

Effect of ethanol extract of propolis (EEP) on interleukin 8 release by human gastric adenocarcinoma cells (AGS) infected with *Helicobacter pylori*

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Abstract

Mucosal chemokines are considered to be involved in the pathogenesis of *Helicobacter pylori* associated gastritis as well as gastric adenocarcinoma as a consequence of chronic inflammation process. Interleukin 8 (IL-8) produced by infected epithelial cells plays an important role in active inflammation by the induction of neutrophil migration and activation.

The aim of a study was to investigate the effect of ethanol extract of propolis from Poland (EEPP) on IL-8 release by *H. pylori* infected human gastric adenocarcinoma cells (AGS).

Materials and methods: The AGS line cells was incubated with bacteria (bacteria to cell ratio was 100 : 1) in the absence (control) or the presence of tested solutions of EEPP (7.5-60 µg/ml) for 16 hours. The concentrations of IL-8 were determined in culture supernatants by ELISA method.

Results: Incubation of AGS cells in presence of EEPP significantly suppressed IL-8 release in comparison with the control ($p < 0.01$). Obtained data has shown that IL-8 inhibition of EEPP was dose dependent. Additionally, 90 µg/ml of EEPP significantly inhibited growth of *H. pylori* ($p < 0.05$). These results indicate that EEPP preparations might partially inhibit *H. pylori*-induced gastric inflammation by decreasing IL-8 production.

Key words: propolis, *Helicobacter pylori*, interleukin 8, AGS cell line.

(Centr Eur J Immunol 2011; 36 (2): 65-69)

Introduction

The Gram-negative bacteria *Helicobacter pylori* are potent agents of gastritis and peptic ulcer [1-3]. Inflammatory response to this germ activates immunological system and due to infiltration of neutrophils, monocytes and lymphocytes into the gastric mucosa [4, 5]. Interleukin 8 (IL-8) produced by infected epithelial cells [6-10] is a key cytokine responsible for neutrophil migration from mucosal vessels into the gastric epithelium and their activation. Local production of inflammatory mediators by activated cells, not only pro-inflammatory chemokines but also reactive oxygen and nitrogen species, is likely to be responsible for mucosal damage.

Propolis, a resinous hive product collected by honeybees from various plants sources is known for anti-inflammatory and immunomodulatory activities [11-17]. Extracts of propolis show its hepatoprotective [18-20] and anti-oxidative effects both *in vivo* [21] and *in vitro* [22, 23]. Recent observations suggest that extracts of propolis might down-regulate inflammatory mediators gene expression, such as inducible nitric oxide synthase and IL-1 β genes [24, 25].

The aim of this study was to investigate the effect of ethanolic extracts of propolis from Poland on IL-8 release by AGS cells infected with *H. pylori*.

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Materials and methods

Ethanol extract of propolis

Polish propolis was collected manually in the bee-hive of the University's farm and kept desiccated pending its processing. Propolis was extracted in 95% (v/v) ethyl alcohol in a hermetically closed glass vessel for 4 days at 37°C, under occasional shaking. The ethanolic extract were then filtrated through a Whatman # 4 filter paper and evaporated under vacuum. Immediately prior to use, ethanol extract of propolis (EEP) samples were weighed, dissolved in Dimethylsulphoxide (DMSO; Sigma Chemical Company, St. Louis, MO, USA) and diluted with culture medium into appropriate concentrations. The final concentration of DMSO was adjusted to 0.2% (v/v). The control AGS cells and bacteria received the same amount of DMSO.

AGS cell culture

The human gastric adenocarcinoma cells (AGS) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in the atmosphere of 5% CO₂ at 37°C in F12K medium (Vitacell, ATCC, Manassas, VA, USA) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco BRL Life Technologies, Paisley, UK). The cells were cultured in 50 ml plastic flasks (Nunc A/S Roskilde, Denmark) and passaged 3 times a week (continuous cell culture). Cell viability was determined by Trypan blue (Sigma Chemical Company, St. Louis, MO, USA). The concentration of cell suspension used was 10⁵ cells/ml.

Bacterial culture

Helicobacter pylori (strain CCUG 17874) was obtained from Deutsche Sammlung von Microorganismen und Zellkulturen (Braunschweig, Germany). The bacteria were grown on Columbia agar plates (Bio Merieux, Marcy l'Etoile, France) supplemented with 5% sheep blood at 37°C under microaerophilic conditions (85% N₂, 10% CO₂, 5% O₂) generated with Genbox microaer (Bio Merieux) using an anaerobic chamber.

Antibacterial activity

Helicobacter pylori were suspended in brain-heart infusion (5 × 10⁵ colony forming units/ml) and 1 ml of aliquots was inoculated on Columbia agar plates with appropriate concentrations of EEP preparations (15-90 µg/ml). The control plates contained medium alone and medium plus DMSO. After 3 days of incubation at 37°C under microaerophilic conditions, bacteria were harvested and the growth inhibition was determined by an optical density method (OD at 595 nm) using a microplate reader Elx800 (Bio-Tek Instruments, Inc., Winooski, VT, USA).

Procedure of infection AGS cell lines incubated with different concentrations of EEP with *Helicobacter pylori*

AGS cells were suspended in antibiotics-free medium (5 × 10⁵ cells/ml), seeded in 24-well plates (Nunc A/S Roskilde, Denmark) and cultured to confluence. Then, monolayers were treated with increased concentrations of tested compounds of EEP (7.5, 15.0, 30.0 and 60.0 µg/ml) for 30 min and then infected with *H. pylori* (bacteria cell to AGS cell ratio of 100 : 1 in a 1 ml volume). A 2-days culture of *H. pylori* was used. After 16 hours at 37°C in 5% CO₂ the culture supernatants were collected after centrifugation and stored at -70°C until measurement. As a control cells not pretreated with EEP preparations were used.

Determination of interleukin-8 release

The concentration of IL-8 in culture supernatants was quantitated by ELISA method (R and D Systems Minneapolis, MN, USA), using human recombinant IL-8 as a standard, following the manufacturer protocol. The absorbance values were measured at 450 nm. The sensitivity of detection for this assay was less than 10 pg/ml.

Statistical analysis

All obtained values are expressed as means ± SD. Student unpaired t-test was used to assess the statistical significance of differences. A P value less than 0.05 was considered significant.

Results

Cytotoxicity and antibacterial activity of EEP

AGS cell viability and *H. pylori* growth were not affected by polish ethanol extract of propolis (EPPP) at the concentrations of between 7.5 and 60 µg/ml. A significant decrease of bacterial growth by a higher dose of EPPP (90 µg/ml) was observed (P < 0.05) (Fig. 1). The viability of infected AGS cells was greater than 95% in all performed experiments.

Effect of EPPP on interleukin-8 release

The ability of EPPP to demonstrate an influence on IL-8 synthesis by infected AGS cells was investigated. The concentrations of IL-8 in culture supernatants were measured by ELISA method. Uninfected AGS cells, after 16 hours incubation in medium alone, produced background levels of IL-8 (57.8 ± 8.0 pg/ml). Cell incubation with tested preparations of EPPP or DMSO (0.2%) did not increase IL-8 release. After cell infection with *H. pylori* the concentration of IL-8 markedly increased (894.6 ± 58.9 pg/ml). Pretreatment of infected cells with tested EPPP preparations significantly reduced IL-8 secretion in

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comparison with the control ($p < 0.01$) (Table 1, Fig. 2), and more, this effect of EEPP action was dose dependent.

Discussion

The aim of this study was to investigate the effect of ethanolic extracts of propolis from Poland on IL-8 release by AGS cells infected with *H. pylori*. These cells are useful gastric epithelial cell line *in vitro*, mimicking IL-8 secretion of normal mucosa stimulated by different damaging agents [26]. This study has shown that EEPP can potently reduce secretion of IL-8 by AGS cells. It is conceivable that the observed action of propolis might be due, at least in part, to the content of flavone derivatives and other flavonoids [23, 27-29], which are able to inhibit inflammatory mediators gene expression in activated cells [30, 31]. Interleukin-8 production is controlled by transcription factor NF- κ B [9, 10, 32]. This factor also regulates the expression of other pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6 [33]. NF- κ B is sequestered in the cytoplasm as an inactive complex with the inhibitory protein I κ B family. Upon induction by bacterial products its inhibitory subunit is phosphorylated and degraded. Released active subunit NF- κ B is translocated to the nucleus [33, 35, 36]. *Helicobacter pylori* infection greatly increases the number of cells containing active NF- κ B, and the activation occurs predominantly in cells of epithelium [34, 35]. Moreover, the epithelial NF- κ B activation correlated with neutrophil influx in *H. pylori*-associated gastritis [35] and with the histological degree of gastric inflammation [36]. Keats *et al.* reported that inhibition of NF- κ B potently decreased IL-8 release by infected epithelial cells [34]. Previous study revealed that propolis significantly suppressed NF- κ B activation in RAW 264.7 macrophages [24]. This action might be referred to caffeic acid phenethyl ester (CAPE), an active component of propolis. It has been reported that CAPE potently suppressed NF- κ B activation [37, 38]. Some other flavone derivatives are also able to prevent NF- κ B activation [39, 40]. Because of its key role in inflammation, NF- κ B might be a target for new types of anti-inflammatory treatment. Their blocking might prevent the early events in the inflammatory cascade, decreasing *H. pylori* induced gastric injury. The data obtained from this study has shown that EEPP possess anti-*H. pylori* activity. Previously it has been reported that propolis preparations are active against Gram-positive bacteria and less against Gram-negative bacteria [34, 41-43, 45]. This activity is dependent on the chemical composition of samples collected in different geographical areas. Antibacterial properties of propolis are mainly attributed to a significant part of flavonoids, phenolic acids and their esters. Our results obtained indicate that EEPP preparations might partially attenuate *H. pylori*-induced gastric inflammation by decreasing IL-8 synthesis and release. Furthermore, natural substances such as propolis might be suitable for medical purposes.

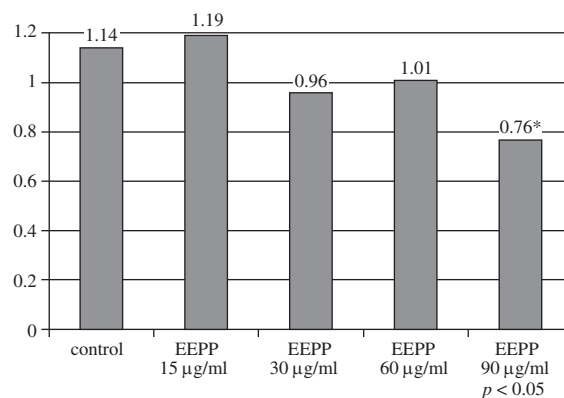


Fig. 1. Effect of ethanol extract of propolis from Poland (EEPP) on the growth of *Helicobacter pylori*. The optical density of bacterial cultures was determined after 72 hours of incubation in the absence (control) or presence of tested preparations (15-90 µg/ml EEPP) in microaerobial conditions at 37°C; * $p < 0.05$

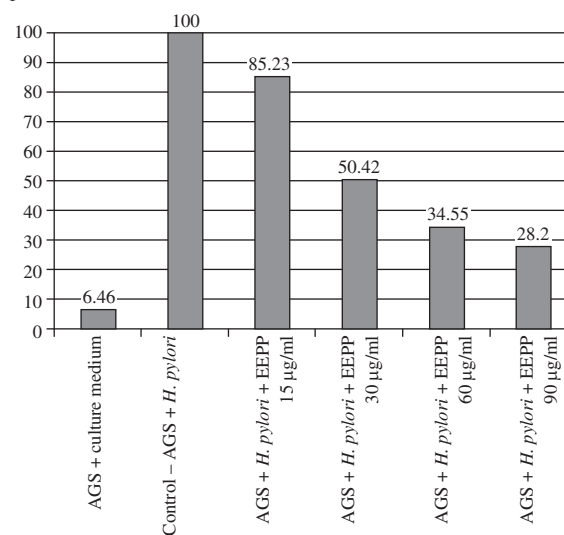


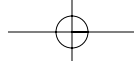
Fig. 2. Effect of ethanol extract of propolis from Poland (EEPP) on IL-8 release by AGS cells infected with *Helicobacter pylori*

Table 1. The effect of ethanol extract of propolis from Poland (EEPP) on IL-8 release by AGS cells infected with *Helicobacter pylori*

	IL-8 (pg/ml)	SD	P
AGS + culture medium	57.8	8.0	
Control			
AGS + <i>H. pylori</i>	894.6	58.9	
AGS + <i>H. pylori</i> + EEPP			
7.5 µg/ml	762.5	21.6	< 0.05
15.0 µg/ml	451.1	11.2	< 0.001
30.0 µg/ml	309.1	9.1	< 0.001
60.0 µg/ml	252.3	26.2	< 0.001

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