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Original Article

Immunomodulatory Effect of Propolis on Foxp3 Gene Expression in Human Peripheral Blood Mononuclear Cells Stimulated *in vitro* with Pseudomonas Aeruginosa Ag

Alwaeely, F. A[1 *], Madlum, K. N[1], Alsaadi, M. A[1]

1. College of Medicine, University of Babylon, Hilla, Iraq

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Corresponding Author: farahawaeely.89@gmail.com

Abstract

Immune balance during infection is critical for both supporting the defense of the immune system of the body and preventing an overly aggressive immune response. Foxp3, a transcription factor of regulatory T cells, plays a critical role in balancing the immune system of the body. Propolis has been shown to affect Foxp3 expression. This study aimed to verify the effect of propolis extracts on *in vitro* Foxp3 gene expression in peripheral blood mononuclear cells (PBMCs) stimulated with *Pseudomonas aeruginosa* Ag. In this study, a total of 20 apparently healthy volunteers were included, with 10 males and 10 females within the age range of 20-40 years old. Five ml of blood were drawn from each participant to assess Foxp3 gene expression in PBMCs using density gradient lymphoprep and stimulated with *P.aeruginosa* lipopolysaccharide (LPS) *in vitro*. The samples were divided into four distinct groups as follows: LPS stimulated PBMCs, ethanol-extracted propolis (EEP) + LPS stimulated PBMCs, and water-extracted propolis (WEP) + LPS stimulated PBMCs and PBMCs as the control group. The Foxp3 gene expression level was estimated in all four groups following a period of 48 h of cultivation by real-time polymerase chain reaction technique using SYBR green dye. Results of the study indicated that propolis had a great effect on the mRNA Foxp3 expression. Both EEP and WEP 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 exhibited an immunoregulatory effect which was the same with ethanol and water extracts on Foxp3 mRNA gene expression.

Keywords: Foxp3, Gene expression, Immunomodulatory, Propolis

1. Introduction

Since regulatory T cells (Tregs) play a pivotal role in immunological homeostasis, several studies have been performed to investigate their role during infection. During an infection, the body should manage the immunological responses that recognize and control the microbial attack; therefore, induction of Treg cells is the 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 and 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 role 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 (e.g., 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 anti-cancer qualities.

Propolis contains more than 100 distinct ingredients (8). The majority of the ethanolic extracts of green propolis are made up of cinnamic acid derivatives, flavonoids, and caffeoylquinic acids (CQA) derivatives (9). Propolis has an anti-inflammatory mechanism that involves a combination of Th1/Th2 balance (10), anti-leukotriene (11), and antihistamine (12), and macrophage activity modulation (13). Artepillin C is a main component of propolis and has a considerable effect on Tregs. This shows that propolis and artepillin C may have the ability to activate Treg cells (14). The biological action of artepillin 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). This study aimed to investigate how propolis affected the expression of Foxp3 mRNA in peripheral blood mononuclear cells (PBMCs) stimulated with *P. aeruginosa* Ag.

2. Material and Methods

This study was performed on 20 apparently healthy volunteers consisting of 10 males and 10 females within the age range of20-40 years old. The information for each volunteer was recorded including the name, gender, and age of each volunteer. The blood samples were collected from the volunteers from December 2019 to April 2020.

2.1. Included and Excluded Criteria

In this study, the inclusion criterion was being healthy. The exclusion criteria were having infections or diseases.

2.2. Samples Preparation

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

2.3. Study design

2.3.1. Sample Processing

2.3.1.1. Ethanolic Extract of Propolis

The EEP was obtained using the methodology of Paviani, Dariva (16).

2.3.1.2. Water Extracted Propolis

The WEP was prepared according to the methodology used by Contari (17).

2.3.2. Pseudomonas Aeruginosa Outer Membrane Isolation

Extraction of pseudomonas outer membrane proteins was carried out using the procedure used by Carlone, Thomas (18).

2.4. Blood Samples Processing

For the purposes of the study, 5 ml of blood was defibrinated by putting in anticoagulant tubes containing heparin to isolate the mononuclear cells of peripheral blood from the whole blood cells by density

gradient medium. The blood samples were handled within 1 h after drawing blood to ensure good separation and also a high percentage of viability of isolated cells.

2.5. Isolation of Peripheral Blood Mononuclear Cellsby Lymphosep

The PBMCs were isolated from heparinized venous blood using lymphoprep density gradient medium according to the methodology used by Böyum (19).

2.6. Culturing and Stimulation of Peripheral Blood Mononuclear Cells

After isolation of PBMCs from heparinized blood, these cells were re-suspended at a final concentration of 1×10^6 cells mL^{-1} in RPMI 1640 complete medium and supplemented with 10% fetal bovine serum and 5% penicillin and streptomycin. Afterward, they were cultured in 24-well tissue culture plates at 37ºC and 5% CO_2 for 16 h. Next, each sample was divided into four groups of Ag stimulated PBMCs as the positive control, EEP and Ag stimulated PBMCs, WEP and Ag stimulated PBMCs and the negative control group represented by PBMCs only. In the first three groups, 40 µl of *P. aeruginosa* bacterial Ag was added to 360 µl of the isolated PBMCs. Moreover, 100 µl 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 48 h at 37ºC and 5% CO_2.

2.7. RNA Isolation and Real-time Reverse Transcription-Polymerase Chain Reaction

Total RNA from blood cells was prepared by using the Trizol reagent according to the protocol of the manufacturer (ZYMO RESEARCH). The cDNA was synthesized with the first-strand cDNA synthesis kit and oligo (dT) primers (Fermentas, Hanover, MD). The primer sequences were FW-TTTA CTCGCATGTTCGCCTACTT and RV-TCAAATTCATCTA CGGTCCACAC. The polymerase chain reaction (PCR) reaction using SYBR® Green PCR Master Mix (Macrogen) and *GADPH* gene was chosen as an internal standard normalized by *GADPH* preceding calculation of mRNA level.

2.8. Statistical Analysis

The comparative data were analyzed in SPSS software (version 26.0) to explain the differences in 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% interquartile ranges or means with standard deviations. Moreover, t-test was used to compare two independent groups as appropriate. In addition, the Kruskal-Wallis test was used to compare three or more independent groups where indicated. It should be mentioned that a p-value of less than0.05 was considered statistically significant.

3. Results

The median concentration of Foxp3 gene expression level was significantly increased in EEP + Ag 4.80 (2.78), compared to the control 2.25 (1.83), Ag 1.35 (2.30), and WEP + Ag 3.05 (2.32). Moreover, the median concentration level of Foxp3 gene expression was significantly increased in WEP + Ag, compared to the control and Ag groups. Finally, the median concentration of Foxp3 gene expression significantly decreased in Ag in comparison with the control group ($P\leq0.05$) (Table 1and Figure1).

3.1. Association between Gene Expression and the Gender

The results of the present study indicated a non-significant association between the mean concentration of Foxp3 gene expression and gender. Accordingly, the mean value of gene expression increased in males, compared to females in the control group (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). Moreover, there was a non-significant decrease in males versus females in the Ag group (1.81±1.93 versus 1.85±0.92) (Table 2).

Table 1. Foxp3 gene expression in the study groups.

	Comparison				p-value		
Gene expression	**Control n=20**	**Ag n=20**	**EEP+Ag n=20**	**WEP+Ag n=20**	**P1**	**P2**	**P3**
Range	1.00-6.8	0.1-5.10	2.40-7.80	1.60-7.80	0.026S	0.001HS>	0.74 NS
Median (IQR)	2.25(1.83)	1.35(2.30)	4.80(2.78)	3.05(2.32)	P4	P5	P6
Total p-value			† 0.001> HS		0.001HS>	0.001S	0.006 S

IQR: interquartile 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.ethanol-extracted propolis (EEP); P3: control vs.water-extracted propolis (WEP); P4: Ag vs. EEP; P5: Ag vs. WEP; P6: EEP vs.WEP.

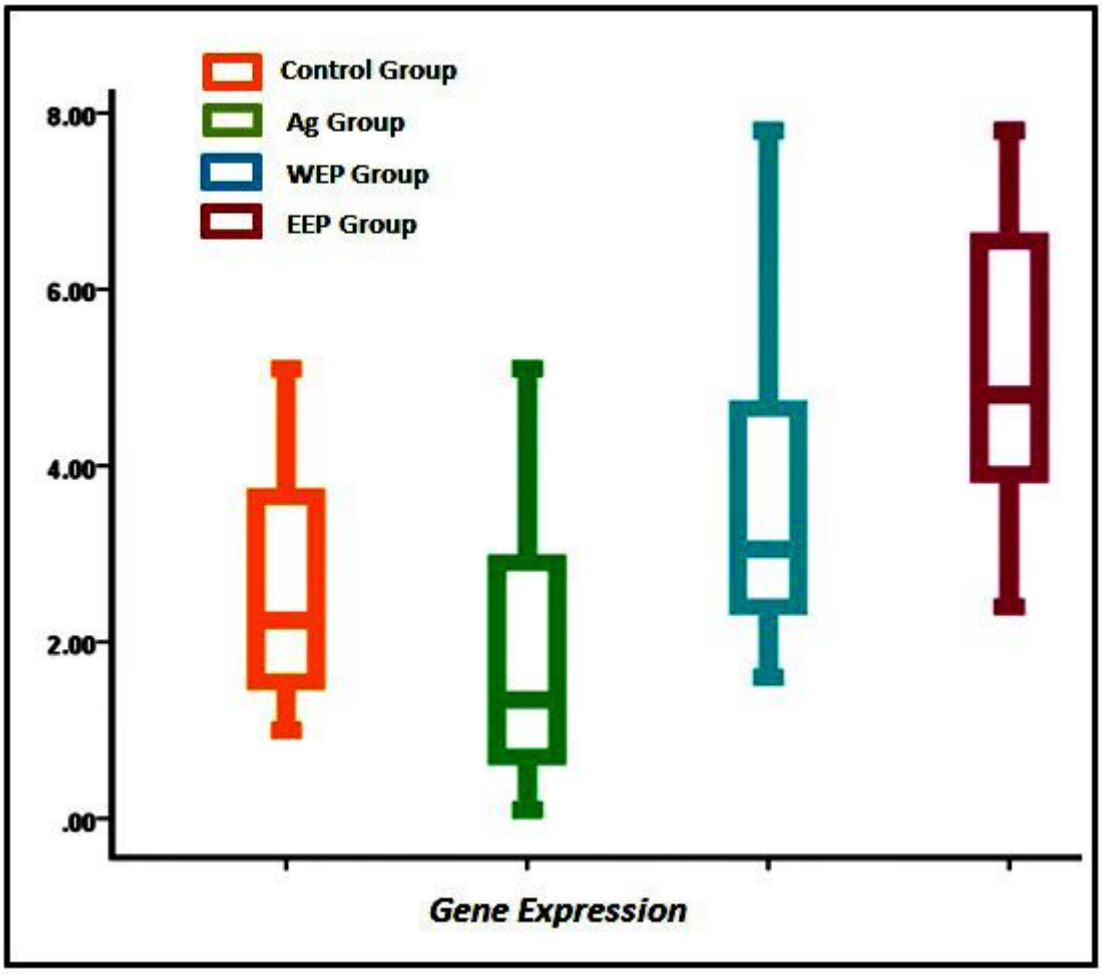

Figure 1. Distribution of groups with Ag, ethanol-extracted propolis (EEP)+Ag, ethanol-extracted propolis (WEP)+Ag, and control groups according to the level of Foxp3 gene expression.

Table 2. Relationship between Foxp3 gene expression levels and gender.

Gene expression	Male	Female	P
Control			
Mean±SD	2.84±1.92	2.74±2.35	
Range	1.00-6.80	1.50-4.80	0.891† NS
N	10	10	
Ag			
Mean±SD	1.93±1.81	1.85±0.92	
Range	0.10-5.10	1.10-3.90	0.953† NS
N	10	10	
EEP			
Mean±SD	5.29±1.62	5.02±1.8	
Range	2.40-7.80	2.90-7.80	0.729† NS
N	10	10	
WEP			
Mean±SD	3.87±2.05	3.42±1.47	
Range	1.60-7.80	1.90-6.80	0.580† NS
N	10	10	

NS: not significant; †: Kruskal-Wallis test

4. Discussion

The recorded data from this research showed that the intervention groups that were given EEP supplemented with Ag, and WEP supplemented with Ag showed an increment in the level of Foxp3 mRNA expression, compared to the negative control group. The positive control group that contains PBMCs which were stimulated with *P. aeruginosa* Ag showed a dramatic reduction in the level of Foxp3 mRNA expression. This reduced the expression level ofFoxp3 mRNA which was lower than that of the intervention groups.

The action of pro-inflammatory cytokines was found to correlate with the systemic inflammatory response syndrome in response to *P. 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). The 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 leads to an increase in the frequency of Treg cells and its mechanism would involve the activation of Smad3 (pSmad3).

The 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 the research performed by Benson, Murray (23), who discovered that acute responses to bacterial Ag lead to a transient decrease in the frequency and the total population of Treg cells, as well as Foxp3 gene expression. As a response to *P. 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. In this case, due to an increase in Treg cells, there was a significant increase in 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 (7). As a result, discovering natural resource-derived compounds that modulate the function of Treg cells 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 been used for a long time as a folk remedy for a wide range of ailments, with antimicrobial, antioxidant, anti-inflammatory, and antitumor properties. It has been found to contain over a hundred different constituents (8).

The results of this study showed that an EEP had a more significant immunomodulation effect on Foxp3 mRNA gene expression in the stimulated PBMCs with bacterial antigen 48 h after the *P. aeruginosa* Ag induction, compared to PBMCs treated with bacterial Ag alone. Propolis promoted Treg expansion and activation by increasing Foxp3 expression. Propolis and its constituents have the potential to activate Tregs through Foxp3 expression. Foxp3 expression is required during bacterial infection to restore immune homeostasis. The ability of honey propolis to increase TGF-β is being investigated as a potential mechanism by which propolis affects Foxp3 (24). The evidence from this study revealed that propolis could increase the Foxp3 mRNA expression. The data in this study simply indicated the potentiality of propolis to increase Foxp3 mRNA expression.

Authors' Contribution

Study concept and design: F. A. A. and K. N. M.
Acquisition of data: F. A. A.
Analysis and interpretation of data: F. A. A. and M. A. A.
Drafting of the manuscript: F. A. A.
Critical revision of the manuscript for important intellectual content: K. N. M.
Statistical analysis: F. A. A.
Administrative, technical, and material support: F. A. A.

Ethics

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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