Mitochondrial DNA Efflux Maintained in Gingival Fibroblasts of Patients with Periodontitis through ROS/mPTP Pathway

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Mitochondria have their own mitochondrial DNA (mtDNA). Aberrant mtDNA is associated with inflammatory diseases. mtDNA is believed to induce inflammation via the abnormal mtDNA release. Periodontitis is an infectious, oral inflammatory disease. Human gingival fibroblasts (HGFs) from patients with chronic periodontitis (CP) have shown to generate higher reactive oxygen species (ROS) that cause oxidative stress and have decreased mtDNA copy number. Firstly, cell-free mtDNA was identified in plasma from CP mice through qRT-PCR. Next, we investigated whether mtDNA efflux was maintained in primary cultures of HGFs from CP patients and the possible underlying mechanisms using adenovirus-mediated transduction live cell imaging and qRT-PCR analysis. Here, we reported that mtDNA was increased in plasma from CP mice. Additionally, we confirmed that CP HGFs had significant mtDNA efflux from mitochondria compared with healthy HGFs. Mechanistically, LPS upregulated ROS levels and mitochondrial permeability transition pore (mPTP) opening by inhibition of pyruvate dehydrogenase kinase (PDK)2 expression, resulting in mtDNA release. Importantly, mtDNA efflux was even persistent in HGFs after LPS was removed and cells were passaged to the next three generations, indicating that mtDNA abnormalities were retained in HGFs in vitro, similar to the primary hosts. Taken together, our results elucidate that mtDNA efflux was maintained in HGFs from periodontitis patients through abnormal ROS/mPTP activity. Therefore, our work indicates that persistent mtDNA efflux may be a possible diagnostic and therapeutic target for patients with periodontitis.

1. Introduction

Periodontal inflammation is known to affect 20%-50% of the global population, and it often interacts with other inflammatory diseases such as heart disease and diabetes [1–3]. Periodontitis is associated with lipopolysaccharide (LPS) from the cell walls of gram-negative bacteria-mediated inflammatory responses and represents the most common cause of teeth loss [1, 4, 5]. Increasing evidences suggest that mitochondrial dysfunction appears to result in periodontitis during LPS stimulus [6–8]. As such, abnormal mitochondria are considered to be one of the major contributors to the periodontitis development. Several mitochondrial components, including mitochondrial DNA (mtDNA), have also been implicated in inflammatory responses [9].

mtDNA exists in mitochondrial matrix and is in intimate contact with the electron transport chain, one of the principal sources of reactive oxygen species (ROS). Therefore, mtDNA is particularly susceptible to oxidation, which can cause mutations and damages, leading to the pathogenesis of inflammation [10]. ROS production and ROS produced in mitochondria (mtROS) are indeed significantly enhanced in human gingival fibroblasts (HGFs) after LPS stimulation or from periodontitis hosts [11]. These results indicate that oxidative stress is induced during periodontitis [12]. According to current studies linking oxidative stress to
decreased mtDNA copy number [13, 14], it is becoming clear that mtDNA disruption may be associated with chronic inflammation [15]. Consistent with this assumption, previous research has also demonstrated that mtDNA deletion is present in the gingival tissues of patients with periodontitis [16]. Moreover, a decrease in mtDNA in periodontitis patients appeared to retain many of the damaged mitochondria in vitro had decreased mtDNA levels and decreased mitochondrial matrix protein expression, especially in pyruvate dehydrogenase kinase 2 (PDK2) when compared with those from healthy subjects [8], suggesting that mtDNA and mitochondria disruption in peripheral HGFs might replicate the mitochondrial dysfunction observed in vivo during periodontitis development. Therefore, we hypothesized that abnormal mtDNA might be maintained in HGFs in vitro connecting the disease in vivo with a certain mechanism. Answering these problems will improve our understanding of the periodontitis etiology, and it might lead to new treatment options.

Superresolution imaging demonstrated that mtDNA constitutes one copy of mtDNA and a number of different proteins, presenting densely compacted nucleoids [17]. Mitochondrial transcription factor A (TFAM) is the most notable mtDNA nucleoid protein that can be assumed to specifically recognize mtDNA [15]. mtDNA can escape from mitochondria and release into the cytoplasm under various pathological situations [18, 19]. Multiple major factors have been attributed to driving mtDNA release from damaged mitochondria, including the opening of mitochondrial permeability transition pore (mPTP), mitochondrial stress, and calcium overload [20–22]. Nonetheless, the biological mechanisms provide limited information on this process during periodontitis conditions. Our recent study demonstrated that mitochondria in HGFs from periodontitis patients appeared to retain many of the damaged features, as observed in donors [8]. In addition, previous studies have suggested that the primary host has profound influence on the cells in vitro, such as higher oxidative stress in HGFs of periodontitis patients than that of healthy individuals [8, 23]. However, differences of mtDNA release in HGFs from chronic periodontitis (CP) patients and healthy HGFs have not been tested and if this abnormal intracellular mtDNA activity would last need to be further elucidated. Thus, therapies targeting mtDNA may become a potential approach to patients suffering from severe recurrent periodontitis.

The mPTP spans the mitochondrial inner membrane, and its formation is associated with various cellular stresses [24, 25]. Interestingly, the opening of mPTP has been detected in the metabolic stress observed in inflammatory diseases [26]. More recently, using an in vitro and in vivo approach, studies have shown that genetic removal of one of the mPTP component proteins ameliorated mtDNA release into the cytoplasm during the neuroinflammatory response [20]. Pharmacological inhibition of mPTP by cyclosporin A (CsA) has also been shown to be effective in preventing mtDNA leakage into the cytoplasm [20]. Despite these results in previous studies, the notion that the opening of mPTP may directly drive mtDNA efflux remains controversial and is still unclear in periodontitis. It has been reported that ROS contributes directly to mPTP opening during ischemia-reperfusion [27]. As a result of cellular ROS and mtROS outburst, mPTP opening can be activated. Nevertheless, its association with mPTP involved in mtDNA efflux in periodontitis is scarcely understood.

In this study, we discovered the differences in the mtDNA efflux process, ROS levels, and mPTP opening between primary HGFs, isolated from patients with CP and age-matched periodontally healthy patients. This elevated mtDNA efflux together with high ROS levels, and mPTP opening in CP HGFs could be more enhanced in response to LPS. Furthermore, this identified mtDNA efflux as an important modifier could be maintained in HGFs even withdrawing the LPS stimulation after passages. Consequently, we explored if the ROS/mPTP pathway involving in the mtDNA efflux along with the progression of periodontitis. This may be a promising target for early diagnosing periodontitis and provides preclinical evidence for therapeutic strategy to people with periodontal inflammation tolerating common anti-microorganism therapy.

2. Materials and Methods

2.1. Ethics Approval. The study was approved by the Review Board and Ethics Committee of Peking University Health Science Center (PKUS-SIRB-2013017) and conducted in agreement with the Declaration of Helsinki II. Written informed consent was obtained from all subjects before inclusion in the study. All animal work was approved by the Review Board and Ethics Committee of Peking University Health Science Center (LA2018076).

2.2. Animals and Experimental Groups. Specific-pathogen-free male C57BL/6 wild-type mice (6-wk-old) (Figure 1(a)) were purchased from Experimental Animal Laboratory, Peking University Health Science Center, in compliance with established polices. All mice were randomly divided into the normal control groups or CP groups of four mice each. The control group was left untreated, and the CP group had their maxillary second molar tooth ligated with established polices. All mice were randomly divided into the normal control groups or CP groups of four mice each. The control group was left untreated, and the CP group had their maxillary second molar tooth ligated with a 5-0 silk ligature (Roboz Surgical Instrument Co, MD, USA) (Figure 1(a)). The ligatures remained in place in CP groups throughout the experimental period. All mice were sacrificed at three weeks postligation (Figure 1(a)). Microcomputed tomography (CT) was used for assuring the CP model was established successfully.

2.3. Microcomputed Tomography. In brief, after sacrificing the mice, the maxillary teeth were carefully dissected and soft tissues were removed. The sample was fixed with 4% paraformaldehyde for 24 h, and scanned using the μCT50 (Scanco Medical) with a resolution of 1024 × 1024, pixel size of 15 × 15 μm, and layer spacing of 15 μm. The region of interest was assessed by 3D reconstructed. Bone loss was evaluated by 3D micro-CT.
2.4. Human Subjects. HGFs were obtained from six CP patients and six age-matched healthy donors. These participants were recruited from the Department of Periodontology, Peking University School and Hospital of Stomatology. The exclusion criteria included smoking and systemic health issues including hypertension, diabetes, and immune-related diseases within the past six months. CP was defined according to the American Academy of Periodontology and European Federation of Periodontology criteria based on staging and grading [28]. CP patients included in this study were grade B and stage III. Gingival tissues from CP were acquired through flap surgery, with PD \( \geq 6 \) mm. Tissues in the healthy group were harvested during crown lengthening surgery, with PD < 4 mm. Table 1 lists the detailed characteristics of the participants.

2.5. Primary Culture of HGFs. HGFs were prepared from the gingival tissues of six CP patients and six healthy controls during periodontal surgery. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) and 1% penicillin-streptomycin. The cells were incubated at 37°C with 5% CO₂. The medium was changed after a week. In approximately two weeks, the cells reached subconfluency and the pieces of gingival tissue were removed from the culture flask. Cells from the third to the eighth passage were used in the subsequent study.

<p>| Table 1: Clinical characteristics at surgery site of patients included in this study. |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Number of patients</th>
<th>Range of age</th>
<th>Percent women</th>
<th>BI PD (mm)</th>
<th>CAL (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>6</td>
<td>27-40</td>
<td>50</td>
<td>1-2</td>
<td>0-0.5</td>
</tr>
<tr>
<td>CP</td>
<td>6</td>
<td>33-45</td>
<td>66.7</td>
<td>6-10</td>
<td>4-7</td>
</tr>
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</table>

2.6. Cell Treatment and Stimulation. HGFs from healthy and CP patients were stimulated with or without 5 μg/mL LPS from Porphyromonas gingivalis (P.g) (ATCC33277, Standard, InvivoGen, San Diego, CA, USA) for 24 h. To investigate whether inflammatory features of donors were retained in HGFs, HGFs from healthy donors were treated with 5 μg/mL of LPS for 24 h, followed by discarding the medium then passing to the next three generations for analysis. The cells were assessed by indicated assays and compared with cells from healthy donors that were directly stimulated with the same amount of LPS for the same time.
following the manufacturer’s instructions. The mtDNA levels were assessed according to a standard curve based on ND1 plasmid (Sangon, Shanghai, China) levels. The mtDNA levels in the samples were analyzed by real-time PCR (TaqMan, Applied Biosystems, Foster City, CA) using the Universal Genomic DNA Kit (ZOMAN, Beijing, China), following the manufacturer’s instructions. The mtDNA levels were assessed according to a standard curve based on ND1 plasmid (Sangon, Shanghai, China) levels.

### 2.7. Cellular ROS and Mitochondrial ROS (mtROS) Detection

2',7'-Dichlorodihydrofluorescein diacetate (H2DCF-DA) (Sigma-Aldrich, St. Louis, MO) and MitoSOX Red (Invitrogen, Carlsbad, CA) were used to detect total ROS and mtROS, respectively, as previously described [11]. HGFs were loaded with H2DCF-DA (10 μM) or MitoSOX Red (5 μM) for 30 min and then observed using a microscope [11]. To inhibit ROS levels, HGFs were preincubated with 3 mM N-acetylcyesteine (NAC) (Sigma-Aldrich, St. Louis, MO) for 2 h. The mtROS scavenger 50 μM Mitotempo (Santa Cruz Biotech, Dallas, TX) were pretreated for 2 h.

### 2.8. Western Blotting

Proteins were extracted from HGFs using ice-cold radioimmunoprecipitation (RIPA) lysis buffer (Solarbio). After being quantified by BCA (Thermo Fisher Scientific), the protein samples were mixed with loading buffer (Solarbio), separated by electrophoresis on SDS-PAGE. The proteins in the gel were transferred on a polyvinylidene fluoride (PVDF) membrane (Beyotime). The membranes were blocked with 5% skimmed milk (Solarbio) and incubated overnight at 4°C with primary antibody. The membranes were washed with Tris-bufered saline and incubated with secondary antibody for 90 min at room temperature. The PVDF membranes were subjected to chemiluminescence detection using an ECL Western Blotting Detection Kit (Solarbio).

### 2.9. DNA Isolation and mtDNA Quantification by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Genomic DNA from HGFs was extracted using the Universal Genomic DNA Kit (ZOMAN, Beijing, China), following the manufacturer’s instructions. The mtDNA levels in HGFs were assessed using primers against mitochondrial genes (ND1), while nuclear 18S rRNA served as a loading control. Detailed ND1 and 18S rRNA sequences are presented in Table 2. Cytosolic mtDNA extraction was performed according to the methods established by West et al. [29]. The plasma from the mice was centrifuged at 1000 g for 5 min, and then the supernatant was centrifuged a second time at 5000 g for 10 min. The top 80% of the volume can be used for cell-free (cf-) mtDNA quantification. DNA from cell supernatants, cf-mtDNA in plasma, and cytosol DNA (200 μL) were isolated using the QIAamp DNA Mini Kit (Qiagen, Germany). ND1 levels in the samples were analyzed according to a standard curve based on ND1 plasmid (Sangon, Shanghai, China) levels.

### 2.10. Adenovirus Transduction for Mitochondria and mtDNA Detection

HGFs were transduced with adenovirus encoding the mitochondrial outer-membrane protein Tomm 20 bearing a mCherry fluorescence protein. mtDNA was detected by coexpression of TFAM, tagged with the green fluorescent protein (GFP) variant mNeonGreen. HGFs were seeded on 10 mm round confocal glass coverslips at a density of 50% and were infected with specified amounts of the Tomm 20-mCherry and TFAM-mNeonGreen adenoviruses. Forty-eight hours after transduction, the medium was changed, and the cells were processed for further analysis.

### 2.11. Live Cell Imaging Microscopy

Live cells were captured using a fluorescence microscope (TCS-STED; Leica, Wetzlar, Germany) with a 63 x oil immersion objective. For all experiments, HGFs were grown in 10 mm round glass bottom confocal wells (Cedarlane, Southern Ontario, Canada). Laser excitation was achieved at 488 nm for mNeonGreen and 561 nm for mCherry. LPS treatment was performed after sample mounting in the medium chamber, if needed. HGFs expressing Tomm 20-mCherry and TFAM-mNeonGreen were imaged serially at every 10 s for 10-15 min. Image processing and analysis were performed using ImageJ (NIH, http://rsb.info.nih.gov/ij/) and Huygens Professional software (Scientific Volume Imaging, Amsterdam, Holland).

### 2.12. Detection of mPTP Opening

HGFs were incubated with 50 mM cobalt chloride for 15 min, before treatment with 1 μM Calcein Green AM (Solarbio, Beijing, China) for 30 min. Free Calcein quenching by cobalt chloride preserved mitochondrial integrity, which could be used to indicate mPTP opening. Calcein fluorescence was detected by confocal microscopy (Leica) using a 488 nm excitation wavelength. Quantification of the Calcein fluorescence intensity was conducted by analyzing 20 cells for every indicated condition using ImageJ software. To prevent nPTP opening, HGFs were preincubated with 0.5 μM cyclosporine A (CsA; Sigma) for 2 h, following the manufacturer’s recommendations.

### 2.13. Flow Cytometric Analysis

Cells were briefly washed with 1 × phosphate-buffered saline (PBS), resuspended in 1 × binding buffer, and centrifuged at 300 × g for 10 min. The pellets were resuspended with 1 x binding buffer at a density of 1 x 10⁶ cells/mL. Cells were replated in a flow cytometric tube at a density of 1 x 10⁵ cells/mL and processed for Annexin V-FITC staining (Solarbio, Beijing, China) for 10 min at 20-25°C. Subsequently, the cells were

### Table 2: List of primers for real-time PCR studies.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>ND 1</td>
<td>Forward primer</td>
<td>5'-CACACTAGCAGACCCAACCGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-CGGCTATGAAGAATAGGGCGA-3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>Forward primer</td>
<td>5'-GACTCAAACGGGAAACTCCC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-ACCAGACAAATCGCTCCACCA-3'</td>
</tr>
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Figure 2: Continued.
stained with propidium iodide (PI) for 5 min at 20-25°C and analyzed for apoptosis by flow cytometry.

2.14. Statistical Analysis. Data are expressed as the mean ± standard error (SE). All p values were determined by two-way Student's t-test or one-way analysis of variance (ANOVA) with a post hoc Student Newman-Keuls test for multiple comparisons. Significant differences were accepted at p < 0.05. Statistical analysis was performed using GraphPad Prism software (version 9.00; GraphPad Software).

3. Results

3.1. mtDNA Release from Mitochondria during Periodontitis Development. Micro-CT results revealed that alveolar bone around the ligated molar was significantly reduced in CP mice compared to control mice, suggesting experimental periodontitis in the CP group established (Figures 1(b) and 1(c)). Intriguingly, mtDNA in plasma from CP mice were enriched compared to age-matched wild-type control mice (Figure 1(d)). These results indicated that mtDNA release might be involved in periodontitis development. However, mtDNA efflux in HGFs during periodontitis is still unclear. Next, we transduced primary HGFs with adenovirus encoding Tomm 20-mCherry and TFAM-mNeonGreen to show mitochondria and mtDNA, respectively (Figure 2(a)). mtDNA were detected robust release into the cytoplasm in CP HGFs (Figure 2(b)). This process was also found by real-time microscopy (Figure 2(c), Movie 1). In contrast, no mtDNA efflux was detected in healthy HGFs (Movie S1). LPS caused remarkable mtDNA release in healthy HGFs and led to more significant mtDNA release in periodontitis-affected samples (Figures 2(b)–2(d), and 2(e), Movie 2 and 3). Next, we calculated a significant increase in the percentage of HGFs with mtDNA efflux in CP HGFs as compared with that in control HGFs (Figure 2(f)). LPS treatment caused marked increase in the percentage of HGFs with mtDNA efflux compared with those without LPS treatment in healthy and CP states (Figure 2(f)). Moreover, qRT-PCR confirmed that mtDNA release into cytosol and out of cells during periodontitis (Figures 2(g)–2(i)). These results
indicated that mtDNA release might be involved in periodontitis development.

3.2. mtDNA Efflux Maintained in HGFs during Periodontitis. LPS is a principal factor that determines the periodontal inflammation; we decided to clarify if LPS causes mtDNA efflux maintained in HGFs. In these experiments, healthy HGFs were exposed to LPS stimulation for 24 h. Next, LPS was removed and HGFs were cultured into the next three generations for analysis (Figure 3(a)). In contrast, healthy HGFs were directly treated with LPS for 24 h (Figure 3(b)).

The results showed that LPS reinforced the mtDNA efflux effect even in the next three generational HGFs (Figures 3(c)–3(e)). No significant differences were observed compared to the LPS directly treated HGFs (Figure 3(f)). Next, we examined the mtDNA levels in the cytosol using qRT-PCR analyzing these groups. LPS directly stimulated HGFs, and LPS treatment following passages of HGFs were both enriched in cytosolic mtDNA (Figure 3(g)). In addition, the percentages of HGFs with mtDNA efflux between LPS direct treatment and LPS treatment followed by HGFs passages were similar (Figure 3(h)). These results suggest that LPS treatment can
**Figure 4: Continued.**

(a) Representative images of fluorescence intensity showing Cytoplasmic ROS and Mitochondrial ROS with control, CP, LPS, and LPS treated cells with 1, 2, and 3 passages.

(b) Bar graph showing RFU values for Cytoplasmic ROS with control, CP, LPS, CP-LPS, LPS and 1 passage, LPS and 2 passage, LPS and 3 passage.

(c) Bar graph showing RFU values for Mitochondrial ROS with control, CP, LPS, CP-LPS, LPS and 1 passage, LPS and 2 passage, LPS and 3 passage.

(d) Western blot images for PDK2 and GAPDH with control, CP, LPS, CP-LPS for 1, 2, and 3 passages.

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**Figure 4 Continued:**

- **Cytoplasmic ROS**
  - Control
  - CP
  - LPS
  - CP-LPS
  - LPS and 1 passage
  - LPS and 2 passage
  - LPS and 3 passage

- **Mitochondrial ROS**
  - Control
  - CP
  - LPS
  - CP-LPS
  - LPS and 1 passage
  - LPS and 2 passage
  - LPS and 3 passage

- **Western Blot**
  - PDK2
  - GAPDH
Patients. To investigate in further details how mtDNA e
3.3. ROS and mtROS Is Overproduction in HGFs from CP
mtDNA release of CP HGFs and CP mice.
the next-generations HGFs, which is consistent with those
mtDNA release e
ff
DA) (10 μM, 30 minutes) to indicate the ROS levels (green) in HGFs (scale bars: 75 μm). HGFs were incubated with MitoSOX Red
(5 μM, 30 minutes) to visualize mitochondrial ROS (mtROS) levels (red) (scale bars: 50 μm). (b, c) The arbitrary fluorescence intensity of
ROS and mtROS in (a) were calculated by ImageJ based on per 10 cells in each group from (a). Data represent the
mean ± standard
error (SE) from 10 cells from each group. (d) Western blot to evaluate the protein expression of pyruvate dehydrogenase kinase 2
error
ROS and mtROS in (a) were calculated by ImageJ based on per 10 cells in each group from (a). Data represent the
mean ± standard
error (SE) from 10 cells from each group. (d) Western blot to evaluate the protein expression of pyruvate dehydrogenase kinase 2
expression. Its regulation may contribute to this mtDNA
ux phenomenon, and the facilitative
mtDNA release effects can be maintained in HGFs even in the next-generations HGFs, which is consistent with those
mtDNA release of CP HGFs and CP mice.

3.3. ROS and mtROS Is Overproduction in HGFs from CP Patients. To investigate in further details how mtDNA efflux
effect remained during periodontitis at cellular level, we
firstly analyzed the ROS and mtROS levels in HGFs from
different hosts. Control healthy HGFs had the lowest ROS
and mtROS levels (Figure 4(a)). HGFs from CP had signifi-
cantly greater levels of ROS and mtROS (Figure 4(a)).
We found that the ROS and mtROS were more activated in the
presence of LPS compared to those in the absence of LPS
groups (Figure 4(a)). In addition, LPS can affect ROS and
mtROS even in the next three generations HGFs (Figure 4(a)).
Furthermore, the levels of these fluorescent signals reflecting ROS and mtROS levels in Figure 4(a) were calcu-
lated by ImageJ (Figures 4(b) and 4(c)). Western blot analy-
sis showed PDK2 exhibited decreased expression in CP
HGFs (Figures 4(d) and 4(e)). Meanwhile, the expression
levels of PDK2 were also reduced after LPS stimulation and
showed low levels even in the next three-generation
HGFs (Figures 4(d) and 4(e)). In summary, CP HGFs are
primed for ROS activation, and LPS can persistently upreg-
ulate the ROS levels in HGFs by suppressing the PDK2
expression. Its regulation may contribute to this mtDNA
efflux process.

3.4. mPTP Opening in HGFs from CP Patients via ROS
Activation. mPTP opening in HGFs was indicative using
Calcein AM fluorescence (Figure 5(a)). Control HGFs
showed strong green fluorescence (Figure 5(a)), suggesting
that mPTP remained in a closed state under normal condi-
tion [30]. However, the fluorescence was hardly detected in
CP HGFs (Figure 5(a)). LPS further resulted in a much more
decrease in fluorescence in the control and CP groups
(Figure 5(a)). Decreased level of fluorescence signal was also
detected in the LPS treated following passaging three gener-
ational HGFs (Figure 5(b)). A significant increase in fluores-
cence was observed in HGFs in the presence of CsA when
compared with that in the absence of CsA (Figures 5(a)–5
d(d)). It was shown that inhibition of ROS and mtROS activa-
tion contributes to suppression of mPTP opening (Figures 5
(a)–5(d)). Collectively, these data show that CP HGFs dis-
play mPTP opening and that mPTP opening in the LPS-
treated HGFs was maintained within the HGFs even in the
later three generations. Additionally, this observed mPTP
opening is dependent on ROS activation.

3.5. mtDNA Release in CP HGFs via ROS and mPTP
Opening. We performed real-time fluorescent microscopy
for control, CP, LPS treatment, and CP LPS HGFs in the
presence of CsA (Figure 6(a)). It was observed that mtDNA
displayed mild or no efflux in the four CsA-treated groups
of HGFs (Figure 6(a)). These data demonstrated that mPTP
was critical for the mtDNA release under these conditions.
Figure 5: Continued.
We performed qRT-PCR to detect the cytosolic mtDNA levels by the inhibitors of mPTP, ROS, and mtROS. CsA, NAC, and Mito-TEMPO all decreased the cytosolic mtDNA levels in the CP, LPS, and CP LPS groups when compared with the three groups without any treatment, whereas the control group showed similar cytosolic mtDNA levels in the presence or absence of lipopolysaccharide (LPS) treatment (5 μg/mL, 24 h). The opening of mPTP in HGFs was measured after cyclosporin A (CsA) (0.5 μM, 2 h) or N-acetylcysteine (NAC) (3 mM, 2 h) or Mito-TEMPO (50 μM, 2 h) treatment (scale bars: 25 μm). (b) Images for mPTP opening in the control, LPS-treated, and LPS-treated group after passaging three generations (scale bars: 25 μm). (c) Quantification of the observed Calcein green signal in HGFs from (a, b). Mean ± SE are indicated (n = 20 cells). The CP group was observed a lower signal; LPS was also observed a lower signal compared with control HGFs. LPS can aggravate this lower signal in the control and CP groups, and this phenomenon can be retained in HGFs after LPS was removed and passaged to next three generations as compared with the control group. (d) The intensity of the indicated Calcein green signal was detected per 20 cells from the control, CP, LPS, and CP LPS groups with CsA, NAC, and Mito-TEMPO treatment compared to HGFs without any treatment, respectively. CsA, NAC, and Mito-TEMPO all downregulated the signal in the LPS, CP, and CP LPS groups, while they fail to induce this phenomenon in control HGFs. p values were determined by 1-way analysis of variance followed by post hoc tests. **p < 0.01 and ****p < 0.0001; ns: not significant.

**Figure 5:** Human gingival fibroblasts (HGFs) from chronic periodontitis presented active mitochondrial permeability transition pore opening via reactive oxygen species. (a) Human gingival fibroblasts (HGFs) were loaded with cobalt chloride (50 mM, 15 minutes) and Calcein AM (green) to determine the opening of the mitochondrial permeability transition pore (mPTP) in HGFs in the presence or absence of lipopolysaccharide (LPS) treatment (5 μg/mL, 24 h). The opening of mPTP in HGFs was measured after cyclosporin A (CsA) (0.5 μM, 2 h) or N-acetylcysteine (NAC) (3 mM, 2 h) or Mito-TEMPO (50 μM, 2 h) treatment (scale bars: 25 μm). (b) Images for mPTP opening in the control, LPS-treated, and LPS-treated group after passaging three generations (scale bars: 25 μm). (c) Quantification of the observed Calcein green signal in HGFs from (a, b). Mean ± SE are indicated (n = 20 cells). The CP group was observed a lower signal; LPS was also observed a lower signal compared with control HGFs. LPS can aggravate this lower signal in the control and CP groups, and this phenomenon can be retained in HGFs after LPS was removed and passaged to next three generations as compared with the control group. (d) The intensity of the indicated Calcein green signal was detected per 20 cells from the control, CP, LPS, and CP LPS groups with CsA, NAC, and Mito-TEMPO treatment compared to HGFs without any treatment, respectively. CsA, NAC, and Mito-TEMPO all downregulated the signal in the LPS, CP, and CP LPS groups, while they fail to induce this phenomenon in control HGFs. p values were determined by 1-way analysis of variance followed by post hoc tests. **p < 0.01 and ****p < 0.0001; ns: not significant.
Periodontitis is a kind of chronic inflammatory disease driving the destruction of soft and hard periodontal tissues such as gingiva recession and alveolar bone loss [5], suggesting a role for mtDNA efflux in the periodontitis. Consistent with the reported mtDNA efflux in other studies, we identified a significant increased mtDNA levels in the plasma from CP mice, implying an association with periodontitis and this mtDNA efflux. One study demonstrated that the mtDNA outside of mitochondria was found to be crucial for inflammation via inducing bone-destructing immunity [38]. Owing to the mtDNA accumulation in the plasma of CP mice, little is known about the mtDNA function and activity in HGFs during periodontitis. In the context of periodontitis, in vitro studies of periodontitis patients have confirmed alterations in mitochondrial structure, function, and hyperoxidative stress in HGFs and gingival tissues compared to normal individuals [8, 12], which indicates that there may be a correlation between periodontitis progression and mitochondrial dysfunction in HGFs from different hosts. Interestingly, we confirmed that aberrant mtDNA release into cytosol and supernatants of HGFs from CP patients. It is evident that LPS stimulation could also induce this phenomenon in healthy HGFs. The observed high mtDNA levels in the cytosol among four groups of HGFs with or without CsA, N-acetylcysteine (NAC) (3 mM, 2 h), and Mito-TEMPO (50 μM, 2 h) treatment. All quantified data represent the mean ± SE. p values were determined by 1-way analysis of variance followed by post hoc tests. Graphs represent at least 3 independent experiments. *p < 0.05, ** p < 0.01, ***p < 0.001, and ****p < 0.0001; ns: not significant.
PDK2 activation has beneficial effects on ROS suppression [39]. Thus, we reasoned that LPS might mediate irreversible high ROS generation by downregulation of PDK2 expression [6, 40], leading to sustained increased mtDNA release activity even in the next-generation HGFs without LPS stimulation. As widely reported in the literature, LPS activated Toll-like receptor (TLR) was abundantly expressed in the inflammatory cells, leading to the ROS production as well as the lower PDK2 expression [8, 41]. Some reported that ROS triggered mtDNA damages and release into cytosol in cancer [42]. Another study showed that mitochondrial ROS induced inflammation dependent on disrupting mtDNA maintenance [15]. In agreement, other study detected that LPS induced accumulation of free mtDNA outside of mitochondria contributing to inflammation via TLR9 activation [43]. We provided herein the proof of mtDNA efflux arising from LPS mediating ROS activation by blocking PDK2 in HGFs. The exact reason for this phenomenon is unclear. It is crucial to note that studies have observed the transfer of entire mitochondria between cells [44]. However, whether the entire mitochondria or mtDNA is transferred is controversial [45]. Therefore, mtDNA is thought to be a signal molecule that spreads inflammatory signals across a population of cells. This suggests that inflammation could spread between cells via the detection of mtDNA [9]. Based on these results, we propose that LPS modulates mtDNA efflux remained in HGFs closely linked to sustained ROS overproduction.

Some studies have concluded mtDNA release in the context of cell apoptosis [46], while other studies have indicated that mPTP opening leads to increased mtDNA release [46]. Given that cell apoptosis was similar among our divided groups, the role of mPTP opening in mtDNA release in HGFs was focused. Based on the inhibitory effect of CsA on mPTP opening, CsA successfully restored mtDNA efflux from mitochondria and reduced mtDNA levels in cytosol within inflamed HGFs. These results highlight that mPTP opening potentially modulates mtDNA release in HGFs with periodontitis. One of the possible physiological mechanisms mediating increased mPTP opening could be linked to ROS increase in inflammatory HGFs. Exceeding levels of ROS can trigger mPTP opening via mitochondrial ATP-sensitive potassium channels, and voltage-dependent anion channel-1 oligomerization, suggesting that ROS works as an important molecular leading to downstream mPTP opening and eventually disruption of cellular functions [47, 48]. Of note, earlier, Bullon’s work together with our recent work demonstrated that HGFs and gingival tissues from CP patients were observed impaired mitochondria and higher oxidative stress [8, 12, 49]. As a result of cellular ROS and mtROS outburst, mPTP opening can be activated. According to our data, we confirmed positive relationship between ROS overproduction and mPTP opening in inflammatory HGFs. In addition, ROS and mPTP both played a critical role in mtDNA release during periodontitis. Our results highlight that ROS could be one possible explanation for mPTP opening, contributing to mtDNA release in HGFs during periodontitis.

5. Conclusion

In summary, mtDNA efflux maintained in primary HGFs could reflect mitochondrial dysfunction detected in periodontitis. This work provides initial preclinical evidence for a new candidate biomarker for mtDNA efflux in HGFs predicting periodontitis. In addition to this focused investigation of mtDNA efflux in HGFs during inflammation, our results also indicate that ROS/mPTP pathway could be the principal mediator of mtDNA efflux in inflamed HGFs. Further investigation is needed to determine how mtDNA release causes periodontitis, which may reveal new therapeutic strategies for the treatment of patients with periodontitis.

Data Availability

The datasets generated and analyzed during the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors’ Contributions

J. Liu, M. Zheng, and Q. Luan designed the study. J. Liu performed the experiments. J. Liu, X. Wang, P. Zou, Y. Wang, and Q. Shi analyzed the data. J. Liu wrote the manuscript. All authors contributed to editing the manuscript and approved the final manuscript.

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Supplementary Materials

**Supplementary 1.** Fig. S1: measurement of cell apoptosis by Annexin V binding and propidium iodide (PI) uptake. Human gingival fibroblasts (HGFs) were from control donors (A) or chronic periodontitis (CP) patients (B). Control cells were treated with lipopolysaccharide (LPS) (5 µg/mL, 24 h) (C), and CP cells were treated with LPS (5 µg/mL, 24 h) (D). These four groups of HGFs were harvested for cell apoptosis analysis by flow cytometry 24 h after LPS treatment or without LPS treatment.

**Supplementary 2.** Movie. S1: mtDNA does not release from mitochondria in human gingival fibroblasts from healthy control donors in real time movie. Human gingival fibroblasts (HGFs) from control hosts were transduced with vector encoding the mitochondrial outer-membrane protein Tomm 20 bearing a mCherry fluorescence to illustrate mitochondria (red). Meanwhile, HGFs were also transduced with mitochondrial transcription factor A (TFAM) tagged with the green fluorescent protein (GFP) variant mNeonGreen to detect mtDNA (green) (scale bar: 7.5 µm).

**Supplementary 3.** Measurement of cell apoptosis by Annexin V binding and propidium iodide (PI) uptake. Human gingival fibroblasts (HGFs) were from control donors (A) or chronic periodontitis (CP) patients (B). Control cells were treated with lipopolysaccharide (LPS) (5 µg/mL, 24 h) (C), and CP cells were treated with LPS (5 µg/mL, 24 h) (D). These four groups of HGFs were harvested for cell apoptosis analysis by flow cytometry 24 h after LPS treatment or without LPS treatment.

**Supplementary 4.** Movie. S1: mtDNA does not release from mitochondria in human gingival fibroblasts from healthy control donors in real time movie. Human gingival fibroblasts (HGFs) from control hosts were transduced with vector encoding the mitochondrial outer-membrane protein Tomm 20 bearing a mCherry fluorescence to illustrate mitochondria (red). Meanwhile, HGFs were also transduced with mitochondrial transcription factor A (TFAM) tagged with the green fluorescent protein (GFP) variant mNeonGreen to detect mtDNA (green) (scale bar: 7.5 µm).

**Supplementary 5.** Data. S1: measurement of cell apoptosis by Annexin V binding and propidium iodide (PI) uptake. Human gingival fibroblasts (HGFs) were from control donors (A) or chronic periodontitis (CP) patients (B). Control cells were treated with lipopolysaccharide (LPS) (5 µg/mL, 24 h) (C), and CP cells were treated with LPS (5 µg/mL, 24 h) (D). These four groups of HGFs were harvested for cell apoptosis analysis by flow cytometry 24 h after LPS treatment or without LPS treatment.

**Supplementary 6.** Movie. S1: mtDNA does not release from mitochondria in human gingival fibroblasts from healthy control donors in real time movie. Human gingival fibroblasts (HGFs) from control hosts were transduced with vector encoding the mitochondrial outer-membrane protein Tomm 20 bearing a mCherry fluorescence to illustrate mitochondria (red). Meanwhile, HGFs were also transduced with mitochondrial transcription factor A (TFAM) tagged with the green fluorescent protein (GFP) variant mNeonGreen to detect mtDNA (green) (scale bar: 7.5 µm).
Oxidative Medicine and Cellular Longevity

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