Effect of apigenin on surface-associated characteristics and adherence of Streptococcus mutans

Yinchen LIU¹, Lin HAN², Hongye YANG³, Siying LIU³ and Cui HUANG³

¹ Department of Stomatology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China
² Department of Dermatology, CR and WISCO General Hospital, Wuhan, China
³ The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) and Key Laboratory of Oral Biomedicine Ministry of Education, School and Hospital of Stomatology, Wuhan University, Wuhan, China

Corresponding authors, Siying LIU; E-mail: liusiying0502@whu.edu.cn, Cui HUANG; E-mail: huangcui@whu.edu.cn

Apigenin is a type of flavonols that exhibits anti-caries properties. Bacterial adherence is the initial step in the forming of a stable biofilm that leads to caries. Bacterial adherence is affected by surface characteristics, including hydrophobicity and bacterial aggregation. However, the effect of apigenin on surface characteristics of cariogenic bacteria has not been reported. We aimed to examine the effects of apigenin on adherence and biofilm formation of Streptococcus mutans UA159. Hydrophobicity and bacterial aggregation, pac and gbpC gene expressions, and cytotoxicity on human dental pulp cells were also determined. Apigenin significantly inhibited the adherence and biofilm formation of S. mutans. Hydrophobicity decreased, whereas the aggregation rate was significantly increased compared with the control. Apigenin significantly suppressed pac and gbpC gene expressions. Apigenin exhibited acceptable biocompatibility on hDPCs. Thus, apigenin may affect adherence and biofilm formation by altering the surface properties of S. mutans without obvious adverse effect on hDPCs.

Keywords: Streptococcus mutans, Apigenin, Hydrophobicity, Bacterial aggregation

INTRODUCTION

Dental caries is an important health problem worldwide and highly related to biofilm formed by microorganisms in the oral cavity⁴. Oral biofilm comprises complex microorganisms, but Streptococcus mutans (S. mutans) is the major etiological microbe of dental caries in humans⁵. Adherence, the initiative step in caries formation, involves complicated interactions between oral surfaces and bacteria⁶. Surfaces-associated properties including bacterial surface hydrophobicity and cellular aggregation affect the adherence process⁴,⁶,⁷.

Bacterial surface hydrophobicity is determined by the presence of bacterial surface antigen, which is referred to as protein antigen (PAc) or antigen I/II (Ag I/II)⁴. PAc functions as an adhesin, which forms hydrophobic interactions between bacteria and tooth surfaces in the absence of sucrose, hereby promoting bacterial adherence⁴,⁶,⁷.

The attachment of S. mutans aggregates on tooth surface starts the process of dental caries⁷. This process depends on glucans synthesized in situ via the activation of glucosyltransferases (Gtfs) gene expressions when sucrose becomes available⁶. However, over-aggregated bacterial clumps show a decline in adherence⁶. Studies on the S. mutans aggregation properties lead to the isolation of several non-Gtf glucan-binding proteins (Gbps). Four types of Gbps have been discovered, among which the cell-surface-anchored GbpC is dominant as the bond between glucans and bacteria⁶,⁷. GbpC contributes to the virulence of S. mutans¹¹ and is associated with dextran-dependent aggregation¹¹. A mutant of S. mutans (GMS900) showed increased expression of gbpC and formed more cellular aggregates than the wild type¹³. Considering the function of aggregation in adherence, the chemicals that alter the expression of gbpC might affect the formation ability of aggregates, thereby affecting the adherence process.

The effects of numerous natural agents on cariogenic bacteria have been investigated to determine their multiple bioactivities, less adverse effects and resistance problems¹³. Apigenin is chemically known as 4',5,7-trihydroxyflavone. It is ubiquitous in plants, propolis and honey¹⁰. Apigenin exhibits multiple bioactivities, including anti-oxidation, anti-cancer, and anti-inflammation¹⁵. It is the most effective agent found in propolis for inhibiting Gtf activity and for suppressing gtf gene expression. Apigenin also inhibits biofilm accumulation and polysaccharide production without showing any bactericidal effect against S. mutans¹⁰. However, information is lacking on the effect of apigenin on surface-associated properties, including hydrophobicity and cell aggregation, as well as on the gene expression of pac and gbpC coding for PAc and GbpC, respectively. In addition, the initial adherence of S. mutans treated with apigenin has not been documented. We aimed to investigate the effect of apigenin on (1) initial adherence and biofilm formation, (2) hydrophobicity and cell aggregation, (3) the gene expression of pac and gbpC of S. mutans, and (4) the cytotoxicity of human dental pulp stem cells (hDPCs).
MATERIALS AND METHODS

Chemical agents, test bacterium and growth conditions
Apigenin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO). The apigenin concentration was adjusted to 25 μg/mL, and the solution was stored at −20°C prior to use. S. mutans UA159 (ATCC #700610) was cultured in brain heart infusion broth (BHI) (BD, Sparks, MD, USA) at 37°C. Unless otherwise mentioned, the final apigenin concentrations used in the experiment were obtained via two-fold serial dilutions to 312, 156, 78 and 39 μg/mL, and assigned as A312, A156, A78 and A39 accordingly. In our preliminary study, proper vehicle controls were also prepared to demonstrate the effect of DMSO on UA159 and hDPCs. One point two five percent DMSO neither caused a significant change to the bacterial growth and biofilm formation, nor exhibited obvious cytotoxicity on hDPCs compared with the non-treated group.

Initial adherence and biofilm formation
Initial adherence and biofilm formation assay were performed in a 96-well microtiter plate (Corning, NY, USA). UA159 was cultured in BHI at 37°C overnight, and the optical density (OD) of the bacterial suspension was adjusted to 0.3 at 600 nm (3×10⁷ CFU/mL). The inoculation medium was obtained by diluting this suspension to 1:200 with fresh BHI supplemented with 1% sucrose (BHIS) and serially diluted apigenin. UA159 cultured with BHIS were used as controls. After incubation in an anaerobic environment at 37°C for 4 and 24 h, the culture supernatant in each well was discarded and non-adherent bacteria was removed by washing each well with sterile PBS twice.

The acquired biofilm was fixed with methanol for 10 min and stained with crystal violet (0.1% w/v) for 5 min at room temperature. Subsequently, the wells were washed for several times with deionized water until the discarded water appeared clear. DMSO (200 μL) was added to each well, and the plate was shaken in the dark for 30 min. The optical absorbance at 570 nm was determined with a spectrophotometer (Powerwave XS2, Bio-Tek Instruments, Winooski, VT, USA).

Field emission scanning electron microscopy (FESEM)
The biofilm structure of UA159 was prepared for the FESEM observations in the presence of different concentrations of apigenin. The samples were prepared on glass discs placed in a 96-well plate according to the biofilm formation assay. The wells were washed with sterile PBS thrice and fixed with 2.5% glutaric dialdehyde for 4 h. The biofilm was dehydrated gradually with ethyl alcohol (50, 60 70, 80, 90% once, and 100% twice) and placed in a desiccator for 24 h. The samples were sputter-coated with gold and observed by SEM (Quanta 450 FEG, FEI, Eindhoven, the Netherlands).

Confocal laser scanning microscopy (CLSM)
CLSM was performed to observe the adhesion and biofilm structure of UA159 in the presence of apigenin. UA159 was dispersed into confocal Petri dishes (Corning) according to the biofilm formation assay. Apigenin was diluted until the concentrations reached 156 and 312 μg/mL, respectively. UA159 cultured with BHIS only were used as controls. After incubating for 4 or 24 h at 37°C under anaerobic conditions, the medium was removed. The dishes were rinsed with sterile PBS thrice. A LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was used for bacteria staining according to the manufacturer’s instructions. The dishes were subsequently observed via a confocal laser scanning microscope (63×, oil immersion). Dual-channel scanning observations were made for a green channel at the excitation wavelength of 488 nm and a red channel at 543 nm. Six representative fields for each sample were established. The images were transferred to BioImageL software to calculate the total biomass and the percentage of green proportion (%).

Bacterial surface hydrophobicity
Surface hydrophobicity of UA159 was determined using the microbial adhesion to hydrocarbon test (MATH), as previously reported11. The bacterial suspension was obtained and the OD was adjusted with BHI to 0.5 at 600 nm. Apigenin was diluted with BHI and mixed with the bacterial suspension at the ratio of 1:1. The mixed solutions were centrifuged at 2,655 g at 4°C for 5 min after treatment with apigenin for 10 or 30 min. The supernatants were discarded, and the acquired pellets were washed twice and resuspended with 0.85% saline solution. Initial OD was determined spectrophotometrically at 570 nm. The suspension (1 mL) was placed in a conical tube, and 200 μL xylene was added. The tubes were agitated vigorously in a vortex mixer for 2 min and left to settle for 10 min at room temperature. After the two phases were separated, the OD of the aqueous phase was determined at 570 nm.

Bacterial hydrophobicity index (HI) was calculated as: \[ \Delta HI = HI_{\text{exp}} - HI_{\text{blank}} \times 100\% \]. The difference in the hydrophobicity between 10 and 30 min was calculated as: \[ \Delta HI = HI_{10 \text{ min}} - HI_{30 \text{ min}} \].

Cellular aggregation
Cellular aggregation assay was performed according to a previously reported method9. The UA159 in mid-log phase was obtained by centrifugation and then resuspended in BHIS supplemented with 0.1% sucrose to obtain an OD of 0.3 at 600 nm. The suspension (100 μL) was mixed with 100 μL of the serially diluted apigenin in BHIS in a 96-well plate and incubated at 37°C for 2 h. Subsequently, 100 μL of the mixture was centrifuged carefully from the well and transferred to a new plate without disturbing the precipitated cells at the bottom. OD was determined at 600 nm. Aggregation percentage was calculated as: \[ 1 - (OD_{\text{exp}} - OD_{\text{blank}})/(OD_{\text{con}} - OD_{\text{blank}}) \times 100\% \].

Real-time quantitative polymerase chain reaction (qRT-PCR)
The effect of apigenin on the gene expression of pac and gbpC of UA159 was determined. UA159 was diluted to
1×10^6 CFU/mL, cultured in BHI with 1% sucrose and various concentrations of apigenin, and then incubated in 24-well plates (Corning) in an anaerobic environment at 37°C for 24 h. The biofilm was collected, washed with PBS twice, and pelleted via centrifugation. The cells were resuspended in lysozyme buffer and sonicated on ice. Four cycles of ultrasonication were performed for 3 s. The total RNA was purified via TRIzol (Invitrogen), extracted using chloroform, and precipitated with alcohol. The cDNA for each experimental group was synthesized from 1 μg of RNA using a RevertAid First Strand cDNA synthesis kit (Fermentas, Burlington, Canada).

To quantify the pac and gbpC mRNA expression, we performed qRT–PCR using 16S rRNA as an internal control. The sequences of the primers are listed in Table 1. Amplification was performed with ABI-7500 (Applied Biosystems, New York, NY, USA). The reaction mixture (20 μL) contained 1 μL of cDNA and 10 μL of the UltraSYBR mixture (with Rox) (CoWin Biotech, Beijing, China), as well as forward and reverse primers (10 μmol/L). PCR conditions were in accordance to the manufacturer’s recommendations for determining the threshold cycle values (Ct), and the 2^−\Delta\Delta Ct method was used to analyze the data.

Cytotoxicity test on hDPCs

MTT method was performed to assess the cytotoxicity of apigenin on hDPCs. The dental pulp tissues of extracted premolars (All donors gave their informed consent approved by the Ethics Committee of the School and Hospital of Stomatoloy, Wuhan University, China) were harvested, minced, and transferred to 10-cm dishes. The hDPCs from pulp tissues were cultured in α-minimum essential media (α-MEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL Life Technologies, Paisley, UK), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 5% CO2 at 37°C. The hDPCs were cultured in 96-well plates at a density of 2×10^3 cells per well. Apigenin and controls were set as above. After incubation at 37°C with 5% CO2 for 24 h, cells were washed, and MTT solution (5.0 mg/mL) was added. This mixture was incubated for 4 h, then the MTT solution was discarded and the wells were washed with sterile PBS. DMSO (200 μL) was added to each well, and the plate was shaken in the dark for 30 min. Finally, optical the absorbance at 570 nm was determined. Cytotoxicity assays were performed in triplicate, and each concentration was tested in three wells.

Statistical analysis

All experiments were repeated thrice with triple or quadruple replicates in each group. One-way analysis of variance (ANOVA) was performed using SPSS software. Post-hoc Tukey test was used when the ANOVA test indicated significant difference. The level of significance was set at 5%.

RESULTS

Initial bacterial adherence and biofilm formation

Apigenin reduced the initial bacterial adherence and biofilm formation in a dose-dependent manner (Fig. 1). Apigenin significantly reduced adherence at 4 h even at a concentration of 39 μg/ml (42.4%). However, this effect was less evident after the biofilm was allowed to form and mature for 24 h. Absorbance was reduced by 36.3%

Table 1 Primers used for real-time PCR

| Primers | Sequences(5’→3’) |
|---------|-----------------|
| 16S rRNA-F | CCTACGGGAGGCAGCAGTAG |
| 16S rRNA-R | CAACAGAGCTTTACGATCCG |
| pac-F | AAAGCCCTTGCTTATCA |
| pac-R | ATATTCAGCTTTGGCCGTTG |
| gbpC-F | GGCATCGTGTGGAAAAAGT |
| gbpC-R | ATAATAAGCCGTCGCAGCAC |

Fig. 1 Inhibition effect of apigenin on initial adherence and biofilm formation of S. mutans UA159. Values represented the mean±standard deviation of quadruplicates from three independent experiments. Values marked with an asterisk are significantly different from that of the control group (p<0.05).
and 76.4% in groups treated with apigenin at 156 and 312 μg/mL compared with the control group (p<0.05) at 24 h. However, apigenin at 39 and 78 μg/mL was sub-effective.

**SEM observation**

The biofilm structure of *S. mutans* treated with apigenin at 24 h was observed by scanning electron microscopy (SEM) (Fig. 2). The biofilm in the control group showed a relatively uniform appearance with big patches covering the glass slide (Figs. 2a, e). The biofilm in the A78 group showed a more porous appearance (Figs. 2b, f). The bacteria were more aggregated into small clumps, left more uncovered areas in the glass slide than those in the

![Fig. 2 SEM micrographs of *S. mutans* biofilms on glass discs in the treated groups (b, f: A78; c, g: A156; d, h: A312) and control group (a, e). The images were obtained at magnification of 1,000× (a, b, c, d; scale bar: 100 μm) and 5,000× (e, f, g, h; scale bar: 20 μm).](image1)

![Fig. 3 CLSM micrographs of *S. mutans* biofilms in the treated groups (a–d: 4 h treatment; e–h: 24 h treatment; b, f: A78; c, g: A156; d, h: A312) and control group (a, e). i: total biomass and green proportion of UA159 at 4 h; j: total biomass and green proportion of UA159 at 24 h. Values marked with an asterisk are significantly different from that of the control group (p<0.05).](image2)
control. Moreover, large aggregates were formed in the A156 group (Figs. 2c, g). Few bacteria were distributed on the surface of the glass slide in A312 treatment group (Figs. 2d, h).

**CLSM observation**

Images at 4 and 24 h of apigenin treatment showed a significant inhibitory effect on UA159 adherence and biofilm formation (Figs. 3a–h). The total biomass at both 4 h (Fig. 3i) and 24 h (Fig. 3j) decreased with increased apigenin concentrations adding to the growth medium. The green proportion of UA159 also decreased with increasing amount of apigenin. However, it was significantly different from that of the control group in A312 group at 24 h (Fig. 3j).

**Bacterial surface hydrophobicity**

The effect of apigenin on the hydrophobicity of *S. mutans* is shown in Fig. 4. In all groups, the reduction of hydrophobicity was more pronounced when increasing concentrations of apigenin were added. However, apigenin was not significantly effective at 39 and 78 μg/mL within a short time frame.

**Cellular aggregation**

As is shown in Fig. 5, bacterial aggregation significantly increased (21.8 to 32.5%) after treatment with apigenin in A78 to A312 group compared with the control (5.5%). However, apigenin at 39 μg/mL failed to achieve a significant difference.

**Gene expression analysis of pac and gbpC**

We tested the *pac* and *gbpC* gene expressions before and after treatment with apigenin. Compared with the control group, the expression level of *pac* was

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**Fig. 4** Effects of apigenin on the decrement of hydrophobicity of *S. mutans* UA159.

Values represented the mean±standard deviation of triplicates from three independent experiments. Values marked with an asterisk means there was a significant decrease in hydrophobicity than that of the control group ($p<0.05$).

**Fig. 5** Effects of apigenin on the bacterial aggregation of *S. mutans* UA159.

Values represented the mean±standard deviation of triplicates from three independent experiments. Values marked with an asterisk means there was a significant decrease in hydrophobicity than that of the control group ($p<0.05$).

**Fig. 6** Effects of apigenin on gene expression profile of *pac* (a) and *gbpC* (b).

Values represented the mean±standard deviation of triplicates from three independent experiments. Values marked with an asterisk are significantly different from that of the control group ($p<0.05$).
significantly inhibited by 39.8, 81.9, and 76.2% after treatment in A78, A156, and A312 group, respectively (Fig. 6a). Meanwhile, apigenin significantly suppressed the gene expression of gbpC by 94.1% at 156.3 μg/mL and by 95.3% in A312 group (Fig. 6b).

Cytotoxicity assays on hDPSCs
The cytotoxicity of apigenin on hDPSCs is shown in Fig. 7. A312 group exhibited significant inhibitory effect on hDPSCs, while the control group and other experiment groups showed no significant cytotoxicity on hDPSCs.

DISCUSSION
Strategies against oral bacteria should focus on inhibiting virulence factors rather than simply reducing their population. Using purified compounds such as apigenin for controlling or preventing dental caries can specifically interfere with the bioactivities of bacteria without disrupting the balance among the resident microflora. Preventing adherence and biofilm formation could be a prophylaxis against S. mutans virulence, and such approach is a major method for preventing dental caries. In this study, we found that apigenin affected the initial adherence and biofilm formation of S. mutans, exhibited evident effects on cell surface-associated characters such as hydrophobicity and cellular aggregation, and on the gene expressions of pac and gbpC.

We demonstrated that apigenin had a more pronounced effect on initial bacterial adherence and biofilm formation as the concentration increased, but S. mutans showed more resistance to apigenin in the same concentration after biofilm formation. This finding can be explained by the differences in characteristics of the biofilm-forming bacteria from those of their planktonic counterparts. The biofilm-forming bacteria showed more resistance to the changing environment and drugs. Mutant strains of S. mutans and S. sanguis, which have lost their surface hydrophobicity, cannot adhere to saliva-coated hydroxyapatite beads. Hydrophobic strains of S. mutans exhibit good caries-inducing ability in humans. Compounds that diminished the surface hydrophobicity of S. mutans have been reported to inhibit bacterial adherence in vitro and reduce cariogenicity in an animal model. The cell surface hydrophobicity of S. mutans is mainly associated with its cell surface proteins, and PAc is a major factor. In this study, the result was shown as the decrement in hydrophobicity between 10 and 30 min to eliminate the influence of apigenin itself during absorbance reading. The surface hydrophobicity of UA159 was found to be reduced by apigenin. Besides, the pac gene expression significantly decreased after apigenin treatment. The reduction of hydrophobicity was probably related to the reduced PAc synthesis due to suppressed pac gene expression.

The relationship between cariogenicity and Gbps emphasizes the importance of the biofilm structure. Apigenin treatment altered the biofilm to a more porous appearance, and this was also more obvious with increased concentration. Apigenin at 78.2 μg/mL failed to significantly reduce absorbance compared with the control, but the biofilm structure formed by UA159 was different from that of the control group. The changes in the biofilm structure might facilitate the exchange of acids produced by S. mutans through the biofilm via the enlarged water channels and the reduced acid accumulation in its environment, thereby lowering the risk of caries even without significantly reducing the quantity of cariogenic bacteria.

Intra- and inter-species bacterial aggregations are naturally occurring phenomena that affect adherence and biofilm formation. The small aggregates containing two or three times more cells than the non-aggregated adherent units increase the bacterial number, whereas the big aggregates reduce the amount of adherent bacteria. Apigenin had been shown to affect biofilm structure of S. mutans, resulting in a reduced thickness due to cellular aggregation. In our study, apigenin showed significant effect on the aggregation rate as well as the biofilm structure. Besides, apigenin is an effective agent for inhibiting gtf expression, Gtf levels and their activities. The synergy among these factors could explain the reduction of adherence at a low apigenin concentration where the effect of apigenin on hydrophobicity and aggregation was not that obvious.

We also found that apigenin suppressed the gene expression of gbpC at 156 μg/mL. While apigenin showed no effect in inhibiting growth of S. mutans, growth conditions and various cellular stresses may affect the expression of gbpC. The GbpC has a specific binding domain for soluble glucans. It is a major cell surface glucan receptor that is closely related to the cariogenicity of S. mutans. The relationship between the gbpC-defective strain and its compromised caries-inducing property was also revealed. A less cariogenic strain GS-5 with a point mutation in the gbpC gene was
reported\textsuperscript{18}. In \textit{in vitro} experiments, GbpC-deficient \textit{S. mutans} exhibited reduction in biofilm formation\textsuperscript{20}. In this study, the suppressed expression of \textit{gbpC} might contribute to the affected adherence and altered biofilm of \textit{S. mutans}. However, the exact mechanism underlying apigenin’s suppression of \textit{gbpC} expression remains unknown.

GbpC shares sequence homology with the V region of Pac and the antisera for Pac cross-reacted with the GbpC protein\textsuperscript{35}. Pac is reportedly not involved in dextran-induced aggregation\textsuperscript{25}, but its existence is associated with a high degree of aggregation\textsuperscript{23}. Moreover, the \textit{gbpC} mutant of \textit{S. mutans} shows the lowest levels of dextran-binding and adherence capability and hydrophobicity\textsuperscript{11}. In this study, the aggregation percentage increased after the treatment with higher concentrations of apigenin, regardless of the reduced hydrophobicity and the low expression level of \textit{gbpC}. Flavonols are known to bind to proteins, and the aggregation process between flavonols and proteins possibly explains their bioactivities\textsuperscript{23}\textsuperscript{35}. In our study, apigenin might bind to the surface proteins of bacteria to form larger aggregates given the \textit{gbpC} expression was suppressed. This finding could be another advantage in forming “fragile” biofilm in addition to the reduced production of adherent, sticky, and water-insoluble glucans. Moreover, saliva induces bacterial aggregation and causes the clearance of bacteria from oral cavity, and these activities could be host defensive mechanisms. Given the role of saliva in aggregation and the subsequent clearance of bacteria, apigenin could promote this process at a relatively low concentration. Low concentration is more favorable for intra-oral application given that a high concentration of apigenin in the oral cavity is difficult to maintain because of the diluting effect of saliva.

\textbf{Cytotoxicity assay} is an important determinant procedure for evaluation of biocompatibility potential of natural extracts. Apigenin should exhibit minimal cytotoxic effect after hours of contact in the oral cavity. In this study, hDPCs is significantly affected by Apigenin at 312 µg/mL, while other groups showed similar results with the control group. Although apigenin has been shown to be toxic to erythrocytes \textit{in vitro}\textsuperscript{34}, different kinds of cells could react to apigenin differently\textsuperscript{35}. Besides, variance in drug administration could affect the pharmaceutical effects of drugs. As for intraoral infections, localized drug administration might be more reliable to enhance antibacterial effects at minimal concentrations. In addition to that, drugs in the form of wax or gel guarantee a prolonged period \textit{in situ}. Thus, more \textit{in vitro} and \textit{in vivo} studies are needed to investigate apigenin used intraorally to find out the proper concentrations, dosage form and even synergy effect with other drugs.

In conclusion, we demonstrated that apigenin could inhibit initial adherence and biofilm formation of \textit{S. mutans} in several ways, including decreasing the bacterial aggregation and hydrophobicity, and the suppression of pac and gbpC. Besides, apigenin exerted favorable biocompatible features under certain concentrations. Apigenin could be a promising anti-caries agent even at a relatively low concentration.

\textbf{ACKNOWLEDGMENTS}

This work was supported by the National Natural Science Foundation of China (81371191, 81600894), and the Natural Science Foundation of Hubei Province (2017CFB798). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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