CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

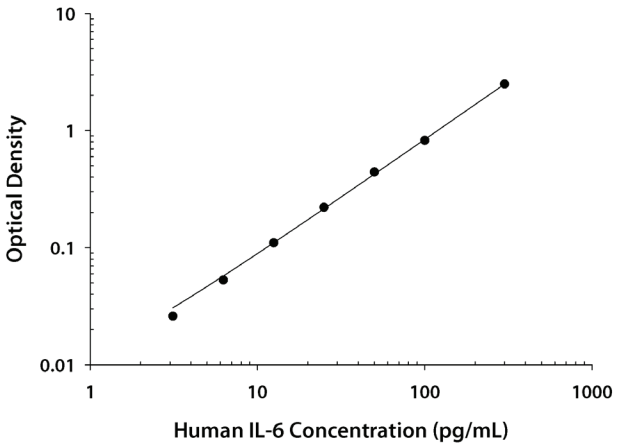
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human IL-6 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

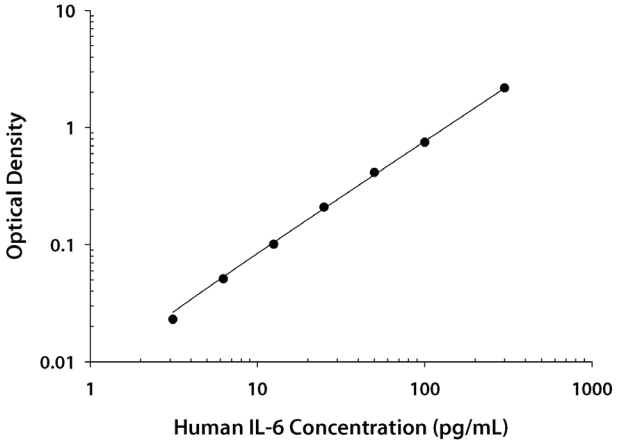
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.022 0.028	0.025	—
3.13	0.050 0.052	0.051	0.026
6.25	0.078 0.078	0.078	0.053
12.5	0.134 0.136	0.135	0.110
25	0.247 0.245	0.246	0.221
50	0.472 0.465	0.468	0.443
100	0.865 0.836	0.850	0.825
300	2.524 2.515	2.520	2.495

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.025 0.029	0.027	—
3.13	0.049 0.051	0.050	0.023
6.25	0.078 0.077	0.078	0.051
12.5	0.127 0.129	0.128	0.101
25	0.236 0.236	0.236	0.209
50	0.438 0.442	0.440	0.413
100	0.780 0.773	0.776	0.749
300	2.176 2.221	2.198	2.171

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	15.8	95.6	179	16.4	98.8	188
Standard deviation	0.7	3.0	3.1	0.6	2.5	3.7
CV (%)	4.4	3.1	1.7	3.7	2.5	2.0

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	16.8	97.7	186	17.2	101	191
Standard deviation	0.7	1.6	3.8	1.1	3.3	7.2
CV (%)	4.2	1.6	2.0	6.4	3.3	3.8

RECOVERY

The recovery of human IL-6 spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=5)	98	94-103%
Serum (n=5)	93	86- 99%
EDTA plasma (n=5)	95	84-101%
Heparin plasma (n=5)	90	88-98%
Citrate plasma (n=5)	91	82-95%

SENSITIVITY

The minimum detectable dose (MDD) of human IL-6 is typically less than 0.70 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, samples were spiked with high concentrations of human IL-6 in various matrices and diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Citrate plasma (n=4)
1:2	Average % of Expected	99	97	101	103	101
	Range (%)	96-101	92-100	98-105	96-109	96-106
1:4	Average % of Expected	100	101	104	106	105
	Range (%)	93-110	93-107	97-110	97-113	101-109
1:8	Average % of Expected	96	102	100	104	106
	Range (%)	92-100	96-108	86-112	93-111	101-111
1:16	Average % of Expected	94	103	99	105	101
	Range (%)	83-108	93-111	90-110	99-107	90-114

CALIBRATION

This immunoassay is calibrated against highly purified *E. coli*-expressed recombinant human IL-6 produced at R&D Systems. The NIBSC/WHO 1st International Standard for IL-6 (89/548), which was intended as a potency standard, was evaluated in this kit. The NIBSC/WHO standard is a CHO cell-derived recombinant human IL-6.

The dose response curve of the International Standard (89/548) parallels the Quantikine standard curve. To convert sample values obtained with the Quantikine Human IL-6 kit to approximate NIBSC 89/548 units, use the equation below.

NIBSC (89/548) approximate value (IU/mL)=0.131 x Quantikine Human IL-6 value (pg/mL)

SAMPLE VALUES

Serum/Plasma - Forty serum and plasma samples from apparently healthy volunteers were evaluated for the presence of human IL-6 in this assay. Thirty-three samples measured less than the lowest standard, 3.13 pg/mL. Seven samples measured between 3.13 and 12.5 pg/mL. No medical histories were available for the donors used in this study.

Cell Culture Supernates - Human peripheral blood mononuclear cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate and stimulated for 1, 3, and 5 days with 10 µg/mL PHA. Aliquots of the culture supernates were removed on days 1, 3, and 5 and assayed for levels of natural human IL-6. Results are listed in the following table.

Condition	Day 1 (pg/mL)	Day 3 (pg/mL)	Day 5 (pg/mL)
Unstimulated	575	311	660
Stimulated	17,130	17,520	16,340

SPECIFICITY

This assay recognizes natural and recombinant human IL-6.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5T and at 100 ng/mL in Calibrator Diluent RD6F and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range rhIL-6 control prepared in Calibrator Diluent RD5T and 100 ng/mL in a mid-range IL-6 control prepared in Calibrator Diluent RD6F were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

CNTF
G-CSF
GM-CSF
sgp130
IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-6 Rα
IL-6 Rα/sgp130
IL-7
IL-8
IL-11
IL-12
LIF
LIF R
OSM
TNF-α
TNF-β

Recombinant mouse:

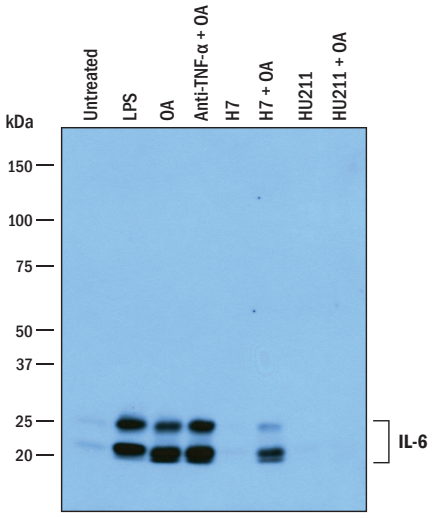
GM-CSF
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-11
IL-12

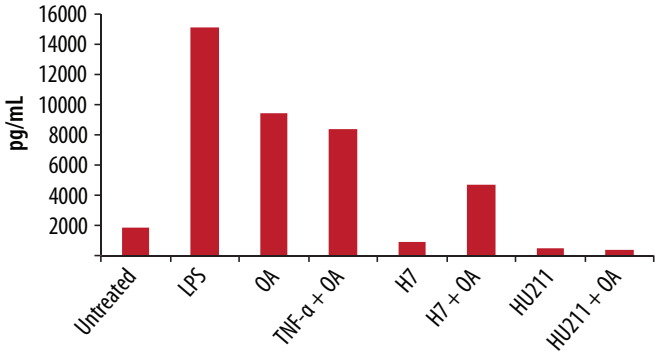
Recombinant rat:

CNTF

Natural proteins:

bovine FGF acidic
bovine FGF basic
human PDGF
porcine PDGF
human TGF-β1
porcine TGF-β1.2
porcine TGF-β2





Monocytes were prepared from human PBMCs by adherence to plastic. Adherent monocytes were washed, replated, and allowed to rest for 24 hours. Pretreatments were for 30 minutes: neutralizing anti-human TNF-α (R&D Systems, Catalog # MAB610) at 5 µg/mL, H7 serine kinase inhibitor (Tocris, Catalog # 0542) at 10 µM, or HU211 NFκB inhibitor (Tocris, Catalog # 2861) at 10 µM. Following the pretreatment, 500 ng/mL LPS or 30 ng/mL okadaic acid (OA, Tocris, Catalog # 1136) was added for 20 hours as indicated. Conditioned media was tested in the Quantikine ELISA, resolved by SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with the detection antibody used in this kit. The immunoprecipitation/ Western blot shows direct correlation with the ELISA value for these samples.

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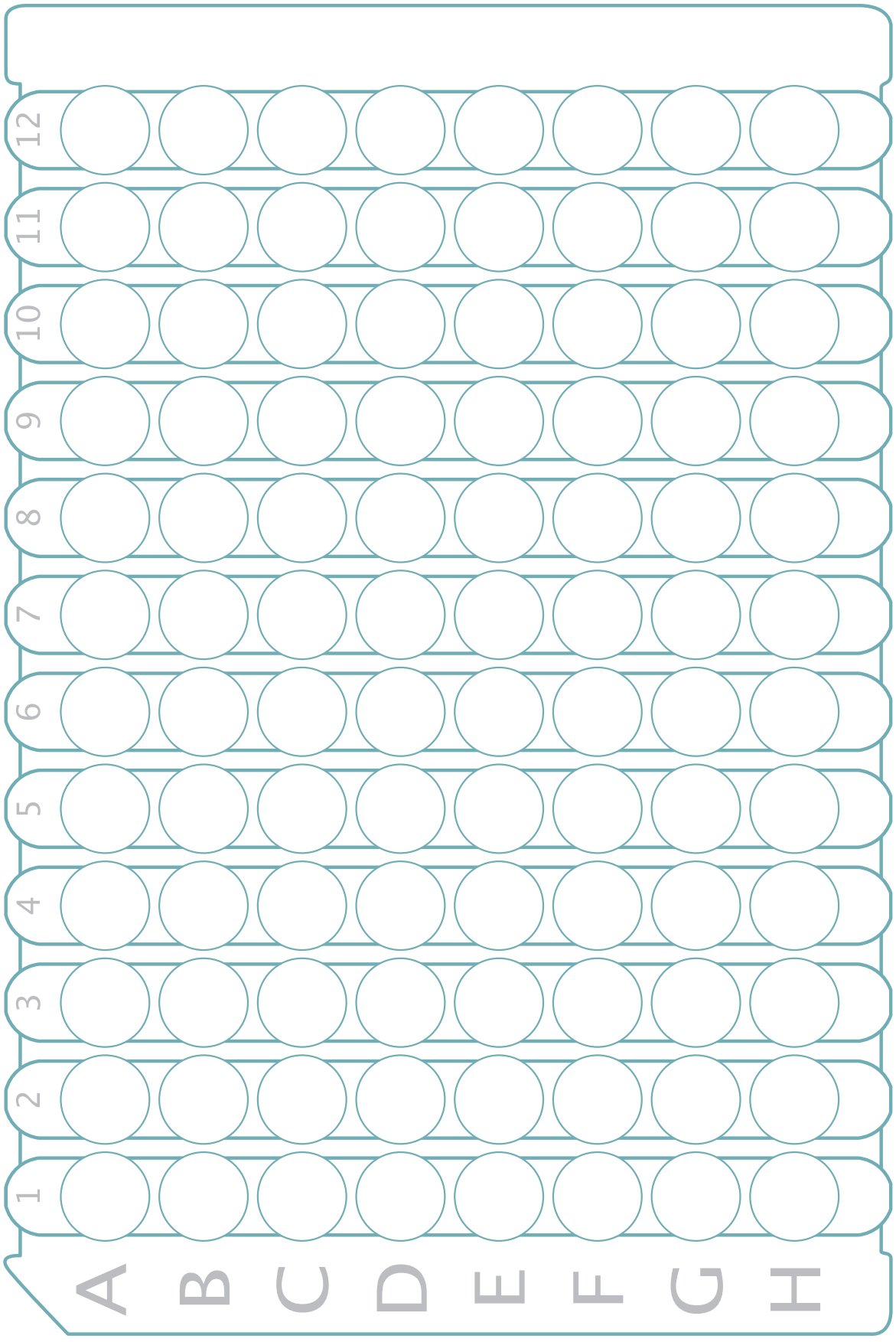
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES

NOTES

PEPROTECH

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Human BD-2
ELISA Development Kit
900-K172 Lot# 0512172
Expiration one year from date of receipt

Description: Human BD-2 ELISA development kit contains the key components required for the quantitative measurement of natural and/or recombinant hBD-2 in a sandwich ELISA format within the range of 16–2000pg/ml. Using the ELISA protocol described below, the recommended microplates, reagents and solutions, the components supplied in this kit are sufficient to assay hBD-2 in approximately 1000 ELISA plate wells.

RECONSTITUTION & STORAGE

Capture Antibody: 50µg of antigen-affinity purified goat anti-hBD-2 + 2.5mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 0.5ml sterile water for a concentration of 100µg/ml.

Detection Antibody: 50µg of biotinylated antigen-affinity purified goat anti-hBD-2 + 2.5mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 0.5ml sterile water for a concentration of 100µg/ml.

Human BD-2 Standard: 1µg of recombinant hBD-2 + 2.2mg BSA + 11.0mg D-mannitol. Centrifuge vial prior to opening. Reconstitute in 1ml sterile water for a concentration of 1µg/ml.

Note: The reconstituted components are stable for 2 weeks when stored at 2-8°C. Components that have been reconstituted and aliquoted can be stored at -20°C for up to 6 months.

Avidin-HRP Conjugate: 60µl vial. Upon receipt, avidin-HRP conjugate should be aliquoted into ten 6µl vials and stored at ≤-20°C. Aliquots stored frozen at ≤-20°C are stable for up to 2 years form date of receipt. Avoid more than one freeze-thaw cycle. **Avidin should be used in conjunction with ABTS only.**

RECOMMENDED MATERIALS (or purchase PeproTech's ELISA Buffer Kit: Cat. # 900-K00)

ELISA microplates (Nunc MaxiSorp Prod. # 439454, or Corning Prod # 3590);
Tween-20 (Sigma Cat. # P-7949);
BSA (Sigma Cat # A-7030);
ABTS Liquid Substrate Solution (Sigma Cat. # A3219);
Dulbecco's PBS [10x] (Gibco BRL Cat. # 14200-075).

RECOMMENDED SOLUTIONS

All solutions should be at ambient temperature prior to use.
PBS: dilute 10xPBS to 1xPBS, pH 7.20 in sterile water.
Wash Buffer: 0.05% Tween-20 in PBS
Block Buffer: 1% BSA in PBS *
Diluent: 0.05% Tween-20, 0.1% BSA in PBS *
**** Sterile filter and store at 4°C for up to 1 week.***

PLATE PREPARATION

1. Dilute capture antibody with PBS to a concentration of 0.5µg/ml. Immediately, add 100µl to each ELISA plate well. Seal the plate and incubate overnight at room temperature.
2. Aspirate the wells to remove liquid and wash the plate 4 times using 300µl of wash buffer per well. After the last wash invert plate to remove residual buffer and blot on paper towel.
3. Add 300µl block buffer to each well. Incubate for at least 1 hour at room temperature.
4. Aspirate and wash plate 4 times.

ELISA PROTOCOL

Standard/Sample: Dilute standard from 2ng/ml to zero in diluent. Immediately add 100µl of standard or sample to each well in triplicate. Incubate at room temperature for at least 2 hours.

Detection: Aspirate and wash plate 4 times. Dilute detection antibody in diluent to a concentration of 0.5µg/ml. Add 100µl per well. Incubate at room temperature for 2 hours.

Avidin Peroxidase: Aspirate and wash plate 4 times. Dilute one 5.5µl aliquot of Avidin Peroxidase 1:2000 in diluent for total volume of 11ml. Add 100µl per well. Incubate 30 minutes at room temperature.

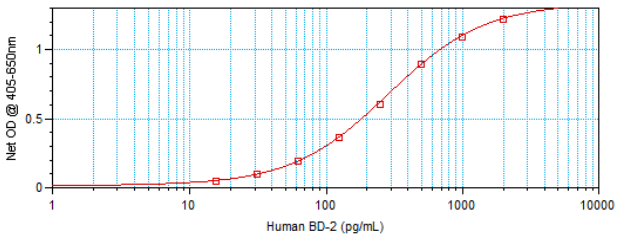
ABTS Liquid Substrate:
(ABTS Substrate should be at ambient temperature prior to use)
Aspirate and wash plate 4 times. Add 100µl of substrate solution to each well. Incubate at room temperature for color development. Monitor color development with an ELISA plate reader at 405 nm with wavelength correction set at 650 nm.

NOTE: Reliable standard curves are obtained when either O.D. readings do not exceed 0.2 units for the zero standard concentrations, or 1.2 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 20 minutes.

CROSS REACTIVITY

When tested at 50ng/ml the following antigens did not exhibit significant cross reactivity:

Human BD-1 (36a.a.), BD-1 (47a.a.), BD-3, BD-4, NP-1



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α-tocopherol decreases interleukin-1β and -6 and increases human β-defensin-1 and -2 secretion in human gingival fibroblasts stimulated with *Porphyromonas gingivalis* lipopolysaccharide

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Derradjia A, Alanazi H, Park HJ, Djeribi R, Semlali A, Rouabhia M. α-tocopherol decreases interleukin-1β and -6 and increases human β-defensin-1 and -2 secretion in human gingival fibroblasts stimulated with Porphyromonas gingivalis lipopolysaccharide. J Periodont Res 2015; doi: 10.1111/jre.12308.

Background and Objective: Periodontitis, a disease associated with chronic inflammation, results in significant destruction of periodontal tissues. Uncontrolled, periodontal disease negatively affects general patient health. We sought to evaluate the effect of α-tocopherol on gingival fibroblast behavior following exposure to *Porphyromonas gingivalis* lipopolysaccharide (LPS).

Material and Methods: Primary human gingival fibroblasts were cultured for 24 and 48 h with α-tocopherol at various concentrations (0, 50, 100 and 200 μM) in the presence or absence of 1 μg/mL of LPS. At the end of each time point, cell adhesion and growth were evaluated by means of optical microscope observations and MTT assay. The secretion levels of cytokines interleukin (IL)-1β and IL-6 and human β-defensins 1 and 2 were measured by specific enzyme-linked immunosorbent assay. Finally, an *in vitro* scratch wound assay was performed to investigate the effect of α-tocopherol on fibroblast migration.

Results: α-tocopherol alone had no adverse effect on cell adhesion and morphology. Fibroblast proliferation increased in the presence of α-tocopherol with and without LPS. α-tocopherol alone had no effect on inflammatory cytokine (IL-1β and IL-6) secretion. Interestingly, following cell exposure to *P. gingivalis* LPS, α-tocopherol significantly ($p < 0.01$) decreased the secretion of these two cytokines and increased human β-defensin-1 and -2 secretion. Finally, α-tocopherol increased the healing rate of the gingival fibroblasts from 12 h up to 48 h.

Conclusion: These results suggest that α-tocopherol may play an active role in countering the damaging effect of LPS by reducing inflammatory cytokines, increasing β-defensins and promoting fibroblast growth, migration and wound healing.

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Key words: cytokines; gingival fibroblasts; lipopolysaccharide; wound healing; α-tocopherol; β-defensins

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Periodontitis, a chronic infectious disease that leads to tissue destruction and possible tooth loss, is in fact a host-mediated inflammatory response to pathogenic microflora residing in periodontal pockets (1,2). Bacterial species associated with periodontitis include *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola* and *Aggregatibacter actinomycetemcomitans* (2–5). Pathogenicity of *P. gingivalis* involves different virulence factors, including fimbriae, capsule polysaccharide, cysteine proteases and lipopolysaccharide (LPS) (6). Various cells in the periodontium, such as periodontal ligament fibroblasts, gingival fibroblasts and gingival epithelial cells, are involved in the host immune response in periodontitis (7–9). These cells are involved in homeostasis of periodontal ligament and alveolar bone remodeling (10). In periodontitis, gingival fibroblasts may be involved in regulating the inflammatory response against such oral pathogens as *P. gingivalis* and their virulence factors, such as LPS (11,12). These virulence factors are involved in tissue colonization and can alter host defenses (13). Both *P. gingivalis* and its LPS are potent stimulators of the host's inflammatory immune response (2,14). This activation of the innate defense system involves cellular receptors that bind conserved structures of microbial origin (15). One important class of gingival cell receptors that recognize pathogen-associated molecular patterns is the family of Toll-like receptors (16). Following bacteria or LPS recognition, cells produce proinflammatory cytokines, such as interleukin (IL)-6 and IL-8 (17), which are involved in periodontal inflammatory response. High levels of interleukin-1β (IL-1β), IL-6 and IL-8 following *P. gingivalis* LPS stimulation were incriminated in alveolar bone resorption and periodontal tissue destruction (1,2,18).

Controlling infection and reducing the inflammatory process both contribute to reducing periodontal disease (19). Antibiotics such as amoxicillin or metronidazole are available to cure infections (20,21), while antioxidants are shown to prevent them (22,23). Vitamin E, a potent antioxidant, acts mainly on the lipid phase of cells to counter the oxidation of polyunsaturated fatty acids (24) and is thus important to the host's antioxidant defense and immune functions (22). It is well known that vitamin E deficiency is associated with increased oxidative stress (24) as well as impaired immune function, including both humoral and cell-mediated immunity (22). Using an animal model, it was demonstrated that vitamin E could play a key role in enhancing host protection against *Heligmosomoides polygyrus* (25). Vitamin E was also shown to decrease bacterial adhesion and biofilm accumulation on the surface of vitamin E-blended polymers (26). These data highlight the potential of vitamin E against periodontal bacteria and such virulence factors as LPS. The aim of the present study was thus to investigate the effect of vitamin E on gingival fibroblast growth, cytokine (IL-1β and IL-6) and antimicrobial peptide (human β-defensin [HBD]-1 and HBD-2) secretions, and cell migration/healing properties following exposure to *P. gingivalis* LPS.

Experimental design

Human gingival fibroblast extraction and culture

Small samples of human gingival mucosa were collected from patients attending the dental clinic at Université Laval's Faculty of Dentistry for treatment. The patients provided free informed consent and the institution's Ethics Committee approved the procedure. Immediately following the biopsies, the epithelium was separated from the lamina propria by thermolysin treatment (500 μg/mL). Gingival fibroblasts were extracted from the lamina propria using 0.125 U/mL of collagenase-P (Boehringer Mannheim, Laval, QC, Canada). These fibroblasts were then cultivated in 5% fetal calf serum supplemented with Dulbecco's modified Eagle medium. When the cultures reached 90% confluence, the cells were detached and used for the study.

Preparation of lipopolysaccharide from *P. gingivalis*

LPS was isolated from *P. gingivalis* (ATCC 33277) using the Darveau-Hancock method (1983) (27), which centers on the protein digestion of a whole cell extract by proteinase K with successive solubilization and precipitation steps. The LPS preparation was then freeze-dried and stored at −20°C until use. The absence of contaminating protein in the preparation was confirmed by a protein assay (Bio-Rad Laboratories, Mississauga, ON, Canada) with bovine serum albumin used as the control. The LPS was subsequently used at a concentration of 1 μg/mL throughout the study.

Tocopherol preparation

α-tocopherol purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) was dissolved in ethanol as 50 mM stock solutions and used thereafter at various concentrations (50, 100 or 200 μM) for the purpose of this study. The culture medium of the control groups and α-tocopherol groups was supplemented with the same volume of ethanol.

Effect of α-tocopherol with or without lipopolysaccharide on gingival cell morphology

Primary human gingival fibroblasts were first seeded into six-well tissue culture plates (Falcon; Becton Dickinson, Lincoln Park, NJ, USA) at 2×10^4 cells/well and subsequently incubated in a 5% CO_2 atmosphere at 37°C for 24 h. They were then stimulated or not with α-tocopherol (50, 100 or 200 μM) in the presence or absence of 1 μg/mL of *P. gingivalis* LPS. LPS is a component of gram-negative bacteria known as a potent inducer of the immune response by various cell types (28). LPS from *P. gingivalis* is strongly associated with chronic and severe adult periodontitis (2,6). The fibroblasts in the presence or absence of α-tocopherol with or without LPS were then cultured for 24 and 48 h. At the end of each incubation period, the stimulated

and non-stimulated fibroblast monolayers were washed three times with phosphate-buffered saline and subjected thereafter to crystal violet staining. One milliliter of 1% w/v crystal violet solution in demineralized water was added and the cultures were further incubated at room temperature for 15 min, after which time the non-bound dye was removed from the wells by thorough washing with demineralized water, followed by drying at 37°C. The stained cells were then observed under an inverted microscope and photographed.

Effect of α-tocopherol with or without lipopolysaccharide on gingival fibroblast growth

Fibroblasts were cultured in the presence or absence α-tocopherol at 50, 100 or 200 μM with or without 1 μg/mL of *P. gingivalis* LPS for 24 and 48 h. Cell proliferation was assessed following each culture period using the assay 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA), which measures cell growth as a function of mitochondrial activity (29). MTT assay is based on hydrolysis of the tetrazolium ring by mitochondrial dehydrogenase, resulting in an insoluble blue reaction product (formazan). Briefly, a stock solution (5 mg/mL) of MTT was prepared in phosphate-buffered saline and added to each culture well at a final concentration of 1% (v/v). Gingival fibroblast cultures were incubated for 4 h at 37°C with the MTT, after which time the supernatant was removed, and the adherent cells were washed twice with warm culture medium. Following the final wash, 2 mL of 0.04 N HCl in isopropanol were added to each culture well, with the incubation extended for another 15 min. At this step, 200 μL (in triplicate) of the reaction mixture was transferred to a 96-well flat-bottom plate and the absorbance (optical density) was measured at 550 nm by means of a microplate reader (Model 680; Bio-Rad Laboratories). Results are reported as the means ± SD of six separate experiments.

Cytokine and β-defensin quantification following fibroblast stimulation with α-tocopherol with or without lipopolysaccharide

Human gingival fibroblasts were cultured for 48 h in the presence or absence of α-tocopherol at 50, 100 or 200 μM, with or without 1 μg/mL of *P. gingivalis* LPS. The supernatant from each condition was then collected to determine IL-1β, IL-6, HBD-1 and HBD-2 levels. The cytokine enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA) while the β-defensin ELISA kits were obtained from Peprotech (Rocky Hill, NJ, USA). The supernatants were first collected in tubes containing 1 μL of a protease inhibitor cocktail (Sigma-Aldrich Canada Ltd.), immediately filtered through 0.22 μm filters, and used thereafter to measure the mediator levels by ELISA assay. The mediator levels were read at 450 nm by means of a microplate reader (Model 680; Bio-Rad Laboratories). The minimum detectable concentrations were less than 1 pg/mL for IL-1β, 0.7 pg/mL for IL-6, 4 pg/mL for HBD-1 and 16 pg/mL for HBD-2, as reported by the manufacturer. Each experiment was repeated four times and the means ± SD were calculated and presented as the levels of cytokines or β-defensins per mg of total protein extracted from the same cell cultures. Indeed, following supernatant collection, the adherent cells were first detached from the culture plate using trypsin then centrifuged for 10 min at 270 *g*, after which time the pellet was resuspended in 300 μL of cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA), incubated 5 min at 4°C, and subsequently spun out for 10 min in a cold microfuge. The collected supernatant was used to qualify the total protein concentration using the Bradford assay (30).

Effect of α-tocopherol with or without lipopolysaccharide on fibroblast monolayer wound repair

In vitro wound repair assays were performed as previously described (31).

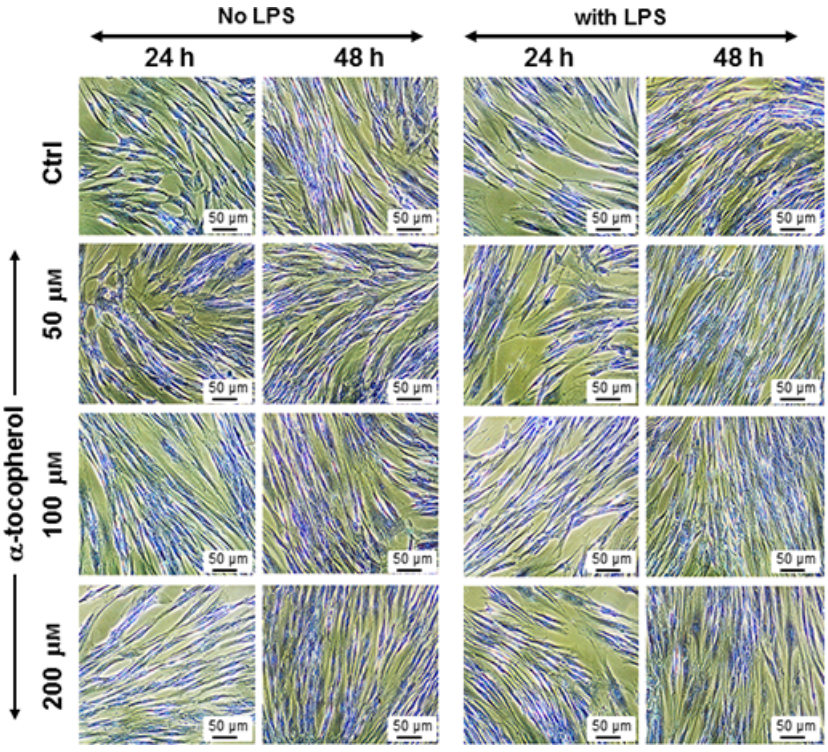

Fig. 1. Effect of α-tocopherol on gingival fibroblast morphology. Following stimulation or not with α-tocopherol with or without LPS, the cells were stained with crystal violet. Photos were taken under an inverted microscope. Scale bars, 50 μm. LPS, lipopolysaccharide.

Briefly, gingival fibroblasts were cultured in the presence or absence of α-tocopherol at various concentrations (0, 50, 100 or 200 μM) with or without LPS (1 μg/mL) until confluence. A scratch (wound) was created on each confluent monolayer using a 200 μL sterile pipette tip (PipetTipFinder, Knoxville, TN, USA) perpendicular to the bottom of the dish. The generated wound was approximately between 0.44 and 0.50 mm in width. The cultures were refreshed with new medium and maintained under incubation. Digital photographs of each wound were taken (Coolpix 950; Nikon Canada, Montréal, QC, Canada) at various time points following the creation of the wound, and the obtained images were used to measure the denuded area by means of the NIH ImageJ public domain image processing program. The experiment was repeated six times independently for statistical analyses. Data are presented as the percentage of the healed wound area at 12, 24 and 48 h divided by the area at time zero (initial wound).

Statistical analysis

Data are presented as the means ± SD of at least four separate experiments. A statistical comparison of the groups was performed using a one-way ANOVA and the statistical difference between two groups was determined by means of the Student's *t*-test. The difference was considered significant when $p \leq 0.05$.

Results

Fibroblast adhesion and morphology following stimulation with α-tocopherol with or without lipopolysaccharide

Various α-tocopherol concentrations were used to treat fibroblast cultures for 24 and 48 h to determine the effect of this molecule on gingival fibroblast attachment and morphology. As shown in Fig. 1, α-tocopherol had no effect on cell adhesion and morphology up to a concentration of 200 μM; the fibroblasts were elongated, with a dense nucleus and a small amount of cytoplasm. In the presence of *P. gingivalis* LPS (1 μg/mL), α-tocopherol also had no adverse effect on cell adhesion and morphology (Fig. 1).

Fibroblast growth following stimulation with α-tocopherol with or without lipopolysaccharide

Quantitative analyses by MTT assay reveal that after stimulation for 24 h, α-tocopherol had no effect on cell adhesion/growth (Fig. 2A), while at 48 h, α-tocopherol significantly ($p < 0.01$) promoted cell growth (Fig. 2A). The addition of LPS alone to the gingival fibroblast culture led to cell growth at 24 and 48 h (Fig. 2B). *P. gingivalis* LPS combined with α-tocopherol promoted greater cell growth than did *P. gingivalis* LPS alone. No difference was observed with higher concentrations of α-tocopherol (Fig. 2B).

α-tocopherol modulated the secretion of proinflammatory cytokines (interleukin-1β and -6) by primary gingival fibroblasts

As shown in Fig. 3, following gingival fibroblast stimulation with α-tocopherol for 48 h, IL-1β secretion remained unchanged, compared to that observed in the control (Fig. 3A). However, the addition of *P. gingivalis* LPS to the culture led to a significant ($p < 0.02$) increase in IL-1β secretion. Interestingly, α-tocopherol was able to downregulate the effect of LPS on

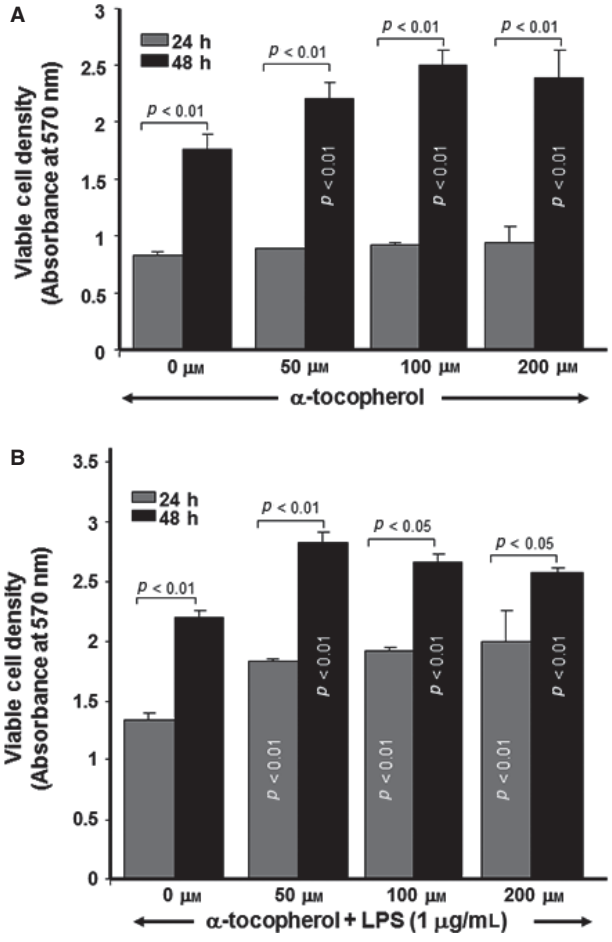

Fig. 2. α-tocopherol with or without LPS modulated gingival fibroblast growth. Cell growth stimulated or not with α-tocopherol (A) or with α-tocopherol and LPS (B) was investigated using MTT. Statistical significance was obtained by comparing the data collected in the presence or absence of α-tocopherol with or without LPS ($n = 4$). LPS, lipopolysaccharide.

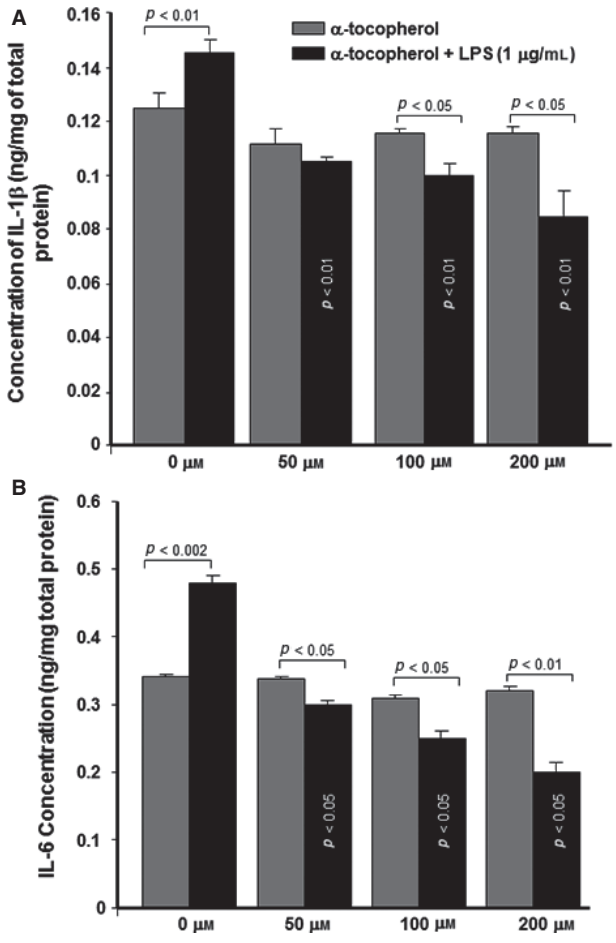

Fig. 3. IL-1β and IL-6 secretion by gingival fibroblasts following stimulation with α-tocopherol alone or with LPS. Cytokine levels in the culture supernatants were measured by enzyme-linked immunosorbent assay. Significance was obtained by comparing the absence (control) and the presence of α-tocopherol in the culture with or without LPS ($n = 5$). IL, interleukin; LPS, lipopolysaccharide.

IL-1β secretion. Figure 3A shows low levels of IL-1β secreted by the cells cultured in the presence of α-tocopherol and LPS, compared to those stimulated with LPS alone. Similar observations were made with IL-6. Figure 3B shows no visible effect of α-tocopherol on IL-6 secretion by the gingival fibroblasts, whereas the cells stimulated with LPS showed increased levels of IL-6. This was significantly ($p < 0.05$) downregulated by α-tocopherol.

α-tocopherol increased human β-defensin-1 and -2 secretion by primary gingival fibroblasts

As shown in Fig. 4A, when primary gingival fibroblasts were cultured for 48 h in the presence of α-tocopherol, the HBD-1 secretion level did not change. In contrast, the addition of LPS to the cell cultures led to a significant increase in HBD-1 secretion. Of interest is that higher levels of secreted HBD-1 were observed when the cells were stimulated with both LPS and α-tocopherol. Figure 4A shows a significant ($p < 0.05$) increase in HBD-1 secretion by gingival fibroblasts following exposure to both LPS and α-tocopherol, compared to LPS alone. HBD-2 secretion was also modulated by α-tocopherol. The addition of α-tocopherol to primary human gingival cultures led to a significant ($p < 0.01$) increase in HBD-2 secretion, compared to that observed in the non-stimulated cultures (Fig. 4B). The effect of α-tocopherol on HBD-2 secretion was greater at 100 and 200 μM than at 50 μM. Cells stimulated with LPS alone were shown to secrete higher levels of HBD-2, in contrast to the control. The effect of LPS on HBD-2 secretion was thus upregulated by α-tocopherol. Figure 4B shows higher HBD-2 levels secreted by cells stimulated with LPS and α-tocopherol than by those stimulated with either α-tocopherol alone or LPS alone.

α-tocopherol enhanced cell migration/wound healing

Figure 5 shows α-tocopherol-modulated fibroblast migration as ascertained by the reduced wound area in the α-tocopherol-treated cultures compared to the non-treated scratched monolayer cultures. The effect of α-tocopherol on cell migration was noticeable beginning at 12 h post-wound and treatment (Fig. 5A). The effects were indeed significant with 100 and 200 μM of α-tocopherol (Fig. 5B). LPS alone did not inhibit or promote cell migration. However, the presence of α-tocopherol in the LPS-stimulated cultures resulted in superior cell migration and wound repair (Fig. 5B). Comparable results were obtained at 24 h post-wound. α-tocopherol alone promoted cell migration to repair the wound (Fig. 5C and 5D), beginning with 50 μM. The presence of LPS alone resulted in significant cell migration at 24 h compared to what was observed in the controls (absence of LPS). This effect was thus upregulated by α-tocopherol. Interestingly, the higher the concentration of α-tocopherol, the greater the wound repair (Fig. 5C). At 48 h, almost the entire wounded area was repaired (Fig. 5D).

Discussion

In this study, we demonstrated that α-tocopherol had no significant effect on gingival fibroblast adhesion and growth during the short culture period and increased cell growth during the longer culture period. This concurs with previous reports stating that

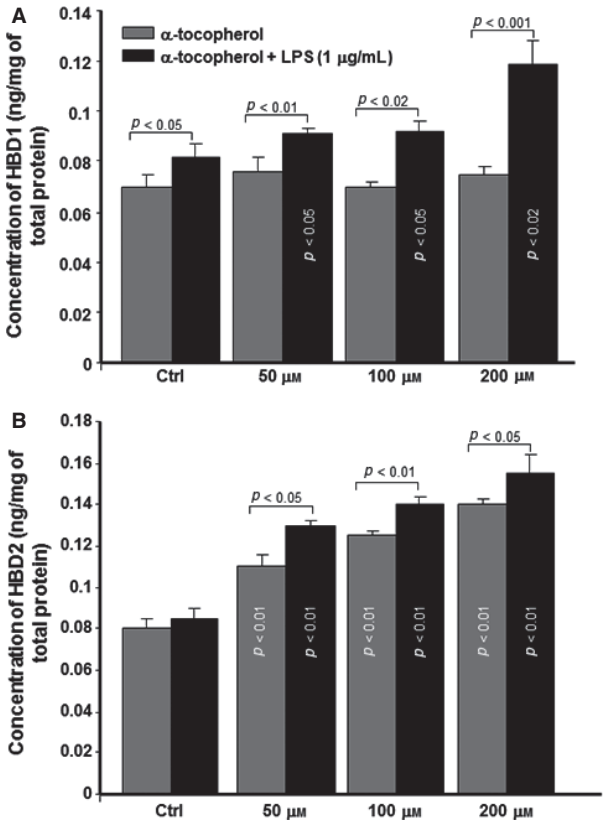

Fig. 4. HBD-1 and HBD-2 secretion by gingival fibroblasts following stimulation with α-tocopherol alone or with LPS. β-defensin levels in the culture supernatants were measured by enzyme-linked immunosorbent assay. Significance was obtained by comparing the absence (control) and the presence of α-tocopherol in the culture with or without LPS ($n = 6$). HBD, human β-defensin; LPS, lipopolysaccharide.

α-tocopherol enhanced the proliferation of cultured endothelial cells (32). However, with other cell types such as human aorta smooth muscle, mouse fibroblasts or cancer cells, α-tocopherol was shown to inhibit cell growth (33,34). The exact reason for this heterogeneity remains rather unclear; the different effects of α-tocopherol on cell proliferation may plausibly be due to cell type. Further standardized studies with various cell types will be required to confirm this hypothesis.

Oral cells are exposed to multiple bacteria and bacterial virulence products, such as LPS, which lead to altered cell properties and functions. *P. gingivalis* LPS was reported to increase human periodontal ligament fibroblast growth (35). This supports our study showing higher gingival fibroblast growth following stimulation with *P. gingivalis* LPS. This overgrowth may be a common cell behavior in response to endotoxin stimulation (36) or possibly a cell-specific behavior against the harmful effect of *P. gingivalis* or its virulence factors, thereby preventing periodontal disease (37). Interestingly, cell growth was promoted by α-tocopherol, which suggests that this molecule may play a preventive role against periodontal disease.

Infections are promoters of proinflammatory mediator secretion by infected cells (38). In the oral cavity, gingival fibroblasts are the main constituent of the periodontium and are key players against infection by producing various proinflammatory cytokines in response to direct contact with periodontal bacteria or their virulence factors, such as LPS (39). We demonstrated increased IL-1β and IL-6 secretion by gingival fibroblasts following stimulation with *P. gingivalis* LPS, as previously reported with *Escherichia coli* LPS and *P. gingivalis* LPS (39,40). Proinflammatory cytokine release can be viewed as a protection process by gingival fibroblasts to fight infection (41). However, multiple studies have reported that increased proinflammatory cytokine secretion may contribute to the progression of periodontal disease (2,42). Downregulating the secretion of proinflammatory mediator may thus be beneficial to the host. This process can occur with the contribution of α-tocopherol. We clearly demonstrated that the presence of α-tocopherol simultaneously with *P. gingivalis* LPS significantly decreased IL-1β and IL-6 secretion by primary gingival fibroblasts. The role of α-tocopherol against the deleterious effects of LPS has been the subject of *in vitro* and *in vivo* studies showing that this vitamin suppresses the inflammatory response and oxidative damage induced by LPS in both cell culture systems and animal experiments (43,44). α-tocopherol was also shown to prevent interferon-γ/LPS-induced dopaminergic neuron degeneration effectively (45) and downregulate LPS-induced lipid peroxidation and IL-6 in murine microglia and brain cells (46). Furthermore, α-tocopherol was found to promote recovery from LPS-induced infection in aged mice (47). These findings and our results therefore highlight the potential of α-tocopherol in the prevention of microbial infections.

Preventing or curing microbial infection can be achieved with chemical antimicrobial molecules, such as antibiotics, but also through the secretion of antimicrobial peptides on host cells (48). The antimicrobial peptide family includes multiple molecules such as HBDs. We thus demonstrated for the first time that primary human gingival fibroblasts secreted HBD-1 and HBD-2 by antimicrobial peptides and that this secretion did not increase following stimulation with α-tocopherol. In contrast, however, *P. gingivalis* LPS alone or in combination with α-tocopherol led to an increase in HBD-1 and HBD-2 secretion by these fibroblasts. HBD-1 has been shown to target a variety of bacteria, including

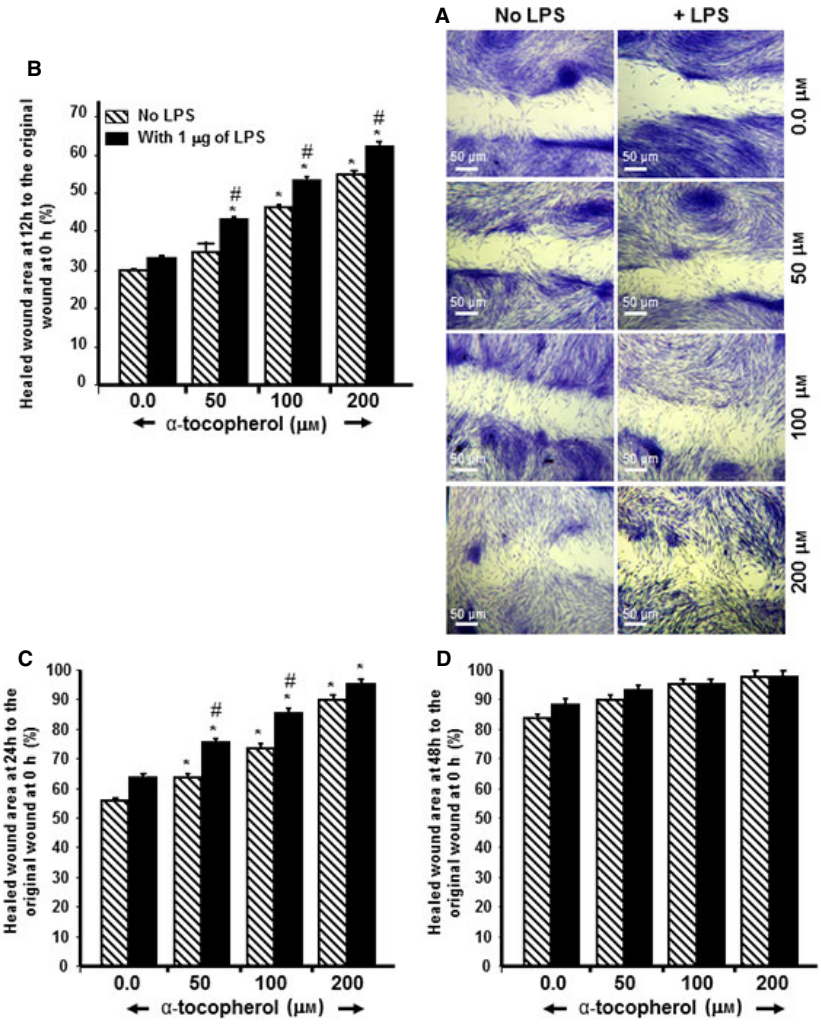

Fig. 5. α-tocopherol with or without LPS increased the wound healing rate in the primary human dermal fibroblast monolayer. Following stimulation and wounding, the percentage changes in wound size (area) over time are presented as the ratio to the initial wound size (time zero after wound). Values are given as means ± SD ($n = 6$). The stimulated and non-stimulated cultures were compared, with the difference considered statistically significant at $p < 0.05$. (A) Wound healing after 12 h (Scale bars, 50 μm). (B) Wound healing after 12 h. (C) Wound healing after 24 h. (D) Wound healing after 48 h. *Statistical significance between the control (0.0 μm of a-tocopherol) and the α-tocopherol-stimulated group. #Statistical significance between the different concentrations of α-tocopherol and the presence or absence of LPS. LPS, lipopolysaccharide.

P. gingivalis, *A. actinomycetemcomitans* and *Fusobacterium nucleatum* (49), while HBD-2 is reported active against gram-negative bacteria and *Candida albicans* but more restricted in its activity against gram-positive bacteria (50,51). This may explain the increased levels of HBD-1 and HBD-2 under *P. gingivalis* LPS stimulation and the observed upregulated activity when α-tocopherol was added to the fibroblast cultures. Our study suggests that the cells activated their defense mechanism against LPS by producing HBD-1 and -2, which was promoted by α-tocopherol. Overall data thus suggest that α-tocopherol may modulate inflammatory reactions and tissue destruction by reducing proinflammatory cytokines and increasing antimicrobial peptides HBD-1 and HBD-2. Further studies are mandatory to elucidate the signaling mechanisms involved with α-tocopherol. Once the inflammation is controlled, the injured periodontal tissue will heal.

One of the key cells involved in periodontal wound healing is the gingival fibroblast (51–53). Fibroblasts have a proliferative phenotype and a high capacity to synthesize and deposit extracellular matrix components necessary for adequate wound healing (51–53).

In vitro, wound-healing models are often used to evaluate the effect of various agents on targeted cells (54). These models consist of scratches made on cell monolayers to create a cell-free area, allowing for an investigation of how cells migrate and cover this area. The scratch cell monolayer assay was used in this study to demonstrate the capability of α-tocopherol to promote cell migration and consequently, wound healing. α-tocopherol-treated cells occupied a significantly larger portion of the wound area, beginning at 12 h up to 48 h, with or without LPS. Results indicate that α-tocopherol may promote periodontal healing at an early stage by stimulating both gingival fibroblast growth and migration, with a noticeably greater effect when α-tocopherol is combined with LPS. This increase may be a way to overcome the negative impact of *P. gingivalis* LPS, which not only promotes inflammation but also the onset of periodontal disease. Laheij *et al.* (55) recently demonstrated that live *P. gingivalis* or its crude culture supernatant inhibited epithelial cell migration. The difference in our study laid in the use of *P. gingivalis* LPS alone, as this bacterium produces different virulence factors, which can be secreted when in contact with cells. Additional studies using live *P. gingivalis* will thus be performed in the future to shed light on the effect of *P. gingivalis* or its virulence factor combination on gingival wound healing. Our findings are in agreement with previously reported studies demonstrating enhanced gingival wound healing following vitamin E use *in vitro* (56) and vitamin E supplementation *in vivo* (57).

Conclusion

This study demonstrated that α-tocopherol downregulated the inflammatory

response of gingival fibroblasts by decreasing IL-1β and IL-6 secretion following stimulation by LPS. Interestingly, α-tocopherol was able to promote cell innate immunity defense by increasing the secretion of HBD-1 and -2. We also demonstrated that α-tocopherol accelerated the *in vitro* wound healing of gingival fibroblasts, even in the presence of LPS. Overall data may suggest a beneficial role of α-tocopherol in preventing/curing periodontal disease.

Acknowledgements

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