Epigallocatechin-3-gallate ameliorates LPS-induced inflammation by inhibiting the phosphorylation of Akt and ERK signaling molecules in rat H9c2 cells

ZHI HUI LI1, ZHANLI SHI1, SHENGJIE TANG2, HANG PING YAO3, XIHUA LIN2 and FANG WU2

1Department of Intensive Care Unit, Hangzhou Red Cross Hospital/Hospital of Integrated Traditional Chinese and Western Medicine in Zhejiang Province, Hangzhou, Zhejiang 310003; 2Department of Endocrinology, The Affiliated Sir Run Run Shaw Hospital, School of Medicine; 3State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Institute of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310016, P.R. China

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Abstract. The inflammatory response has been implicated in various cardiac and systemic diseases. Epigallocatechin-3-gallate (EGCG), the major polyphenol extracted from green tea, has various biological and pharmacological properties, such as anti-inflammation, anti-oxidative and anti-tumorigenesis. To some extent, the mechanism of EGCG in the inflammatory response that characterizes myocardial dysfunction is not fully understood. The present study aimed to investigate the inhibiting effect of EGCG on lipopolysaccharide (LPS)-induced inflammation in vitro. Treatment with LPS affected rat H9c2 cardiomyocytes and induced an inflammatory response. However, the LPS-induced effects were attenuated after treatment with EGCG. The present results demonstrated that EGCG treatment repressed several inflammatory mediators, such as vascular endothelial growth factor, chemokine ligand 5, chemokine ligand 2, intercellular adhesion molecule-1, matrix metalloproteinase-2, tumor necrosis factor-α and nitric oxide (induced by LPS), and the repressing effect of EGCG on inflammatory response was dose-dependent in the range of 6.25-100 µM. EGCG inhibited these marked inflammatory key signaling molecules by reducing the expression of phospho-nuclear factor-κB p65, -Akt, -ERK and -MAPK p38 while the total protein level of these signal proteins were not affected. In conclusion, the present findings suggested that EGCG possesses cardiomyocyte-protective action in reducing the LPS-induced inflammatory response due to the inhibition of the phosphorylation of Akt and ERK signaling molecules.

Introduction

The inflammatory response is the key pathogenesis of the most common forms of heart disease and its processes underlie various conditions related with injury of the cardiac muscle, such as cardiomyopathy, myocardial infarction, sepsis and heart failure (1,2). Lipopolysaccharide (LPS) is a major component of the bacterial outer membrane, and plays a crucial role in the initiation of several diseases (3). Numerous previous studies demonstrated that LPS contributes to inflammation and apoptosis; for example, LPS-induced acute respiratory distress syndrome, systemic inflammation induced by a low dose of LPS in mice and LPS-stimulated acute kidney injury (4,5). LPS can also induce inflammation and apoptosis in cardiomyocytes (6).

LPS, as a stimulus, contributes to pro-inflammatory responses, in addition to the increased expression of numerous inflammatory cytokines, including tumor necrosis factor-α (TNF-α), monocyte chemo-attractant protein (MCP)-1 and intercellular adhesion molecule (ICAM)-1 in the heart (7). A previous study has demonstrated that TNF-α shows direct negative inotropic effects and several features of heart failure (HF). In HF, inflammation might be associated with dysregulation of the TNF-α feedback system (8). A previous study identified that cardiac inflammation is accompanied by overexpression of ICAM-1 and vascular cell adhesion molecule (VCAM)-1 (9). During the development of inflammation, cellular adhesion molecules (CAMs) mediate the transendothelial migration of immune-cells into the cardiac tissue. Those infiltrated cells, as well as cardiomyocytes, produce pro-inflammatory cytokines, such as TNF-α, interleukin (IL)-1β and IL-18. These cytokines...
stimulate the expression of CAMs in a positive feedback system. Furthermore, they have direct and indirect detrimental effects on the heart (10).

Epigallocatechin-3-gallate [EGCG; Fig. 1; (11)] is the major polyphenol extracted from green tea, which is the most abundant and well-studied catechin (12). Previous studies demonstrated the beneficial effects of EGCG, including potential anti-oxidative, anti-inflammatory and anti-tumorigenesis properties in the treatment and prevention of several chronic diseases, including heart diseases, cancer, obesity and endocrine disorders (13,14). Among these effects, the anti-inflammatory activity of EGCG plays a vital role against these diseases.

EGCG was demonstrated to decrease expression of inflammatory genes, such as TNF-α, IL-1β, IL-6 and IL-8, when EGCG (10 µg/ml) was applied to inflamed human corneal epithelial cells (15). Similarly, there was a significant down-regulation of the expression of some kidney injury markers and pro-inflammatory mediators in unilateral ureteral obstruction (16). Previous studies demonstrated that cardiac injuries were associated with the activation of p38, PI3K-Akt and ERK 1/2 (17). The PI3K-Akt pathway plays a vital role in many biological reactions, including inflammatory responses, chemotaxis, cellular activation and apoptosis, which also regulates the expressions of inflammatory genes (18). Previous studies have demonstrated that inflammation stimulates the activation of PI3K/Akt signaling pathway in cardiomyocytes (19,20). A previous study demonstrated that EGCG repressed the PI3K/Akt system in fibroblast cells and phosphorylation of ERK and Akt kinases in epidermal growth factor stimulated cells (21). However, to the best of the authors' knowledge, few studies have investigated the effect of EGCG on LPS-induced inflammation in H9c2 cells.

Therefore, the aim of the present study was to investigate the regulation of EGCG on inflammatory mediators, and examine whether EGCG treatment could ameliorate inflammatory responses induced by LPS in H9c2 and the underlying mechanisms in vitro.

Materials and methods

Reagents. EGCG (purity, 98%) was purchased from Sigma-Aldrich (Merck KGaA). LPS was obtained from Sigma-Aldrich (Merck KGaA). The Cell Titer 96 Aqueous cell viability assay kit was procured from Promega Corporation. ELISA kits for vascular endothelial growth factor (VEGF; cat. no. DY493), Rantes (cat. no. DY478), MCP-1 (cat. no. SMJEO00B), ICAM-1 (cat. no. MIC100), matrix metalloproteinase 2 (MMP-2; cat. no. MMP200) and TNF-α (cat. no. SMTA00B), as well as nitric oxide (NO; cat. no. SKGE001) assay kits were purchased from R&D Systems, Inc. Anti-Akt (cat. no. 4690), anti-nuclear factor-κB (NF-κB) p65 (cat. no. 3034), anti-p38 (cat. no. 9212), anti-Erk (cat. no. 4695), anti-phosphorylated (p)-Akt (cat. no. 4060), anti-p-NF-κB p65 (cat. no. 3033) and anti-p-ERK (cat. no. 4376) were all obtained from Cell Signaling Technology, Inc. Mouse anti-β-actin (cat. no. sc-58673) was obtained from Santa Cruz Biotechnology, Inc.

Cell culture. The rat embryonic-heart derived cell line H9c2 was obtained from The American Type Culture Collection. Cells were cultured in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.), 25 mM D-glucose, 100 U/ml penicillin and 100 U/ml streptomycin, and maintained in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every 2 days.

Viability evaluation. Cell viability was determined by an MTS assay (Promega Corporation). Cultured H9c2 cells (1x10⁴ cells/well in 96-well plate) were treated with EGCG (0, 1.5, 3.0, 6.25, 12.5, 25, 50 and 100 µmol/l) for 24 h. Cultured supernatants were collected and analyzed for the release of cyto- and chemokines using commercial ELISA test systems. Levels of VEGF, Rantes, MCP-1, ICAM-1, MMP-2 and TNF-α were determined using ELISA kits (R&D Systems, Inc.). Absorbance was measured at 450 nm, with the correction wavelength set at 540 or 570 nm.

ELISA analysis. Cultured H9c2 cells were treated with LPS (250 ng/ml) or LPS (250 ng/ml) + EGCG (0, 1.5, 3.0, 6.25, 12.5, 25, 50 and 100 µmol/l) for 24 h. Cultured supernatants were collected and analyzed for the release of cyto- and chemokines using commercial ELISA test systems. Levels of VEGF, Rantes, MCP-1, ICAM-1, MMP-2 and TNF-α were determined using ELISA kits (R&D Systems, Inc.). Absorbance was measured at 450 nm, with the correction wavelength set at 540 or 570 nm.

NO assay. Cultured H9c2 cells were treated with LPS (250 ng/ml) or LPS (250 ng/ml) + EGCG (0, 1.5, 3.0, 6.25, 12.5, 25, 50 and 100 µmol/l) for 24 h. Nitrite determination was detected using Griess reagent. The absorbance was measured at 540 nm using a flow-through spectrophotometer. The sensitivity of the NO assay was <0.78 µmol/l.

Western blot analysis. Cultured H9c2 cells (5x10⁴/10 cm dish) were treated with LPS (250 ng/ml) or LPS (250 ng/ml) + EGCG (0, 1.5, 3.0, 6.25, 12.5, 25, 50 and 100 µmol/l) for 24 h. After treatment, cells were washed twice with cold PBS and lysed with RIPA lysis buffer (Beyotime Institute of Biotechnology) for 30 min at 4°C. Extracted protein in each cell lysate was determined using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts (20 µg) of protein were separated by SDS-PAGE on 10% gels. Proteins were transferred to a PVDF membrane and blocked with 5% non-fat dry milk in PBS with 0.02% v/v Tween-20 for 1 h at room temperature. The membrane was incubated for 16 h with primary antibodies (all 1:1,000) in PBS-Tween at 4°C. The membrane was washed and incubated for 1 h at room temperature with a peroxidase-labeled secondary antibody in PBS-Tween (anti-mouse, cat. no. P0447; anti-rabbit, cat. no. P0448; Dako; Agilent Technologies, Inc.). After further washing, the membrane was detected with ECL chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). ImageQuantTL software (LAS 4000; GE Healthcare) was used for densitometry.

Statistical analysis. All experiments were performed in triplicate. The data are presented as the mean ± SEM. One-way ANOVA and Tukey's test were used to determine the statistical
significance of differences among the experimental groups and the control group. SPSS 20.0 (IBM Corp) was used for statistical analyses. P<0.05 was considered to indicate a statistically significant difference.

Results

**EGCG does not markedly affect cell viability in H9c2 cells.** The effects of EGCG on cell viability in H9c2 were examined by the MTS assay. Cultured H9c2 cells (1x10^4 cells/well in a 96-well plate) were incubated without or with EGCG (1.5, 3.0, 6.25, 12.5, 25, 50, 100 and 200 µmol/l) for 48 h. Cell viability did not change significantly with respect to the control up to 100 µM. However, 200 µM EGCG significantly reduced cell viability and caused cytotoxicity in H9c2 cells (Fig. 2). Therefore, concentrations <200 µM of EGCG were selected for use in the subsequent experiments.

**EGCG attenuates LPS-induced inflammation in H9c2 cardiac cells.** To investigate the effects of EGCG on the inflammatory response in H9c2 cells, inflammatory cytokine expressions were determined using ELISA. LPS (250 ng/ml) significantly increased the TNF-α (Fig. 3A; P<0.001), ICAM-1 (Fig. 3B; P<0.001) and MMP-2 (Fig. 3C; P<0.001) protein levels in the medium supernatant compared with the control, according to ELISA, whereas EGCG suppressed the release of these cytokines in LPS-treated cells in a dose-dependent manner (Table I). The levels of TNF-α and MMP-2 were significantly alleviated after EGCG intervention (≥12.5 µmol/l). Meanwhile, treatment with EGCG (≥6.25 µmol/l) significantly reduced the expression of ICAM-1 induced by LPS.

**EGCG inhibits LPS-induced chemokine expression related to inflammation.** To further analyze the cardioprotective role of EGCG, the concentrations of chemokines MCP-1 and Rantes in the H9c2 cell medium supernatant were analyzed after LPS stimulation. The present data demonstrated that LPS induced a significant upregulation of the expression of MCP-1 (Fig. 3D; P<0.001) and Rantes (Fig. 3E; P<0.001) compared with the control group. EGCG treatment significantly attenuated the LPS-induced increased MCP-1 and Rantes in a dose-dependent manner when the concentrations of EGCG were ≥25 or ≥6.25 µmol/l, respectively (Fig. 3D and E; Table I).

**Inhibitory effect of EGCG on LPS-induced upregulation of VEGF in H9c2 cells.** To evaluate the inhibitory effect of EGCG on the LPS-induced upregulation of VEGF, the levels of VEGF in the supernatant of H9c2 cells were determined by ELISA. Cultured H9c2 cells were treated without or with LPS (250 ng/ml), or LPS (250 ng/ml) + EGCG (6.25, 12.5, 25, 50 and 100 µmol/l) for 24 h. EGCG (≥12.5 µmol/l) significantly diminished the LPS-induced upregulation of VEGF (Fig. 3F).

**EGCG suppresses NO expression in LPS-induced inflammation of H9c2 cardiomyocytes.** The effect of EGCG on the NO content in the culture medium was additionally determined. LPS significantly increased the NO content in the culture medium (Fig. 4; Table II; P<0.001). However, EGCG (≥25 µmol/l) significantly counteracted the induction of NO release into the culture medium (Fig. 4).

Discussion

The present study provided evidence that H9c2 cells with LPS-induced inflammation, exhibited enhanced inflammatory mediators, such as VEGF, Rantes, MCP-1, ICAM-1, MMP-2,
EGCG is the main and most significantly bioactive polyphenol found in solid green tea extract, accounting for ~65% of the catechin content; 250 mg EGCG is present in a brewed cup of green tea (22). Several previous studies showed that EGCG attenuates LPS-induced cytokine release in H9c2 cells. (A) TNF-α content in the culture medium of cultured H9c2 cells. (B) ICAM-1 content in the culture medium of cultured H9c2 cells. (C) MMP-2 content in the culture medium of cultured H9c2 cells. Effects of LPS and EGCG in H9c2 cells and the supernatant levels of (D) MCP-1 and (E) Rantes of H9c2 incubated with different concentrations of EGCG for 24 h were determined by ELISA. (F) EGCG inhibited LPS-induced upregulation of VEGF. Data are presented as the mean ± SEM. n=3 in each group. *P<0.05, **P<0.01, ***P<0.001 vs. control group; #P<0.05, ##P<0.01, ###P<0.001 vs. LPS group. EGCG, epigallocatechin-3-gallate; TNF-α, tumor necrosis factor-α; ICAM-1, intercellular adhesion molecule-1; MMP-2, matrix metalloproteinase 2; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; VEGF, vascular endothelial growth factor.

TNF-α and NO. However, the upregulation of the inflammatory mediators were attenuated by EGCG treatment. Additionally, EGCG suppressed the inflammatory signal pathway by down-regulating p-NF-κB p65, p-Akt, p-ERK and p-p38.
Table I. Effects of LPS and EGCG in H9c2 cells and the supernatant levels of inflammatory mediators of H9c2 incubated with different concentrations of EGCG for 24 h were determined by ELISA.

| Groups                      | Inflammatory mediators Control | 250 ng/ml LPS + 6.25 µmol/l EGCG | 250 ng/ml LPS + 12.5 µmol/l EGCG | 250 ng/ml LPS + 25 µmol/l EGCG | 250 ng/ml LPS + 50 µmol/l EGCG | 250 ng/ml LPS + 100 µmol/l EGCG |
|-----------------------------|-------------------------------|---------------------------------|---------------------------------|--------------------------------|--------------------------------|---------------------------------|
| TNF-α, pg/ml               | 25.67±4.16                   | 117±10.54                      | 99.33±13.58                    | 68.67±12.66                   | 43.67±15.95                     | 38±12.53                       |
| ICAM-1, ng/ml              | 2.04±0.30                    | 11.81±1.13                     | 5.68±0.33                      | 3.96±0.36                     | 5.78±0.29                       | 3.71±0.39                      |
| MMP-2, ng/ml               | 40.67±14.74                  | 242.67±53.01                   | 133.67±55.79                   | 77.66±22.89                   | 57.66±15.95                     | 51.66±13.83                    |
| MCP-1, pg/ml               | 123.67±46.26                 | 498.67±15.95                   | 394.67±15.95                   | 288.33±4.16                   | 194.67±15.82                    | 134.67±13.88                   |
| Rantes, ng/ml              | 25.67±4.16                   | 131.3±46.69                    | 99.3±13.58                     | 68.67±12.66                   | 43.67±15.95                     | 38±12.53                       |
| VEGF, pg/ml                | 39.33±4.07                   | 293.49±11.65                   | 250.49±11.65                   | 142.9±11.65                   | 92.9±11.65                      | 63.67±13.88                    |

Data are presented as the mean ± SD. n=3 in each group. aP<0.05, bP<0.01, cP<0.001 vs. control group; dP<0.05, eP<0.01, fP<0.001 vs. LPS group. EGCG, epigallocatechin-3-gallate; TNF-α, tumor necrosis factor α; ICAM-1, intercellular adhesion molecule-1; MMP-2, matrix metalloproteinase-2; VEGF, vascular endothelial growth factor.

EGCG has important anti-atherogenic and anti-inflammatory properties (23,24). The anti-inflammatory effects of EGCG have been demonstrated in numerous previous studies related to the pathological conditions where inflammation is a core-driving factor (23,24). For example, previous studies identified that EGCG was effective in preventing IL-8 production in airway epithelial cells through stimulation of IL-1β, restraining the development of respiratory inflammation (25,26). Moreover, EGCG treatment ameliorated cigarette smoke (CS)-induced airway inflammation and mucus secretion in a CS-exposed rat model (27). EGCG was also demonstrated to reduce cardiac apoptosis by decreasing the inflammatory response (28) and attenuating the serum level of cardiac function biomarker enzymes in rats (29). However, EGCG possesses several limitations, such as poor stability and low bioavailability, which are associated with the concentration of EGCG. High doses of EGCG achieve a positive result of antioxidant and pro-oxidative properties. However, a series of toxic side effects are induced by high...
Figure 5. EGCG suppresses LPS-induced marked inflammatory protein activation. Cultured H9c2 cells were treated with control or LPS (250 ng/ml) or LPS + EGCG (0, 6.25, 12.5, 25, 50 and 100 µmol/l) for 24 h. After immunoblotting, the phosphorylation or the total levels of Akt, ERK, p38, nuclear factor-κB p65 were identified through their phosphor-specific or non-phosphor-specific antibodies. (A) The protein expressions of phosphorylated/total Akt, ERK, p38, nuclear factor-κB and p65 are presented. The expressions of (B) p-Akt/Akt, (C) p-ERK/ERK, (D) p-p65/p65 and (E) of p-p38/p38 are demonstrated. Data are presented as the mean ± SEM. n=3 in each group. *P<0.05, **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. LPS group. EGCG, epigallocatechin-3-gallate; LPS, lipopolysaccharide; p, phosphorylated.
doses of EGCG (30). Previous clinical studies have identified that the side effects of EGCG include nausea, insomnia and hepatotoxicity (31,32). When the present study was conducted, to the best of the authors’ knowledge there were no previous studies in the literature to check the optimal EGCG concentration. Specific concentrations ranging from 50-400 µM were adopted according to the cell viability experiment in different literatures (33,34). Therefore, in the present study the safest maximum concentration (100 µM) was used in the present experiments. Recent studies on the optimum concentrations have since been conducted, the cell viability and Actin-Tracker Green technique in these recent studies demonstrated that the optimal range of concentration for the protective effects of EGCG was 50-100 µM (35,36).

H9c2 is a traditional cell line used to study myocardial disease, which was preserved in the laboratory of Hangzhou Red Cross Hospital/Hospital of Integrated Traditional Chinese and Western Medicine. Moreover, animal cells are also one of the most important ways to establish disease models, so that research is not limited to using human cell lines. Many previous studies on the effects of LPS on cardiomyocytes also used the H9c2 cell line (37-39). Additionally, subsequent validation experiments were performed in rats in addition to H9c2 cell lines. Therefore, the present study selected the H9c2 cell line for the present investigations.

NO is generated from all cell types composing the myocardium and regulates cardiac function, including coronary vessel tone, proliferative and inflammatory properties (40). Under severe inflammatory conditions, excessive NO production causes decreased vascular tonus and consequently hypotension, which is a characteristic of sepsis (19).

LPS, released from the surface of the cell membrane of Gram-negative bacteria, contributes to an inflammatory response. The cellular response to LPS includes the production of reactive oxygen species and other mediators, such as NO and pro-inflammatory cytokines (41). Oxidative stress, a condition caused by excessive production of free radicals, plays an important role in the progression of an inflammatory condition (42). Previous clinical studies demonstrated that the levels of TNF-α and serum concentration of soluble tumor necrosis factor receptors and IL are increased in patients with chronic HF (43-45). Indeed, concentrations of TNF-α are associated with the stimulation of NO synthase, contributing to reduced cardiac contractility in HF (46). As for NO, three NO synthases support various involvements of NO in cardiac physiology. Induced excessive NO from inflammatory cells and LPS-stimulated cardiomyocytes themselves, may lead to profound cellular disturbances resulting in HF (47). In the present study, with LPS-induced inflammation, H9c2 exhibited upregulated expression of inflammatory factors and oxidative stress molecules, such as Rantes, MCP-1, ICAM-1, MMP-2, TNF-α and NO. Moreover, the effects of LPS (250 µg/ml) on cell viability were examined and it was identified that LPS (250 µg/ml) had no effect on the cell viability rate or cytotoxicity in H9c2 cells compared with the control group (101.52±9.0% vs. 100%; P>0.05; data not shown) and the chosen concentration of LPS (250 µg/ml) was lower the concentration used in literature (48,49). The pro-inflammatory cytokines play an important role in the inhibition of cardiac function and the progression from cardiac injury to failure (50). Treatment with EGCG after LPS-stimulated inflammation significantly decreased the levels of pro-inflammatory cytokines and adhesion molecules, suggesting its anti-inflammatory potential.

To demonstrate how EGCG plays a role in the inflammation process, the effects on the inflammatory response of H9c2 cells stimulated by LPS were investigated. H9c2 cells stimulated by LPS demonstrated an increased activation of p-Akt. However, EGCG treatment inhibited the expression of p-Akt. PI3K/Akt signaling is an important pathway involved in controlling cardiomyocyte function and survival (51). One of the downstream effectors of Akt in the PI3K/Akt pathway is endothelial NO synthase, which after phosphorylation, leads to the production of NO (52). Excessive NO delivery from inflammatory cells and cardiomyocytes may influence cellular abnormal and cardiac contractility (40). The present results demonstrated that EGCG significantly counteracted the induction of NO release into the culture medium. Furthermore, the activation of p-p38, p-NF-xB p65 and p-ERK was increased by stimulation of LPS in H9c2 cells. EGCG suppressed the activation of these phosphorylated proteins. NF-xB, p38 and ERK are the most important factors playing a vital role in mediating inflammatory responses to a variety of signals, including inflammatory cytokines (53). Therefore, these associated pathways may comprise important molecular mechanisms responsible for cardiomyocyte inflammation induced by LPS, and EGCG ameliorates LPS-induced inflammation in H9c2 cells through the PI3K/Akt and p38 signaling pathways. A potential mechanism is that EGCG affects the kinase upstream, and then p38 and ERK regulate the downstream target NF-xB. Furthermore, future studies should broaden the scope to determine the cross-talk between the ERK and p38 pathways in H9c2 cells after EGCG treatment by inhibiting ERK and p38.

In the present study, H9c2 cells were incubated without or with EGCG at different concentrations for 48 h. The MTS assay showed no effect on the cell viability with EGCG at concentrations <200 µM. Although, further investigations will be necessary to further study the effects of EGCG on cell viability in H9c2 cells at multiple time points, such as 24, 48 and 96 h. The present results should also be verified in human cardiomyocytes in future studies.

The present findings provided evidence for the inhibiting effects of EGCG on LPS-stimulated inflammation in H9c2 cells. These results suggested the therapeutic potential of EGCG in cardiac inflammation.

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

ZL, ZS, HY, ST, XL and FW designed the present study, analyzed the data and wrote and revised the manuscript. Moreover, FW gave final approval of the version to be published. 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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