Upregulation of serum and glucocorticoid-regulated kinase 1 (SGK1) ameliorates doxorubicin-induced cardiotoxic injury, apoptosis, inflammation and oxidative stress by suppressing glucose regulated protein 78 (GRP78)-mediated endoplasmic reticulum stress

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ABSTRACT
The clinical application of doxorubicin (Dox) in tumor chemotherapy is limited by time-dependent and dose-dependent cardiotoxicity. Hence, there is an urgent need to elucidate doxorubicin cardiotoxicity and to solve the difficult problem in clinical application. It has been verified that serum and glucocorticoid-regulated kinase 1 (SGK1) possess cardioprotective effects. Here, H9c2 cells were treated with 1 μM doxorubicin for 24 h to establish doxorubicin cardiotoxicity, so as to determine the biological role of SGK1 in doxorubicin cardiomyopathy and to elucidate the underlying molecular mechanism. SGK1 level in doxorubicin-treated H9c2 cells was assessed by performing Western blot assay and RT-qPCR. CCK-8 assay and TUNEL staining were employed to evaluate the cell viability and cell apoptosis. Besides, apoptosis-related proteins were measured by Western blot assay to analyze cell apoptosis. Additionally, the release of TNF-α, IL-1β, IL-6, and IL-10 and the levels of ROS, MDA, and SOD were detected to reflect inflammation and oxidative stress. Moreover, Western blot assay was adopted for determination of ERS-associated proteins. Results revealed that SGK1 was downregulated in doxorubicin-treated H9c2 cells. Upregulation of SGK1 alleviated doxorubicin-induced cardiotoxic injury, cell apoptosis, inflammation and oxidative stress in H9c2 cells. Moreover, SGK1 overexpression mitigated doxorubicin-induced ERS in H9c2 cells. The suppressing effects of SGK1 on doxorubicin-induced cardiotoxic injury, apoptosis, inflammation, oxidative stress and ERS in H9c2 cells were partially abolished upon GRP78 overexpression. To conclude, upregulation of SGK1 may alleviate doxorubicin cardiotoxicity by repressing GRP78-mediated ERS.

Introduction
Doxorubicin (Dox) is an anthracycline antibiotic with strong anti-tumor effect, which is widely used in single or combined chemotherapy for diverse solid tumors and hematological malignancies [1]. Nevertheless, its clinical application is limited by time-dependent and dose-dependent cardiotoxicity [2]. Under the premise of ensuring the anti-tumor effect, reducing the cardiotoxicity of doxorubicin is one of the key points of tumor chemotherapy [3].

Despite a large amount of research on doxorubicin, no suitable target has been found to reduce cardiotoxicity caused by doxorubicin while ensuring the efficacy of chemotherapy [4,5]. It has been verified that doxorubicin cardiomyopathy is related to apoptosis of cardiomyocytes [6]. Endoplasmic reticulum stress (ERS) is a response of cells to relieve stress caused by accumulation of abnormal proteins in the endoplasmic reticulum [7]. Severe or prolonged presence of ERS could promote ERS-associated signaling cascades and stimulate apoptosis [8]. It has been accepted that doxorubicin-induced intrinsic activation of ERS poses important functions in myocardial dysfunction [9]. Glucose regulated protein 78 (GRP78) belongs to the heat shock protein 70 (HSP70) family [10]. It has a highly conserved structure and versatile functions and can participate in the protein folding and assembling as an ER molecular chaperone [11]. Hu [12] et al. hold the view that doxorubicin
elevates the expressions of ERS-related proteins including GRP78, leading to ERS-associated cardiomyocyte apoptosis. Therefore, GRP78-mediated ERS may serve as a critical indicator of doxorubicin cardiomyopathy.

Serum and glucocorticoid-regulated protein kinases (SGKs) are serine-threonine protein kinases of the AGC family (protein kinase A, G, C), which are encoded by three genes located on different chromosomes (SGK1, SGK2, and SGK3) [13]. SGK1 is widely expressed in human body and involved in regulating multiple functions, such as apoptosis, DNA damage, signal transduction, and ion transmembrane transport [14]. In addition, sparse literature confirms that downregulation of SGK1 can induce GRP78-mediated ERS [15]. Furthermore, upregulation of SGK1 has been reported to participate in the protective effects on cardiomyocytes [16].

Herein, SGK1 level in doxorubicin-treated H9c2 cells was assessed and the effects of SGK1 on cell viability, cell apoptosis, inflammation, oxidative stress and ERS in doxorubicin-treated H9c2 cells were also evaluated, so as to demonstrate the biological role of SGK1 in doxorubicin cardiotoxicity. Moreover, the molecular mechanism underlying the participation of ERS in doxorubicin cardiotoxicity was elaborate through a series of functional experiments.

Materials and methods

Cell culture

H9c2 cells were obtained from the American Type Culture Collection (ATCC, VA, USA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA) and 1% penicillin/streptomycin (Gibco, NY, USA) in a humidified incubator with 5% CO₂ at 37°C.

H9c2 cells were treated with the specific SGK1 inhibitor EMD638683 to downregulate SGK1 expression.

Cell transfection

Transfection of pcDNA 3.1 vectors (GenePharma, Shanghai, China) carrying SGK1 gene (Ov-SGK1) or GRP78 gene (Ov-GRP78) or negative control (Ov-NC) was conducted using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) in strict line with the manufacturer’s instructions. Briefly, cells were seeded in a 6-well plate and incubated until they reached ≥85% confluence. 50 nM vectors and 5 μl Lipofectamine 2000 reagent were separately incubated in serum-free Opti-MEM (Gibco, NY, USA) for 5 min and then mixed for another 20 min incubation. Subsequently, the mixture was added into cells and cultured for 8 h. Then, the serum-free medium was replaced with DMEM (10% FBS) and cultured for 48 h. The transfected cells were harvested for further investigation.

Cell counting kit (CCK)-8 assay

H9c2 cells (5 × 10³ cells per well) were seeded in 96-well plates and cultured in a standard atmosphere. After the designed treatment, H9c2 cells were incubated with 10 μl CCK-8 solution (Beyotime, Shanghai, China) for 4 h. Finally, the absorbance at 450 nm of each well was detected using a microplate reader (Bio-Rad, CA, USA).

Evaluation of lactate dehydrogenase (LDH) activity

The release of intracellular LDH was assessed using the biochemical analysis kit (Sigma-Aldrich, MO, USA) in line with the manufacturer’s protocol.

Determination of malondialdehyde (MDA) and superoxide dismutase (SOD)

In line with the protocol recommended by manufacturer, the levels of MDA and SOD were analyzed using MDA assay kit and SOD assay kit (Beyotime, Shanghai, China).
Detection of intracellular reactive oxygen species (ROS)

Intracellular ROS generation was detected by 2′, 7′-dichlorofluorescin-diacetate (DCFH2-DA; Beyotime, Shanghai, China). Briefly, after the designed treatment, H9c2 cells were washed twice with PBS and then incubated with DCFH2-DA (10 µM) for 30 min. Cells were imaged under a fluorescence microscope (Leica, Wetzlar, Germany).

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-1β, IL-6 and IL-10 in cell supernatants were measured by the standard ELISA kits (Roche, Basel, Switzerland) in line with the manufacturer’s instructions. Optical densities were detected using a microplate reader (Bio-Rad, CA, USA).

TUNEL staining

Apoptosis of H9c2 cells was determined by performing TUNEL staining. In brief, H9c2 cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 solution for 5 min on ice. Next, H9c2 cells were incubated with TUNEL reagent (Roche, Basel, Switzerland) for 1 h at 37°C and then counterstained with 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 15 min in the dark. Images were observed under a fluorescence microscope (Leica, Wetzlar, Germany).

Western blot assay

Total proteins were extracted using RIPA lysis buffer (Beyotime, Shanghai, China) and protein concentration was quantified with a BCA protein assay kit (Beyotime, Shanghai, China). Protein samples were isolated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes. After blocking with 5% bovine serum albumin (BSA) for 1 h at room temperature, membranes were incubated overnight at 4°C with primary antibodies against SGK1 (Abcam, ab32374, 1:500), Bcl-2 (Abcam, ab32124, 1:1000), Bax (Abcam, ab243140, 1:1000), Cleaved caspase-3 (Abcam, ab2302, 1:1000), caspase-3 (Abcam, ab184787, 1:2000), Cleaved caspase-9 (Abcam, ab2324, 1:1000), caspase-9 (Abcam, ab32539, 1:5000), GRP78 (Abcam, ab108615, 1:1000), p-PERK (CST company, 3179s, 1:1000) PERK (CST company, 3192s, 1:1000), ATF4 (Abcam, ab184909, 1:1000), CHOP (Abcam, ab11419, 1:1000) and GAPDH (Abcam, ab32124, 1:1000) and incubated with the corresponding secondary antibody (Abcam, ab205718, 1:50,000) for 1.5 h at room temperature. Protein signals were visualized using electrochemiluminescence (ECL; Beyotime, Shanghai, China) method and detected by a Bio-Rad imaging system (Bio-Rad, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA extraction was implemented with TRIzol Reagent (Invitrogen, CA, USA) in compliance with the manufacturer’s instructions. Equal amount of RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) by the PrimeScript RT Kit (Takara, Tokyo, Japan). PCR amplifications were quantified using an SYBR-Green master mix kit (Takara, Tokyo, Japan) in ABI 7500 quantitative PCR instrument (ABI/Perkin Elmer, CA, USA). The PCR conditions were as follows: 94 °C for 10 min, followed by 40 cycles of 94 °C for 15 sec and 60 °C for 60 sec. The sequences of the primers were as follows: SGK1 forward: 5′-ATCGTGTAGCTCAAAGGC-3′, reverse: 5′-GTCTGTGATCAGGCATAGC-3′; GRP78 forward: 5′-CACGCCGGTCTATGTCGC-3′, reverse: 5′-AAATGTCTTTGTTTGCCCACC-3′; GAPDH forward: 5′-TGACTTCAACAGCGACACCCA-3′, reverse: 5′-CACCTGGTTGCTGTAGCCAAA-3′. GAPDH served as the internal control. Relative gene expressions of SGK1 and GRP78 were normalized to GAPDH and calculated by 2−ΔΔCt method.

Statistical analysis

Experimental data of three replicates were expressed as mean values ± standard deviation (SD). One-way analysis of variance followed by Tukey’s post hoc test was employed to test the differences among multiple groups. Differences with statistical significance were set at p < 0.05.
Results

SGK1 was downregulated in doxorubicin-treated H9c2 cells

H9c2 cells were treated with 1 μM doxorubicin for 3, 6, 12, or 24 h. Treatment with doxorubicin for 6, 12, 24 h significantly downregulated SGK1 expression in H9c2 cells (Figure 1(a,b)), prompting the potential role of SGK1 in doxorubicin cardiomyopathy. Then, treatment with doxorubicin for 24 h was selected to establish doxorubicin-induced cardiotoxicity in H9c2 cells for further investigation.

Upregulation of SGK1 alleviated doxorubicin-induced cardiotoxic injury and suppressed the apoptosis of doxorubicin-treated H9c2 cells

In order to demonstrate the biological function of SGK1 in doxorubicin cardiomyopathy, H9c2 cells

Figure 1. SGK1 was downregulated in doxorubicin-treated H9c2 cells. H9c2 cells were treated with 1 μM doxorubicin for 3, 6, 12 or 24 h. (a) Western blot assay for determination of SGK1 protein expression in H9c2 cells. (b) RT-qPCR for determination of SGK1 mRNA level in H9c2 cells. * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 2. Upregulation of SGK1 alleviated doxorubicin-induced cardiotoxic injury. (a) H9c2 cells were transfected with Ov-SGK1 or Ov-NC. Western blot assay for determination of SGK1 protein expression in H9c2 cells. (b) H9c2 cells were transfected with Ov-SGK1 or Ov-NC. RT-qPCR for determination of SGK1 mRNA level in H9c2 cells. (c) Doxorubicin-treated H9c2 cells were transfected with Ov-SGK1 or treated with the specific SGK1 inhibitor EMD638683. CCK-8 assay for determination of the viability of H9c2 cells. (d) Doxorubicin-treated H9c2 cells were transfected with Ov-SGK1 or treated with the specific SGK1 inhibitor EMD638683. LDH assay kit for determination of the production of intracellular LDH. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 3. Upregulation of SGK1 suppressed the apoptosis of doxorubicin-treated H9c2 cells. Doxorubicin-treated H9c2 cells were transfected with Ov-SGK1 or treated with the specific SGK1 inhibitor EMD638683. (a, b) TUNEL staining for determination of the apoptosis of H9c2 cells. (c) Western blot assay for determination of Bcl-2, Bax, Cleaved caspase-3, caspase-3, Cleaved caspase-9 and caspase-9 protein expressions in H9c2 cells. ** p < 0.01, *** p < 0.001.
were transfected with Ov-SGK1 to upregulate SGK1 expression or treated with the specific SGK1 inhibitor EMD638683 to downregulate SGK1 expression. The transfection efficiency of Ov-SGK1 was validated by performing Western blot assay and RT-qPCR (Figure 2(a,b)). Doxorubicin treatment inhibited the viability of H9c2 cells and increased the production of intracellular LDH. Then, SGK1 overexpression enhanced the viability of doxorubicin-treated H9c2 cells and reduced LDH production, mitigating doxorubicin-induced cardiotoxic injury. Additionally, SGK1 inhibitor exerted the opposite effects on the viability of H9c2 cells and LDH production, further intensifying doxorubicin-induced cardiotoxic injury (Figure 2(c,d)). It was observed that cell apoptosis manifested as the number of TUNEL positive cells was extremely increased in doxorubicin-treated H9c2 cells. SGK1 overexpression reduced TUNEL positive cells while SGK1 inhibitor increased TUNEL positive cells in doxorubicin-treated H9c2 cells. Upregulation of SGK1 suppressed the apoptosis of doxorubicin-treated H9c2 cells and downregulation of SGK1 promoted the apoptosis of doxorubicin-treated H9c2 cells (Figure 3(a,b)). Moreover, increased Bcl-2 expression and decreased expressions of Bax, Cleaved caspase-3 and Cleaved caspase-9 also indicated that SGK1 overexpression could repress the apoptosis of doxorubicin-treated H9c2 cells. SGK1 inhibitor exhibited the opposite effects on the expressions of Bcl-2, Bax, Cleaved caspase-3, and Cleaved caspase-9, boosting the apoptosis of doxorubicin-treated H9c2 cells (Figure 3(c)).

**Figure 4.** Upregulation of SGK1 mitigated inflammation and oxidative stress in doxorubicin-treated H9c2 cells. Doxorubicin-treated H9c2 cells were transfected with Ov-SGK1 or treated with the specific SGK1 inhibitor EMD638683. (a) ELISA kits for determination of TNF-α, IL-1β, IL-6 and IL-10 levels. (b) DCFH-DA for determination of ROS content. (c) MDA assay kit and SOD assay kit for determination of MDA and SOD levels. * p < 0.05, ** p < 0.01, *** p < 0.001.
Upregulation of SGK1 mitigated inflammation and oxidative stress in doxorubicin-treated H9c2 cells

Obvious inflammation and oxidative stress was observed in doxorubicin-treated H9c2 cells. SGK1 overexpression decreased the release of TNF-α, IL-1β, and IL-6 and increased IL-10 release, alleviating doxorubicin-induced inflammation in H9c2 cells. SGK1 inhibitor enhanced the release of TNF-α, IL-1β, and IL-6 and reduced IL-10 release, aggravating doxorubicin-induced inflammation in H9c2 cells (Figure 4(a)). Additionally, SGK1 overexpression decreased the levels of ROS and MDA and increased SOD level, relieving doxorubicin-induced oxidative stress in H9c2 cells. SGK1 inhibitor enhanced the levels of ROS and MDA and reduced SOD level, intensifying doxorubicin-induced oxidative stress in H9c2 cells (Figure 4(b,c)).

Upregulation of SGK1 relieved doxorubicin-induced ERS in H9c2 cells

Doxorubicin treatment induced ERS in H9c2 cells. SGK1 overexpression decreased the expressions of GRP78, p-PERK, ATF4, and CHOP, relieving doxorubicin-induced ERS in H9c2 cells. SGK1 inhibitor exerted the opposite effects on the expressions of GRP78, p-PERK, ATF4, and CHOP, exacerbating doxorubicin-induced ERS in H9c2 cells (Figure 5).

SGK1 overexpression alleviated doxorubicin-induced cardiotoxic injury and suppressed the apoptosis of doxorubicin-treated H9c2 cells by repressing GRP78-mediated ERS

To elaborate the molecular mechanism underlying the participation of ERS in doxorubicin cardiotoxicity, H9c2 cells were transfected with Ov-GRP78. Introduction of Ov-GRP78 distinctly upregulated GRP78 protein and mRNA levels (Figure 6(a,b)). Enhanced expressions of GRP78, p-PERK, ATF4, and CHOP upon GRP78 elevation evidenced that upregulation of GRP78 exacerbated ERS, abolishing the suppressing effect of SGK1 on ERS in doxorubicin-treated H9c2 cells (Figure 6(c)). Besides, upregulation of GRP78 reduced the viability of H9c2 cells and increased the production of intracellular LDH, reversing the influence of SGK1 on cell

Figure 5. Upregulation of SGK1 relieved doxorubicin-induced ERS in H9c2 cells. Doxorubicin-treated H9c2 cells were transfected with Ov-SGK1 or treated with the specific SGK1 inhibitor EMD638683. Western blot assay for determination of GRP78, p-PERK, PERK, ATF4 and CHOP protein expressions in H9c2 cells. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 6. SGK1 overexpression alleviated doxorubicin-induced cardiotoxic injury by repressing GRP78-mediated ERS. (a) H9c2 cells were transfected with Ov-GRP78 or Ov-NC. Western blot assay for determination of GRP78 protein expression in H9c2 cells. (b) H9c2 cells were transfected with Ov-GRP78 or Ov-NC. RT-qPCR for determination of GRP78 mRNA level in H9c2 cells. (c) Doxorubicin-treated H9c2 cells were transfected with Ov-SGK1 or co-transfected with Ov-SGK1 and Ov-GRP78. Western blot assay for determination of GRP78, p-PERK, PERK, ATF4 and CHOP protein expressions in H9c2 cells. (d) Doxorubicin-treated H9c2 cells were transfected with Ov-SGK1 or co-transfected with Ov-SGK1 and Ov-GRP78. CCK-8 assay for determination of the viability of H9c2 cells. (e) Doxorubicin-treated H9c2 cells were transfected with Ov-SGK1 or co-transfected with Ov-SGK1 and Ov-GRP78. LDH assay kit for determination of the production of intracellular LDH. * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 7. SGK1 overexpression suppressed the apoptosis of doxorubicin-treated H9c2 cells by repressing GRP78-mediated ERS. Doxorubicin-treated H9c2 cells were transfected with Ov-SGK1 or co-transfected with Ov-SGK1 and Ov-GRP78. (a, b) TUNEL staining for determination of the apoptosis of H9c2 cells. (c) Western blot assay for determination of Bcl-2, Bax, Cleaved caspase-3, caspase-3, Cleaved caspase-9 and caspase-9 protein expressions in H9c2 cells. * p < 0.05, *** p < 0.001.
viability and LDH production in doxorubicin-treated H9c2 cells (Figure 6(d,e)). In addition, increased TUNEL positive cells, decreased Bcl-2 expression as well as increased expressions of Bax, Cleaved caspase-3 and Cleaved caspase-9 jointly confirmed that GRP78 elevation promoted the apoptosis of H9c2 cells, abrogating the inhibition of SGK1 on the apoptosis of doxorubicin-treated H9c2 cells (Figure 7(a-c)).

**SGK1 overexpression mitigated inflammation and oxidative stress in doxorubicin-treated H9c2 cells by repressing GRP78-mediated ERS**

Obvious increases in TNF-α, IL-1β, IL-6 and IL-10 caused by GRP78 overexpression suggested that upregulation of GRP78 aggravated doxorubicin-induced inflammation in H9c2 cells, reversing the inhibition of SGK1 on inflammation in doxorubicin-treated H9c2 cells (Figure 8(a)). Besides, GRP78 overexpression elevated the levels of ROS and MDA and reduced SOD level, abrogating the suppressing effects of SGK1 on oxidative stress in doxorubicin-treated H9c2 cells (Figure 8(b,c)).

**Discussion**

Doxorubicin is widely used in tumor chemotherapy, but doxorubicin cardiotoxicity severely limits its clinical application [2]. Exploration of the molecular mechanism underlying doxorubicin cardiotoxicity is crucial to the development of cancer.
cardiotoxicity. Recently, the understanding of cardiotoxicity has ranged from early clinical and pathological observations to molecular biology and translational medicine research using biomarkers [6,17].

SGK1, a serine/threonine protein kinase, was discovered in 1993 since it was rapidly transcribed in rat breast cancer cells when stimulated by serum and/or glucocorticoids. It has been verified that SGK1 possesses cardioprotective effects [18]. Sun et al. [16] have identified that activation of SGK1 could participate in the protection of cardiomyocytes. In addition, literature reports that SGK1 inhibitor could suppress Angiotension II (Ang II)-induced cardiac fibrosis and remodeling [19]. In this present work, results discovered that SGK1 was downregulated in doxorubicin-treated H9c2 cells. Upregulation of SGK1 alleviated doxorubicin-induced cardiotoxic injury and suppressed the apoptosis of doxorubicin-treated H9c2 cells. Besides, upregulation of SGK1 mitigated doxorubicin-induced inflammation and oxidative stress in H9c2 cells.

Endoplasmic reticulum can avoid the accumulation of misfolded and unfolded proteins by activating a series of signaling pathways, and then maintain eukaryotic cell homeostasis, which is called ERS [20]. The Protein kinase R-like ER kinase (PERK)-mediated pathway is one of the important signaling pathways in ERS [21]. Under the condition of ERS, PERK is phosphorylated and activated when GRP78 dissociates from PERK [21]. Activated PERK phosphorylates eukaryotic translation-initiation factor 2a (eIF2a), which shuts down cap-dependent translation and facilitates the production of activating transcription factor 4 (ATF4) [22]. ATF4 can induce C/EBP homologous protein (CHOP) that is involved in the promotion of apoptosis [22,23]. Previous reports have demonstrated that doxorubicin could enhance the expressions of ERS-related proteins in cardiac tissues, prompting the vital role of ERS in doxorubicin cardiomyopathy [24–26]. In the current research, it was revealed that doxorubicin treatment induced ERS in H9c2 cells. SGK1 overexpression reduced the expressions of GRP78, p-PERK, ATF4, and CHOP, mitigating ERS in doxorubicin-treated H9c2 cells. Moreover, GRP78 elevation abrogated the suppressing effects of SGK1 on doxorubicin-induced cardiotoxic injury, apoptosis, inflammation, oxidative stress, and ERS in H9c2 cells.

**Conclusion**

To sum up, the suppressing effects of SGK1 on doxorubicin-induced cardiotoxic injury, apoptosis, inflammation, oxidative stress, and ERS in H9c2 cells were partially abolished upon GRP78 overexpression. Upregulation of SGK1 may alleviate doxorubicin cardiotoxicity by repressing GRP78-mediated ERS. These findings prompted that SGK1 could serve as a biological indicator in the diagnosis of doxorubicin cardiotoxicity. SGK1 agonists may be a promising approach for the therapies of doxorubicin cardiomyopathy.

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**Consent for publication**

All authors approve to submit the manuscript to your journal for publication.

**Authors’ contributions**

Feng Wang and Lili Han designed study, performed experiments, analyzed data and wrote manuscript.

**Data availability statement**

Data sets generated during the present study are available from the corresponding author on reasonable request.

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