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Research paper

Brazilian propolis promotes immunomodulation on human cells from American Tegumentar Leishmaniasis patients and healthy donors infected with *L. braziliensis*

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ABSTRACT

American Tegumentar Leishmaniasis (ATL) is an infectious disease caused by *Leishmania* parasites with ineffective treatment. The properties of propolis have been studied in different experimental studies, however, few works have investigated the effects of propolis on human-derived peripheral blood mononuclear cells (PBMC) in leishmaniasis models. Thus, we investigate the immunomodulatory effects of propolis treatment on PBMC from ATL patients and on PBMC from healthy donors infected with *Leishmania braziliensis*. Our data demonstrate that propolis pretreatment shows immunomodulatory effects on both healthy donors and ATL patients adherent cells, increasing IL-4 and IL-17 and decreasing IL-10, in either the presence or absence of the *L. braziliensis* infection, demonstrating that propolis contributes with the decrease of the inflammation and could also contribute with parasite control.

1. Introduction

American Tegumentar Leishmaniasis (ATL) is an infectious disease caused by parasites of the genus *Leishmania*, spread by sand fly vectors [1]. This disease can result in a spectrum of clinical manifestations ranging from ulcerative lesions in the skin, mucosal or as no ulcerated nodules [2]. The immune response from the host and the parasite species are the main responsible factors to determine the clinical disease [2].

This parasitic disease is a serious public health problem due to the worldwide distribution in temperate, tropical and subtropical regions. Additionally, the available drugs for treatment are ineffective, its uses are restricted and they possesses high side effects, contributing to the spread of the infection [3,4].

Several studies have demonstrated the importance of a host parasite interaction on leishmaniasis pathogenesis [5,6]. Furthermore, experimental models have shown that the outcome of *Leishmania* infection is dependent on the activation of $CD4^+$ T cell subsets [7,8].

Studies of *L. major* infection in susceptible and resistant mice has demonstrated that protective immune responses against *Leishmania* are linked to development of a Th1 response and IFNγ production. IFNγ trigger macrophage activation leading to upregulation of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) synthesis, promoting the elimination of intracellular parasites and control of disease. On the other hand, the disease progression has been associated with a development of Th2 responses and IL-4 production [5,8].

Besides Th1/Th2 profile, in recent years, Th17 response has been shown in infections caused by *Leishmania* spp. [9–11]. Th17 cells have proinflammatory properties and secrete primarily IL-17. The role of IL-17 during *Leishmania* infection remains controversial and poorly defined. In a model of cutaneous leishmaniasis

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caused by *Leishmania major*, IL-17 production is associated with disease progression [12]; however, in human *L. braziliensis* infections, the opposite holds true [10]. Moreover, higher level of IL-17 was found in the serum of asymptomatic individuals than in serum of *L. donovani* symptomatic patients [9].

Considering the characteristics of this disease and in the search of new drugs, propolis is a candidate because of its several biological properties described already (reviewed in [13]) highlighting its anti-inflammatory [14–16], immunomodulatory [17–19], wound healing and antileishmanial effect [20–25].

The major propolis components of our sample are phenolic compounds (flavonoids, aromatic acids and benzopyranes), di- and triterpenes and essential oils [18]. In the experimental leishmaniasis, propolis was able to kill promastigote and amastigote forms of several *Leishmania* species [20,22–27]. Besides that, propolis components, such as prenylated compounds and benzophenones [22] have already been associated with the inhibition of amastigote proliferation by macrophage activation, or by direct effect on promastigote forms (caffeic acid, p-coumaric acid, aromadendrine-4′-methyl ether, 3-prenyl-p-coumaric and 3,5-diprenyl-p-coumaric) [24].

Some works have reported that the propolis or some of its constituents suppresses inflammatory processes on human PBMC [28–30]. However, there are few studies focusing on the action of propolis on human PBMC in experimental leishmaniasis [31].

Therefore, the aim of this study was to evaluate the immunomodulatory effect of propolis and *L. braziliensis* on adherent cells from ATL patients and healthy donors.

2. Material and methods

2.1. Subjects

This study included patients with ATL (n = 14), diagnosed by indirect immunofluorescence assay from serum, biopsies and clinical parameters, and patients with negative serology for HIV, HBV and HCV at Clinical Hospital of Universidade Estadual de Londrina, Paraná-PR, Brazil. Healthy donors (n = 9) were selected according to similar demographic characteristics and from the same geographic area. Parameters such as age and gender were controlled. All control individuals did not present either clinical symptoms or laboratory parameters of inflammation and negative to the leishmania skin test or other autoimmune diseases.

The peripheral blood of the ATL patients and healthy donors was collected only once, before the beginning of the standard treatment, therefore all assays were conducted only once per donor in triplicate.

All donors and researchers signed a consent term for blood collection. This study was approved by the Human Ethics Committee of the Universidade Estadual de Londrina and followed all institutional guidelines (257/08 – May 22, 2009).

2.2. Hydroalcoholic extract of Brazilian propolis

The propolis sample was collected in the Beekeeping Section of Lageado Farm, Universidade Estadual Paulista (UNESP), Campus of Botucatu, SP, from colonies of *Apis mellifera L.* bees. The extraction method as well as the chemical composition of this sample had been documented in previous studies in which chemical analysis showed that major constituents of this sample are phenolic compounds, such as flavonoids, aromatic acids and benzopyrans, as well as di and tri terpenes and essential oils [14]. The final concentration of ethanol solvent in the experiments did not exceed 0.1%.

2.3. Leishmania braziliensis promastigotes

Leishmania (Viannia) braziliensis in promastigote forms (MHOM/BR/1987/M11272) were maintained in culture medium 199 (GIBCO Invitrogen®, Grand Island, USA), and supplemented with 10% fetal bovine serum (GIBCO Invitrogen®, Grand Island, USA), 10 mM of HEPES biological buffer (AMRESCO®, Solon, USA), 1% human urine, 1% L-glutamine (Synth®, Diadema, Brazil), penicillin and streptomycin (10 U/mL-10 μg/mL, GIBCO Invitrogen®, Grand Island, USA), and 10% sodium bicarbonate (Synth®, Diadema, Brazil). The cell cultures were grown on a BOD type incubator at 24 ± 25 °C in 25 cm^2 culture flask. In all experiments, there were used live promastigote forms in the stationary growth phase (5-day culture)

2.4. Human adherent cells culture and treatments

PBMC enriched monocytes obtained from the heparinized blood of ATL patients and healthy control subjects were separated on Ficoll-Hypaque (Sigma®) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), glutamine (0,1%, Invitrogen-Life Technologies) and penicillin-streptomycin (10 U/mL-10 μg/mL, GIBCO Invitrogen®, Grand Island, USA). Adherent cells (5×10^5 cells/well) were incubated in 24 well plates in the absence or presence of propolis at 5 and 25 μg/mL for 24 h at 37 °C and 5% CO_2. When indicated, after propolis treatment, adherent cells were challenged with viable promastigote forms of *Leishmania braziliensis* (5:1) for 2 h and the supernatants were collected, centrifuged to remove debris and kept at −76 °C until further analysis.

2.5. Cytokine determination

The Cytometric Bead Array Assay (CBA, BD Biosciences®) was used to measure the levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17 in serum or supernatants from adherent cells (patients and healthy controls) following the manufacturer's instructions.

2.6. Determination of nitric oxide levels

NO level was estimated as previously described [32], as total nitrite content in serum or supernatants of both ATL patients and healthy donors. In summary, both the treated and nontreated samples were deproteinized by adding $ZnSO_4$ (50 μL, 75 mM) and NaOH (70 μL, 55 mM). The mixture was centrifuged (10.000 rpm, 5 min). The supernatant was recovered and diluted in glycine buffer solution (45 g/L pH 9.7). It was added cadmium granules previously activated with $CuSO_4$ (5 mM) solution and left for 10 min at room temperature. After incubation, 50 μL of the supernatant aliquots were transferred to 96-well microplates and the same volume of Griess reagent was added. Calibration curve was prepared by dilution of $NaNO_2$ and the absorbances were measured at 550 nm in a microplate reader.

2.7. Statistical analysis

All experiments were conducted in triplicate. Statistical analysis was performed using the software GraphPad Prism 5.0 (Graph Pad, USA). Results were expressed as arithmetic means and standard errors of the means. Differences among the groups were assessed by Student's *t*-test or by One-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All data were checked using the Grubbs test (GraphPad Quickcalcs) to eliminate significant outliers ($p < 0.05$). A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Clinicopathological data of patients

Of the 14 patients available with ATL, 12 were women with age ranging between 33 to 71 years, with anti-*Leishmania* serology titles ranging from 1:40 to 1:320. The biopsies identified the presence of *Leishmania* parasites (15%) and inflammatory infiltrates (30%). In addition, clinical observations showed that 30% of patients were diagnosed with the ulcerous form of the disease, while 70% presented mixed forms (ulcer, crusting or scarring lesions). Furthermore, 40% of patients exhibited lesions in the lower limbs (Table 1).

3.2. NO profile of serum from healthy donors and ATL patients

Serum from both ATL patients and healthy donors was used to assess NO levels. Our results showed that NO production in ATL patients was 50% lower than the results from healthy donors (Fig. 1).

3.3. Effect of propolis extract on NO profile of adherent cells from healthy donors and ATL patients

The NO levels in supernatant of adherent cells culture from ATL patients was reduced in comparison to healthy donors. When the adherent cells culture from healthy donors was treated with propolis 5 μg/mL, the NO level reduced. In cells derived from ATL patients, the treatment with propolis (5 and 25 μg/mL) was not able to restore NO levels (Fig. 2).

3.4. Cytokine profile on adherent cells from healthy donors and ATL patients treated with Brazilian propolis

When analyzed the role of propolis in immunomodulation of adherent cells from ATL patients and healthy donors, initially we observed that there was no difference in the IL-4 levels in adherent cells from healthy donors and ATL patients, however the IL-10 concentration increased in cells from patients (Fig. 3). When examined the immunomodulatory role of propolis on adherent cells from donors (healthy and ATL patients), we found that only the concentration of 25 μg/mL was able to increase the IL-4 levels when compared with their respective controls and the group treated with 5 μg/mL (Fig. 3A). Regarding the IL-10 cytokine, we found that the treatment with 10 μg/mL maintained the high levels of this cytokine as the infected control, however the concentration of 25 μg/mL of propolis was able to reduce the concentration of IL-10 in adherent cells from ATL patients to similar levels found in healthy donors (Fig. 3B).

The *in vitro* treatment of adherent cells from healthy donors and ATL patients with propolis was not able to affect the levels of IL-2, TNF-α, IFN-γ and IL-17. IL-6 level was not altered in the ATL treated group (Supplementary figure).

3.5. Cytokine profile on adherent cells from healthy donors treated with Brazilian propolis and infected with L. braziliensis

On the other hand, when cells from healthy donors were infected with *Leishmania braziliensis*, we observed that the pretreatment with propolis was able to increase IL-6 cytokine levels (5 μg/mL) (Fig. 4A) and IL-17 (5 and 25 μg/mL) (Fig. 4C), but reduced the levels of IL-10 when compared to the infected control (Fig. 4B).

Table 1
Clinicopathological data of ATL patients. Patients diagnosed with American Tegumentar Leishmaniasis (by indirect immunofluorescence assay, biopsies and clinical parameters) from Clinical Hospital of the State University of Londrina, Brazil.

Parameter	Data
Total number of patients	14
Female gender	85%
Male gender	15%
Mean age in years (range)	51 (33–71)
Histopathological findings	15% with the parasite identified in the biopsy 30% inflammatory infiltrate presence
Mean titer of serology for Leishmaniasis	1:124
Type of lesions	30% ulcerous, 70% ulcer, crusting or scarring
Site of infection	40% lower limbs, 60% face and neck

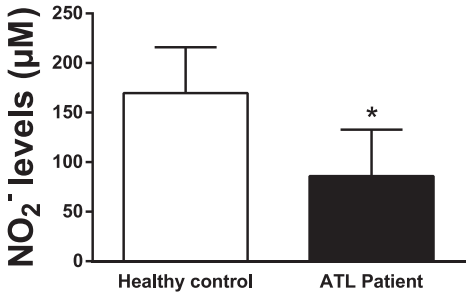

Fig. 1. Profile of NO from serum of healthy donors and ATL patients. The NO levels were evaluated in the serum of healthy donors (n = 9) and ATL patients (n = 14) by Cadmium-copper-Griess reaction. Results represent the mean ± SEM of experiments performed in triplicate. *Significantly different from untreated healthy donors control (Student's *t*-test, $p < 0.05$).

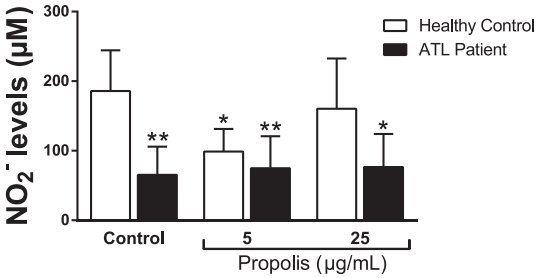

Fig. 2. NO profile of adherent cells from healthy donors and ATL patients treated with Brazilian propolis. The NO levels were evaluated in adherent cells from healthy donors (n = 9) and ATL patients (n = 14) with or without treatment with propolis 5 and 25 μg/mL for 24 h, by Cadmium-copper-Griess reaction. One-way ANOVA followed by Tukey test. Results represent the mean ± SEM of experiments performed in triplicate. Significantly different from untreated healthy donors control (*$p < 0.05$, **$p < 0.01$).

4. Discussion

Leishmania is a parasite able to down regulate the production of several inflammatory mediators including nitric oxide (NO). In fact, several studies have shown that this adjustment can occur either by suppressing iNOS via glycocalyx components, or by consuming the precursor of NO (L-arginine) in its own metabolism, directly influencing the levels of this molecule, resulting in permanent of this pathogen in the host [33,34].

According to the literature, patients with ATL exhibit reduced levels of this mediator when compared to healthy donors. Consistent with the results found in the serum, we also observed reduced levels of NO in supernatant of adherent cells from ATL patients.

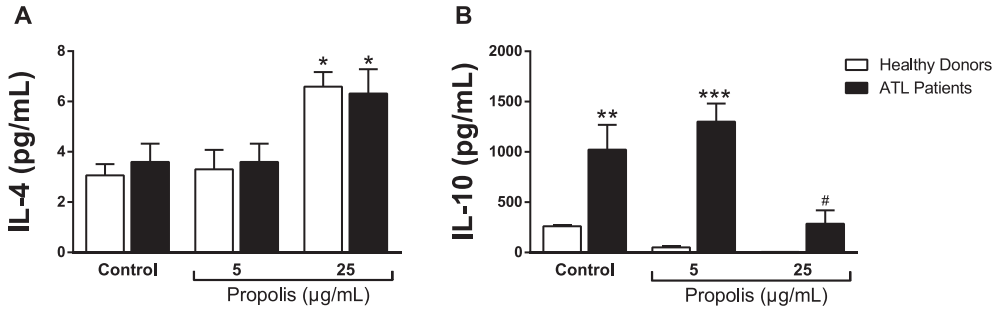

Fig. 3. Effect of Brazilian propolis extract on cytokine profile of adherent cells from healthy donors and ATL patients. IL-4 (A), IL-10 (B) were evaluated in the supernatant of healthy donors (n = 9) and ATL patients (n = 14) adherent cells after treatment with propolis 5 and 25 µg/mL for 24 h, by CBA. One-way ANOVA followed by Tukey test. Results represent the mean ± SEM of experiments performed in triplicate. Significantly different from untreated healthy donors control, ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Significantly different from ATL control ($^{\#}p < 0.001$).

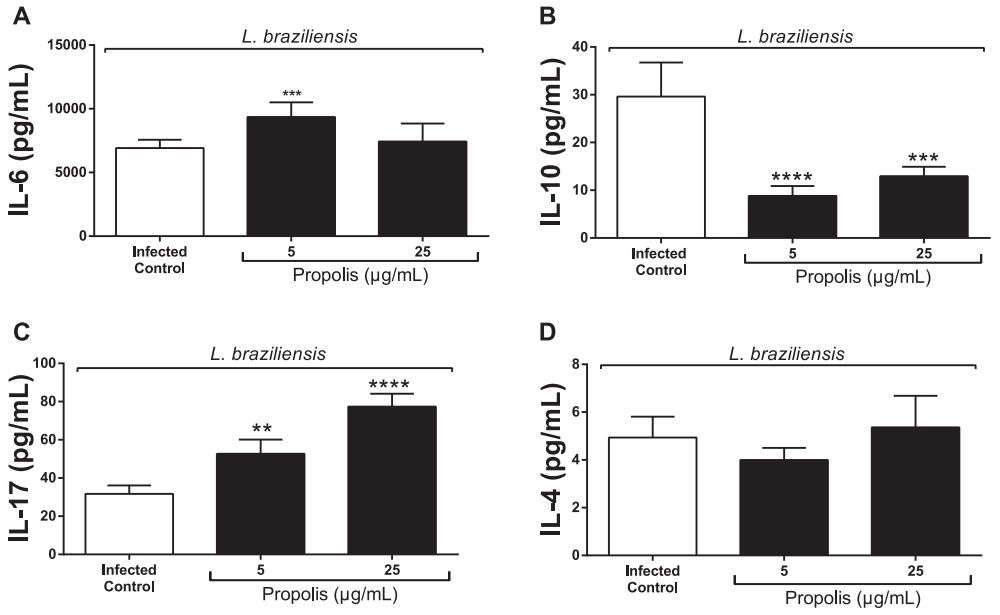

Fig. 4. Effect of propolis extract on cytokine profile on adherent cells from healthy donors treated with Brazilian propolis and infected with *L. braziliensis*. IL-6 (A), IL-17 (B), IL-10 (C) were evaluated in adherent cells supernatant after treatment with propolis 5 and 25 µg/mL for 24 h an posterior infection with *L. braziliensis* for 2 h. One-way ANOVA followed by Tukey test. Results represent the mean ± SEM of 9 independent experiments performed in triplicate. Significantly different from untreated healthy donors control ($^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

Although NO is considered the most relevant microbicidal molecule, once the disease is installed, the presence of this molecule associated with intense activation of many other defense mechanisms, result in a strong inflammatory response, increasing tissue damage and exacerbation of the injury [35,36].

As stated by [37], the development or discovery of drugs that can act on the pathogen's metabolism and also have an effect on the immune component should be subject of several studies.

It is known that propolis has been widely used in folk medicine, showing promising results in several disease models [17–19] and has a great potential to display a protective immune response against leishmaniasis [20,21,38]. We decided to evaluate its activity in adherent cells from healthy donors and ATL patients, and found that the treatment was not able to restore the levels of NO in the ATL group.

These results are explained by the fact that propolis by itself produces an anti-inflammatory effect through the inhibition of NO production [17,39], as we previously demonstrated.

Although propolis act in inhibiting the synthesis of NO, an important molecule for microbicidal host, it presents direct leishmanicidal [20,38] and immunomodulatory effect on experimental *Leishmania*-infection model [20,21,38].

When evaluating the immunomodulatory activity of propolis on adherent cells, we found that IL-4 levels increased significantly (25 µg/mL), whereas the IL-10 production reduced (25 µg/mL). Subsequently, we verified whether the immunomodulation induced by propolis remains in adherent cells after infection with *L. braziliensis*. In cells treated with propolis, the infection was not able to reduce IL-17 levels (5 and 25 µg/mL) and IL-6 (5 µg/mL), while treatment with propolis maintained IL-10 levels reduced even after infection (5 and 25 µg/mL).

Although IL-4 is responsible for parasite resistance inside the infected cells [40] this is an important cytokine in the control of exacerbated inflammation, which occurs in ATL patients lesions [41]. Yao (2005) [42] demonstrated that the presence of IL-4 is responsible for inhibiting IL-10 production, a cytokine which is directly related to the progression of the leishmaniasis [43–45]. It is also known that combination of both cytokines are able to act in controlling inflammation (IL-10) and promotes fibrosis progression (IL-4), by stimulating the synthesis of types I and III collagen [46–49].

IL-6 is a pleiotropic cytokine with several functions in the immune response, including regulating Th17 response [50]. Th17 cells are responsible for synthesising various cytokines, including IL-17. Although it has been demonstrated that this cytokine could induce tissue damage and progression of the injury [51,52], it may also be important in the protection and clearance of the parasite [10,11,53]. Our results corroborate with Born's et al. study, which demonstrated that the IL-17 protects against *Leishmania* infection and is associated with downregulation of regulatory T cells and IL-10 production, benefitting the Th1 response and improving the leishmanicidal activity of macrophages.

5. Conclusion

Altogether, the data shows that propolis didn't restore NO levels on ATL patients adherent cells. However, propolis pretreatment shows immunomodulatory effects on both healthy donors and ATL patients adherent cells, increasing IL-4 and IL-17 and decreasing IL-10, in either the presence or absence of the *L. braziliensis* infection, demonstrating that propolis contributes with the decrease of the inflammation and could also contribute with parasite control.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cellimm.2016.09.014.

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