The Immunomodulatory Effect of Propolis on Foxp3 Gene Expression in Human Peripheral Blood Mononuclear Cells Stimulated in vitro with pseudomonas aeruginosa Ag.

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Abstract:
Immune balance during infection is critical for both supporting the body’s immune system’s defense and preventing an overly aggressive immune response. Foxp3, a transcription factor of regulatory T cells, plays critical roles in balancing the body’s immune system. Propolis has been shown to have an effect on Foxp3 expression. The aim of this study was to verify the effect of propolis extracts on in vitro Foxp3 gene expression in peripheral blood mononuclear cells (PBMCs) stimulated with P. aeruginosa Ag. In this study, a total of 20 apparently healthy volunteers were included, with 10 males and 10 females ranging in age from 20-40 year-olds. Five ml of blood were drawn from each participant in order to assess Foxp3 gene expression in PBMCs using density gradient lymphoprep and stimulated with pseudomonas aeruginosa LPS in vitro. The samples allocated into four distinct groups as follows; LPS stimulated PBMCs, Ethanol extracted propolis EEP+LPS stimulated PBMCs, water extracted propolis WEP+LPS stimulated PBMCs, and PBMCs – as a control group. The Foxp3 gene expression level were estimated, in all the four groups following a period of 48 h of cultivation, by Real time PCR technique uses SYBR green dye. Results of the study indicated that propolis had a great effect on the mRNA Foxp3 expression. Both ethanol extracted propolis and water extracted propolis had immunomodulatory effects through the Foxp3 mRNA expression, both the EEP and WEP could significantly inhibit Foxp3 mRNA gene expression by human PBMCs after stimulation with pseudomonas Ag in vitro. Propolis exhibits an immunoregulatory effect with the same effect with ethanol and water extracts on Foxp3 mRNA gene expression. 

Keywords: Immunomodulatory, Propolis, Foxp3, Gene expression

1. Introduction
Since regulatory T cells (Tregs) play a pivotal role in immunological homeostasis, several researches have been done to investigate their role during infection. During an infection, body requires managing the immunological responses that recognize and control the microbial attack,
therefore induction of Treg cells is a response of hosts to maintain or restore immunological homeostasis while preventing damage of tissues due to excessive immune responses [1]. Foxp3 expression as a transcription factor must be constant and continuous for Treg cell development and function [2]. Foxp3 deficiency affects Treg development and transcription factor induction. Foxp3 has the ability to transform CD4 + T cells into CD4 + CD25- CD25 + Treg cells. Propolis has been proven to have both pro-inflammatory and anti-inflammatory effects on the immune system [3]. Propolis, a natural chemical product of bees, contains polyphenol and flavonoids. Evidence suggests that flavonoids in propolis may be both medicinal and effective against bacterial infections such as pseudomonas aeruginosa [4]. FOXP3-expressing regulatory Tregs are implicated in the good attenuation of immunopathology, they are also engaged in the down-regulation of infection-fighting responses [5]. FOXP3 has been discovered as a good marker for suppressor cells and has been proven to play a direct function in causing immunosuppression. These cells were once assumed to be CD4+ CD25, high-level naturally occurring Tregs in humans, but more recent research has revealed that this is not the case, and FOXP3 is expressed in other cells (such as CD8+) with suppressor roles [6]. In autoimmune illnesses, in vivo Treg expansion appears to be a viable treatment option, with multiple trials demonstrating the efficacy of treatments such as IL-2 administration [7]. As a result, finding natural resource-based molecules that control Treg function is critical for avoiding autoimmune and pathogen-induced inflammatory disorders. Honey bees make propolis from a range of resinous plant secretions such as gums and resins, as well as some plant leaf buds. For decades, propolis has been utilized as a folk remedy for a variety of diseases due to its antibacterial, antioxidant, anti-inflammatory, and anticancer qualities. Propolis contains more than a hundred distinct ingredients [8]. The majority of the ethanolic extracts of green propolis are made up of cinnamic acid derivatives, flavonoids, and caffeoylquinic acid derivatives [9]. Propolis has an anti-inflammatory mechanism that involves a combination of Th1/Th2 balance [10], antileukotriene [11], antihistamine [12] and macrophage activity modulation [13]. Propolis' artemillin C, a main component, had a considerable effect on Tregs. This shows that propolis and artemillin C may have the ability to activate Treg cells [14]. The biological action of artemillin C has been demonstrated to suppress NF-κB on macrophages and boost cytotoxic activity on natural killer cells, resulting in a reduction in inflammatory responses [15]. The goal of this study was to see how propolis affected the expression of Foxp3 mRNA in PBMCs stimulated with Pseudomonas aeruginosa Ag.

2. Material & Methods
In this study a total of 20 apparently healthy volunteers consist of 10 males and 10 females, 20-40 year-olds were included. The information for each volunteer was noted including the name, sex and age of each volunteer. The blood samples were collected from the volunteers during December 2019 to April 2020.

2.1. Included and Excluded Criteria

The enrollment standards of volunteers in this study comprised any person who has apparently healthy. The excluded criteria included any person who had infection or disease.

2.2. Ethical Approval

Volunteers were asked permission prior to take any blood specimen. In addition, the study concept was accepted by the Research Ethical Committee at the College of Medicine / University of Babylon.

2.3. Samples preparation

Propolis sample collected from the hives of honey bees in Karbala City during spring season of 2020. Propolis samples cleaned and divided to small pieces then stored inside a clean container to prepare two types of propolis extract, water extracted propolis (WEP) and ethanol extracted propolis

2.4. Study design

2.4.1. Sample Processing

2.4.1.1. Ethanolic Extract of Propolis (EEP)

Ethanolic extract of the red propolis (EEP) were obtained using the methodology of Paviani et al, [16].

2.4.1.2. Water extracted propolis (WEP)

Water extracted propolis was prepared according to the methodology of [17].

2.4.2. Pseudomonas aeruginosa outer membrane isolation

Extraction of pseudomonas (OMPs) was carried out using the procedure of [18].

2.5. Blood samples processing

5 ml of blood were defibrinated by putting in anticoagulant tubes containing heparin to isolate the mononuclear cells of peripheral blood from whole blood cells by density gradient medium, the
blood samples were handled within one hour after blood drawing to ensure good separation and also high percentage of viability of isolated cells.

2.6. Isolation of PBMCs by lymphosep

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood using lymphoprep density gradient medium according to methodology of [19]

2.7. Culturing and Stimulation of PBMCs

After isolation of PBMCs from heparinized blood these cells were re-suspended at a final concentration of \((1 \times 10^6)\) cells mL\(^{-1}\) in RPMI 1640 complete medium and supplemented with 10% FBS and 5% penicillin and streptomycin and were culturing in 24 well tissue culture plate at 37ºC and 5% CO\(_2\) for 16 h then each sample were divided in to four groups, Ag stimulated PBMCs as a positive control, ethanol extracted propolis and Ag stimulated PBMCs, water extracted propolis and Ag stimulated PBMCs and the negative control group represented by PBMCs only. At each group, to 360 µl of isolated PBMCs in the first three groups 40 µl of \textit{pseudomonas aeruginosa} bacterial Ag were added, while 100µl of 5µg/ml EEP was added to the second group and the same volume and concentration of WEP was added to stimulate the third group for 48hrs.at 37ºC and 5% CO\(_2\).

2.8. RNA Isolation and Real-time RT-PCR

Total RNA from blood cells was prepared by using the Trizol reagent according to the manufacturer’s protocol (ZYMO RESEARCH). cDNA was synthesized with the first-strand cDNA synthesis kit and oligo (dT) primers (Fermentas, Hanover, MD), Primer sequences was FW- TTTA CTCGATGTTGGCCTACTT, RV- TCATTTCATCTA CGTCCACAC. PCR reaction used SYBR® Green PCR Master Mix (Macrogen) and GADPH gene was chosen as an internal standard, normalizing by GADPH preceding calculation of mRNA level.

2.9. Statistical Analysis

Calculation of the comparative data through Software of Statistical Package for the Social Sciences (SPSS), version 26.0, to explain the differences of study parameters between the four groups. Normality of data distribution was tested by the Kolmogorov–Smirnov test. The data were represented as medians with 25% and 75% inter quartile ranges (IQR) or means with standard deviations (SD). t-tests were used to compare two independent groups as appropriate. Kruskal–
Wallis (KW) tests were used to compare three or more independent groups where indicated. It is considered a significant difference when P value < 0.05.

3. Results

The median concentration of Foxp3 gene expression level were significantly increased in EEP+Ag 4.80 (2.78) as compared to control 2.25 (1.83), Ag 1.35 (2.30) and WEP+Ag 3.05 (2.32). Also the median concentration level of Foxp3 gene expression was significantly increased in WEP+Ag compared to control and Ag groups. Finally the median concentration of Foxp3 gene expression were significantly decreased in Ag versus control group (P ≤ 0.05), (Table 1) and (Figure1).

Table 1. Foxp3 gene expression in testing groups.

<table>
<thead>
<tr>
<th>P3</th>
<th>P2</th>
<th>P1</th>
<th>WEP+Ag n=20</th>
<th>EEP+Ag n=20</th>
<th>Ag n = 20</th>
<th>Control n = 20</th>
<th>Gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.74</td>
<td>&gt;</td>
<td>0.026 S</td>
<td>7.80 – 1.60</td>
<td>7.80 – 2.40</td>
<td>5.10 – 0.1</td>
<td>6.8 – 1.00</td>
<td>Range</td>
</tr>
<tr>
<td>0.06</td>
<td>0.001 S</td>
<td>&gt;</td>
<td>(2.32) 3.05</td>
<td>(2.78) 4.80</td>
<td>(2.30) 1.35</td>
<td>(1.83) 2.25</td>
<td>Median (IQR)</td>
</tr>
</tbody>
</table>

IQR: inter-quartile range; †: Kruskal Wallis test; ¥: HS: Highly significant at P ≤ 0.001; NS: not significant at P ≤ 0.05; P1: Control vs Ag; P2: Control vs EEP; P3: control vs WEP; P4: Ag vs EEP; P5: Ag vs WEP; P6: EEP VS WEP.
Figure 1. Distribution of groups with Ag, EEP+Ag, WEP+Ag and control groups according to the level of Foxp3 gene expression

3.1. Association between Gene expression and the gender

The results of present study indicated non-significant association between mean concentration of Foxp3 gene expression and the gender, although the mean of gene expression increased in male compared to female in control (2.84 ± 1.92) versus (2.74 ± 2.35), EEP (5.29 ± 1.62) versus (5.02 ± 1.8), and WEP (3.87 ± 2.05) versus (3.42 ± 1.47), but non-significant decreased in male versus female in Ag groups (1.81 ± 1.93) versus (1.85 ± 0.92), table (2).

Table 2. Relation between Foxp3 gene expression levels and the gender

<table>
<thead>
<tr>
<th></th>
<th>Female</th>
<th>Male</th>
<th>Gene expression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NS</strong></td>
<td>2.35 ± 2.74</td>
<td>1.92 ± 2.84</td>
<td><strong>Mean± SD</strong></td>
</tr>
<tr>
<td></td>
<td>4.80- 1.50</td>
<td>6.80- 1.00</td>
<td><strong>Range</strong></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10</td>
<td><strong>N</strong></td>
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</tbody>
</table>

Table (2)
<table>
<thead>
<tr>
<th></th>
<th>Mean± SD</th>
<th>Mean± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EEP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>† 0.953 NS</td>
<td>0.92 ± 1.85</td>
<td>1.93 ± 1.81</td>
</tr>
<tr>
<td>Range</td>
<td>3.90- 1.10</td>
<td>5.10- 0.10</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><strong>WEP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>† 0.729 NS</td>
<td>1.8 ± 5.02</td>
<td>1.62 ± 5.29</td>
</tr>
<tr>
<td>Range</td>
<td>7.80- 2.90</td>
<td>7.80- 2.40</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><strong>WEP</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>† 0.580 NS</td>
<td>1.47 ± 3.42</td>
<td>2.05 ± 3.87</td>
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<tr>
<td>Range</td>
<td>6.80 - 1.90</td>
<td>1.60-7.80</td>
</tr>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

4. Discussion

The recorded data from this research showed that the intervention groups that were given ethanol extracted propolis supplemented with Ag, and water extracted propolis supplemented with Ag showed an increment in the level of Foxp3 mRNA expression compared with the negative control group. While the positive control group that contains PBMCs which were stimulated with *Pseudomonas aeruginosa* Ag showed a dramatic reduction in the level of Foxp3 mRNA expression, this reduced expression level for Foxp3 mRNA was lower than the intervention groups. The action of pro-inflammatory cytokines found to correlate with the systemic Inflammatory Response Syndrome (SIRS) in response to pseudomonas aeruginosa bacteria and other gram-negative bacteria, such as an increase in interleukin-1, tumor necrosis factor Alpha (TNF-α), and interleukin-6 (IL-6) [20]. Foxp3 expression is required during bacterial infection to maintain immune homeostasis. The ability of propolis to increase TGF-β is being investigated as a potential mechanism for propolis to affect Foxp3 [21]. The systemic increase in TGF-β increasing the frequency of Treg cells, and its mechanism would involve the activation of Smad3 (pSmad3). Induced Smad3 initially binds to the Foxp3 enhancer site in intron 2 and interacts with nuclear factor-kB, NFATc2, and CREB, which would otherwise bind to the Foxp3 promoter [22]. The findings of this study were consistent with those of [23], who discovered that acute responses to bacterial Ag leading to a transient decrease in the frequency and total population of Treg cells, as well as Foxp3 gene expression. As response to Pseudomonas aeruginosa Ag, there was a partial loss of Treg cells, which are required for the onset of a potent Th1 response and host defense.
against this pathogen because an increase in Treg cells in this case resulted in a significantly increased susceptibility to this pathogen. In autoimmune diseases, the in vivo Treg expansion represents a promising therapeutic option, with several studies demonstrating that treatments such as IL-2 administration are effective [24]. As a result, discovering natural resource-derived compounds that modulate Treg cells function is critical for preventing autoimmune or pathogen-induced inflammatory diseases. Propolis is a natural substance made by bees from various resinous plant secretions such as gums and resins, as well as leaf buds of certain plants. Propolis has long used as a folk remedy for a wide range of ailments, with antimicrobial, antioxidant, anti-inflammatory, and antitumor properties. Propolis has been found to contain over a hundred different constituents [8]. The results of this study showed that an ethanolic extract of propolis had a significantly higher immunomodulation effect on Foxp3 mRNA gene expression in the stimulated PBMCs with bacterial antigen following a period of 48 h post pseudomonas aeruginosa Ag induction, compared with PBMCs treated with bacterial Ag alone. Propolis promoted Treg expansion and activation by increasing Foxp3 expression. Through Foxp3 expression, propolis and its constituents have the potential to activate Tregs. Foxp3 expression is required during bacterial infection to restore immune homeostasis. The ability of honey propolis to increase TGF- is being investigated as a potential mechanism by which propolis affects Foxp3 [25]. The evidence from this study revealed that the propolis could increase the Foxp3 mRNA expression. The data in this study simply indicate the potentiality of propolis to increase Foxp3 mRNA expression.

References:
