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Research Article

RESEARCH ARTICLE

Immunomodulatory/anti-inflammatory effects of a propolis-containing mouthwash on human monocytes

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One sentence summary: The combination propolis/chlorhexidine increased the bactericidal activity of human monocytes against *Streptococcus mutans* and stimulated anti-inflammatory cytokine production, which could be beneficial in the treatment of periodontal diseases.

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ABSTRACT

Propolis is a bee product used in folk medicine to improve health and prevent inflammatory diseases. It has attracted the attention of researchers from the odontological field lately, reducing inflammation resulting from surgical procedures and as an antimicrobial agent in the control of bacterial plaque. Thus far, no side-effects that might compromise oral health have been observed. Chlorhexidine is an antimicrobial agent widely used as an antiseptic, but side-effects restrict its use. This work investigated the effects of an odontological product containing propolis in combination with chlorhexidine in lower concentrations on human monocytes. Cell marker expression, the nuclear factor kappa B (NF-κB) signaling pathway, the production of pro- and anti-inflammatory cytokines, and the bactericidal activity of these cells against *Streptococcus mutans* were evaluated. Data showed that the combination of propolis and chlorhexidine may favor the recognition of antigens by monocytes, slightly activates the NF-κB signaling pathway, and increases the bactericidal activity of human monocytes against *S. mutans*. Also, the combination played a role in anti-inflammatory cytokine production, which can be beneficial in the treatment of periodontal diseases. These results may have implications for the development of odontological products with immunomodulatory/anti-inflammatory action, and may have further-reaching implications for the pharmaceutical industry.

Keywords: monocytes; cytokine; propolis; chlorhexidine; odontological product

INTRODUCTION

Propolis is a resinous and balsamic product, collected and prepared by bees from some parts of plants, i.e. leaves, branches, bark, resinous exudates and flower buds, to which the bees add salivary secretions, wax and pollen (Bankova 2005). The color of propolis may vary between yellow, red, brown and green, depending on its botanical origin (Salatino *et al*. 2005). Its chemical composition is extremely complex, varying according to geographical location and the local flora. The main sources of propolis in the apiary of the University (UNESP, Campus of Botucatu) are *Baccharis dracunculifolia* DC, *Araucaria angustifolia* (Bert.) O. Kuntze and *Eucalyptus citriodora* Hook (Bankova *et al*. 1999). The

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composition of our propolis sample was analyzed, revealing that its main groups are: phenolics (flavonoids, aromatic acids, benzopyranes), di- and triterpenes, and essential oils (Sforcin 2007).

Propolis has been used since ancient times in folk medicine, due to its numerous biological properties, such as antimicrobial, anti-inflammatory, immunomodulatory and anti-tumor (Sforcin 2007; Búfalo *et al.* 2009). Moreover, the absence of side-effects after short or long-term propolis administration to rats has been reported (Sforcin, Funari and Novelli 1995; Mani *et al.* 2006). Propolis has attracted the attention of researchers from the odontological area, in order to reduce inflammation resulting from surgical procedures, and as an antimicrobial agent in the control of bacterial plaque, showing no side-effects that could compromise the oral health.

Chlorhexidine (CHX) is a cationic antimicrobial agent and has been widely used as an antiseptic, being active against Gram-positive and Gram-negative bacteria, aerobic and facultative anaerobic, and molds, yeasts and viruses. Its action occurs by adsorption to the cell wall of microorganisms, causing leakage of intracellular components, leading to cell death (Hernandez *et al.* 2005). However, its use as an antimicrobial agent for oral use is still controversial due to the occurrence of side-effects, such as pigmentation of teeth, tongue, restorations and prostheses, desquamation and injury to the oral mucosa, and taste disorders. These side-effects restrict its prolonged use. Thus, the search for new products with significant antimicrobial activity and fewer side-effects has increased, aiming for the preventive or therapeutic control of bacteria that cause periodontal diseases (Libério *et al.* 2009).

Periodontal diseases are characterized by chronic inflammation associated with the presence of specific bacteria in the periodontal pocket, which results in the destruction of soft tissue and the resorption of alveolar bone. The local inflammatory immune response is crucial for protecting periodontal tissues from various pathogens. Gingival epithelial cells play an important role in the innate immune response by producing antimicrobial peptides and chemokines that recruit neutrophils (Matsuyama *et al.* 2005). Monocytes from the gum bleeding come to the inflammatory site as well. Antigen-presenting cells (APCs) remove invading microorganisms such as *Streptococcus mutans* that cause caries by engulfing, processing and presenting them to T cells. Antigen-specific T-cell activation by T-cell receptor/MHC class II engagement requires a co-stimulatory signal which is induced by the binding between cluster of differentiation (CD)28 on T cells and B7-1 (CD80) and B7-2 (CD86) co-stimulatory molecules expressed by APCs, which produce inflammatory mediators that regulate the adaptive immune response (Konermann *et al.* 2012).

Toll-like receptors (TLRs) may bind to products or conserved microbial structures, called pathogen-associated molecular patterns (PAMPs), which are absent in eukaryotic cells but may be present in both pathogenic and non-pathogenic microorganisms (Medzhitov 2001). After recognition of microorganisms, several transcription factors are activated, such as nuclear factor kappa B (NF-κB), leading to the gene expression of tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-10. Individually or in combination, cytokines interact with their specific receptors, modulating cellular function. Cytokines such as TNF-α are important in the activation of the monocyte and macrophage process, whereas other cytokines, such as IL-10, are considered inactivating cell factors. IL-6 exerts both pro- and anti-inflammatory effects, and acts in regenerative processes, in regulating metabolism, and in maintaining bone and neural function (Scheller *et al.* 2011).

Recently, our group produced a propolis-based odontological product whose patent was deposited in February 2015 (BR10 2015 003982-4). This product was tested *in vivo* and beneficial effects were seen in the control of dental plaque in humans. Here we wish to present its immunomodulatory effects *in vitro* on human monocytes. The effects of propolis in combination with lower concentrations of chlorhexidine were assessed regarding the expression of cell markers (TLR-4, human leukocyte antigen (HLA)-DR, CD80, CD86), NF-κB signaling pathway, pro- and anti-inflammatory cytokine (TNF-α, IL-10, IL-6) production by human monocytes and its bactericidal activity against *Streptococcus mutans* – one of the causative agents of dental caries.

MATERIALS AND METHODS

Propolis, chlorhexidine and the combination propolis/chlorhexidine

Propolis was collected in the Beekeeping Section, UNESP, Campus of Botucatu. Propolis was ground and 30% ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 mL with 70% ethanol), in the absence of bright light, at room temperature and with moderate shaking. After a week, extracts were filtered and the dry weight of the extracts was calculated (130 mg mL^{-1}) (Sforcin, Orsi and Bankova 2005). Propolis was diluted in RPMI 1640 culture medium (Cultilab, Campinas, SP, Brazil) containing L-glutamine (0.1 g L^{-1}), sodium bicarbonate (2.2 g L^{-1}), non-essential amino acids (10 mL L^{-1}) and supplemented with 10% fetal calf serum (complete medium). Specific dilutions were prepared for each assay in order to achieve different propolis (P) concentrations (P1: 0.2, P2: 1.0, P3: 2.0, P4: 5.0, P5: 10.0 and P6: 20.0 μg mL^{-1}). The same procedure was carried out with 70% ethanol (propolis solvent).

Chlorhexidine (CHX), 0.12% (Swerts, Costa and Fiorini 2005), was purchased from a local pharmacy, and was diluted in RPMI 1640 culture medium to obtain CHX1: 1.2, CHX2: 6.0, CHX3: 12.0, CHX4: 30.0, CHX5: 60.0 and CHX6: 120.0 μg mL^{-1}.

For the combination propolis/chlorhexidine (P/CHX), the concentrations were P/CHX1: 0.2/1.2, P/CHX2: 1.0/6.0, P/CHX3: 2.0/12.0, P/CHX4: 5.0/30.0, P/CHX5: 10.0/60.0 and P/CHX6: 20.0/120.0 μg mL^{-1}.

Healthy blood donors and isolation of human monocytes

Ten healthy blood donors (aged 20–40 years) from the University Hospital, School of Medicine, UNESP, were included in the present work, which was approved by the Ethics Committee of the School of Medicine (CEP 4077-2011). An informed consent was signed by all blood donors. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized (50 U mL^{-1} heparin) venous blood using Ficoll-Paque (density = 1.077) (GE Healthcare Bio-Sciences, Uppsala, Sweden). Briefly, 20 mL of heparinized blood was added to an equal volume of complete medium. Samples were added to 4 mL of Ficoll-Paque and centrifuged at 400 *g* for 30 min at room temperature. The interface layer of the PBMCs was taken and washed three times with RPMI medium at 300 *g* for 10 min. Cell viability, as determined by neutral red (0.02%) staining, was >95% in all experiments. Cells were resuspended at a final concentration of 1×10^6 monocytes mL^{-1} in complete medium. After 2 h, non-adherent cells were discarded, and monocytes were incubated with the stimuli for 18 h.

Cell viability by MTT assay

Cell viability was performed using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St Louis, MO, USA) colorimetric assay. Monocytes (1×10^6 cells mL^{-1}) were incubated with different concentrations of propolis, chlorhexidine and the combination P/CHX at 37°C and 5% CO_2, in a final volume of 500 μL. Control cells were incubated only with culture medium. After 18 h, the culture medium was removed and 300 μL of MTT (1 mg mL^{-1}) in complete RPMI was added to the culture cells for 3 h. Afterwards, MTT was taken and 200 μL of dimethylsulfoxide (DMSO) was added to dissolve the formazan salt. Optical densities (OD) were read at 540 nm in an enzyme-linked immunosorbent assay (ELISA) reader and the percentage of cell viability was calculated using the formula: [OD test/OD control] × 100. Assays were carried out in duplicate.

Flow-cytometry analysis of TLR-4, HLA-DR, CD86 and CD80 expression by monocytes

The expression of TLR-4, HLA-DR, CD86 and CD80 by human monocytes was assessed using a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson, San Jose, CA, USA). PBMCs containing 1×10^6 cells mL^{-1} were incubated for 18 h at 37°C and 5% CO_2 with complete medium in the presence or absence of different concentrations of propolis, chlorhexidine and the combination P/CHX in polystyrene tubes for cytometric analysis (BD Labware, San Jose, CA, USA). Cells were washed and incubated with monoclonal antibodies (MAbs), according to the manufacturer's instructions: 0.5 μg mL^{-1} of phycoerythrin-CY7 (PE/Cy7)-labeled anti-CD14 (clone M5E2), 1 μg mL^{-1} of phycoerythrin (PE)-labeled anti-TLR-4 (clone HTA125) and 2 μg mL^{-1} of ALEXA Fluor 488-labeled anti-CD86 (clone IT2.2). In other tubes, PBMCs were incubated with 0.5 μg mL^{-1} of PE/Cy7-labeled anti-CD14, 0.5 μg mL^{-1} of FITC-labeled anti-HLA-DR (clone L243) and 1 μg mL^{-1} of PE-labeled anti-CD80 (clone 2D10). Antibodies were purchased from Biolegend (San Diego, CA, USA).

Stained cells were incubated for 30 min in the dark at 4°C, then washed and fixed with 5% paraformaldehyde in PBS. Background staining was determined from cells incubated with 2 μg mL^{-1} of PE/Cy7, FITC and PE-labeled control isotype antibodies. Cell samples were washed with complete medium and analyzed by flow cytometry. Thirty thousand monocyte events, defined as cells with respective side scatter (SSCs) and CD14 staining characteristics, and corresponding levels of fluorescence for TLR-4, HLA-DR, CD86 and CD80, were obtained for the gated $CD14^+$ cells. Results were expressed as percentage of positive CD14 cells of gated events.

NF-κB signaling pathway

Monocytes (1×10^6 cells mL^{-1}) were distributed into 24-well flat-bottomed plates and incubated with different concentrations of propolis, chlorhexidine, combination P/CHX, lipopolysaccharide (10 μg mL^{-1}) and medium alone as control for 45 min at 37°C and 5% CO_2. Then the cells were washed with PBS containing 10% endotoxin-free fetal bovine serum (FBS) on ice. Washed cells were then lysed using a nuclear extract kit (Cayman Chemical Company, Ann Arbor, MI, USA). A nuclear extract for each culture condition was obtained and stored at −80°C until further analysis. Afterwards, the nuclear portion of p65NF-κB was detected by ELISA using a transcription factor ELISA kit (Cayman Chemical Company), according to the manufacturer's instructions, and quantified at 450 nm with a reference wavelength of 655 nm.

Bactericidal activity of human monocytes

Monocytes (2×10^5 cells mL^{-1}) were treated with different concentrations of propolis, chlorhexidine and combination P/CHX for 18 h at 37°C and 5% CO_2. After incubation, stimuli were removed and cells were challenged with 500 μL of a *Streptococcus mutans* suspension (ATCC 25175) in complete medium containing 1×10^6 bacteria mL^{-1} (monocytes/bacteria ratio = 1:5) at 37°C in 5% CO_2. After 2 h, supernatants were collected and Triton X-100 (1%) was used to lyse monocytes, resulting in a final volume of 10 mL. Then, 10 μL was plated on brain–heart infusion (BHI) agar medium (Difco Laboratories, Detroit, MI, USA) in triplicate, and plates were incubated at 37°C. The number of colony-forming units (CFUs) per plate was counted after 48 h. Plates containing monocyte–bacteria cocultures were considered as experimental plates, and plates containing only bacteria were used as control plates. Bactericidal activity percentage (%) was determined by the following formula:

$$\%\,\text{bactericidal activity} = [1 - (\text{mean CFU of experimental cultures} / \text{mean CFU of control plates})] \times 100$$

Cytokine determination by enzyme-linked immunosorbent assay (ELISA)

To evaluate cytokine production, monocytes (1×10^6 cells mL^{-1}) were distributed into 24-well flat-bottomed plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) and incubated with different concentrations of propolis (0.2, 1.0 and 2.0 μg mL^{-1}), chlorhexidine (1.2, 6.0 and 12.0 μg mL^{-1}), combination P/CHX (0.2/1.2, 1.0/6.0, 2.0/12.0 μg mL^{-1}), lipopolysaccharide (LPS, 10 μg mL^{-1}) and only culture medium as control at 37°C and 5% CO_2. Afterwards, the supernatants were harvested for TNF-α, IL-6 and IL-10 measurement by ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, a 96-well flat bottom Maxisorp (Thermo Fisher Scientific, Waltham, MA, USA) was coated with capture antibody specific to each cytokine. The plate was washed and blocked before 100 μL of the supernatants and serially diluted specific standards were added to the respective wells. Following a series of washing, the cytokine was detected using the specific conjugated detection antibody. The substrate reagent was added into each well and, after color development, the plate was read at 450 nm, using an ELISA plate reader.

Statistical analysis

Data were analyzed with Graph Pad statistical software (Graph Pad Software, Inc., San Diego, CA, USA). Analysis of variance (ANOVA) and Dunnett method were employed. Data were expressed as mean ± standard deviation of 10 individuals. A P value of less than 0.05 was considered significant.

RESULTS

Monocyte viability

The effect of propolis, CHX and P/CHX on human monocyte viability was expressed in comparison to control (non-treated

cells). Propolis in all concentrations (0.2, 1.0, 2.0, 5.0, 10.0 and 20.0 μg mL^{-1}) did not affect cell viability (>98%) (Fig. 1A). Ethanol at 70% (in the same concentrations found in propolis) had no cytotoxic effects on monocytes (data not shown).

After incubation with the highest concentrations of chlorhexidine alone (30.0, 60.0 and 120.0 μg mL^{-1}) (Fig. 1B) and combined with propolis P/CHX (5.0/30.0, 10.0/60.0 and 20.0/120.0 μg mL^{-1}) (Fig. 1C), a significant decrease in monocyte viability was observed ($P < 0.0001$). Thus, the assays were carried out using only non-cytotoxic concentrations.

Effect of propolis, chlorhexidine and P/CHX on TLR-4, HLA-DR, CD86 and CD80 expression by monocytes

Propolis, CHX and P/CHX upregulated TLR-4 expression by human monocytes at all concentrations compared to control, although no difference was seen between them (Fig. 2A). The treatments had no effect on HLA-DR expression, since it was expressed in high percentage even in control (87.5%) (Fig. 2B).

With regard to the co-stimulatory molecules, propolis (at all concentrations) upregulated CD86 expression, whereas chlorhexidine and P/CHX had no effect on CD86 level. Also, propolis (0.2 and 2.0 μg mL^{-1}) induced a higher expression of CD86 compared to the combinations containing the respective concentrations of propolis ($P < 0.01$ and $P < 0.001$, respectively) (Fig. 2C). Unexpectedly, chlorhexidine with or without propolis downregulated CD80 expression by human monocytes, whereas propolis alone had no effects on its expression. Comparing the combinations with its components alone, only the 2.0 μg mL^{-1} concentration of propolis increased CD80 expression ($P < 0.001$) (Fig. 2D).

NF-κB signaling pathway

LPS upregulated the NF-κB activation, as expected. Compared to the control (medium alone), the incubation of human monocytes with propolis (0.2 and 2.0 μg mL^{-1}), chlorhexidine (6.0 and 12.0 μg mL^{-1}) and P/CHX (0.2/1.2 μg mL^{-1}) slightly increased the activation of this transcription factor ($P < 0.01$) (Fig. 3).

Bactericidal activity

Non-stimulated monocytes exhibited a bactericidal activity (24%) against *S. mutans*. This activity was increased after incubation with propolis, CHX and the combination P/CHX, although the highest bactericidal activity was seen after incubation with the combination at all concentrations (Fig. 4). P/CHX3 was significantly different from P3 but not from CHX3.

Cytokine production

Chlorhexidine (at all concentrations) increased TNF-α production compared to control. Propolis had no effect on TNF-α production; however, its combination with CHX prevented the increase induced by chlorhexidine ($P < 0.001$ and $P < 0.0001$) (Fig. 5A).

Regarding IL-10 production, although propolis did not affect the release of this cytokine, an increased IL-10 production was seen after incubation with the combination (at all concentrations) compared to its separate components ($P < 0.0001$) (Fig. 5B).

IL-6 levels were not altered after incubation with propolis (Fig. 5C), and its production was similar to control. On the other hand, CHX in combination with propolis (1.0/6.0 μg mL^{-1}) significantly inhibited the production of IL-6 compared to the same propolis concentration (1.0 μg mL^{-1}).

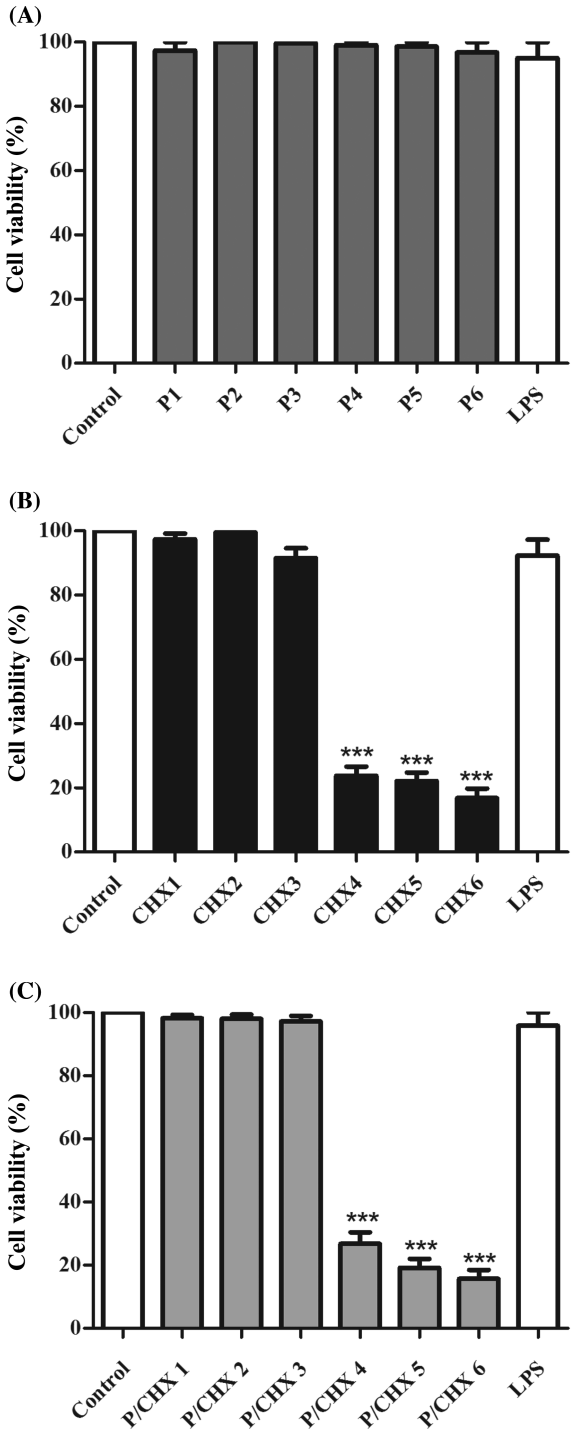

Figure 1. Viability (%) of human monocytes incubated with medium alone (control), (**A**) propolis P1 (0.2), P2 (1.0), P3 (2.0), P4 (5.0), P5 (10.0) and P6 (20.0 μg mL^{-1}), (**B**) chlorhexidine CHX1 (1.2), CHX2 (6.0), CHX3 (12.0), CHX4 (30.0), CHX5 (60.0) and CHX6 (120.0 μg mL^{-1}), (**C**) combination P/CHX1 (0.5/1.2), P/CHX2 (1.0/6.0), P/CHX3 (2.0/12.0), P/CHX4 (5.0/30.0), P/CHX5 (10.0/60.0) and P/CHX6 (20.0/120.0 μg mL^{-1}) and lipopolysaccharide (LPS, 10 μg mL^{-1}) for 18 h. ***$P < 0.0001$ *vs.* control (mean ± SD; $n = 10$ subjects).

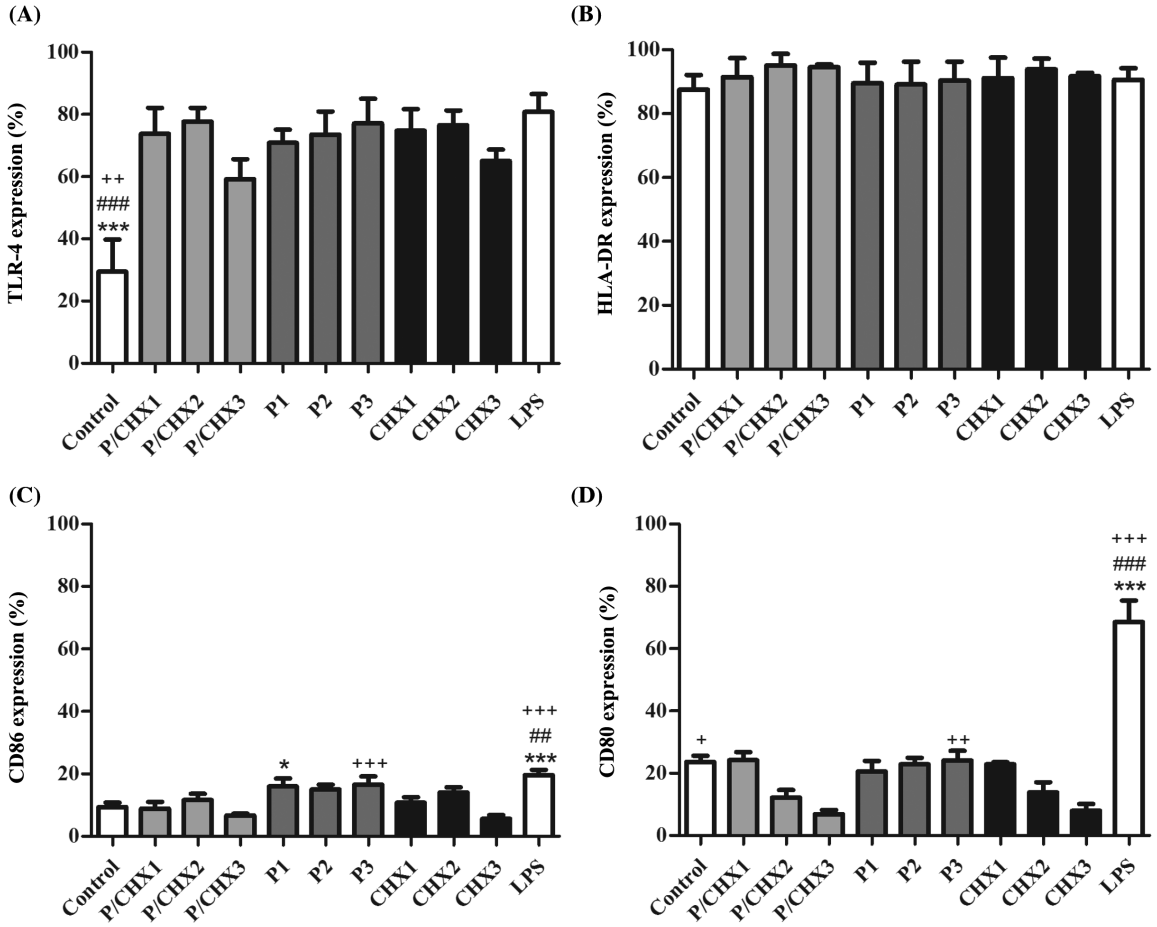

Figure 2. Percentage (%) of (**A**) TLR4, (**B**) HLA-DR, (**C**) CD86 and (**D**) CD80 expression of human monocytes incubated with medium alone (control), propolis P1 (0.2), P2 (1.0) and P3 (2.0 μg mL^{-1}), chlorhexidine CHX1 (1.2), CHX2 (6.0) and CHX3 (12.0 μg mL^{-1}), combination P/CHX1 (0.2/1.2), P/CHX2 (1.0/6.0) and P/CHX3 (2.0/12.0 μg mL^{-1}) and lipopolysaccharide (LPS, 10 μg/ml) for 18 h. The effects of P/CHX were compared to P and CHX alone, in the respective concentration. $^{*}P < 0.01$ *vs.* P/CHX1; $^{++}P < 0.001$, $^{+++}P < 0.0001$ *vs.* P/CHX3; $^{\#\#\#}P < 0.0001$ *vs.* P/CHX2 (mean ± SD; $n = 10$ subjects).

LPS (positive control) stimulated all cytokine production by human monocytes.

DISCUSSION

A propolis-containing mouthwash was investigated concerning its immunomodulatory action. First, propolis alone did not affect cell viability. These findings are in agreement with other works from our group evaluating the effects of propolis and its constituents on the viability of human monocytes (Conti *et al.* 2013; Búfalo *et al.* 2014; Búfalo and Sforcin 2015). Likewise, no cytotoxic effect was seen on peritoneal macrophages from BALB/c and C57BL/6 mice (Missima *et al.* 2009; Pagliarone *et al.* 2009; Orsatti *et al.* 2010). Regarding chlorhexidine effects, its highest concentrations affected cell viability (24%, 22% and 17%) even associated with propolis, leading to 27%, 19% and 16% viability. In an attempt to verify whether the cell viability affected by chlorhexidine concentrations could be due to the presence of distilled water, its effect alone on monocytes was investigated, and the viability remained unaltered (data not shown). Thus, one may conclude that the low viability of monocytes was exclusively due to chlorhexidine.

Several studies have reported that chlorhexidine may be both cytotoxic and genotoxic (Grassi *et al.* 2007; Lee *et al.* 2010). Bonacorsi, Raddi and Carlos (2004) observed a reduced viability of macrophages exposed to chlorhexidine after 1 h (using concentrations higher than 20 μg mL^{-1}) and 24 h (higher than 5 μg mL^{-1}). Li *et al.* (2014) observed a cytotoxic effect on RAW 264.7 cells after 1 h incubation with chlorhexidine 0.001%, with a maximum cytotoxic effect after 2 h, in a time- and dose-dependent manner. Thus, our experiments were carried out using only non-cytotoxic concentrations of the combination P/CHX.

During the triggering of the immune response, there is an increased expression of certain genes such as cytokines, chemokines, histocompatibility molecules, and important co-stimulatory molecules for T cell activation such as CD80 (B7-1) and CD86 (B7-2) (Swerts, Costa and Fiorini 2005). Here, the combination P/CHX enhanced TLR-4 expression by human monocytes compared to non-treated cells and propolis may be involved in this effect, since previous works from our group reported an increased TLR-4 expression by propolis-stimulated monocytes (Búfalo *et al.* 2014). Propolis-containing products diminished CD80 expression without affecting CD86 and HLA-DR expression. These results suggested that the combination did not affect either the antigen recognition by monocytes or the action of co-stimulatory molecules. A possible explanation could be the fact that the combination P/CHX increased IL-10 production, which inhibits the expression of co-stimulatory

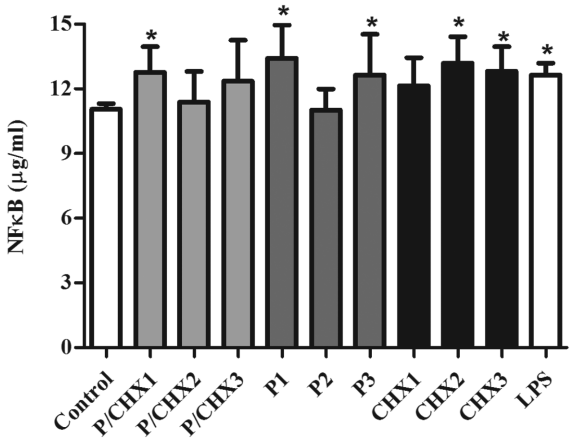

Figure 3. NF-κB quantification (μg mL^{-1}) by human monocytes incubated with medium alone (control), propolis P1 (0.2), P2 (1.0) and P3 (2.0 μg mL^{-1}), chlorhexidine CHX1 (1.2), CHX2 (6.0) and CHX3 (12.0 μg mL^{-1}), combination P/CHX1 (0.2/1.2), P/CHX2 (1.0/6.0) and P/CHX3 (2.0/12.0 μg mL^{-1}), and lipopolysaccharide (LPS, 10 μg mL^{-1}) for 45 min. $^{*}P < 0.01$ *vs.* control (mean ± SD; $n = 10$ subjects).

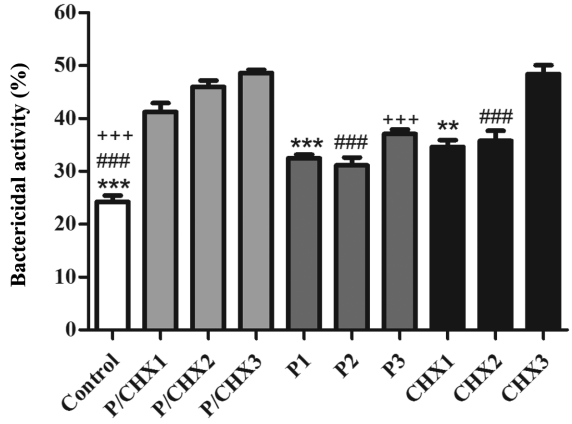

Figure 4. Percentage (%) of bactericidal activity of human monocytes incubated with medium alone (control), propolis P1 (0.2), P2 (1.0) and P3 (2.0 μg mL^{-1}), chlorhexidine CHX1 (1.2), CHX2 (6.0) and CHX3 (12.0 μg mL^{-1}), and combination P/CHX1 (0.2/1.2), P/CHX2 (1.0/6.0) and P/CHX3 (2.0/12.0 μg mL^{-1}) for 18 h and challenged with *Streptococcus mutans* for 2 h. The effects of P/CHX were compared to P and CHX alone, in the respective concentration. $^{**}P < 0.001$; $^{***}P < 0.0001$ *vs.* P/CHX1; $^{\#\#\#}P < 0.0001$ *vs.* P/CHX2; $^{+++}P < 0.0001$ *vs.* P/CHX3 (mean ± SD; $n = 10$ subjects).

molecules and class II histocompatibility molecules (Norkina *et al.* 2007).

The activation of TLRs can initiate multiple signaling pathways such as the transcription factor NF-κB, which controls the gene expression of several cytokines related to physiological or pathological processes (Kawai and Akira 2007). The combination P/CHX induced the activation of the NF-κB signaling pathway slightly, although its quantification was already high in control cells and similar to the LPS-induced one. Thus, although a significant increase was seen in treated cells, one may speculate that this increase was biologically discreet.

The bactericidal activity against *S. mutans* increased after incubation of monocytes with the combination P/CHX, although propolis and CHX alone induced it as well. Recent studies of our group with human monocytes revealed that propolis, as well as the isolates cinnamic acid and caffeic acid, increased the fungicidal activity of monocytes against *Candida albicans*

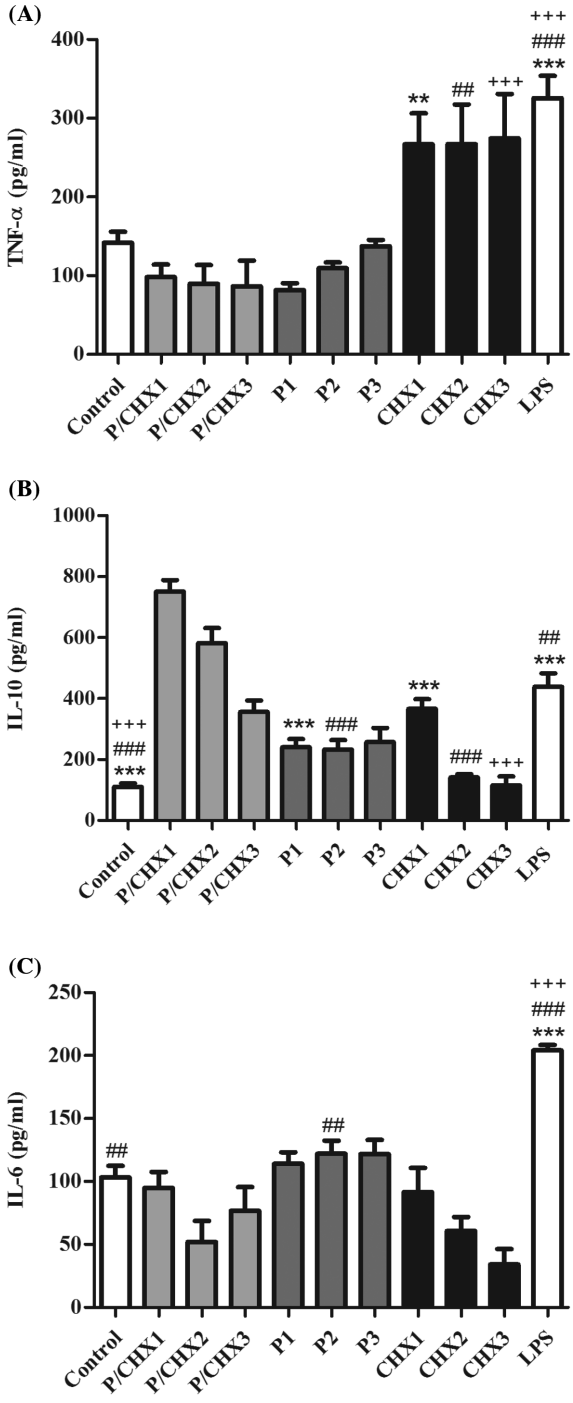

Figure 5. (**A**) TNF-α, (**B**) IL-10 and (**C**) IL-6 production (pg mL^{-1}) by human monocytes incubated with medium alone (control), propolis P1 (0.2), P2 (1.0) and P3 (2.0 μg mL^{-1}), chlorhexidine CHX1 (1.2), CHX2 (6.0) and CHX3 (12.0 μg mL^{-1}), combination P/CHX1 (0.2/1.2), P/CHX2 (1.0/6.0) and P/CHX3 (2.0/12.0 μg mL^{-1}), and lipopolysaccharide (LPS, 10 μg mL^{-1}) for 18 h. The effects of P/CHX were compared to P and CHX alone, in the respective concentration. $^{**}P < 0.001$, $^{***}P < 0.0001$ *vs.* P/CHX1; $^{\#\#}P < 0.001$, $^{\#\#\#}P < 0.0001$ *vs.* P/CHX2; $^{+++}P < 0.0001$ *vs.* P/CHX3 (mean ± SD; $n = 10$ subjects).

(Conti *et al.* 2013; Búfalo *et al.* 2014; Búfalo and Sforcin 2015). Our data are important, since there are no data in the literature investigating the combination P/CHX effects on human monocytes. Although the mechanisms by which our treatments increased the microbicidal activity of monocytes were not investigated, one may speculate that the combination P/CHX might upregulate the production of reactive oxygen species (ROS) by human monocytes. Propolis increased hydrogen peroxide (H_2O_2) production by murine peritoneal macrophages (Orsi *et al.* 2000), while no effect was seen incubating these cells with chlorhexidine (Bonacorsi, Raddi and Carlos 2004). However, both monocytes and macrophages can also destroy microorganisms by non-oxidative mechanisms, such as acidification of phagosomes, increased lysosomal content and interaction with the complement system components (Moonis, Ahmad and Bochhowot 1992). Thus, our findings open the possibility of further investigation in this research field, since the presence of propolis could exert a compensatory effect in combination with chlorhexidine most probably inducing ROS generation, which is desirable since this product increased the bactericidal effect of human monocytes using lower concentrations of chlorhexidine.

Our results showed that the combination P/CHX prevented the increase of TNF-α production induced by chlorhexidine in monocytes, suggesting a possible anti-inflammatory effect. TNF-α is a multifunctional cytokine, inducing other cytokine and chemokine production, the expression of adhesion molecules and the increased permeability of the endothelium, favoring monocyte arrival at the inflammation site (Hehlgans and Pfeffer 2005).

Although propolis did not affect the release of IL-10, there was an increase in the concentration of this cytokine when it was co-administered with chlorhexidine. IL-10 displays inhibitory effects on activated macrophages and dendritic cells, controlling innate and cell-mediated immunity. IL-10 inhibits the expression of co-stimulatory molecules and class II histocompatibility molecules, promotes the humoral immune response and inhibits the cellular immune response, decreasing the cytokine production of the Th1 profile, the proliferation of antigen-specific T cells, and the levels of pro-inflammatory cytokines such as TNF-α (Norkina *et al.* 2007). Since IL-10 exerts an anti-inflammatory action, our findings suggest that P/CHX may display an anti-inflammatory role, which may be beneficial in the treatment of periodontal diseases.

Propolis (P2) induced IL-6 production compared to P/CHX2, but chlorhexidine did not affect the production of this cytokine, irrespective of the presence of propolis. IL-6 plays an important role in the transition from innate to acquired immunity. In innate immunity, it acts as a stimulus for neutrophil production and synthesis of acute phase proteins. In addition to its chemoattractant role for monocytes, IL-6 supports the differentiation of monocytes to macrophages. After activation by APCs, T helper cells can differentiate into Th1, Th2, Th17 and regulatory T cells. IL-6 has been identified as a cytokine able to promote the differentiation of T cells in Th2 and Th17 (Scheller *et al.* 2011). Moreover, IL-6 acts on the differentiation of B lymphocytes into plasma cells (Yoon *et al.* 2009). There has been an increasing number of studies that demonstrate the ambiguity of IL-6 in possessing both anti-inflammatory and proinflammatory effects. IL-6 is involved not only in inflammatory and infectious processes, but also in the regulation of metabolic and regenerative processes (Scheller *et al.* 2011).

Our propolis sample has a chemically characterized composition (Conti, Bankova and Sforcin 2015) and the odontological product contains lower concentrations of chlorhexidine, diminishing side-effects. Taken together, the data indicate that the combination P/CHX inhibits TNF-α and IL-6 production and stimulates IL-10 production by human monocytes, suggesting its putative anti-inflammatory effects on the monocytes through the mucous membrane of the oral cavity, reinforcing its beneficial application as an odontological product to be used in the treatment of periodontal diseases. In addition, the bactericidal activity against *S. mutans* increased after incubation of monocytes with the combination P/CHX. One may verify that the odontological product exerted an important role, not only increasing the microbicidal activity but also for its anti-inflammatory effects.

CONCLUSION

In summary, propolis and chlorhexidine in combination may enhance the recognition of antigens by monocytes, mildly activate the transcription factor NF-κB and increase the bactericidal activity of human monocytes against *S. mutans*. The alterations in cytokine concentrations are in accord with an anti-inflammatory action, and this may be useful to treat periodontal diseases. These findings open new perspectives for further research, which indicates the practical application of our work and its possible use by the pharmaceutical industry.

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