Free Radical Biology & Medicine 50 (2011) 1336–1343

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Mitochondrial dysfunction promoted by *Porphyromonas gingivalis* lipopolysaccharide as a possible link between cardiovascular disease and periodontitis

Pedro Bullon [a], Mario David Cordero [b], José Luis Quiles [c], Juan Manuel Morillo [a], Maria del Carmen Ramirez-Tortosa [d], Maurizio Battino [e,*]

[a] Department of Periodontology, Dental School, University of Sevilla, Sevilla, Spain
[b] Centro Andaluz de Biología del Desarrollo, Universidad Pablo de Olavide–CSIC, and Centro de Investigación Biomédica en Red de Enfermedades Raras, ISCIII, Sevilla 41013, Spain
[c] Department of Physiology, Institute of Nutrition and Food Technology "José Mataix," Biomedical Research Center, University of Granada, Granada, Spain
[d] Department of Biochemistry and Molecular Biology II, Institute of Nutrition and Food Technology "José Mataix," Biomedical Research Center, University of Granada, Granada, Spain
[e] Department of Biochemistry, Biology, and Genetics, Faculty of Medicine, Università Politecnica delle Marche, 60100 Ancona, Italy

ARTICLE INFO

Article history:
Received 11 October 2010
Revised 3 February 2011
Accepted 15 February 2011
Available online 24 February 2011

Keywords:
Periodontitis
CoQ_{10}
Mitochondrial membrane potential
Apoptosis
Reactive oxygen species
Cardiovascular disease
Free radicals

ABSTRACT

Oxidative stress is one of the factors that could explain the pathophysiological mechanism of inflammatory conditions that occur in cardiovascular disease (CVD) and periodontitis. Such inflammatory response is often evoked by specific bacteria, as the lipopolysaccharide (LPS) of *Porphyromonas gingivalis* is a key factor in this process. The aim of this research was to study the role of mitochondrial dysfunction in peripheral blood mononuclear cells (PBMCs) from periodontitis patients and to evaluate the influence of LPS on fibroblasts to better understand the pathophysiology of periodontitis and its relationship with CVD. PBMCs from patients showed lower CoQ_{10} levels and citrate synthase activity, together with high levels of ROS production. LPS-treated fibroblasts provoked increased oxidative stress and mitochondrial dysfunction by a decrease in mitochondrial protein expression, mitochondrial mass, and mitochondrial membrane potential. Our study supports the hypothesis that LPS-mediated mitochondrial dysfunction could be at the origin of oxidative stress in periodontal patients. Abnormal PBMC performance may promote oxidative stress and alter cytokine homeostasis. In conclusion, mitochondrial dysfunction could represent a possible link to understanding the interrelationships between two prominent inflammatory diseases: periodontitis and CVD.

Within most cells, the mitochondrion is the main source of reactive species, which are by-products of cell energy production. Inside mitochondria the primary reactive oxygen species (ROS) produced is superoxide, most of which is converted to hydrogen peroxide by the action of superoxide dismutase. The mitochondrial production of superoxide has been ascribed to several electron transport chain enzymes, including complex I and complex III. These complexes along with coenzyme Q_{10} (CoQ_{10}) may leak electrons, which in turn may interact with oxygen, thus forming ROS [1,2]. All conditions able to alter mitochondrial efficiency can enhance ROS production, with a direct and critical effect on oxidative stress. In this respect, CoQ_{10} also has an important lipid-soluble antioxidant activity, in addition to its well-known redox role as electron/proton carrier, and is synthesized by the organism under physiological conditions.

Oxidative stress is defined as a persistent imbalance between the production of highly reactive molecular species (e.g., ROS and reactive nitrogen species (RNS)) and antioxidant defenses [3]. When the production of ROS/RNS exceeds the capacity of antioxidant defenses, oxidative stress may have a harmful effect on the functional and structural integrity of biological tissue. Oxidative stress is involved in atherosclerosis, hypertension, insulin resistance, heart failure, and the aging process [3]. Specifically, mitochondrial oxidative stress has been related to myocardial dysfunction and heart failure [4]. Most of the oxidative stress diseases are considered chronic diseases and their influence on morbidity and mortality, in the future, will be a critical public health topic [5]. Among them, cardiovascular diseases (CVDs) are considered the major cause of death in Western countries [6] and are related to some risk factors such as obesity, diabetes, insulin resistance, and metabolic syndrome. Recently, CVDs have also been associated with an oral disease: periodontitis [7–11]. Periodontitis is a generally chronic disorder characterized by the breakdown of the tooth-supporting tissues producing the loss of dentition. The cause is due to an ecological imbalance between the microbial biofilm on teeth and an impaired host inflammatory response. One of the main challenges is the search for factors that may explain these relationships. There is evidence supporting the role of inflammation in all the

Abbreviations: CAL, clinical attachment level; CoQ_{10}, coenzyme Q_{10}; CVD, cardiovascular disease; GM, gingival margin; LPS, lipopolysaccharide; PD, periodontal probing depth; PBMC, peripheral blood mononuclear cell; RNS, reactive nitrogen species; ROS, reactive oxygen species.

* Corresponding author. Fax: +39 0712204123.
E-mail address: m.a.battino@univpm.it (M. Battino).

0891-5849/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2011.02.018

previously indicated pathological events as a meeting point [11]. Oxidative stress is one of the main factors studied that may be able to explain the pathophysiological mechanism of inflammatory conditions that occur in atherosclerosis, CVD, and periodontitis. Several studies have demonstrated an increase in products from oxidative damage in plasma and serum of subjects with periodontitis compared with healthy individuals [12–14]. Moreover, there is evidence for a decreased antioxidant capacity in subjects with periodontitis, evaluated by various assays [15–17].

Periodontitis, as an infectious disease, has been related also to a specific group of bacteria, three of which have been considered the main periodontal pathogens: *Tannerella forsythia, Aggregatibacter actinomycetemcomitans,* and *Porphyromonas gingivalis* [18]. In the oral environment the inflammatory response is often evoked by specific bacteria, such as *P. gingivalis,* able to produce an inflammatory response that can be correlated with atherosclerosis and ultimately thrombotic complications [19]. *P. gingivalis* bacterial DNA has been found in coronary artery biopsy samples [20]. The lipopolysaccharide (LPS) of *P. gingivalis* is a key factor in the development of periodontitis. Gingival fibroblasts, which are the major constituents of gingival connective tissue, may directly interact with bacteria and bacterial products, including LPS, in periodontal lesions. It has been suggested that gingival fibroblasts play an important role in the host responses to LPS in periodontal disease [21].

The purpose of this study was to study the role of mitochondrial dysfunction in peripheral blood mononuclear cells (PBMCs) from periodontitis patients. The influence of *P. gingivalis* LPS was also investigated in cultured human primary fibroblasts to provide new approaches that could contribute to the comprehension of the pathophysiology of periodontitis and its relationship with CVD.

Materials and methods

Patients

A total of 58 patients, all over 35 years of age, attending Seville University Dental School, were enrolled in the study. All patients who were accepted gave voluntary written informed consent. Protocol and consent forms had been previously approved by the Committee of Ethics and Research of Sevilla University (16 December 2006). All patients met the following inclusion criteria: they had more than 20 teeth, they had not taken antibiotics or anti-inflammatory drugs in the previous 6 months, they were not affected by immunodeficiency, they were generally healthy, and they had undergone no previous periodontal treatment. Patients were recruited over a period of 10 months and one blood sample was taken from each patient as he or she was recruited.

Routine laboratory tests yielded normal results for glucose 94.7 ± 8.7 mg/dl (normal values (nv) 76–110), urea 36.3 ± 10.10 mg/dl (nv 10–45), uric acid 5.3 ± 1.2 mg/dl (nv 2.5–7.5), total protein 7.1 ± 0.4 g/dl (nv 6.6–8.7), creatinine 0.5 ± 0.1 mg/dl (nv 0.5–1.1), aspartate aminotransferase 24.3 ± 12.5 mU/ml (nv 10–40), alanine aminotransferase 25.2 ± 15.1 mU/ml (nv 10–40), cholesterol 192.7 ± 42.3 mg/dl (nv <220), and triglycerides 97.1 ± 44.8 mg/dl (nv 70–170). Also, blood pressure yielded normal results (systolic blood pressure 115 ± 10 mm Hg and diastolic blood pressure 70 ± 12 mm Hg).

A baseline periodontal examination was performed, and a single examiner collected full medical and dental histories. A single trained dental examiner recorded periodontal data. The periodontal probing depth (PD) and the recession of the gingival margin (GM) relative to the cementoenamel junction at six sites per tooth were recorded. Clinical attachment level (CAL) was calculated by adding recession to PD. PD and CAL were recorded to the nearest highest millimeter by means of the North Carolina periodontal probe (Hu-Friedy, Chicago, IL, USA), 15 mm in length and 0.35 mm in diameter. According to the criteria established by Machtei et al. [22], the clinical entity of periodontitis is based on the presence of CAL ≥ 6 mm in two or more teeth and one or more sites with PD ≥ 5 mm. Patients were divided into two groups: one with periodontitis ($n = 38$) and the other without periodontitis ($n = 20$).

Blood mononuclear cell and fibroblast cultures

Heparinized and coagulated blood samples were collected from each patient and centrifuged at 3800 *g* for 5 min, and the separated plasma and serum were stored at −80 °C. PBMCs were purified by isopycnic centrifugation using Histopaque-1119 and Histopaque-1077 (Sigma Chemical Co., St. Louis, MO, USA). Mononuclear cells were cultured at 37 °C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with L-glutamine, an antibiotic/antimycotic solution (Sigma Chemical Co.), and 10% fetal bovine serum (Gibco, Invitrogen, Eugene, OR, USA).

Fibroblasts from the skin of healthy volunteers (30 years of age) were cultured in DMEM (4500 mg/L glucose, L-glutamine, pyruvate; Gibco, Invitrogen) supplemented with 20% fetal bovine serum (FBS; Gibco, Invitrogen) and antibiotics (Sigma Chemical Co.). Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Fibroblast treatment

Fibroblasts were cultured with 10 μg/ml LPS of *P. gingivalis* (Nucliber S.A., Spain) in the absence or presence of a known dose, 30 μM, of CoQ_{10} for 24 h [23]. LPS used for treatment of cell cultures was diluted with fetal bovine serum.

Measurement of CoQ_{10} levels

Lipid extraction from PBMCs and skin fibroblasts was performed as described previously [23]. Coenzyme Q_9 was used as an internal standard. Briefly, cells were lysed with 1% SDS and vortexed for 1 min. A mixture of ethanol:isopropanol (95:5) was added and the samples were vortexed for 1 min. To recover CoQ_{10}, 5 ml of hexane was added and samples were centrifuged at 1000 *g* for 5 min at 4 °C. The upper phases from three different extractions were recovered and dried by a rotatory evaporator (Rotavapor R-210; Büchi Labortechnik AG, Flawil, Switzerland). Lipid extract was resuspended in 1 ml ethanol, dried in a Speed-Vac (Express SC250EXP; Thermo Fisher Scientific. Waltham, MA, USA), and kept at −20 °C until used. Samples were suspended in 60 μl of ethanol before HPLC injection. Lipid components were separated by a Beckmann 166–126 HPLC system equipped with a 15-cm Kromasil C-18 column in a column oven set to 40 °C, with a flow rate of 1 ml/min and a mobile phase containing 65:35 methanol:*n*-propanol and 1.42 mM lithium perchlorate. CoQ_{10} levels were analyzed with a UV detector (System Gold 168; Beckman Coulter, Brea, CA, USA).

Mitochondrial membrane potential ($\Delta\Psi_m$)

PBMCs and fibroblasts were cultured in six-well plates (35-mm-diameter well) until confluent. MitoTracker (100 nM; MitoTracker Red CMXRos packing aging; Molecular Probes, Eugene, OR, USA) was added and incubated for 30 min. Once the incubation was finished, the cells were harvested, incubated with fresh medium, washed, centrifuged (500 *g*), resuspended in RPMI medium, and analyzed by flow cytometry in an Epics XL cytometer (Beckman Coulter; excitation wavelength 579 nm, emission wavelength 599 nm).

Measurement of citrate synthase activity

Citrate synthase-specific activity in whole-cell extracts prepared from PBMCs and fibroblasts was measured at 412 nm minus 360 nm ($13.6\ mM^{-1}\ cm^{-1}$) using 5,5-dithiobis-(2-nitrobenzoic acid) to detect free sulfhydryl groups in coenzyme A as described previously [24].

Table 1
Periodontal data, CoQ_{10} level, and citrate synthase activity in periodontitis and nonperiodontitis patients.

	Periodontitis ($n=38$)	Nonperiodontitis ($n=20$)
Age (years)	45 ± 11	44.3 ± 8
BMI (kg/m^2)	27.7 ± 2.7	24.6 ± 1.6
CoQ_{10} (pmol Q/mg protein)	60.2 ± 16*	150.4 ± 13.5
Citrate synthase (EA)	1.9 ± 0.6*	8.6 ± 0.3
Periodontal data		
GM	0.79 ± 0.08*	0.18 ± 0.02
PD	3.5 ± 0.5*	1.9 ± 0.2
CAL	4 ± 0.3*	2.1 ± 0.45
Dental plaque	45.1 ± 4.8*	26.4 ± 2.21
Gingival bleeding	60.3 ± 5.3*	41.31 ± 5.9

Data represent the means ± SD. BMI, body mass index; CoQ_{10}, coenzyme Q_{10}; EA, enzymatic activity; CAL, clinical attachment level; GM, recession of the gingival margin; PD, periodontal probing depth.
*$P<0.001$; significantly different between periodontitis and nonperiodontitis.

Western blotting for mitochondrial protein

Whole cellular lysate from fibroblasts was prepared by gentle shaking with a buffer containing 0.9% NaCl, 20 mM Tris–HCl, pH 7.6, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.01% leupeptin. Electrophoresis was carried out by 10–15% acrylamide SDS–PAGE. Proteins were transferred to Immobilon membranes (Amersham Pharmacia, Piscataway, NJ, USA). Mouse anti-complex I (39-kDa subunit) and mouse anti-complex III (core 1 subunit) antibodies were used to detect proteins by Western blotting. Proteins were electrophoresed, transferred to nitrocellulose membranes, and, after being blocked overnight at 4 °C, incubated with the respective antibody solution diluted at 1:1000. Membranes were then probed with their respective secondary antibody (1:2500). Immunolabeled proteins were detected by using a chemiluminescence method (Immun Star HRP substrate kit; Bio-Rad Laboratories, Hercules, CA, USA). Protein was determined by the Bradford method [25].

Mitochondrial ROS production

Mitochondrial ROS generation in PBMCs and fibroblasts was assessed using MitoSOX red, a red mitochondrial superoxide indicator. MitoSOX red is a novel fluorogenic dye recently developed and validated for highly selective detection of superoxide in the mitochondria of live cells [26]. MitoSOX red reagent is live-cell permeative and is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX red reagent is oxidized by superoxide and exhibits red fluorescence.

Fluorescence microscopy

Cells grown on microscope slides in six-well plates for 24 h were incubated with MitoSOX red for 30 min at 37 °C, washed twice in PBS, fixed with 4% paraformaldehyde in PBS for 0.5–1 h at room temperature, and washed twice with PBS. Cells were then incubated for 10 min at 37 °C with anti-cytochrome *c* antibody (Invitrogen, Barcelona, Spain) to label mitochondria. Slides were analyzed by immunofluorescence microscopy (MitoSOX red; excitation wavelength 555/28; emission wavelength 617/73).

Flow cytometry

Approximately 1×10^6 cells were incubated with 1 μM MitoSOX red for 30 min at 37 °C, washed twice with PBS, resuspended in 500 μl of PBS, and analyzed by flow cytometry in an Epics XL cytometer (Beckman Coulter; excitation at 510 nm and fluorescence detection at 580 nm).

Analysis of apoptosis

Apoptosis in fibroblasts was assessed by observing nuclei condensation by Hoechst 33342 (Invitrogen, Molecular Probes) staining (0.05 μg/ml) and anti-active caspase-3 (Cell Signaling Technology, Beverly, MA, USA) activation by immunofluorescence microscopy and Western blot. Ten random fields and more than 500 cells were counted in each experiment to obtain cell death percentage considering the number of condensed nuclei. Cells were grown on 1-mm^2 glass coverslips for 24 h in DMEM culture medium containing 10% FBS. After 24 h treatment, cells were rinsed once with PBS, fixed in 3.8% paraformaldehyde for 5 min at room temperature, and permeabilized in 0.1% saponin for 5 min. For immunostaining, glass coverslips were incubated with anti-active caspase-3 (17 kDa) antibodies diluted 1:100 in PBS for 1–2 h at 37 °C in a humidified chamber. Excess antibody binding was removed by washing the coverslips with PBS (three times, 5 min). The secondary antibodies, a tetramethylrhodamine goat anti-rabbit IgG (Molecular Probes) diluted 1:100 in PBS, were added and incubated for 1 h at 37 °C. Coverslips were then rinsed with PBS for 3 min, incubated for 1 min with PBS containing Hoechst 33342 (1 μg/ml), and washed with PBS (three times, 5 min). Finally, the coverslips were mounted onto microscope slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and analyzed using a fluorescence microscope (BX 41, Olympus, Barcelona, Spain). Western blotting was performed using standard methods previously described.

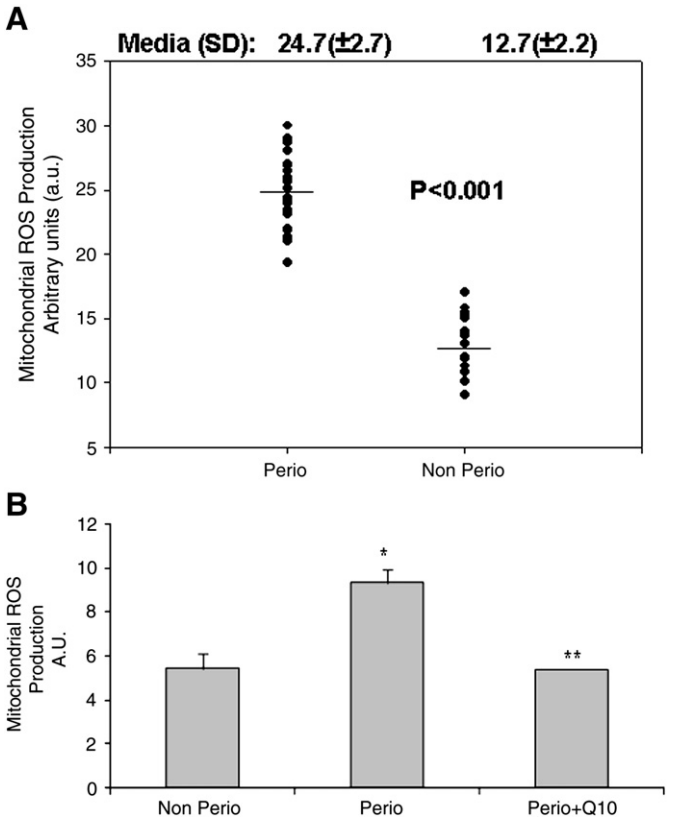

Fig. 1. ROS production in periodontitis patients. (A) ROS production was analyzed in PBMCs from periodontitis and nonperiodontitis patients by flow cytometry as described under Materials and methods. Data represent the means ± SD of three separate experiments. *$P<0.001$ between periodontitis and nonperiodontitis patients. (B) Effect of CoQ10 on ROS generation. PBMCs of a representative periodontitis patient and a nonperiodontitis patient were treated with 30 μM CoQ_{10} for 24 h. Data represent the means ± SD of three separate experiments. *$P<0.001$ between periodontitis and nonperiodontitis; **$P<0.001$ between the absence and the presence of CoQ_{10}.

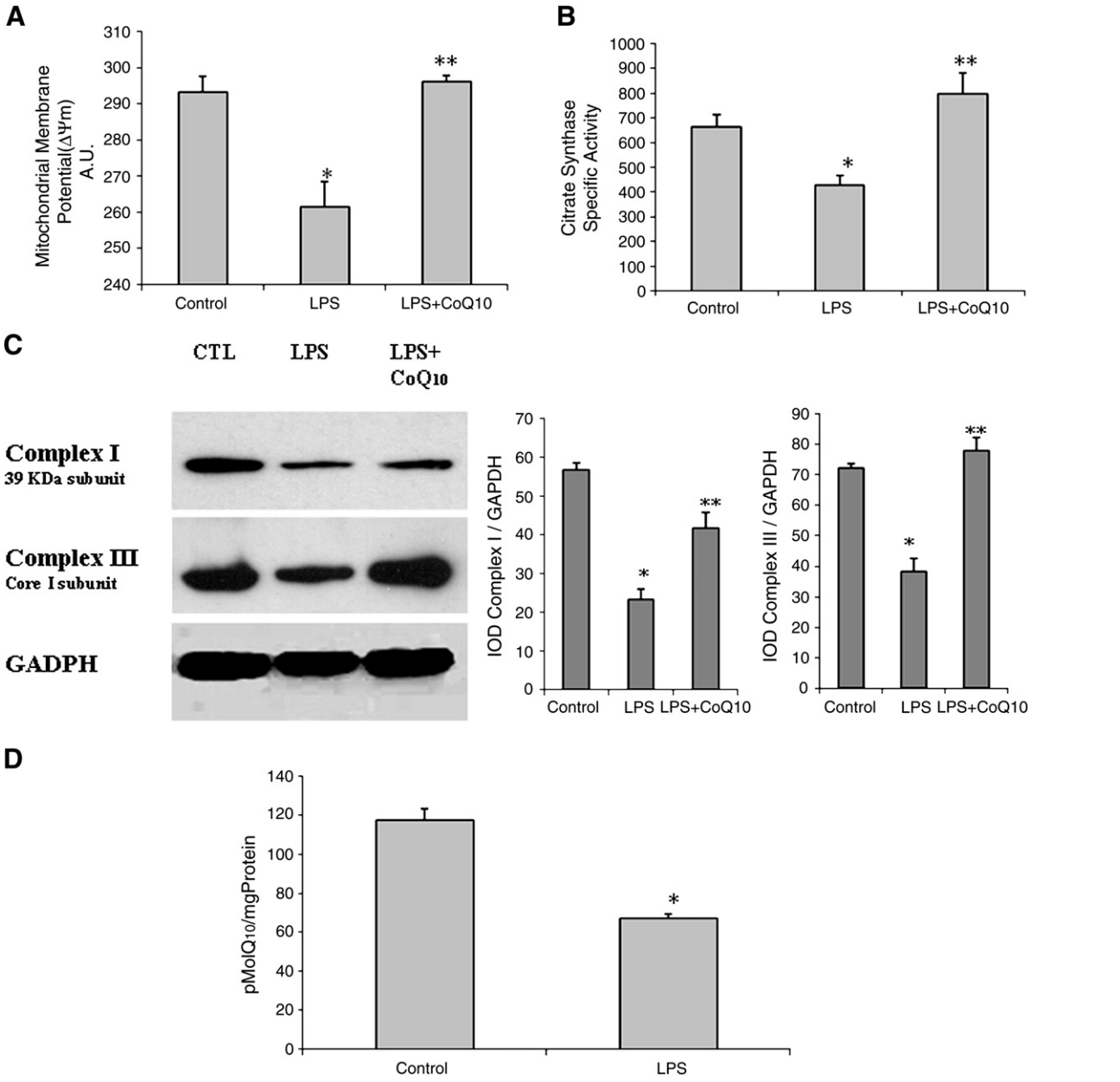

Fig. 2. Effect of LPS on mitochondrial components. (A) Effect of LPS on mitochondrial membrane potential ($\Delta\Psi_m$). Fibroblasts were treated with 10 μg/ml LPS or 30 μM CoQ_{10} + 10 μg/ml LPS for 24 h. (B) Citrate synthase specific activity in control and LPS-treated PBMCs was assayed as described under Materials and methods. (C) Mitochondrial protein expression levels in LPS-treated cells. Proteins (50 μg) from control and treated fibroblast extracts were immunostained with antibodies against complexes I (39-kDa subunit) and III (core 1 subunit). Protein levels were determined by densitometric analysis (IOD, integrated optical intensity) of three different Western blots and normalized to GAPDH signal. *$P<0.05$, between control and LPS-treated cells; **$P<0.05$, between the absence and the presence of CoQ_{10}. (D) CoQ_{10} level after LPS treatment. Results are expressed as pmol of CoQ_{10} per mg of protein. Data represent the means ± SD of three separate experiments. *$P<0.01$.

Statistical analysis

All results are expressed as means ± SD unless stated otherwise. The unpaired Student *t* test was used to evaluate the significance of differences between groups, accepting $P<0.05$ as the level of significance.

Results

Clinical data

Thirty-eight of the 58 patients who met the inclusion criteria and accepted to participate in the study were diagnosed with periodontitis. Table 1 summarizes the results of the periodontal examination with significant differences in all the parameters studied ($P<0.001$ for GM, PD, CAL, dental plaque, and gingival bleeding determinations), whereas no significant differences were found for age or body mass index among the considered groups.

Mitochondrial dysfunction in periodontitis patients

CoQ_{10} levels, determined in PBMCs isolated from the 38 periodontal patients, were about 56% lower than in nonperiodontitis patients (Table 1). To further examine mitochondrial dysfunction in PBMCs from periodontitis patients, we determined mitochondrial mass by citrate synthase activity. Table 1 shows a statistically significant low level of citrate synthase activity in PBMC in periodontitis patients (1.99 ± 0.59 sp act) compared to nonperiodontitis patients (8.61 ± 0.27 sp act).

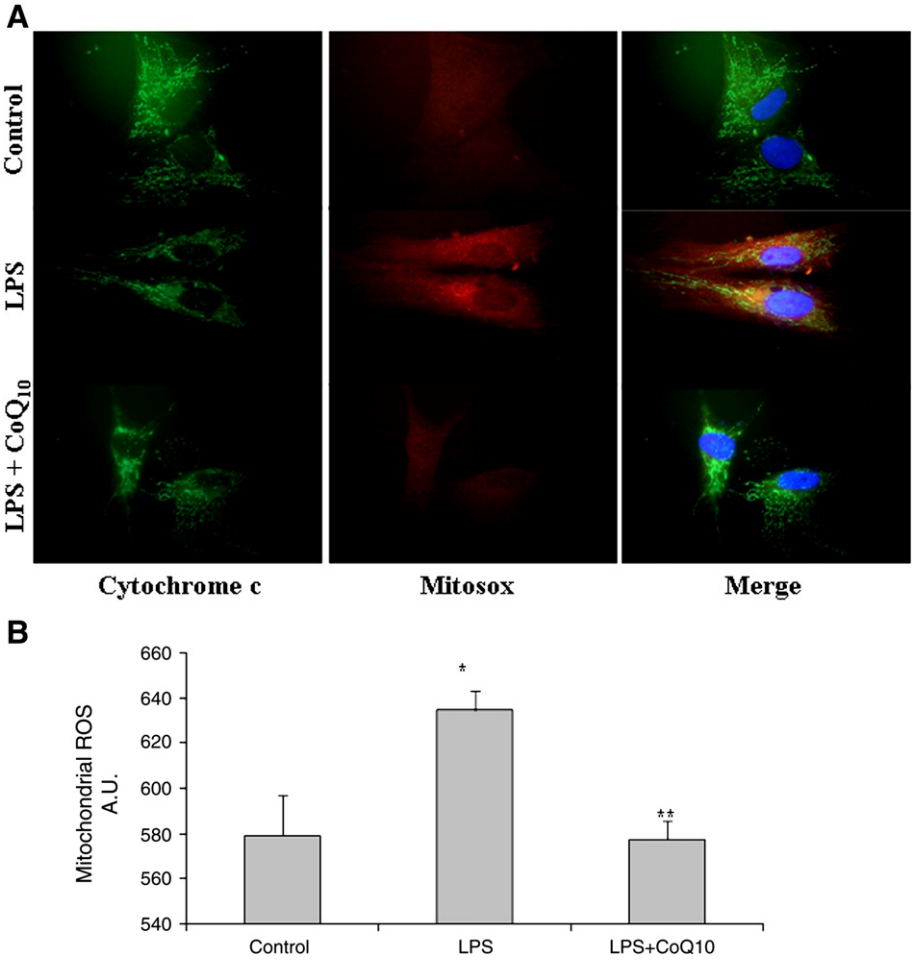

Fig. 3. Effect of LPS on mitochondrial ROS generation. (A) MitoSOX red stain revealed increased superoxide anion. MitoSOX red colocalized with cytochrome *c* in merged images, indicating that superoxide anion production occurred mainly in mitochondria. (B) Flow cytometry quantification of ROS production. Data represent the means ± SD of three separate experiments. $^{*}P<0.001$, between control and LPS-treated cells; $^{**}P<0.001$, between the absence and the presence of CoQ_{10}.

Oxidative stress in periodontitis patients

Quantification of ROS production by flow cytometry analysis showed high levels of mitochondrial ROS production in PBMCs from periodontitis patients compared to nonperiodontitis patients (Fig. 1A).

To further examine the role of ROS generation in periodontitis, PBMCs of one representative patient were incubated with CoQ_{10}, and mitochondrial ROS production (Fig. 1B) was monitored, showing a significant reduction in mitochondrial ROS production in the presence of CoQ_{10}.

LPS induces mitochondrial dysfunction in fibroblasts

The $\Delta\Psi_m$ was significantly reduced by LPS treatment. Flow cytometry analysis showed a $\Delta\Psi_m$ decrease of about 35% compared to untreated fibroblasts (Fig. 2A). LPS-induced $\Delta\Psi_m$ decrease was also partially prevented by CoQ_{10}. Fig. 2B shows a statistically significant decrease in citrate synthase activity after LPS treatment compared to controls, also partially prevented by CoQ_{10}. Enzymatic activity data were 293.3 ± 4.3 for control, 261.6 ± 7 for LPS, and 296.4 ± 1.5 for LPS + CoQ_{10}. LPS treatment induced a significant decrease in complex I (39-kDa subunit) and complex III (core 1 subunit) expression levels with a concomitant decrease in CoQ_{10} levels compared to control fibroblasts (67.29 ± 0.8 and 117.244 ± 5.2 pmol/mg protein, respectively; Figs. 2C and D). CoQ_{10} supplementation efficiently prevented LPS-induced down-regulation of mitochondrial proteins.

LPS induces a high level of mitochondrial ROS production in fibroblasts

MitoSOX red fluorescence colocalized with mitochondrial cytochrome *c* oxidase (Fig. 3A). Quantification of ROS production by flow cytometric analysis indicated that LPS treatment induced a significant increment in mitochondrial ROS production (634.65 ± 9 arbitrary units) compared to control (579.25 ± 18) (Fig. 3B). The addition of a widely recognized membrane antioxidant, such as CoQ_{10}, attenuated ROS detection (577.7 ± 7).

LPS initiates the intrinsic pathway of caspase-3-dependent apoptosis in fibroblasts

LPS caused an increment in apoptotic nuclei condensation and caspase-3 activation. In contrast, CoQ_{10} highly prevented the LPS-induced caspase-3 activation and apoptosis rate (Figs. 4A, B, and C).

Discussion

Oxidative stress and free radical generation, as primary or secondary events, play important roles in the development of systemic diseases such as type 2 diabetes, atherosclerosis, and cardiovascular diseases [27,28], as mitochondria are the major source of ROS. CoQ_{10} levels have been suggested to be useful as a mitochondrial dysfunction marker [29] and CoQ_{10} deficiency has been found in gingival biopsies and leukocytes from periodontal patients [30,31]. To assess mitochondrial dysfunction in periodontitis,

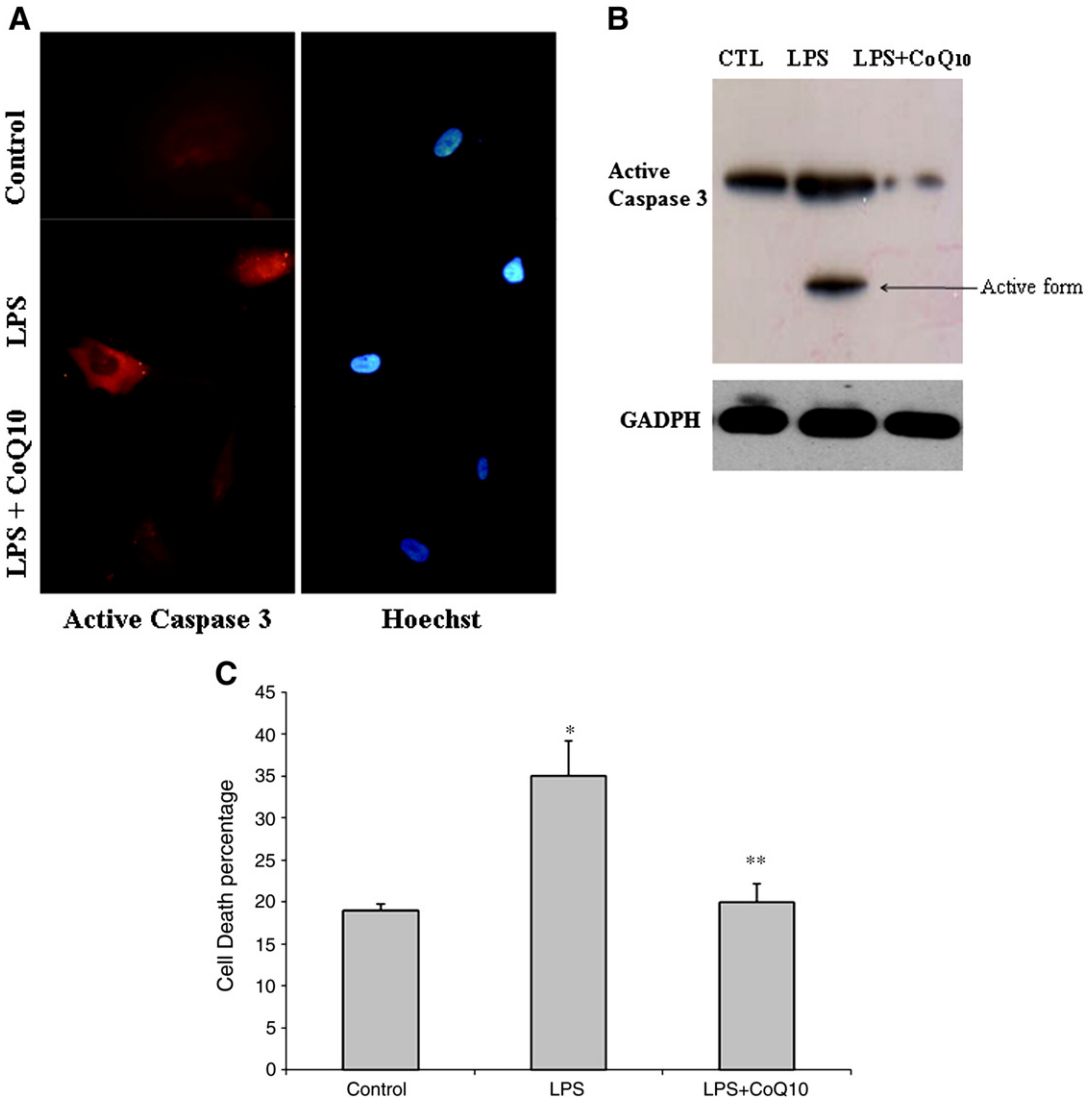

Fig. 4. LPS induced apoptosis in fibroblasts treated with LPS 10 μg/ml. (A and B) LPS-induced apoptosis was assessed by caspase-3 activation by immunofluorescence microscopy and Western blot as described under Materials and methods. (C) LPS-induced apoptosis is prevented by CoQ_{10}. Fibroblasts were treated with 10 μg/ml LPS in the absence or presence of 30 μM CoQ_{10} for 24 h. Apoptosis was assessed as described under Materials and methods. Data represent the means ± SD of three separate experiments. $^{*}P<0.01$, between control and LPS-treated cells; $^{**}P<0.01$, between the absence and the presence of CoQ_{10}.

PBMCs of periodontal patients were studied, showing a decrease in CoQ_{10} levels and citrate synthase activity. Interestingly, a positive correlation between the content of CoQ_{10} in PBMCs and skeletal muscle and fibroblasts [32,33] was demonstrated, therefore suggesting that CoQ_{10} deficiency and mitochondrial dysfunction in periodontal patients could also be present in other cells and tissues. Furthermore, fibroblasts of some patients with CoQ_{10} deficiency show a higher production of ROS in mitochondria [34]. In this respect, high levels of mitochondrial ROS production were observed in PBMCs of our periodontitis patients.

Previous work has shown that *P. gingivalis,* one of the key etiological factors in periodontal pathology, induces oxidative stress in vitro [35], with bacterial LPS playing a major role in the pathogenesis of periodontal pathology [36].

To assess the mitochondrial damage induced by LPS, we determined mitochondrial $\Delta\Psi_m$ and mitochondrial mass in both control and LPS-treated fibroblasts. Citrate synthase is a mitochondrial matrix protein whose activity has been shown to correlate well with mitochondrial mass [24]. We found that LPS treatment in cultured fibroblasts provoked increased oxidative stress and mitochondrial dysfunction, characterized by a decrease in mitochondrial protein expression, mitochondrial mass, and CoQ_{10} levels, as well as a reduction in the mitochondrial membrane potential.

It has been reported that CoQ_{10} deficiency determined a decrease in complex II + III, complex III, and complex IV activities; a decrease in the expression of mitochondrial proteins involved in oxidative phosphorylation; a decrease in the mitochondrial $\Delta\Psi_m$; and an increase in ROS production [34]. To study the pathophysiological mechanisms of LPS-induced mitochondrial dysfunction in cultured primary fibroblasts, we assessed the expression levels of critical components of the mitochondrial respiratory chain, observing a decrement in expression levels of proteins of complex I (39-kDa subunit) and complex III (core 1 subunit), together with a reduction in CoQ_{10} levels.

Next, to investigate the possible mitochondrial origin of ROS production in relation to periodontitis, LPS-treated and control fibroblasts were exposed to MitoSOX red, a fluorochrome specific for superoxide anion, which is produced in the inner mitochondrial

compartment, showing high levels of mitochondrial ROS by LPS treatment. In agreement with the hypothesis that ROS production was caused by a mitochondrial dysfunction, we found that ROS were mainly generated in mitochondria of LPS-treated fibroblasts.

It should be taken into account that ROS are essential in cells and tissues for many life-sustaining processes, but they can also induce cell damage and death [37], especially when their activities are uncontrolled. ROS can be released into the cytosol and trigger "ROS-induced ROS release" in neighboring mitochondria. This mitochondrion-to-mitochondrion ROS signaling constitutes a positive feedback mechanism for enhanced ROS production potentially leading to significant mitochondrial injury [38]. In this context, cytochrome *c* is released and procaspase-9, caspase-3, and endonuclease G are activated, resulting in DNA degradation and apoptotic death. In our investigation, an increase in apoptosis was observed in LPS-treated fibroblasts by caspase-3 activation, suggesting that LPS treatment induces apoptosis by the activation of at least the intrinsic pathway.

Finally, it can be considered that oxidative stress is one of the key factors explaining some of the pathophysiological mechanisms associated with inflammatory conditions such as CVD and periodontitis [39,40], as lipid peroxidation is one of its most well known effects. In fact, increased lipid peroxidation has been observed in periodontitis [8], and it is accepted that lipid peroxidation indirectly reflects intracellular ROS generation. It is interesting to note that superoxide plays a major role in the release of cytokines (for example, TNF-α, IL-1β, and IL-6) [41], which are involved in the pathogenesis of periodontal disease and CVD [7] and this is also induced by *P. gingivalis* LPS treatment. The findings of this study show that CoQ_{10} treatment ameliorated mitochondria dysfunction and oxidative stress and reduced cell death. Because CoQ_{10} is a pivotal element in the mitochondrial respiratory chain and, at the same time, is an important antioxidant, these results suggest that mitochondrial dysfunction is crucial in the pathophysiology of periodontal disease.

Our study supports the hypothesis that LPS-mediated mitochondrial dysfunction could be at the origin of oxidative stress in periodontal patients. Abnormal PBMC performance may promote oxidative stress and alter cytokine homeostasis. In conclusion, mitochondrial dysfunction could represent a possible link to understanding the interrelationships between two prominent inflammatory diseases: periodontitis and CVD.

Acknowledgment

The authors are indebted with Ms. M. Glebocki for extensive editing of the manuscript.

References

[1] Kowaltowski, A. J.; de Souza-Pinto, N. C.; Castilho, R. F.; Vercesi, A. E. Mitochondria and reactive oxygen species. *Free Radic. Biol. Med.* **47:**333–343; 2009.
[2] Lambert, A. J.; Brand, M. D. Reactive oxygen species production by mitochondria. *Meth. Mol. Biol.* **554:**165–181; 2009.
[3] Halliwell, B. Reactive oxygen species in living systems: source, biochemistry, and role in human disease. *Am. J. Med.* **91:**14–22; 1991.
[4] Tsutsui, H.; Kinugawa, S.; Matsushima, S. Mitochondrial oxidative stress and dysfunction in myocardial remodelling. *Cardiovasc. Res.* **81:**449–456; 2009.
[5] Strong, K.; Mathers, C.; Leeder, S.; Beaglehole, R. Preventing chronic diseases: how many lives can we save? *Lancet* **366:**1578–1582; 2005.
[6] World Health Organization The World Health Organization warns of the rising threat of heart disease and stroke as overweight and obesity rapidly increase Available at http://www.who.int/mediacentre/news/releases/2005/pr44/en/index.htm Accessed on 5 September 2010.
[7] Kuo, L. C.; Polson, A. M.; Kang, T. Associations between periodontal diseases and systemic diseases: a review of the inter-relationships and interactions with diabetes, respiratory diseases, cardiovascular diseases and osteoporosis. *Public Health* **122:**417–433; 2008.
[8] Pischon, T.; Hu, F. B.; Rexrode, K. M., et al. Inflammation, the metabolic syndrome, and risk of coronary heart disease in women and men. *Atherosclerosis* **197:** 392–399; 2008.
[9] Ford, P. J.; Yamazaki, K.; Seymour, G. J. Cardiovascular and oral disease interactions: what is the evidence? *Prim. Dent. Care* **14:**59–66; 2007.
[10] Friedewald, V. E.; Kornman, K. S.; Beck, J. D.; Genco, R.; Goldfine, A.; Libby, P.; Offenbacher, S.; Ridker, P. M.; Van Dyke, T. E.; Roberts, W. C. The American Journal of Cardiology and Journal of Periodontology Editors' Consensus: periodontitis and atherosclerotic cardiovascular disease. *Am. J. Cardiol.* **104:**59–68; 2009.
[11] Paquette, D. W.; Brodala, N.; Nichols, T. C. Cardiovascular disease, inflammation, and periodontal infection. *Periodontology 2000* **44:**113–126; 2007.
[12] Battino, M.; Ferreiro, M. S.; Bompadre, S.; Leone, L.; Mosca, F.; Bullon, P. Elevated hydroperoxide levels and antioxidant patterns in Papillon–Lefèvre syndrome. *J. Periodontol.* **72:**1760–1766; 2001.
[13] Baltacioglu, E.; Akalin, F. A.; Alver, A.; Deger, O.; Karabulut, E. Protein carbonyl levels in serum and gingival crevicular fluid in patients with chronic periodontitis. *Arch. Oral Biol.* **53:**716–722; 2008.
[14] Uno, K.; Nicholls, S. J. Biomarkers of inflammation and oxidative stress in atherosclerosis. *Biomark. Med.* **4:**361–373; 2010.
[15] Battino, M.; Ferreiro, M. S.; Quiles, J. L.; Bompadre, S.; Leone, L.; Bullon, P. Alterations in the oxidation products, antioxidant markers, antioxidant capacity and lipid patterns in plasma of patients affected by Papillon–Lefèvre syndrome. *Free Radic. Res.* **37:**603–609; 2003.
[16] Chapple, I. L.; Milward, M. R.; Dietrich, T. The prevalence of inflammatory periodontitis is negatively associated with serum antioxidant concentrations. *J. Nutr.* **137:**657–664; 2007.
[17] Ramirez-Tortosa, M. C.; Quiles, J. L.; Battino, M.; Granados, S.; Morillo, J. M.; Bompadre, S.; Newman, H. N.; Bullon, P. Periodontitis is associated with altered plasma fatty acids and cardiovascular risk markers. *Nutr. Metab. Cardiovasc. Dis.* **20:**133–139; 2010.
[18] Armitage, G. C. Comparison of the microbiological features of chronic and aggressive periodontitis. *Periodontology 2000* **2000** (53)**:**70–88; 2010.
[19] Gibson III, F. C.; Ukai, T.; Genco, C. A. Engagement of specific innate immune signaling pathways during Porphyromonas gingivalis induced chronic inflammation and atherosclerosis. *Front. Biosci.* **13:**2041–2059; 2008.
[20] Pucar, A.; Milasin, J.; Lekovic, V.; Vukadinovic, M.; Ristic, M.; Putnik, S.; Kenney, E. B. Correlation between atherosclerosis and periodontal putative pathogenic bacterial infections in coronary and internal mammary arteries. *J. Periodontol.* **78:**677–682; 2007.
[21] Wang, P. L.; Ohura, K. Porphyromonas gingivalis lipopolysaccharide signaling in gingival fibroblasts—CD14 and Toll-like receptors. *Crit. Rev. Oral Biol. Med.* **13:** 132–142; 2002.
[22] Machtei, E. E.; Christersson, L. A.; Grossi, S. G.; Dunford, R.; Zambon, J. J.; Genco, R. J. Clinical criteria for the definition of "established periodontitis". *J. Periodontol.* **63:** 206–214; 1992.
[23] Cordero, M. D.; Moreno-Fernández, A. M.; Gomez-Skarmeta, J. L.; de Miguel, M.; Garrido-Maraver, J.; Oropesa-Avila, M.; Rodríguez-Hernández, A.; Navas, P.; Sánchez-Alcázar, J. A. Coenzyme Q10 and α-tocopherol protect against amitriptyline toxicity. *Toxicol. Appl. Pharmacol.* **235:**329–337; 2009.
[24] Garrabou, G.; Soriano, A.; Lopez, S.; Guallar, J. P.; Giralt, M.; Villarroya, F., et al. Reversible inhibition of mitochondrial protein synthesis during linezolid-related hyperlactatemia. *Antimicrob. Agents Chemother.* **5:**962–967; 2007.
[25] Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **72:**248–254; 1976.
[26] Mukhopadhyay, P.; Rajesh, M.; Yoshihiro, K.; Hasko, G.; Pacher, P. Simple quantitative detection of mitochondrial superoxide production in live cells. *Biochem. Biophys. Res. Commun.* **358:**203–208; 2007.
[27] Kaneto, H.; Katakami, N.; Matsuhisa, M.; Matsuoka, T. A. Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. *Mediat. Inflamm.* **2010:**453892; 2010.
[28] Heistad, D. D.; Wakisaka, Y.; Miller, J.; Chu, Y.; Pena-Silva, R. Novel aspects of oxidative stress in cardiovascular diseases. *Circ. J.* **73:**201–207; 2009.
[29] Haas, R. H.; Parikh, S.; Falk, M. J.; Saneto, R. P.; Wolf, N. I.; Darin, N., et al. The in-depth evaluation of suspected mitochondrial disease. *Mol. Genet. Metab.* **94:**16–37; 2008.
[30] Hansen, I. L.; Iwamoto, Y.; Kishi, T.; Folkers, K.; Thompson, L. E. Bioenergetics in clinical medicine. IX. Gingival and leucocytic deficiencies of coenzyme Q10 in patients with periodontal disease. *Res. Commun. Chem. Pathol. Pharmacol.* **14:** 729–738; 1976.
[31] Nakamura, R.; Littarru, G. P.; Folkers, K.; Wilkinson, E. G. Study of CoQ10-enzymes in gingiva from patients with periodontal disease and evidence for a deficiency of coenzyme Q10. *Proc. Natl. Acad. Sci. USA* **71:**1456–1460; 1974.
[32] Duncan, A. J.; Heales, S. J.; Mills, K.; Eaton, S.; Land, J. M.; Hargreaves, I. P. Determination of coenzyme Q10 status in blood mononuclear cells, skeletal muscle, and plasma by HPLC with di-propoxy-coenzyme Q10 as an internal standard. *Clin. Chem.* **51:**2380–2382; 2005.
[33] Land, J. M.; Heales, S. J.; Duncan, A. J.; Hargreaves, I. P. Some observations upon biochemical causes of ataxia and a new disease entity ubiquinone, CoQ10 deficiency. *Neurochem. Res.* **32:**837–843; 2007.
[34] Quinzii, C. M.; Lopez, L. C.; Von-Moltke, J.; Naini, A.; Krishna, S.; Schuelke, M.; Salviati, L.; Navas, P.; Dimauro, S.; Hirano, M. Respiratory chain dysfunction and oxidative stress correlate with severity of primary CoQ10 deficiency. *FASEB J.* **22:** 1874–1885; 2008.
[35] Staudte, H.; Güntsch, A.; Völpel, A.; Sigusch, B. W. Vitamin C attenuates the cytotoxic effects of Porphyromonas gingivalis on human gingival fibroblasts. *Arch. Oral Biol.* **55:**40–45; 2010.
[36] Wiesner, P.; Choi, S. H.; Almazan, F.; Benner, C.; Huang, W.; Diehl, C. J.; Gonen, A.; Butler, S.; Witztum, J. L.; Glass, C. K.; Miller, Y. I. Low doses of lipopolysaccharide and minimally oxidized low-density lipoprotein cooperatively activate

macrophages via nuclear factor kappa B and activator protein-1: possible mechanism for acceleration of atherosclerosis by subclinical endotoxemia. *Circ. Res.* **107:**56–65; 2010.

[37] Battino, M.; Bullon, P.; Wilson, M.; Newman, H. Oxidative injury and inflammatory periodontal diseases: the challenge of anti-oxidants to free radicals and reactive oxygen species. *Crit. Rev. Oral Biol. Med.* **10:**458–476; 1999.

[38] Zorov, D. B.; Juhaszova, M.; Sollott, S. J. Mitochondrial ROS-induced ROS release: an update and review. *Biochim. Biophys. Acta* **1757:**509–517; 2006.

[39] Shen, G. X. Oxidative stress and diabetic cardiovascular disorders: roles of mitochondria and NADPH oxidase. *Can. J. Physiol. Pharmacol.* **88:**241–248; 2010.

[40] Bullon, P.; Morillo, J. M.; Ramirez-Tortosa, M. C.; Quiles, J. L.; Newman, H. N.; Battino, M. Metabolic syndrome and periodontitis: is oxidative stress a common link? *J. Dent. Res.* **88:**503–518; 2009.

[41] Wang, Z. Q.; Porreca, F.; Cuzzocrea, S.; Galen, K.; Lightfoot, R.; Masini, E., et al. A newly identified role for superoxide in inflammatory pain. *J. Pharmacol. Exp. Ther.* **309:**869–878; 2004.