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ORIGINAL ARTICLE

Oxidative stress in human gingival fibroblasts from periodontitis versus healthy counterparts

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Abstract

Objective: Elevated p53 promotes oxidative stress and production of pro-inflammatory cytokines in liposaccharide (LPS)-treated healthy human gingival fibroblasts (HGFs). This study compared oxidative stress, production of inflammatory cytokines, and p53 expression in HGFs from patients with chronic periodontitis (CP) and healthy subjects in vitro upon LPS from *Porphyromonas gingivalis* challenge.

Methods: Human gingival fibroblasts were isolated from 6 biopsies—3 from healthy donors and 3 from diseased area in CP (Grade B, Stage III). HGFs were cultured with or without 1 μg/ml 24 h LPS. Oxidative stress was assessed by analyzing the level of reactive oxygen species (ROS). Mitochondrial membrane potential and respiration were determined by immunofluorescence and respirometry, respectively. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β were determined by enzyme-linked immunosorbent assay. P53 expression was monitored by Western blot and immunofluorescence.

Results: Human gingival fibroblasts from CP exhibited increased levels of mitochondrial p53, enhanced ROS production, decreased mitochondrial membrane potential, increased mitochondrial oxygen consumption, and increased secretion of TNF-α, IL-6, and IL-1β, as compared to HGFs from healthy donors. Moreover, LPS exacerbated these changes.

Conclusion: Human gingival fibroblasts from CP exhibited stronger basal and LPS-inducible oxidative stress and inflammatory response as compared to HGFs from healthy subjects by increased p53 in mitochondria.

KEYWORDS
chronic periodontitis, cytokines, fibroblast, inflammation, lipopolysaccharide, reactive oxygen species

1 | INTRODUCTION

Reactive oxygen species (ROS) are major normal byproducts of aerobic metabolism, and their levels are highly increased under oxidative stress conditions (Xu et al., 2020). The increased levels of ROS usually suggest activated mitochondrial oxidative phosphorylation, led to the accumulation of inflammatory responses (Li & Qin, 2018). Many factors such as periodontal pathogenic bacteria and the host immune response contributing to chronic periodontitis (CP) are associated with oxidative stress (Chen et al., 2019). Lipopolysaccharide (LPS) from *Porphyromonas gingivalis* is a major inducer of periodontitis and promotes oxidative stress (Liu et al., 2018). LPS activates the

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transcription factor, nuclear factor-kappa B (NF-κB), which in turn induces the production of pro-inflammatory cytokines (Gorbunov et al., 2013; Kajiura et al., 2018). In addition, high ROS levels could also activate NF-κB signaling (Liu & Du, 2015), indicating the role of ROS in the regulation of cytokine production. The balance between pro-oxidant and antioxidant events determines the impact of ROS on cell physiology (Fernando et al., 2019). Indeed, extrinsic factors such as radiation, hypoxia, smoking, and infection may induce ROS formation and result in the activation or suppression of multiple cellular functions, ultimately promoting the progression of oral and dental diseases, such as periodontitis and mucosal inflammation, as well as tumor development (Kanzaki et al., 2017; Kesarwala et al., 2016). Therefore, protective cellular mechanisms have evolved to limit the deleterious effects of excessive ROS levels. In mitochondria, metabolic uncoupling is a critical modulator of ROS production. Different systems contribute to ROS scavenging, including superoxide dismutase (SOD), catalase (CAT), and the glutathione system (Kanzaki et al., 2017; Liang et al., 2019). In this regard, the development of ROS-inhibiting antioxidant drugs or micronutrients for periodontal treatment in conditions such as tolerance for common anti-microorganism therapy is recently attracting great attention. Although a few studies demonstrated that the administration of antioxidants by mouth rinsing or toothpastes could limit ROS levels and attenuate the inflammatory state (Miguel et al., 2011). Nevertheless, the effects of ROS inhibition on periodontitis are still unclear (AlQranei et al., 2020; Bhattarai et al., 2017; Lee et al., 2020). Based on this, there are also animal studies testing the administration of pharmacological agents controlling ROS on periodontitis progression (Bhattarai et al., 2019; Kasuyama et al., 2011). However, the underlying mechanisms of ROS inhibition on periodontitis are still unknown.

The tumor suppressor protein, p53, contributes to the regulation of cell survival or cell death and modulates many metabolic pathways (Rao et al., 2019). It affects the balance between glycolysis and oxidative phosphorylation, thus influencing the generation of ROS and inducing various cellular responses in the presence of strong metabolic stress (Rao et al., 2019). Redox enzymes such as SOD, cytochrome c oxidase 2 (SCO2), pyruvate dehydrogenase kinase 2 (PDK2), glutaminase 2 (GLS2), and phosphoglycerate mutase (PGM) are regulated by p53, most likely via a context-dependent mechanism (Beyfuss & Hood, 2018). Thus, p53 activation can regulate different target genes and may produce diverse, sometimes even opposite, downstream effects. Given that, it is important to clarify the mechanism by which ROS and p53 affect the cellular response to LPS stimulation. Intercellular Toll-like receptor (TLR)3 and TLR9 are transcriptional targets of p53 on the plasma membrane (Menendez et al., 2019), suggesting that p53 may stimulate inflammation under certain conditions. In addition, a recent study from our laboratory showed that p53 promotes ROS formation in healthy human gingival fibroblasts (HGFs), resulting in increased generation of pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β (Liu et al., 2018). However, the role of p53 in the modulation of periodontitis-related oxidative stress is currently unknown.

Periodontitis is an inflammatory lesion associated with the response to bacterial infection and involves the tissues surrounding the teeth (Rich et al., 2017). It causes the destruction of the alveolar bone and connective tissue (Kuo et al., 2017). HGFs are the predominant cells in periodontal soft tissues and are the object of extensive investigation. They protect the periodontal tissues, mainly serving as a barrier, and control the effects of inflammation and infection (Sun et al., 2016). Furthermore, HGFs consistently exposed to oral pathogens or their products can secret more IL-1β and TNF-α, further exacerbating inflammation (Naruishi & Nagata, 2018). HGFs can also maintain, remodel, and repair periodontal tissues (Gao et al., 2018). Previous studies have shown that increased oxidative stress in HGFs may aggravate periodontal tissue damage (Bhattarai et al., 2017). Interestingly, these effects in HGFs are reversed by antioxidant treatments (Bhattarai et al., 2017). These findings support the crucial role of HGFs in periodontal diseases. Therefore, it may be important to determine whether and how HGFs isolated from patients with CP differ from control HGFs at baseline or in the response to inflammatory stimuli such as LPS. It has been reported that HGFs and gingival tissues obtained from patients with CP showed abnormal mitochondrial structure and mitochondrial dysfunction compared with HGFs and gingival tissues from controls (Liu et al., 2021). These results indicate that the disruption of mitochondria is retained in HGFs in vitro similar to that in vivo during periodontitis. Mitochondria play a key role in oxidative stress. As a result, we aimed to comparatively evaluate the differences in oxidative stress between HGFs from CP and from healthy individuals.

Herein, we focus on assessing if various HGFs' responses and periodontally diseased or healthy hosts origin are closely linked. It might be related to the oxidative stress and mitochondrial dysfunction retained in hosts. This will provide a knowledge required for the development of effective prevention and treatment of oxidative stress disorders in periodontitis patients. These findings implicate that the cellular response could be affected by host for a long time, which will shed light on the relationship between oxidative stress and periodontitis.

2 | MATERIALS AND METHODS

2.1 | Clinical parameters at surgery site of patients included in the study

The clinical characteristics at surgery site from patients are shown in Table 1. Three gingival specimens from healthy group were all collected during crown lengthening surgery of teeth from 13 to 23. The interdental clinical attachment loss (CAL) of periodontally healthy donors is less than 2 non-adjacent teeth. The buccal or oral CAL ≥3 mm with pocketing >3 mm is <2 teeth. Three gingival specimens from patients with chronic periodontitis who were all under Grade B, Stage III in the current classification of periodontitis were acquired through flap surgery. The vitality of the tooth in diseased area is available, and the furcation involvement of the inclusion tooth is

TABLE 1 Clinical parameters at surgery site of patients included in this study

Group	Age	Gender	Smoking	GI	BOP	PD	CAL	Teeth	Vitality	FI
Con 1	35	Male	No	1	1	2–3 mm	0	13–23	Available	–
Con 2	31	Female	No	0	0	3 mm	0	13–23	Available	–
Con 3	33	Male	No	0.5	1	3 mm	0	13–23	Available	–
CP 1	34	Female	No	2	2	6–8 mm	5 mm	31–32	Available	–
CP 2	45	Male	No	1.5	2	6–7 mm	5 mm	13,17	Available	2
CP 3	42	Female	No	1.75	2	8–10 mm	7 mm	17	Available	2

Abbreviations: BOP, bleeding on probing; CAL, clinical attachment level; Con, control group; CP, chronic periodontitis; FI, furcation involvement; GI, gingival index; PD, probing depth.

≤II. All donors in periodontally diseased group have completed scaling and root planning (SRP) before the surgery. Specimen from one tooth was about 0.5 × 5 mm.

2.2 | Subjects, cell isolation and culture, cell treatment

The experimental procedures were reviewed and approved by the Review Board and Ethics Committee of Peking University Health Science Center (PKUS-SIRB-2013017) and were conducted in accordance with the Helsinki Declaration of 1975, as revised in 2013. All subjects enrolled in this study were provided written informed consent. Three gingival tissue specimens were harvested from three healthy individuals (2 men and 1 woman) without gingival or periodontal diseases, during crown lengthening surgery with periodontal depth (PD) lower than 4 mm. Another three tissue specimens were collected during flap surgery with PD of at least 6 mm from three patients with chronic periodontitis (Grade B, Stage III) (1 man and 2 women). Healthy donors and patients had no history or present signs of systemic diseases and received no medication within the previous 6 months. Primary HGFs, deriving from gingival tissue samples, were cultured by tissue explant culture method. HGFs were maintained in Dulbecco's modified Eagle's medium (DMEM) (HyClone Laboratories) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin as previously described in detail (Liu et al., 2018). HGFs between Passages 3 and 8 were used for the experiments. The following experiments among four groups were conducted only for the same passage each time. The cells were treated with or without 1 μg/ml LPS from *P. gingivalis* (InvivoGen, ATCCs 33277, Standard, France) for 24 h.

2.3 | Detection of cytoplasmic and mitochondrial ROS

Mitochondrial ROS (mtROS) production was evaluated by MitoSOX Red (Invitrogen). Cytoplasmic ROS were examined via 2′,7′-dichlor odihydrofluorescein diacetate (H2DCF-DA) (Sigma-Aldrich). After a 30 min's pretreatment in the dark with 10 μM H2DCF-DA (excitation at 488 nm, emission at 535 nm) or 5 μM MitoSOX Red (excitation at 510 nm, emission at 580 nm) in Dulbecco's modified Eagle's medium (DMEM), the cells were monitored with a fluorescence microscope (TCS-SP82; Leica), and later, the fluorescence intensity based on the image was analyzed by ImageJ (NIH, http://rsb.info.nih.gov/ij/). A multi-model microplate reader (EnSpire, PerkinElmer) was also performed for directly measuring fluorescence intensity of cells as optical density (OD) at 488 nm and 510 nm, respectively.

2.4 | Mitochondrial function assay

To test the level of mitochondrial respiration, the basal and maximal respirational capacity of HGFs was measured by a high-resolution respirometer (Oxygraph-2K; Oroboros Instruments Corp). HGFs from healthy subjects or periodontal patients were exposed to 1 μg/ml LPS in essential medium and cultured for 24 h. Then, the cells in each chamber of the respirometer received injections of oligomycin, carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP), rotenone, and antimycin A, as previously described (Liu et al., 2018).

The mitochondrial membrane potential was evaluated by using tetramethylrhodamine methyl ester (TMRM) (Invitrogen). HGFs were seeded into confocal plates containing 1 ml of growth medium and grew overnight. The cells were treated with 200nM TMRM for 15 min and washed, and images were captured by using a fluorescence microscope (Leica) with excitation and emission wavelengths of 549 nm and 573 nm, respectively.

2.5 | Enzyme-linked immunosorbent assay

IL-1β, IL-6, and TNF-α quantifications in the cell supernatant were performed by using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) following the manufacturer's protocols.

2.6 | Real-time quantitative reverse transcription polymerase chain reaction analysis

For gene expression analysis, total RNA from HGFs was extracted with TRIzol (TRIzol, Life Technologies, Thermo Fisher

Scientific) and reverse transcribed into cDNA by using a qPCR RT Master Mix (ReverTra Ace, Toyobo). Real-time PCR array was performed with the qPCR Mix SYBR (Toyobo). Primer sequences were as follows: p53 (TCCTCCCCAACATCTTATCC and GCACAAACACGAACCTCAAA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GGAGCGAGATCCCTCCAAAAT and GGCTGTTGTCATACTTCTCATGG), SOD2 (CCTGCTCCC CGCGCTTTCTT and CGGGGAGGCTGTGCTTCTGC), TNF-α (CCCAGGCAGTCAGATCATCTTC and AGCTGCCCCTCAGCTTGA), IL-1β (ATGGCTTATTACAGTGGCAATGAG and GTAGTGGT GGTCGGAGATTCG), IL-6 (ATGAACTCCTTCTCCACAAGCGC and GGGAAGGCAGCAGGCAACAC).

2.7 | Western blot

For the isolation of mitochondria from HGFs, the cells were harvested in 1.5 ml of 100 nM phenylmethanesulfonyl fluoride (PMSF). The Cell Mitochondria Isolation Kit (Beyotime) was used according to the manufacturer's instruction, and lysates were passed 10–20 times through a needle. Finally, mitochondria-enriched fractions were obtained. Total and mitochondrial proteins were quantified by BCA protein assay (Pierce Biotech) and analyzed by Western blot as previously described (Liu et al., 2018). Primary antibodies against p53 and GAPDH (Cell Signaling Technology), SOD2 and Tom 20 (ProteinTech), and horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibodies from Cell Signaling Technology were utilized.

2.8 | Immunofluorescence

Human gingival fibroblasts were seeded into confocal dishes and maintained in serum-containing essential medium until 70% confluence. Then, they were treated with 200 nM MitoTracker Red (Invitrogen) for 30 min, washed, fixed with 4% paraformaldehyde (PFA), permeabilized, and washed again. After blocking with 5% bovine serum albumin, the cells were incubated with primary antibodies specific for p53 for 2 h at 37°C, washed, and incubated for

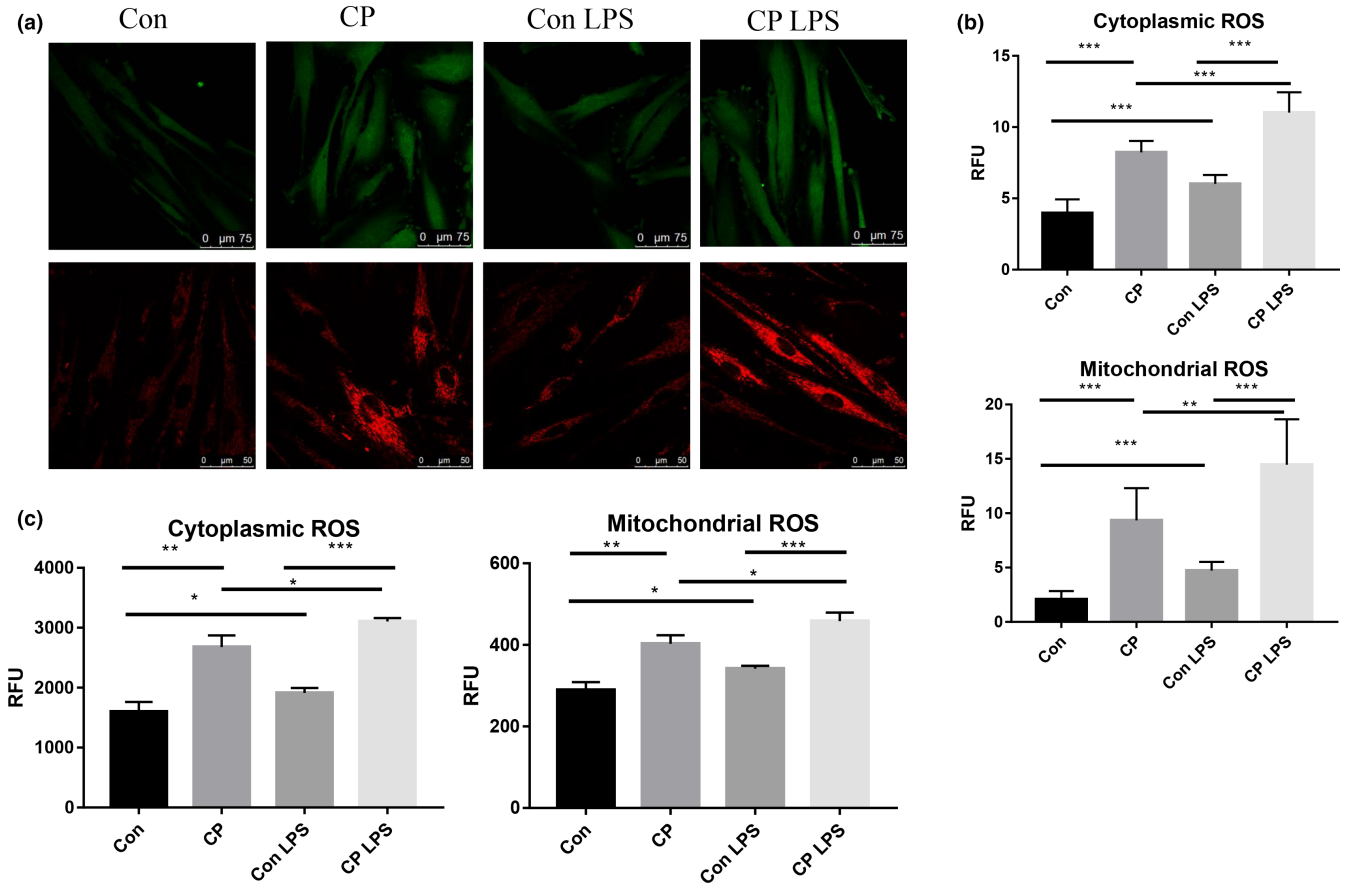

FIGURE 1 Cytoplasmic and mitochondrial reactive oxygen species (ROS) levels in human gingival fibroblasts (HGFs) from patients with periodontitis and healthy subjects. (a) Confocal images of cytoplasmic and mitochondrial ROS staining by 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) (green) and MitoSOX Red (red) in HGFs with and without lipopolysaccharide (LPS) stimulation (1 μg/ml, 24 h). Scale bars: 75 μm (cytoplasmic ROS), 50 μm (mitochondrial ROS). (b) Average fluorescence intensity from confocal images in A ($n = 10$ images per group). (c) Quantification of cytoplasmic and mitochondrial ROS with a multi-model microplate reader in HGFs from chronic periodontitis (CP) and control group (Con) ($n = 3$). *Significant ($p < 0.05$); **highly significant ($p < 0.01$); ***$p < 0.001$. RFU, relative fluorescence units

1 h with Andy Fluor 488 fluorescent-labeled secondary antibody (GeneCopoeia). The nuclei were stained with DAPI (4'-6-diamidino -2-phenylindole). Images of fluorescence staining were taken with a confocal microscope (Leica). P53 and mitochondrial colocalization percentages were assessed by using the "colocalization rate" in the LAS X software (Leica).

2.9 | Statistical analysis

The data were expressed as mean ±SE fold change, and Student's *t* test was used to analyze the differences between the two experimental groups, setting the significance threshold at $p < 0.05$. Multiple comparisons were analyzed with ANOVA followed by Student–Newman–Keuls post hoc test. The experiments were repeated at least 3 times, and *p* values <0.05 were considered statistically significant. Analyses were performed with GraphPad Prism 7.04 software.

3 | RESULTS

3.1 | HGFs from patients with periodontitis exhibit higher ROS levels compared with those from healthy subjects

Human gingival fibroblasts were stained with H2DCF-DA and MitoSOX-Red for visualization of cytosolic and mitochondrial ROS, respectively. Confocal images showed that, in HGFs from CP patients, both cytosolic and mitochondrial ROS levels were higher than in HGFs from healthy individuals (Con) under basal conditions (Figure 1a). LPS stimulation increased cytosolic and mitochondrial ROS levels in Con HGFs, in line with our previous findings (Figure 1a). Moreover, LPS stimulation further increased cytosolic and mitochondrial ROS levels in HGFs from CP patients (Figure 1a).

Total and mitochondrial ROS levels in HGFs were quantified based on fluorescence intensity in confocal images (Figure 1b). CP

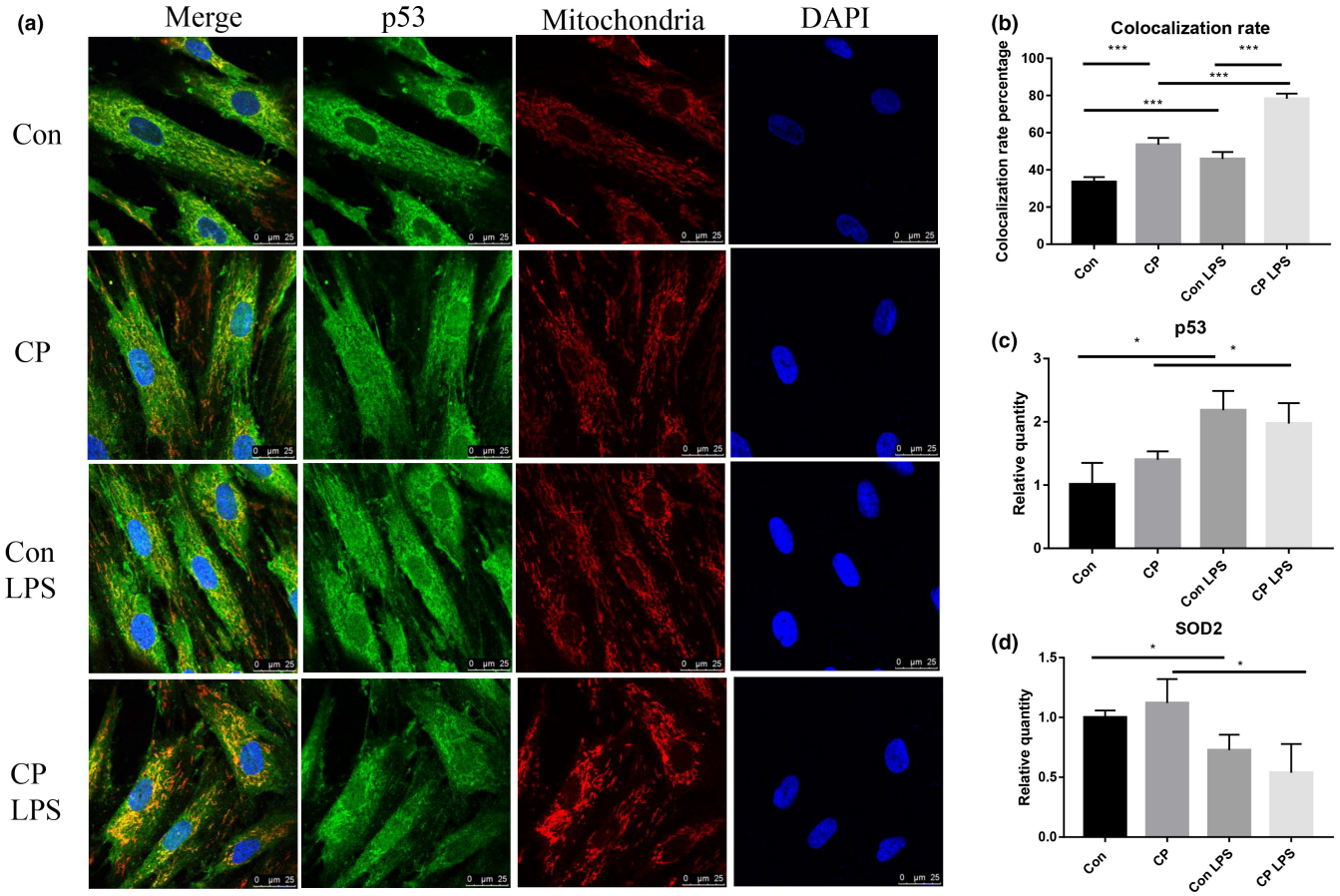

FIGURE 2 Determination of p53 level by fluorescence staining in HGFs from periodontal patients and healthy subjects with or without LPS stimulation (1 μg/ml, 24 h). (a) Confocal images show the localization of p53 in HGFs after staining with anti-p53 antibody (green), MitoTracker Red for mitochondria (red), and 4′-6-diamidino-2-phenylindole (DAPI) for nuclei (blue). Scale bars: 25 μm. (b) Colocalization of p53 with mitochondria in HGFs based on the confocal fluorescent signals shown in (a). The data are presented as mean ± *SE*; $n = 15$ images per group. (c) Total level of p53 mRNA determined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control ($n = 3$). (d) qRT-PCR was performed with total mRNA level of SOD2 ($n = 3$). $^{*}p$ values <0.05 were considered significant. $^{***}p < 0.001$ were considered highly statistically significant

HGFs exhibited higher ROS levels compared with Con HGFs under basal conditions. Notably, a 24-h stimulation with LPS caused a stronger increase in ROS production in CP HGFs than in Con HGFs (Figure 1b). The analysis of total and mitochondrial ROS levels by an alternative method based on the use of a multi-model microplate reader confirmed the above findings (Figure 1c).

3.2 | Mitochondrial p53 levels are increased in CP HGFs, as well as in response to LPS stimulation

Staining with anti-p53 antibodies showed that CP HGFs contained a higher level of mitochondrial p53 compared with Con HGFs (Figure 2a and b). LPS treatment increased the mitochondrial p53 proportion in HGFs from both CP and healthy donors (Figure 2a and b), indicating that inflammatory stimulation induced the translocation of p53 from the cytosol to mitochondria.

We then used qRT-PCR to verify whether p53 transcription was also increased in response to inflammatory simulation. Although the level of p53 mRNA was slightly higher in CP HGFs compared with Con HGFs, p53 expression was significantly increased by LPS in both HGF populations (Figure 2c), in line with our previous findings (Liu et al., 2018). We also observed that LPS causes a major decrease in the mRNA level of SOD2 in both HGF groups (Figure 2d). Consistently, under basal conditions, the total level of p53 protein was not significantly different in CP and Con HGFs, while LPS stimulation resulted in a dramatic elevation of p53 protein level in both cell populations (Figure 3b). In addition, in isolated mitochondrial fractions, p53 protein level was higher in CP HGFs than in Con HGFs, and LPS stimulation further increased the mitochondrial p53 level in both groups (Figure 3a). These data indicated that p53 in mitochondria was increased in CP HGFs, resulting from increased mitochondrial p53 translocation from cytosol to mitochondria, and that inflammatory stimulation may promote p53 total expression and mitochondrial translocation. Interestingly, we found that increases in either mitochondrial or total p53 were always accompanied by reduced levels of SOD2, in both CP and LPS-challenged HGFs, as compared to untreated HGFs (Con) (Figure 3a and b).

3.3 | The mitochondrial function is damaged in CP- and LPS-stimulated HGFs

To investigate the impact of increased p53 translocation to mitochondria on mitochondrial function, the mitochondrial membrane potential in HGFs was measured by cell staining with the TMRM dye. The mitochondrial membrane potential was significantly decreased in both CP- and LPS-stimulated HGFs, as compared to untreated HGFs (Figure 4a and b). Notably, LPS stimulation exacerbated the decline in mitochondrial membrane potential in CP HGFs (Figure 4a and b).

Next, mitochondrial respiration was investigated in CP and Con HGFs, with or without LPS stimulation. The measurement of oxygen

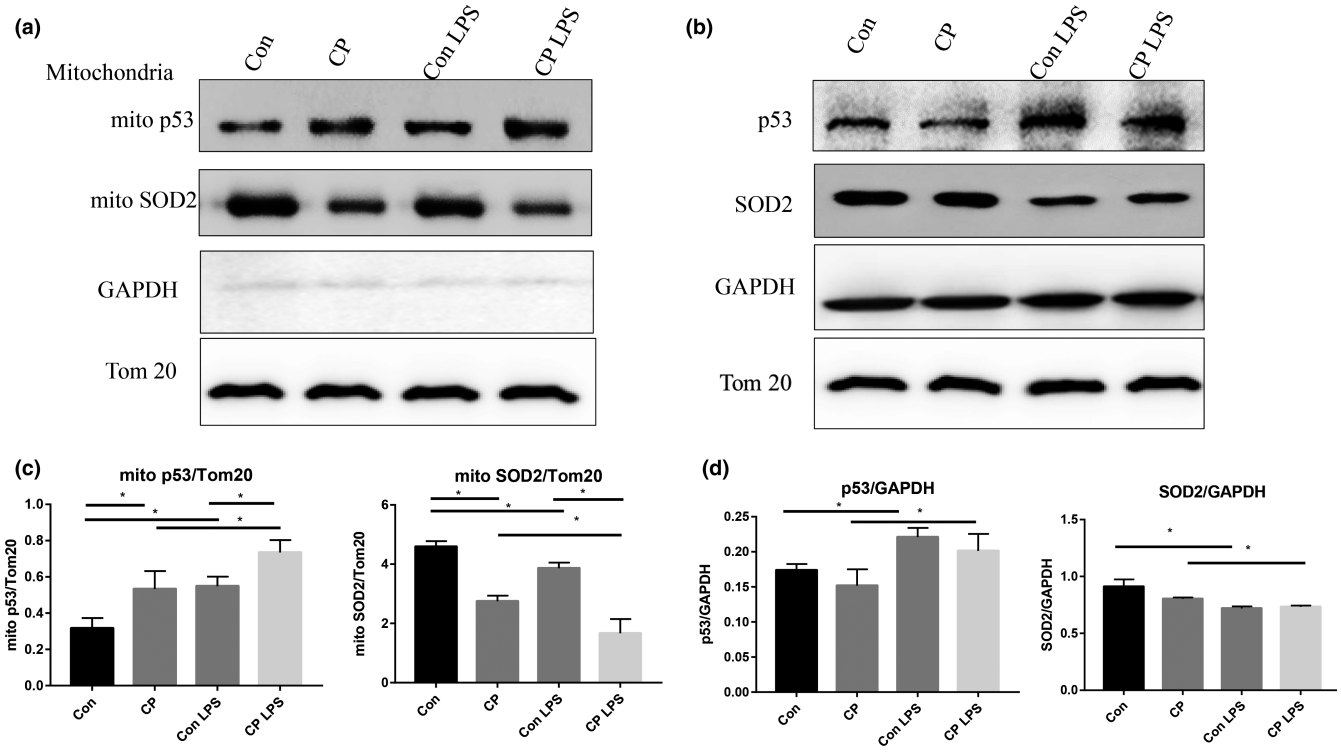

FIGURE 3 Determination of p53 and SOD2 levels by immunoblotting in HGFs from CP patients and healthy subjects with or without LPS stimulation (1 μg/ml, 24 h). (a) Mitochondrial levels of p53 and SOD2 proteins (mito p53 and mito SOD2). (b) Total levels of p53 and SOD2 proteins. Tom 20 and GAPDH were used as mitochondrial and total cellular protein controls, respectively. (c) Average data from (a). (d) Average data from (b; $n = 3$). $^{*}p < 0.05$

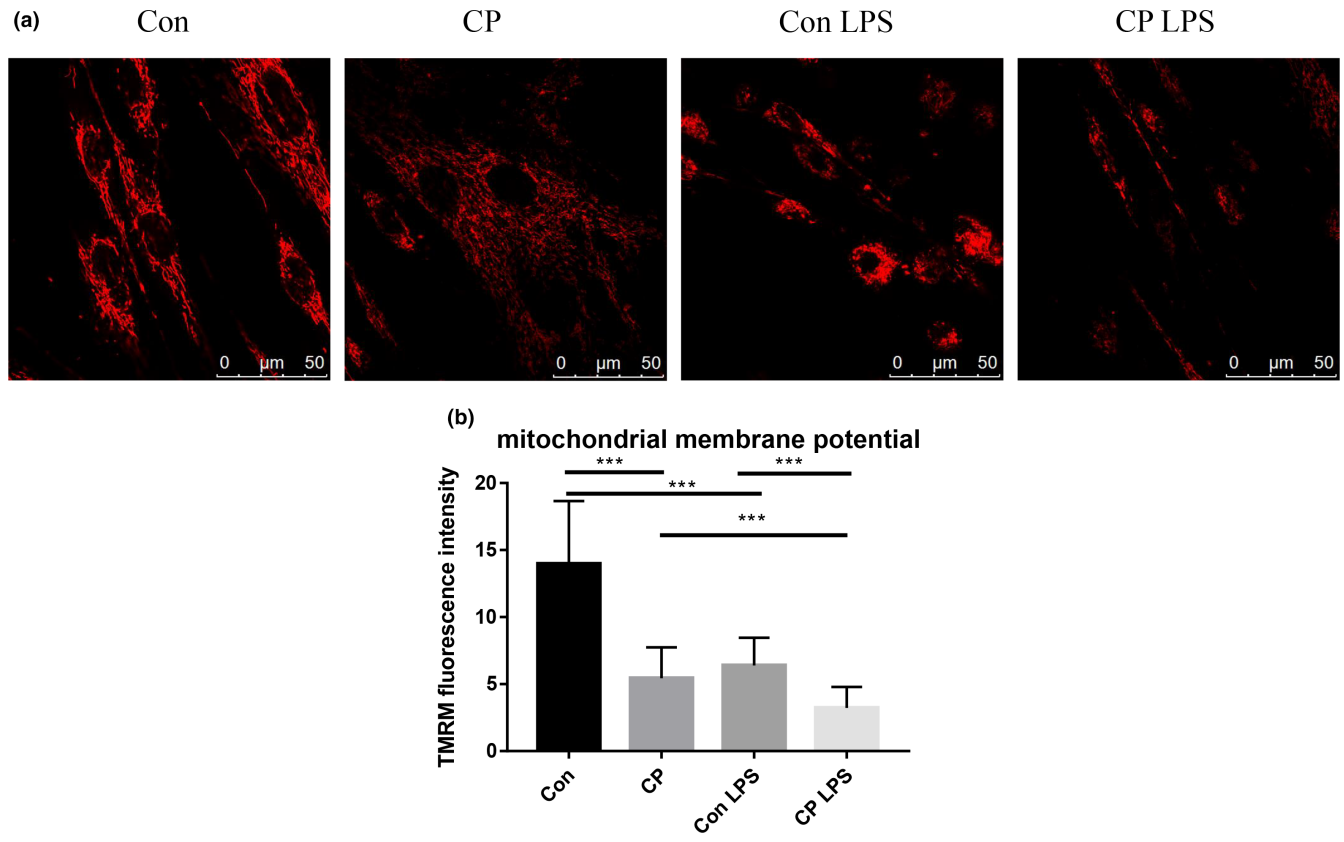

FIGURE 4 Mitochondrial membrane potential in HGFs from periodontal patients and healthy individuals with or without LPS stimulation (1 μg/ml, 24 h). (a) Confocal images after staining with tetramethylrhodamine methyl ester (TMRM) (red); scale bars: 50 μm. (b) TMRM fluorescence intensities referred to images in A ($n = 18$ images per group). $^{***}p < 0.001$. oligo: oligomycin; FCCP: carbonyl cyanide 4-trifluoromethoxy phenylhydrazone; A: antimycin A; R: rotenone

consumption rate showed that both basal and maximal mitochondrial respiration were higher in CP compared with Con HGFs, and that LPS stimulation further increased these indexes (Figure 5a and b).

3.4 | The expression of inflammatory cytokines is upregulated in HGFs from patients with chronic periodontitis

We compared the mRNA levels and secretion of IL-1β, TNF-α, and IL-6 by qRT-PCR and ELISA, respectively, in CP and healthy HGFs. We found that, under basal conditions, CP HGFs were observed higher levels of inflammatory cytokines compared with control HGFs, as determined by qRT-PCR and ELISA (Figure 6a and b). Moreover, although LPS increased the release of inflammatory factors in both HGF populations, this change was more pronounced in CP compared with control HGFs (Figure 6a and b).

4 | DISCUSSION

Oxidative stress is important in the etiology and pathogenesis of inflammatory conditions such as periodontitis (Waddington et al., 2000). The initial description of its participation in the wide periodontal damage is marked by identification reactive oxygen and antioxidant species in the periodontium related to the respiratory burst in polymorphonuclear lymphocytes (PMNLs) (Chapple & Matthews, 2007). This raised the possibility that ROS accumulation in cells and tissues may lead to pathologic inflammatory responses and induce periodontal disease (Kanzaki et al., 2017; White et al., 2016). In our previous report, elevated ROS promoted the p53-mediated release of inflammatory cytokines in healthy HGFs, such as IL-6, IL-1β, and TNF-α (Liu et al., 2018). This evidence suggests that oxidative stress plays a crucial role in healthy HGFs' responses during periodontitis development.

To the best of our knowledge, there is limited information addressing the differences in oxidative stress and cellular responses between CP patients and healthy host. In a previous study, it was reported that gingival fibroblasts from periodontal patients could maintain inflammatory characteristics (Baek et al., 2013). This finding is similar to another study, which showed that differential response to LPS exhibited in healthy and inflamed gingival fibroblasts (Kang et al., 2016). These findings suggest that different cellular responses in vitro are with respect to various host types. Consistent with these observations, our present finding demonstrated that HGFs from CP patients increased mRNA levels of TNF-α, IL-1β, and IL-6 and secreted higher levels of these three cytokines compared with HGFs from controls even in the absence of LPS. In addition,

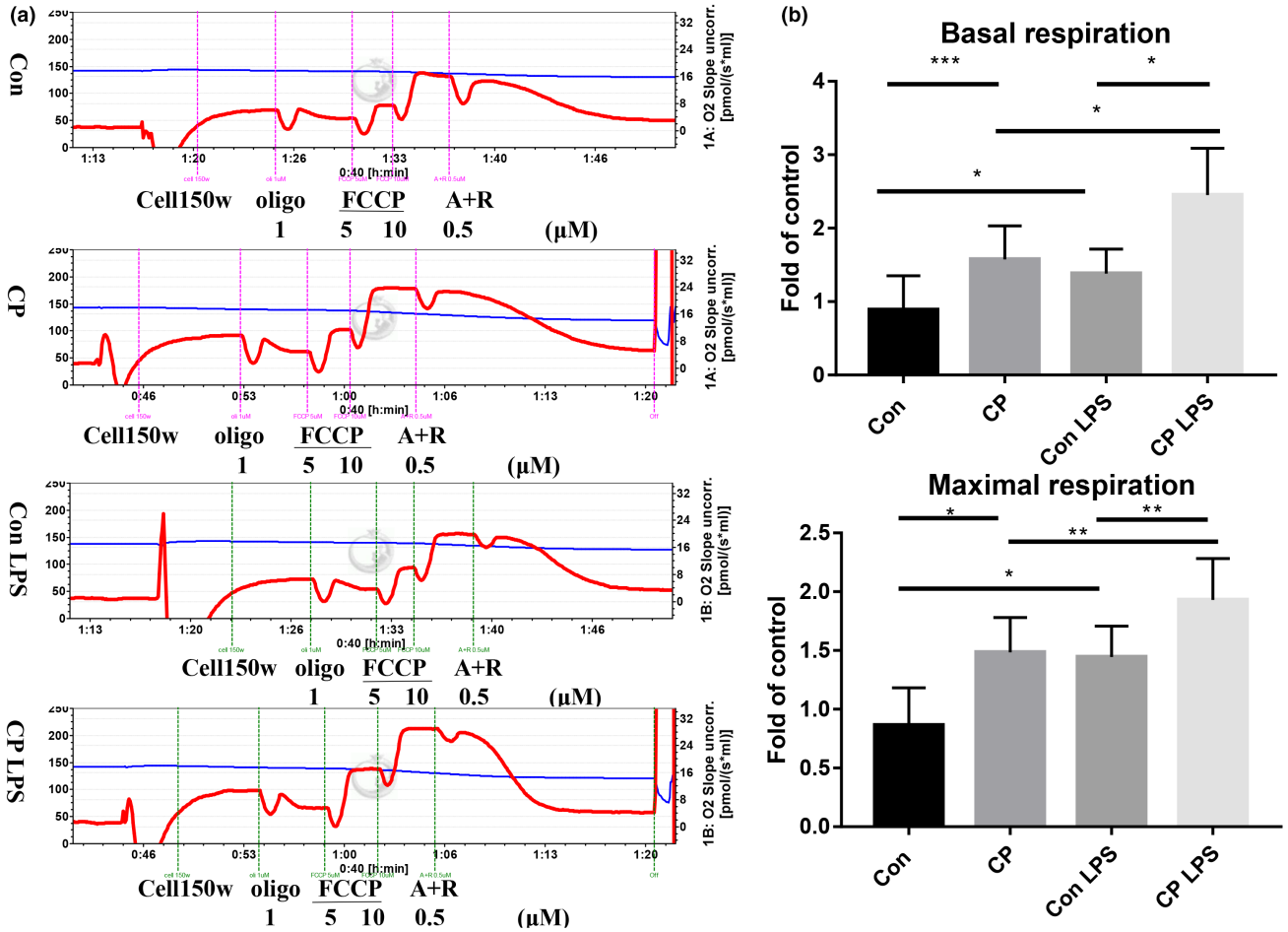

FIGURE 5 Mitochondrial respiration in HGFs from periodontal patients and healthy subjects in the presence or absence of LPS stimulation (1 μg/ml, 24 h). (a) Mitochondrial respiration in HGFs is determined by measurement of oxygen consumption with a respirometer. (b) Average values of basal and maximal respiration capacity calculated from A ($n = 5$). *Significant ($p < 0.05$); **highly significant ($p < 0.01$); $^{***}p < 0.001$

LPS led to more significant increase in these cytokines' mRNA and secretion levels in HGFs from CP patients compared with HGFs from controls. Enhanced levels of these inflammatory cytokines subsequently can result in alveolar bone destruction and periodontitis progression, suggesting that inflammatory characteristics potential of periodontal inflammation in vivo are kept in HGFs in vitro. We also observed increased ROS levels and mitochondrial p53 protein expression in CP HGFs in the presence of LPS and in the absence of LPS compared with HGFs from controls at the same situation. These data suggest that ROS, oxidative stress, and p53 expression in HGFs between patients with periodontitis and healthy subjects with or without LPS induction decide inflammatory state in HGFs. One plausible explanation for these differences is that the relative higher level of oxidative stress was retained in HGFs from inflammatory hosts, and it affects net cell inflammatory responses after passages. Notably, ROS are known to cause mitochondrial impairment, lead to the activation of NF-κB signaling pathway, and enhancement of the downstream generation for pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Early et al., 2018; Gao et al., 2020; Mills et al., 2016). However, further investigations will be required to clarify in detail the relationship between oxidative stress and the changes observed in HGFs between CP patients and healthy donors.

Reactive oxygen species is well known for the target in the redox imbalance and mitochondrial impairment (Rovira-Llopis et al., 2017; Wang & Tang, 2019). Regarding mitochondrial impairment, we previously reported that LPS treatment induced higher level of ROS, mitochondrial membrane potential reduction, and increase in mitochondrial respiration in healthy HGFs (Liu et al., 2018). In our present study, HGFs derived from CP patients showed higher ROS level, attenuated mitochondrial membrane potential, and increased mitochondrial respiration activity compared with control HGFs. Further, LPS treatment promoted these changes more significantly in CP HGFs than control HGFs. These evidences confirm the difference in ROS levels and mitochondrial function of HGFs between periodontally healthy individuals and CP patients. Despite certain distinctions of mitochondria existed between periodontally diseased and periodontally healthy samples (Liu et al., 2021), there are limited available data showing the reason for the discrepancy of ROS in HGFs among CP patients and healthy donors. SOD2 is an antioxidant enzyme playing an

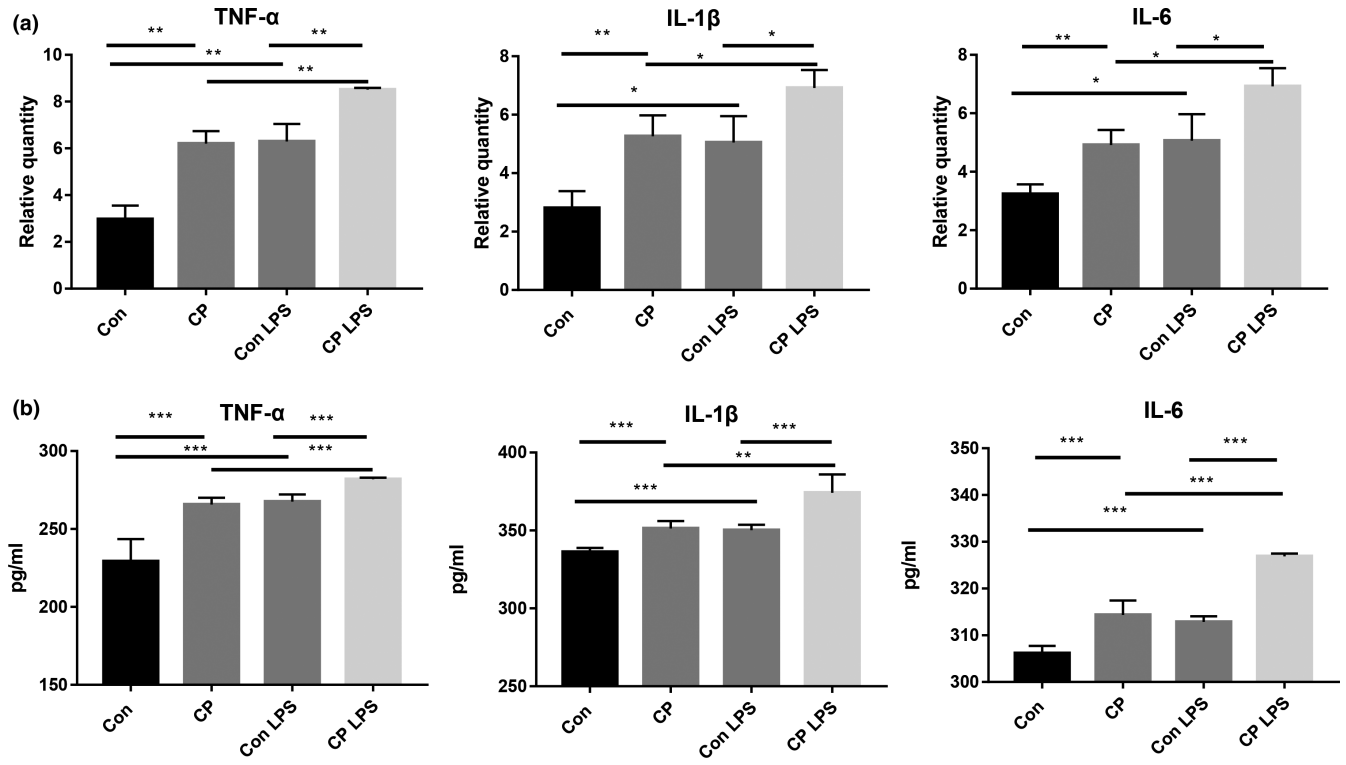

FIGURE 6 mRNA levels and secretion of inflammatory cytokines in HGFs from CP patients and healthy donors with or without LPS stimulation (1 μg/ml, 24 h). (a) qRT-PCR analysis of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 expression in HGFs ($n = 3$), GAPDH mRNA was used as a control. (b) TNF-α, IL-1β, and IL-6 secretion by HGFs as assessed by enzyme-linked immunosorbent assay (ELISA) ($n = 6$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

important role in the regulation to oxidative stress (Dikalova et al., 2017; Kim et al., 2017; Pi et al., 2015; Russo et al., 2019). We demonstrated that mitochondrial SOD2 expression tends to decline in various hosts' cells after exposure to LPS. In addition, the mitochondrial SOD2 expression was inhibited more significantly in CP HGFs than control HGFs. These findings may imply that the balance between antioxidant and redox systems is dysregulated after LPS infection and in periodontitis-affected HGFs. These findings are in keeping with earlier reports that LPS-induced higher oxidative stress is considered as a major determinant in periodontitis and other types of inflammatory conditions (Bhattarai et al., 2017; Le Sage et al., 2017). However, to date, the contribution of oxidative stress to inflammatory responses has been mainly considered to be restricted to cells from a single type of host such as healthy HGFs (Houde et al., 2006). It will then be beneficial to know the impact of and difference in oxidative stress in HGFs from CP patients and healthy individuals in vitro.

p53 is an important regulator of redox homeostasis and inflammation (Beyfuss & Hood, 2018; Reuter et al., 2010). Furthermore, p53 was found in mitochondrial matrix under specific stress conditions (Neitemeier et al., 2014; Park et al., 2005). The increased level of mitochondrial p53 might contribute to the loss of mitochondrial membrane potential (Lee et al., 2010; Marchenko et al., 2000) and affected the mitochondrial permeability transition during sustained inflammation (Bonora et al., 2013; Choudhury et al., 2016). These results of higher p53 expression in mitochondria suggested that p53 might have a role in ROS generation, mitochondrial dysfunction, and inflammation (Zhang et al., 2017; Zhou et al., 2018). Notably, a specific involvement of mitochondrial p53 in oxidative stress under inflammatory conditions, causing mitochondrial dysfunction and increased ROS formation, has been previously reported in other cell types (Beyfuss, & Hood, 2018; Qian et al., 2019). These findings correlated with our results regarding p53 protein expression in mitochondria, p53 translocation to mitochondria, and mitochondrial function evaluation in HGFs from CP patients. Moreover, cellular redox homeostasis can also be directly regulated by p53 through modified expression of pro- and antioxidant proteins (Wang et al., 2014). For example, under inflammatory conditions induced by bacterial infection, p53 upregulation promoted ROS formation by regulating SOD2 expression (Garrison et al., 2010; Jeong & Cho, 2015). Previous reports also suggested that the pro-inflammatory effects of ROS were partly due to increased mitochondrial oxidative phosphorylation (OXPHPS) (Cadenas, 2018). Similar to these reports, higher mitochondrial p53 expression in the current study was a key event in oxidative stress and inflammatory responses of CP fibroblasts. This suggests us that increased p53 in mitochondria may contribute to the increase in mitochondrial respiration, as well as to the loss of mitochondrial membrane potential by repressing SOD2 expression in mitochondria that we detected in periodontitis-affected HGFs and in LPS-challenged HGFs in the present study. The finding is based on the vitro model from CP patients and

periodontally healthy donors, which provides the linkage between irreversible oxidative stress and inflammatory responses in HGFs. Additional analysis in antioxidant response element (ARE) differences in HGFs from healthy individuals and CP patients will provide deep insights regarding the role of antioxidant defense in treating periodontitis.

In summary, we demonstrated that HGFs from patients with chronic periodontitis showed p53 upregulation in mitochondria, leading to oxidative stress, mitochondrial dysfunction, and increased ROS generation. This might be responsible for cytokines production, ultimately enhancing the inflammatory response.

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CONFLICT OF INTEREST

The authors report no conflicts of interest related to this study.

AUTHOR CONTRIBUTIONS

Jia Liu: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing. **Xiaoxuan Wang:** Formal analysis; Methodology. **Ming Zheng:** Funding acquisition; Project administration; Resources; Supervision. **Qing-Xian Luan:** Funding acquisition; Project administration; Resources; Supervision.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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