knocked down to detect the cell viability, apoptosis, LDH level and the expression levels of inflammatory factors and Grp78. The results demonstrated that in the H/R group, cell viability was lower and apoptosis was higher, and higher levels of LDH and inflammatory factors were present compared with those in the Dex+H/R group. Silencing of CHOP significantly reversed the H/R-reduced cell viability, high apoptotic rate and LDH levels, as well as the elevated expression levels of inflammatory factors and Grp78 caused by H/R injury, whereas the overexpression of CHOP inhibited cell viability and promoted apoptosis, elevated LDH level and expression of inflammatory factors and Grp78 compared with the negative control. Additionally, pretreatment with Dex significantly alleviated the H/R injury; thus, Dex may protect H9C2 cells against H/R induced cell injury, possibly by suppressing the CHOP signaling pathway.

Introduction

Ischemic reperfusion (I/R) commonly occurs after shock therapy, artery bypass and cardiopulmonary cerebral resuscitation (1-3). The functions of tissues and organs normally recover after I/R; however, I/R injury may aggravate and cause severe complications, including stroke (4). The outcome of myocardial surgery I/R is closely associated with the occurrence of myocardial ischemic reperfusion injury (MIRI). Previous studies have reported that oxygen free radical injury (5), energy metabolism disorder in cardiomyocytes (6), calcium overload (7), apoptosis of cardiomyocytes and other inflammatory diseases (8-10) serve important roles in MIRI. During reperfusion, a large amount of inflammatory factors is produced to promote tissue infiltration of inflammatory cells, thus affecting cell structures and functions (11). Hypoxia/reoxygenation (H/R) in cells is a major characteristic of I/R, and is frequently used to simulate the activity of I/R (12,13). Low cell viability, high apoptosis and inflammatory responses occur after cell injury induced by H/R (14).

Dexmedetomidine (Dex) is a new α2-adrenergic receptor agonist with high selectivity that and exerts analgesic (15), anti-stress (16) and anti-inflammatory (17) effects. Chen et al (18) have demonstrated that Dex exerts protective effects on postoperative cognitive dysfunction by downregulating the expression levels of inflammatory factors interleukin (IL)-1β, tumor necrosis factor (TNF)-α and NF-κB in rats. Wang et al (19) have investigated the mechanism of lidocaine-induced cytotoxicity and reported that the anti-inflammatory effect of Dex may be achieved by suppressing the mitogen-activated protein kinase (MAPK) signaling pathway to affect the expression of caspase-3 and Bcl-2 and reduce apoptosis. Additionally, Dex has been reported to reduce the expression of macrophage inflammatory protein-2 and other cytokines (TNF-α, IL-6 and IL-1β) in rat lung cells (20). Through regulating the peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α signaling pathway, Dex alleviates encephala edema and apoptosis in the
presence of PGC-1α expression, thus protecting neurons from oxidative stress (21). In addition, Liu et al. (22) explored the effects of Dex on injury caused by ischemia in neuronal cells, and the results demonstrated that Dex promoted neuronal cell viability and reduced apoptosis, as well as the ratio of Bax/Bcl-2 expression levels. In the present study, an H/R-induced H9C2 cell model was constructed and pretreated with Dex to investigate the protective effects of Dex on H/R injury in cardiomyocytes and its mechanism in the C/EBP-homologous protein (CHOP) signaling pathway.

Materials and methods

Cell culture and grouping. The H9C2 (2-1) cell line was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (cat. no. GNR 5). The cells were incubated in 90% DMEM (GIBCO; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA) with 5% CO₂ at 37°C. The cells were divided into control, Dex, H/R, and Dex+H/R groups. The control group was normally cultured; Dex group was pretreated with 1 µM Dex (Beijing Huamaike Biotechnology Co., Ltd.) for 1 h and cultured normally; the H/R group was pretreated with 1 µM Dex followed by H/R treatment.

Cell transfection. Cell transfection was performed as previously described (23). Small interfering (si)RNA targeting CHOP (siCHOP; cat. no. siB078266608‑1‑5; Guangzhou RiboBio Co.), si-negative control (NC; siNC; cat. no. siN0000002‑1‑5), pCMV6‑XL5‑CHOP (cat. no. SC117581; Origene Technologies, Inc.) and its nc overexpression vector (pCMV6‑XL5; cat. no. PCMV6XL5; Origene Technologies, Inc.) were dissolved in 50 µl DMEM (HyClone; GE Healthcare Life Sciences) and used to overexpress or knock down the expression of CHOP. Prior to cell transfection, the cells were digested, thoroughly mixed, seeded into a 6-well plate (1x10⁶ cells/ml) and evenly distributed in an orifice plate. After culturing overnight until the cells reached 60-70% confluency, the cells were transfected with 20 pmol sicHoP, cHoP sinc, and nc in DMEM using lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc.) for 48 h at 37°C. At 24 h post-transfection, reverse transcription-quantitative PCR (RT-qPCR) and western blotting were used to detect the transfection efficiency. After the transfection, the H9C2 cells were divided into control, H/R, Dex+H/R, siNC+c+Dex+H/R and siCHOP+Dex+H/R groups to detect the effects of silencing CHOP on H/R injury to the cells, whereas those in the H/R, Dex+H/R, NC+Dex+H/R, CHOP+Dex+H/R groups were used to explore the effects of CHOP overexpression in H9C2 cells.

MTT assay. H9C2 cells were seeded (2x10⁴ cells/well) into 96-well plates. MTT (10 µl; Amresco) was added to each well and cultured for 4 h at room temperature. Subsequently, 100 µl DMSO (Sigma-Aldrich; Merck KGaA) was added into each well and gently agitated for 10 min at room temperature. The optical density (OD) was detected using a microplate reader at 490 nm.

Flow cytometry. Early and late apoptosis were detected by flow cytometry to explore the effects of Dex, overexpression or knockdown of CHOP on H9C2 cells using the FITC/propidium iodide (PI) Apoptosis Detection Kit [cat. no. 70-AP101-100; Multisciences (Lianke) Biotech Co., Ltd.] and Annexin V-fluorescein isothiocyanate and PI. Finally, the cells were analyzed by flow cytometry (BD FACScanto II; BD Biosciences) and FlowJo software (version 7.6.1; FlowJo LLC).

RT-qPCR. The relative mRNA expression levels of TNF-α, IL-1β, IL-6, CHOP and 78 kDa glucose-regulated protein (Grp78) in H9C2 cells were determined by RT-qPCR. Total RNAs were extracted from H9C2 cells using QIAshredder and an RNeasy Kit (Qiagen GmbH), and the RNA concentration was detected. A total of 1.0 µg RNA was reverse transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Thermo Fisher Scientific, Inc.) with 5 µl First-Strand Buffer, 1 µl dNTP mix and 100 ng primers at 37°C for 60 min and 4°C for 5 min. Subsequently, qPCR was performed using SYBR Fast qPCR Mix (Invitrogen; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation at 95°C for 15 min; denaturation 95°C for 10 sec; 40 cycles at 60°C for 30 sec (annealing) and at 72°C for 30 sec (extension); and final extension at 72°C for 7 min.

Table I. Primer sequences used for RT-qPCR.

| Primer | Sequences (5’→3’) |
|--------|------------------|
| TNF-α  | Forward: ATGGGCTCCCTCTCATCAGT | Reverse: GCTTGGTGTGTTGCTACGAC |
| IL-1β  | Forward: TCCTCGTGACTGTTGGGAT | Reverse: GGTTGCGAGTCGCCGGTTCCAG |
| IL-6   | Forward: CCAGTTGGCTTCTGGGACT | Reverse: GGTTCGTGTGGTGTTGATTC |
| Grp78  | Forward: GTGGCACGAGCATCAAGTT | Reverse: CCCACCTCCATAATCACTTGA |
| CHOP   | Forward: CTGGAAGGGCTGGTATGAGGT | Reverse: CAGGGTCAGAGTGAATGAGGT |
| GAPDH  | Forward: AGAAGGGCTTG GGCTCATTTG | Reverse: AGGGGCGCCTAC CAGATCTTC |

2,4-dinitrophenylhydrazine colorimetric assay. The cells were collected and centrifuged at 255 x g for 2 min at room temperature to obtain the supernatant. The lactate dehydrogenase (LDH) levels were determined by 2,4-dinitrophenylhydrazine colorimetric assay (cat. no. A020-1-2; Nanjing Jiansheng Bioengineering Institute) according to the manufacturer's instructions. The OD value was determined using a microplate reader at 440 nm.

2,4-dinitrophenylhydrazine colorimetric assay. The cells were collected and centrifuged at 255 x g for 2 min at room temperature to obtain the supernatant. The lactate dehydrogenase (LDH) levels were determined by 2,4-dinitrophenylhydrazine colorimetric assay (cat. no. A020-1-2; Nanjing Jiansheng Bioengineering Institute) according to the manufacturer's instructions. The OD value was determined using a microplate reader at 440 nm.

2,4-dinitrophenylhydrazine colorimetric assay. The cells were collected and centrifuged at 255 x g for 2 min at room temperature to obtain the supernatant. The lactate dehydrogenase (LDH) levels were determined by 2,4-dinitrophenylhydrazine colorimetric assay (cat. no. A020-1-2; Nanjing Jiansheng Bioengineering Institute) according to the manufacturer's instructions. The OD value was determined using a microplate reader at 440 nm.
The expression levels of rT-qPCR products were determined by the 2-ΔΔcq method (24). GADPH served as the internal reference, and the primer sequences are presented in Table I.

Western blotting. The protein expression levels of CHOP and Grp78 were measured by western blotting. The total proteins of H9c2 cells were harvested by radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology), and the protein concentration was measured by a bicinchoninic acid protein assay (Bio-rad laboratories, inc.). The proteins (20 µg/lane) were separated by SDS-PAGE and electrotransferred to PVDF membranes, which were blocked with 5% skimmed milk at room temperature for 1 h. The membranes were then incubated with CHOP (1:200; cat. no. ab11419; Abcam), Grp78 (1:100; cat. no. ab21685; Abcam) and GAPDH (1:10,000; cat. no. ab181602; Abcam) primary antibodies at 4˚C overnight.

The membranes were rinsed with TBST (0.05% Tween-20) and incubated with the specific horseradish peroxidase-conjugated secondary antibodies for CHOP (goat anti-mouse; 1:2,000; cat. no. ab205719; Abcam), Grp78 (goat anti-rabbit; 1:5,000; cat. no. ab205718; Abcam) and GAPDH (goat anti-rabbit; 1:1,000; cat. no. ab6721; Abcam) for 2 h at room temperature. The protein bands were visualized by enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.). Protein expression was quantified using Quantity One software (version 4.4; Bio-Rad Laboratories, Inc.).

ELISA. The cells were treated and suspended, the culture supernatants were collected into a 1-ml centrifuge tube and centrifuged at 3,000 x g for 10 min at room temperature, and the supernatant was collected. The levels of superoxide dismutase (SOD), nitric oxide (NO) and malondialdelyde (MDA) were measured using ELISA kits (SOD, cat. no. 19160; Sigma-Aldrich; Merck KGaA; NO, cat. no. S0021; Beyotime Institute of Biotechnology; MDA, cat. no. MAK085c; Sigma-Aldrich; Merck KGaA) according to the manufacturers' instructions. The absorbance was measured at 450 nm using an Infinite M200 PRO microplate reader (Tecan Group, Ltd.).

Statistical analysis. SPSS 17.0 (SPSS, Inc.) software was used for data analysis. The data are presented as the mean ± standard deviation from three independent experiments. One-way ANOVA with Tukey's post hoc test was used for multiple group analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of Dex on cell viability, LDH level, apoptosis and expression of inflammatory factors in H9c2 cells. The H9c2 cells were induced by H/R, and cell viability was detected by MTT assay. The results demonstrated that H9c2 cell viability was significantly lower in the H/R group compared with that in the control group, and that the dex+H/R group exhibited significantly higher cell viability compared with that in the H/R group (Fig. 1A). Additionally, LDH levels were significantly higher in the H/R group compared with the control and Dex+H/R groups (Fig. 1B). The results also demonstrated a higher apoptotic rate in the H/R group compared with that in the control group, and the apoptotic rate was significantly lower in the Dex+H/R group compared with those in the H/R group (Fig. 1C and D). In the H/R group, the expression levels of TNF-α, IL-1β and IL-6 were significantly higher compared with those in the control group, whereas the levels of TNF-α, IL-1β and IL-6 were significantly lower in the Dex+H/R group compared with those in the H/R group (Fig. 2A).

Dex affects the CHOP signaling pathway. CHOP and Grp78 expression levels were determined by RT-qPCR and western blotting. Both mRNA and protein expression levels of CHOP...
and Grp78 were significantly upregulated in the H/R group compared with the control group, whereas in the Dex+H/R group, CHOP and Grp78 expression levels were significantly lower compared with those in the H/R group (Fig. 2B-D).

Effects of silencing CHOP on cell viability, LDH level, apoptosis and expression of inflammatory factors and Grp78 in H9C2 cells. siCHOP was transfected into H9C2 cells, and the transfection efficiency was measured by western blotting. The expression of CHOP in the siCHOP group was significantly lower compared with the control and sinc groups (Fig. 3). In addition, MTT assay revealed that the cell viability was significantly higher in the siCHOP+Dex+H/R group compared with the sinc+dex+H/R group (Fig. 4a). In addition, the LDH levels and apoptotic rates were significantly lower in the siCHOP+Dex+H/R group compared with the H/R and sinc+Dex+H/R groups (Fig. 4B-D). In the H/R group, the expression levels of TNF-α, IL-1β and IL-6 were significantly higher compared with the control group; by contrast, the expression levels of TNF-α, IL-1β and IL-6 were significantly lower in the Dex+H/R and siCHOP+Dex+H/R groups compared with the H/R and sinc+Dex+H/R groups (Fig. 5A). Furthermore, the protein and mRNA expression levels of Grp78 were significantly elevated in the H/R group compared with the control, whereas the expression levels of Grp78 were lower in the Dex+H/R and siCHOP+Dex+H/R groups compared with the H/R and sinc+H/R groups (Fig. 5B-D).

Effects of CHOP overexpression on cell viability, LDH level, apoptosis and expression of inflammatory factors and Grp78 in H9C2 cells. The cHoP overexpression plasmid was transfected into H9C2 cells, and significantly higher levels of cHoP were observed in the cHoP group compared with the control and nc groups (Fig. 6a-c). The effect of cHoP
overexpression on cell viability was then measured; compared with the H/R and Dex+H/R groups, cell viability was significantly lower in the CHOP+Dex+H/R group (Fig. 6D). The CHOP+Dex+H/R group exhibited significantly higher LDH levels and apoptotic rates compared with the H/R and Dex+H/R groups (Fig. 6E-G). Furthermore, the expression levels of TNF-α, IL-1β and IL-6 were significantly lower in the Dex+H/R and NC+Dex+H/R groups, but significantly higher in the CHOP+Dex+H/R group compared with the H/R group (Fig. 7A). The Grp78 protein and mRNA expression levels were significantly higher in the CHOP+Dex+H/R group compared with the H/R and Dex+H/R groups (Fig. 6E-G). Furthermore, the expression levels of TNF-α, IL-1β and IL-6 were significantly lower in the Dex+H/R and NC+Dex+H/R groups, but significantly higher in the CHOP+Dex+H/R group compared with the H/R group (Fig. 7A). The Grp78 protein and mRNA expression levels were significantly higher in the CHOP+Dex+H/R group compared with the H/R and Dex+H/R groups (Fig. 6E-G).
Figure 6. Transfection efficiency of CHOP and effects of CHOP overexpression on cell viability, LDH level and apoptosis in H9C2 cells. (A-C) Expression levels of CHOP were determined by (A and B) western blotting and (C) RT-qPCR. (D) Cell viability of H9C2 cells was determined by MTT assay at 24 h. (E) LDH levels of H9C2 cells determined by 2,4-dinitrophenylhydrazine colorimetric assay. (F and G) Apoptotic rates of H9C2 cells were determined by flow cytometry. **P<0.001 vs. Control; ##P<0.001 vs. NC or H/R; ###P<0.001 vs. Dex+H/R; ^P<0.001 vs. NC+Dex+H/R. CHOP, c/eBP-homologous protein; RT-qPCR, reverse transcription-quantitative PCR; LDH, lactate dehydrogenase; H/R, hypoxia/reoxygenation; Dex, dexmedetomidine; NC, negative control; OD, optical density; PI, propidium iodide.

Figure 7. Effects of CHOP overexpression on the expression levels of inflammatory factors and Grp78 in H9C2 cells. (A) Expressions of TNF-α, IL-1β and IL-6 in H9C2 were detected by RT-qPCR. (B and C) Protein and (D) mRNA expression levels of Grp78 in H9C2 cells were determined by (B and C) western blotting and (D) RT-qPCR. **P<0.001 vs. H/R; #P<0.001 vs. Dex+H/R; ^P<0.001 vs. NC+Dex+H/R. TNF, tumor necrosis factor; IL, interleukin; RT-qPCR, reverse transcription-quantitative PCR; Dex, dexmedetomidine; H/R, hypoxia/reoxygenation; NC, negative control; CHOP, c/eBP-homologous protein.
levels were significantly higher in the CHOP+Dex+H/R group compared with the Dex+H/R group (Fig. 7B-D).

**Effects of silencing CHOP on the SOD activity and NO and MDA levels in H/R-induced H9C2 cells.** The effects of silencing CHOP on H/R-induced H9C2 cells were further observed by measuring SOD activity, as well as MDA and NO levels. The results demonstrated that the SOD activity was significantly decreased in the H/R group compared with the control group, and that the SOD activity was significantly higher in the Dex+H/R and siCHOP+Dex+H/R groups compared with that in the H/R and siNC+Dex+H/R groups, respectively (Fig. 8A). In addition, the NO and MDA levels were significantly higher in the H/R group compared with the control group, but significantly lower in the Dex+H/R and siCHOP+Dex+H/R groups compared with the HR and siNC+Dex+H/R groups (Fig. 8B and C).

**Discussion**

Dex has been demonstrated to reduce H/R-induced damage in primary neonatal rat cardiomyocytes (25). In addition, Dex reduces H$_2$O$_2$-induced cardiomyocyte apoptosis in neonatal rats through mitochondrial and endoplasmic reticulum-mediated oxidative stress pathways (26). To explore the mechanism of Dex pretreatment in the present study, an H/R model was constructed in H9C2 cells using sodium dithionite. Sodium dithionite depletes oxygen in the culture medium and simulates an anoxic environment, which causes hypoxic injury in cells without damaging the cell membrane; thus, it is frequently used to induce H/R injury in cells (27,28). In the present study, the H/R model was established with or without the pretreatment of Dex; the results demonstrated that H/R significantly reduced the viability of H9C2 cells, promoted apoptosis and increased the expression levels of LDH and inflammatory factors, whereas Dex pretreatment prior to H/R increased the cell viability and suppressed the release of LDH and inflammatory factors. LDH occurs ubiquitously in cardiomyocytes and is released into the blood upon damage to the cell membrane (29). Accordingly, the level of LDH in the supernatant of the culture medium may reflect the degree of cardiomyocyte injury (30-32). In corroborarion with the results of the present study, previous studies have demonstrated that the level of LDH is significantly higher in the H/R-induced I/R injury compared with the control in cardiomyocytes (33-35). The inflammatory factors TNF-α, IL-1β and IL-6 are commonly recognized as inflammatory indicators (36). TNF-α and IL-1β are responsible for the stimulation of chemokines and the secretion of adhesion molecules in ischemic tissues, and IL-6 is responsible for the regulation of inflammation (37,38). Previous studies have reported that Dex induces multiple protective effects on MIRI (39), and such findings were further supported by the present study. Yu et al (40) have reported that microRNA-665 and Dex protect cardiomyocytes against I/R injury caused by oxide stress and suppress apoptosis. Additionally, by inhibiting the expression of inflammatory cytokines, Dex attenuates the I/R injury induced by bilateral renal pedicle clamping (41).

The present study further explored the effects of CHOP on H9C2 cells induced by H/R. Yang et al (42) have observed that the expression levels of CHOP and Grp78 are significantly upregulated in cardiomyocytes following I/R. During the H/R process, high CHOP and Grp78 expression levels in H9C2 cells are present (43). CHOP serves a role in the apoptosis signaling pathways (44,45). CHOP and Grp78 are endoplasmic reticulum (ER) stress proteins and are widely recognized as ER stress markers (46). Evidence suggests important roles for ER stress proteins in the cardiomyocyte apoptosis caused by H/R process and its regulation on CHOP in ER stress-induced autophagy (47,48). In H/R-induced H9C2 cell autophagy, the high expression level of CHOP is reduced by the inhibition of ER stress (49). Liu et al (26) have reported that Dex alleviates cardiomyocyte apoptosis induced by H$_2$O$_2$ via ER stress pathways. Furthermore, Dex treatment has been observed to attenuate cerebral I/R injury by moderating the protein kinase RNA-like endoplasmic reticulum kinase/CHOP/Caspase-11 pathway through inhibition of the expression of ER stress-related apoptotic pathway proteins (50). In human umbilical vein endothelial cells, pretreatment with Dex increases cell survival rate, decreases expression of CHOP and caspase-3 protein, and the apoptotic rate of H/R-induced cells, suggesting a possible association between Dex and the CHOP signaling pathway in H/R-induced apoptosis (51). In the present study,
western blotting and RT-qPCR demonstrated that pretreatment with Dex significantly suppressed the H/R-induced high expression levels of CHOP and Grp78, suggesting a potential association between Dex and the CHOP signaling pathway. Additionally, in H/R-injured cells, knockdown of CHOP expression promoted cell viability, inhibited apoptosis and suppressed the release of LDH and expression of inflammatory factors and Grp78, whereas the overexpression of CHOP aggravated the cell injury induced by H/R. These results suggested that the block of CHOP may attenuate the negative effects of H/R on cell viability and apoptosis, release of LDH and the expression of inflammatory factors and Grp78 of H9C2 cells, and that the overexpression of CHOP inhibited the protective effects of Dex on cardiomyocytes.

However, the present study is still not rigorous enough; the effects of Dex on the cardiomyocytes of H/R-injured neonatal mice need further research. In future studies, an H/R injury mouse model will be established or neonatal mouse cardiomyocytes will be selected for research.

In conclusion, the present study explored the effects of Dex on H/R-induced H9C2 cells and observed that the pretreatment with Dex may alleviate H/R-induced cell injury through regulating the CHOP pathway.

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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
XS, ZL and JL made substantial contributions to the conception and design of the study, data acquisition, analysis and interpretation, drafted the manuscript and critically revised it for important intellectual content. All authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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