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Research Paper

Aspergillus fumigatus conidia stimulate lung epithelial cells (TC-1 JHU-1) to produce IL-12, IFN γ , IL-13 and IL-17 cytokines: Modulatory effect of propolis extract

Effet de la propolis sur les cytokines induites par Aspergillus fumigatus

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ARTICLE INFO

Article history:

Received 24 January 2018

Accepted 25 September 2018

Available online xxx

Keywords:

Aspergillus fumigatus

Propolis

Lung epithelial cells

IL-13

IL-17

IL-12

IFN γ

ABSTRACT

Aspergillus fumigatus conidia are the most prevalent indoors fungal allergens. The interaction between *Aspergillus* antigens and lung epithelial cells (LECs) result in innate immune functions. The association between *Aspergillus* conidia and allergic reactions, like allergic bronchopulmonary aspergillosis (ABPA) and asthma have been repeatedly reported. Since conventional therapies for allergy and asthma are limited, finding new promising treatments are inevitable. This study was designed to evaluate the effect of *A. fumigatus* conidia on IL-12, IFN γ , IL-13 and IL-17 release from mouse LECs and to investigate the effect of propolis on cytokines modulation. Cells were divided to two groups, one was exposed to 3×10^4 conidia of *Aspergillus fumigatus* and another group was treated by propolis (25 μ g/mL) as well as exposed to *A. fumigatus* conidia. Cytokines IL-13, IL-12, IFN γ and IL-17 were measured at times 0, 6 and 12 hours after exposure using ELISA assay. The results indicated that *A. fumigatus* could increase the release of the cytokines with IL-13 and IL-17 being the most affected ones whilst treatment with propolis decreased the effects of *A. fumigatus* on IL-13 and IL-17 production. The results showed that propolis has down regulatory effects on Th2 cytokine, IL-13, and IL-17 production, whereas it caused a significant induction of IL-12, as an important Th1 cytokines by LECs. With respect to the obtained results, propolis extract might be contributed to decrease Th2 responses in allergic asthma phenomenon. However more investigations must be done in future to fully understand its efficacy.

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1. Introduction

Allergy is hypersensitive response of immune system to inhaled or ingested proteins, or allergens [1]. Fungi are found everywhere. They produce airborne conidia that are found in outdoor and indoor air in important amounts [2]. Most fungi are considered allergenic [3]. *Aspergillus spp* are among allergens that can aggravate asthmatic responses [4]. *Aspergillus* conidia are mainly found indoor and are associated with asthma [5]. Major characteristics of *Aspergillus*-induced allergy and asthma include Th2 cytokine generation, increase in IgE and IgG levels, eosinophilia, airway hyper-responsiveness and airway remodeling.

Allergic disorders caused by *Aspergillus spp* include asthma, allergic bronchopulmonary aspergillosis (ABPA) and allergic *Aspergillus* sinusitis [6,7]. The relationship between *Aspergillus* and asthma is not fully understood yet [7]. However it is believed that indoor exposure to airborne fungal conidia is related to development and exaggeration of asthma [6].

Airways epithelial cells are the first line of defense against pathogens. They produce cytokines including IL-25, IL-33, and IL-17 [8] Kao et al., 2004 showed that IL-17 stimulates production of hBD-2 from lung epithelial cells however they did not indicated that whether this cytokine is secreted from lung epithelial cells [9]. Conventional therapies for allergy and allergic asthma including corticosteroids remove symptoms temporary and have many known side effects such as immunosuppression [10]. Immunotherapy for allergy and asthma has limitations as well [11,12]. Thus finding new therapeutics is necessary. Propolis is a

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natural product derived from honey bees. Propolis is composed of 50% resin and balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, and 5% other substances. It has lots of properties including anti-inflammatory. It is shown that propolis could inhibit acute and chronic inflammation [13–16]. Therefore it could be a potential product for allergy and asthma treatment. This study was done with the purpose to study the effect of *Aspergillus* conidia on LECs' cytokine production as the preventive effect of propolis on allergy induction.

2. Materials and methods

2.1. Cell culture

Mouse lung epithelial cells (TC-1 JHU-1) were used in this study. Cells were purchased from Pasteur institute, Tehran, Iran. Cells were cultured on DMEM medium supplemented with 5% FBS and penicillin/streptomycin 100 IU/mL and incubated at 37 °C, 5% CO₂. Cells concentration was adjusted to 3×10^5 cells/mL and were incubated overnight in 6well plates before tests. The treatment groups were cells treated by 25 µg/mL propolis alone; cells treated with propolis and exposed to *A. fumigatus* conidia; Cells exposed to *A. fumigatus* conidia.

2.2. Preparation of *Aspergillus fumigatus* conidia

Aspergillus fumigatus (ATCC 204305) was cultured on sabouraud's glucose agar at 28 °C for 5 days. Conidia were harvested by washing culture with PBS containing 0.5% tween 20. Conidia suspension was then centrifuged at 1,000 g for 5 min and resuspended in PBS solution containing 0.1% tween 20. Number of conidia was evaluated using a haemocytometer and a concentration of 3×10^4 cells/mL was made. This concentration was added to LEC cultures along with propolis extract.

2.3. Preparation of ethanolic extract of Propolis

The propolis sample was collected from hives of *Apis mellifera* L. bees of southern Iran (Alikhani Farm), packed in a sealed plastic bag and stored at 20 °C. The propolis (30 g) was ground, added to 90 mL of 95% ethanol in the absence of bright light at 25 °C and moderately shaken 10 min twice a day. After 10 days, the extract was filtered through a Whatman No. 4 filter paper. Then, the solvent was evaporated at 25 °C in sterile condition. The resulting dried matter was dissolved in phosphate buffer saline (PBS) solution (pH 6.2). Propolis was added to cells (25 µg/mL) simultaneously with *A. fumigatus* conidia. All experiments were done in three replicates.

Table 1
Chemical composition of propolis ethanolic extract obtained by GC-MS.

Peak number	Compound Name
Aromatic acids	
1	Benzoic acid
3	p-Methoxy-Cinnamic acid
10	3,4-Dimethoxy-Cinnamic acid
12	Ferulic acid
13	Isoferulic acid
14	4-Methoxy-Cinnamic acid
15	Caffeic acid
Aliphatic acids	
3	Succinic acid
12	Palmitic acid
16	Octadecadienoic acid
Sugars	
6	D-Fructose
7	Glucofuranose
9	Talose
10	Treitol Polycyclic aromatic hydrocarbons (PAH)
Polycyclic aromatic hydrocarbons	
8	7,14-Dihydro-dibenz[a,j]anthracene
37	(R)-(1)-2-Amino-20-(isopropylamino)-1,10-binaphthyl
33	1,3,6,7,8-Pentamethyl-5,6,7,8-tetrahydro-2,4(1H,3H)-pteridindione
39	syn-Phenanthro[9,10:10,20][2.2]metacyclophan-1-ene
43	2-Imino-5-phenyl-1,2-dihydro-3H-1,3,4-benzotriazepine Phenolics
Phenolic acids	
17	3-Methyl-3-butenyl isoferulate
19	2,6-Di-t-butyl-4-nitro-phenol (DBNP)
40	5-(Hydroxymethyl)resorcinol
Other compounds	
45	Methylindan
21	Phoratoxon sulfone
25	Benzazepin derivatives
34	Pentachlorobenzenamine
40	2,5-Bis(4-methylphenyl)-1,4-dithiin
5	Methylthiouracil
18	p-Toluidine
20	Pinostrobin chalcone
22	4-Methyl-6,7-dimethoxy-2H-1,3-benzothiazine
27	Chalcone
29	2,4-Bis(methylthio)-5,6-dihydro-8-methoxybenz[f]isoquinoline
31	9,10-Bis(7-deuterobicyclo[4.1.0]hept-7-yl)-9,10-dihydro-anthracene
32	2-1-(methylethyl)-8-oxo-1,2-dihydrofurano[2,3-H] 2H-chromene
41	Buxozine-C
42	Salbutamol
46	Delta 1-tetrahydrocannabinolic acid
47	Guaifenesin
23, 24, 26, 28, 30, 35, 36, 38, 44	Unknown components

All chemicals used, unless otherwise stated, were obtained from Merck Company (Darmstadt, Germany) [17].

2.4. Determination of propolis extract's chemical composition by GC-MS analysis

Chemical composition of the ethanolic extract was determined using gas chromatography mass spectrometry as described previously [18]. In brief, the column temperature was initially held at 100 °C for 2 min, then raised to 280 °C at a rate of 20 °C/min and held for 5 min. The total run-time was 16 min. The temperatures of the injector port and the interface were set at 250 and 280 °C, respectively. The carrier gas (helium) flow rate was 1.0 mL/min. The ionization energy was set at 70 eV. The mass spectra were collected by scanning from m/z 50 to m/z 550 at two second intervals. Various compounds present in propolis sample were identified by computer search on MS database libraries on the basis of mass spectral fragmentation.

2.5. Measurement of cytokines

Levels of IL-17, IL-13, IL-12 and IFN γ were measured at 0, 6 and 12 hrs in the supernatants of cultivated cells after exposure to Propolis/*A. fumigatus* conidia and *A. fumigatus* conidia along with propolis. Different wells were considered for each mentioned time and all experiments were done in three replications. Cytokines were measured using Enzyme-linked immunosorbent assay (ELISA, eBiosciences, USA). Briefly, a 96-well flat bottom was coated with capture antibody specific to each cytokine. The plate was washed and blocked before 100 μ L of the supernatants and serially diluted specific standards were added to the respective

wells. Following a series of washing, the captured cytokine was detected using the specific conjugated detection antibody. The chromogen/substrate reagent was added into each well, and after color development, the plate was read at 450 nm using an Elisa plate reader [17].

2.6. Statistical analysis

Data were analyzed using SPSS version 21. T-student and Chi² tests were used to compare data and *P* value less than 0.05 was considered significant.

3. Results

Table 1 shows the chemical composition of propolis ethanolic extract obtained by GC-MS. Our extract was mainly composed of phenolic acids followed by aromatic acids, aliphatic acids, sugars and polycyclic aromatic hydrocarbons. As it is shown in Fig. 1, measurement of understudy cytokines demonstrated that exposure to *Aspergillus* conidia increased production of IL-12, IFN γ , IL-17 and IL-13 after 6 hours. This increase was significant for IL-17, IL-13 and IFN γ ($P < 0.05$). However the production of IL-13, IL-12 and IFN γ decreased 12 hours after exposure and this decrease was significant for IL13 ($P < 0.05$). Addition of propolis (25 μ g/mL) to LEC cultures resulted in elevation of IL12 and IFN γ levels in comparison to cells exposed to *Aspergillus* conidia alone, although this increase was not significant between groups ($P > 0.05$). Propolis addition resulted in less increase in IL-17 and decrease in IL-13 levels than *Aspergillus* conidia alone treated cells. The decrease in IL-13 levels was significant in hours 6 and 12 after exposure in comparison to IL-13 induced by *Aspergillus* conidia

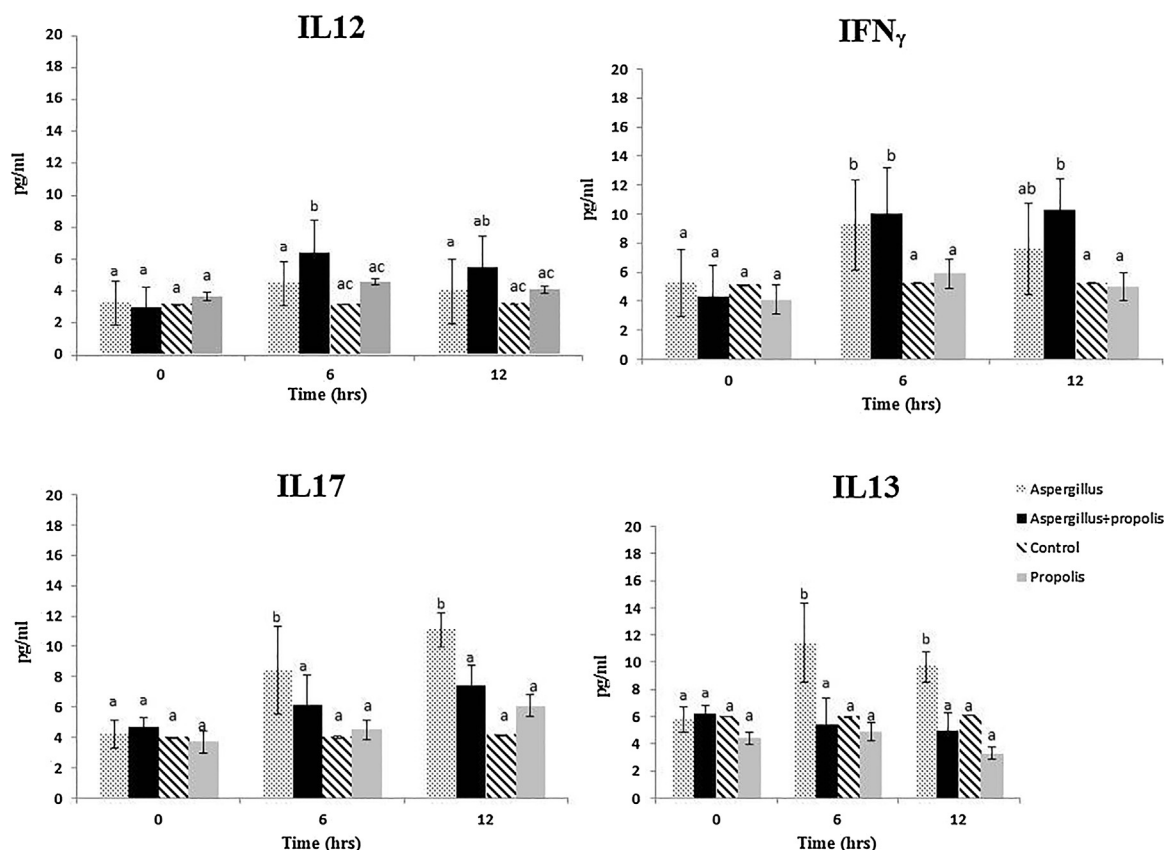


Fig. 1. Secretion of IL12, IFN γ , IL13 and IL17 by epithelial cells (TC-1 JHU-1) when exposed to live *A. fumigatus* conidia and after treatment with propolis (25 μ g/mL). Each experiment was performed in three replications. Different letters indicate significant differences between groups ($P < 0.05$).

($P < 0.05$). The decrease in IL-17 secretion was also significant comparing the cells exposed to propolis and *Aspergillus* conidia with cells exposed to *Aspergillus* conidia alone ($P < 0.05$).

4. Discussion

Aspergillus fumigatus is the most prevalent airborne fungal pathogen. Allergic responses to *Aspergillus* species play an important role in pathogenesis of asthma and cystic fibrosis [19]. Immune response to *Aspergillus* allergens include IgE elevation, increase in Th2 cytokines such as IL-4, IL-5 and IL-13, eosinophil infiltration, and profound airway remodeling [19–21]. The exposure of *Aspergillus* conidia with respiratory epithelium results in the release of proteins such as superoxide dismutases, catalases, proteases, ribotoxin and gliotoxin. These proteins affect pulmonary epithelium and macrophages and destroy epithelium integrity. This induces an inflammatory response and activation of Th2 cells [22,23].

In this study we investigated the effect of *A. fumigatus* conidia on release of IL-13, IL-12, IFN γ and IL-17 by LECs. Our results showed that 6 hours after exposure to *A. fumigatus* conidia release of all understudy cytokines increased by LECs but this increase was not significant for IL-12. IFN γ , which plays an important role in Macrophage activation, increased significantly 6 hours after exposure to *A. fumigatus* [24]. This result is in accordance with other investigators [24–28]. IFN γ has protective effect against aspergillosis [29]. IL-13 is a Th2 response cytokine which plays a role in allergic reactions to *Aspergillus* [30,31]. In our study stimulation of LEC with *Aspergillus* conidia caused to increase IL-13, 6 hours after exposure. IL-17 is a proinflammatory cytokine which is related to allergic responses. It contributes the production of inflammatory cytokines, such as IL-6, IL-1 β and TNF α ; and prostaglandins, which act in airway remodeling [32,33]. Our study indicates that *A. fumigatus* conidia stimulates LECs to significantly release IL17 6 & 12 hrs after exposure. Regarding the above mentioned points, *A. fumigatus* conidia contributes to play an important role in allergic phenomenon by enhancing IL-17 production.

Propolis is a natural product with antimicrobial, anti-inflammatory and anticancer properties [34]. The anti-inflammatory properties of propolis are mostly due to its flavonoids [35,36]. We studied the anti-inflammatory properties of propolis extract on LECs line (TC-1 JHU-1). During allergic responses, IL-33 induces the secretion pro-inflammatory mediators like IL-6, IL-1, and TNF α as well as Th2 cytokines such as IL5 and IL13 from different cell types [37,38]. In this study, treatment of LECs with ethanolic extract of propolis caused increase in IL-12 levels in cells' supernatants 6 hrs after treatment, but a reduction of this cytokine was observed at hour 12. The effect of propolis on LECs stimulation is not fully understood and more studies are necessary to describe these phenomena. IFN γ production was increased in propolis treated group but not significant to control group (time 0 after treatment). There was a significant reduction in IL13 level in LEC propolis treated group. This reduction is compatible with other studies in which propolis reduced secretion of IL-13 and IL-5 [39–41]. There was an increase in IL-17 levels 6 and 12 hours after cells treated by propolis, but this increase was not significant between different times. In comparison to cells exposed to *Aspergillus* conidia, IL-17 levels had decreased. This could be as a result of anti-inflammatory effect of propolis compounds [42,43].

5. Conclusion

According to our results, *Aspergillus fumigatus* conidia could induce Th2 cytokine profile in LEC; and propolis treatment caused

modulation of these responses. With respect to the obtained results, propolis extract might be contributed to decrease Th2 responses in allergic asthma phenomenon. However, more investigations must be done in future to fully understand its efficacy.

Disclosure of interest

The authors declare that they have no competing interest.

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