AJCMI
Avicenna Journal of Clinical Microbiology and Infection
Avicenna J Clin Microbiol Infect, 2022; 9(3):115-118. doi:10.34172/ajcmi.2022.3399
http://ajcmi.umsha.ac.ir

Original Article

Royal Jelly Feeding as a Main Inducer of Bcl-2-associated X Protein in the Peripheral Blood Immune Cells of Patients With Chronic Hepatitis B

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Article history:
Received: July 21, 2022
Accepted: September 23, 2022
ePublished: September 29, 2022

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Abstract

Aim: Chemokines, cytokines, and their related molecules play crucial roles in the fight against the hepatitis B virus (HBV) and its related complications. Royal jelly (RJ) is considered an immunomodulatory factor for humans. This clinical trial study aimed to explore the RJ effects on the relative expression of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, BAX, and MAVS in chronic hepatitis B (CHB) patients.

Methods: The CHB patients were under one month of RJ treatment, 1 g/d. The relative expressions of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, BAX, and MAVS were evaluated using the real-time *polymerase chain reaction* (PCR) technique.

Results: RJ feeding significantly increased the expression of BAX in the peripheral blood immune cells of CHB patients. However, relative expressions of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, and MAVS were not altered following RJ feeding.

Conclusion: RJ can modulate immune responses via induction of homeostasis in the peripheral blood immune cells of CHB patients. Reduced inflammation following RJ feeding may be a result of homeostasis in the peripheral blood immune cells.

Keywords: Chronic hepatitis B, Royal jelly, Chemokine, Gene expression

Please cite this article as follows: Bahaaldin-Beygi M, Kariminik A, Kazemi Arababadi M. Royal jelly feeding as a main inducer of bcl-2-associated x protein in the peripheral blood immune cells of patients with chronic hepatitis b. Avicenna J Clin Microbiol Infect. 2022; 9(3):115-118. doi:10.34172/ajcmi.2022.3399

Introduction

Cytokines and chemokines are the main parts of the immune responses against the hepatitis B virus (HBV) (1). The molecules play several roles in the immune system, including chemotaxis, angiogenesis/stasis, activation of immune cells, and tissue remodeling (2). Among the chemokines, CC ligand (CCL) 2, 3, and 8, which are known as monocyte chemoattractant protein 1, macrophage inflammatory protein 1-alpha, and monocyte chemoattractant protein 2, respectively, play key roles in the chemotaxis and activation of macrophages against HBV (3, 4). Interferon-beta (IFN-β) is a member of interferons, a cytokine family that plays a key anti-viral role against HBV (5). The molecules also participate in HBV-related complications such as liver fibrosis and hepatocellular carcinoma (6,7). Additionally, intracellular viral sensors play key roles in the induction of chemokine and cytokine production. For example, mitochondrial antiviral-signaling protein (MAVS) is a target of a group of cytosolic proteins that detect the presence of the virus (8). Upon binding the cytosolic proteins to MAVS, the sensor will be activated, inducing the virally infected cell to secrete chemokines (8). Therefore, the environmental factors that affect the expression of the molecules during HBV infection can impact the disease compilations. In addition, it has been demonstrated that the Bcl-2-associated X (BAX) protein, NANOG, and octamer binding transcription factor 4 (OCT4) molecules participate in the survival of immune cells and prolonged responses to HBV (9,10). Accordingly, BAX can induce apoptosis, and both NANOG and OCT4 can induce the survival of the cells. Hence, increasing the BAX and decreasing the NANOG and OCT4 expressions are associated with homeostasis and decreased inflammatory responses during chronic hepatitis B (CHB). Therefore, the supplementary foods that alter the expression of the molecules can be considered plausible therapeutic strategies during CHB infection.

It has been reported that a natural product from

worker bees (*Apis mellifera*), royal jelly (RJ) (11), can be used as a dietary supplement (12). RJ plays potentially immunomodulatory roles in the immune system and also has several antimicrobial and antioxidant effects (12-16). Thus, it has been hypothesized that RJ may be useful to regulate immune responses and regulate the molecules involved in HBV-related liver complications. Accordingly, this study was designed to examine the effects of one month of RJ feeding on the relative expressions of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, BAX, and MAVS in the peripheral blood immune cells of CHB patients.

Materials and Methods

In this clinical trial study, relative expression of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, BAX, and MAVS were explored in 30 (13 men and 17 women) CHB patients who were referred to Samenal-Hojaj hospital in Kerman, Iran. They were under RJ feeding of 0.013 g/kg per day on an average of 1 g/d (17). The patients had normal ranges of liver enzymes (using the commercial kits from MAN Company); hence, they were not under anti-HBV therapy. The patients were entered into the study as the "Guide of Prevention and Treatment in Viral Hepatitis" (18). The patients with microbial co-infections, under treatment of antiviral and immunosuppressive drugs, history of liver disorders, mental disorders, and breastfeeding or pregnancy were excluded from the study. The patient's blood samples were collected, and the relative expressions of the molecules were evaluated just before and 1 month after RJ feeding (Pars Asal Company, Shiraz, Iran).

RNA Extraction and Complementary DNA Synthesizes

Total mRNA was extracted and converted to complementary DNA using commercial kits from Karmania Pars Gene Company, Kerman, Iran. Briefly, the peripheral blood immune cells were lysed, and then the RNA was precipitated and moved to the high absorbance column. After centrifugation, a wash buffer was used to wash the columns. Finally, the elution buffer was used to collect the total mRNA. The extracted mRNA (1 µg) was added to the 15 µL master mix and adjusted to 20 µL by RNase/DNase-free water, then it was incubated at 40°C for 60 minutes followed by 5 minutes at 70°C.

Real-Time Polymerase Chain Reaction

Relative expression of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, BAX, and MAVS was carried out using SYBR Green real-time polymerase chain reaction (PCR) technique. Consequently, a master mix was used in a Rotor-Gene Q *instrument using* the following program: 95°C for 3 minutes and then 40 cycles with 95°C for 15 seconds/60°C for 35 seconds, followed by a melting curve ranging from 60°C to 95°C (acquiring fluorescence data every 0.3°C). This master mix was obtained from Biosystem Company in association with specific primers (Table 1) and was designed by Primer 3 software. To verify specific amplification, in addition to the melting curve step during the run, the assay also confirmed the amplicon sizes by 1% agarose gel electrophoresis. Finally, the raw data were analyzed by the $2^{-\Delta\Delta Ct}$ formula using glyceraldehyde-3-phosphate dehydrogenase as a housekeeping gene.

Table 1. Primer Sequences Used in Real-time PCR Assays

Gene	Primer Sequences (5'-3')
CCL2	ATGAAAGTCTCTGCCGCCCTTCTGT AGTCTTCGGAGTTTGGGTTTGCTTG
CCL3	ATGCAGGTCTCCACTGCTGCCCTT GCACTCAGCTCCAGGTCGCTGACAT
CCL8	TATCCAGAGGCTGGAGAGCTAC TGGAATCCCTGACCCATCTCTC
IFN-β	CTTGGATTCCTACAAAGAAGCAGC TCCTCCTTCTGGAACTGCTGCA
NANOG	ATACCTCAGCCTCCAGCAGA GCTCCAGGTTGAATTGTTCC
OCT4	ATTCAGCCAAACGACCATCT TCTCCAGGTTGCCTCTCACT
BAX	CCAAGAAGCTGAGCGAGTGT CAGTTGAAGTTGCCGTCAGA
MAVS	AGCAAGAGACCAGGATCGAC GGGTATTGAAGAGATGCCAGAG
GAPDH	GGATTTGGTCGTATTGGG GGAAGATGGTGATGGGATT

Note. PCR: *Polymerase chain reaction;* CCL: CC Ligand; IFN-β: Interferon-beta; BAX: Bcl-2-associated X; OCT4: Octamer binding transcription factor; MAVS: Mitochondrial antiviral signaling.

Data Analysis and Statistical Methods

Data analysis was performed using SPSS software version 18 and the dependent paired *t* test to analyze mRNA levels of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, BAX, and MAVS before and after RJ feeding. *P* value was considered significant at < 0.05.

Results

The results indicated that feeding with RJ led to significant upregulation of BAX ($P = 0.003$). Accordingly, relative expressions of BAX before and after RJ feeding were 1.00 ± 0.43 and 3.84 ± 0.84, respectively. However, RJ feeding did not alter relative expression of CCL2 ($P = 0.160$), CCL3 ($P = 0.997$), CCL8 ($P = 0.237$), IFN-β ($P = 0.083$), NANOG ($P = 0.660$), OCT4 ($P = 0.519$), and MAVS ($P = 0.155$) in a significant manner. Figure 1 presents the relative expression of the molecules before and after RJ feeding in detail.

Discussion

The results demonstrated that RJ feeding led to significantly increased expression of BAX in the peripheral blood immune cells. However, RJ feeding was not associated with significant alteration in the expression of the chemokines, IFN-β, NANOG, OCT4, and MAVS. Since BAX plays a key role in the induction of apoptosis, upregulation of the molecule following RJ feeding demonstrated that RJ can induce homeostasis in the activated immune cells and modulate immune responses independent of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, and MAVS. To

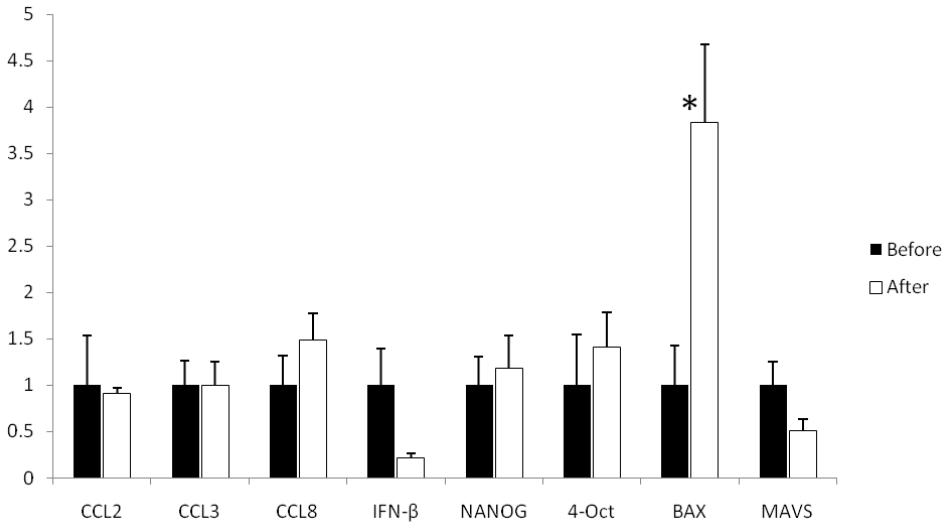

Figure 1. Relative expression of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, BAX, and MAVS Before and after RJ Feeding. The paired *t* test revealed that the relative expression of BAX significantly increased after one month of RJ feeding. Relative expression of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, and MAVS was not different when compared to before and after RJ feeding. *Note.* CCL: CC Ligand; IFN-β: Interferon-beta; BAX: Bcl-2-associated X; OCT4: Octamer binding transcription factor; MAVS: Mitochondrial antiviral signaling

the best of our knowledge, this is the first study on RJ feeding in CHB patients to explore the expression of CCL2, CCL3, CCL8, IFN-β, NANOG, OCT4, BAX, and MAVS molecules. However, the anti-HBV effects of RJ have been reported previously (19,20). RJ also plays significant roles in reducing inflammation, protecting the liver, and improving its functions during CHB and non-infectious diseases (21-23). It appears that RJ applies several mechanisms to reduce inflammation. However, according to the results of the present study, it appears that RJ increases apoptosis in the human peripheral blood immune cells to induce homeostasis and decreases the number of immune cells to reduce inflammation. In line with the current results, an *in vitro* investigation revealed that RJ increases BAX levels in human lymphocytes (24). Another animal model also confirmed the roles played by RJ in increasing the expression of BAX in the heart tissue (25). However, several reports demonstrated that RJ can decrease the expression of BAX in a dose-dependent manner (26,27). According to the best of our knowledge, the present study is unique as it was performed on CHB patients; thus, it may be concluded that the responses of the immune cells for expression of BAX with regard to the effects of RJ are dependent on the infection with HBV. Accordingly, in physiological conditions, RJ may lead to decreased apoptosis as reported by Veshkini et al (26) and Hashem et al (27). Therefore, it may be concluded that the effects of RJ on the expression of BAX are dose-dependent and also depend on the pathological/physiological conditions. According to the obtained results in this study, it may be concluded that RJ is an inducer of apoptosis in the human blood immune cells in 1 gram/day dose, which may decrease inflammation in CHB patients. Previous investigations proved that RJ uses several mechanisms, including suppression of nuclear factor kappa-light-chain-enhancer of activated B cells and other related signaling pathway phosphorylation (28-31).

Conclusion

It seems that RJ effects on the immune system of HBV-infected patients are complicated and need more investigations. Based on the obtained results, RJ is unable to alter the expression of the IFN-β, CCL2, CCL3, and CCL8 as parts of immune responses against HBV. Additionally, the cytokine and chemokines are involved in inflammation; hence, it appears that the immunoregulatory effects of RJ are independent of the molecules. Moreover, the survival of immune cells during RJ feeding is not affected by alteration in the expression of OCT4 and NANOG. The recognition of HBV molecules by MAVS is not also impacted by RJ feeding. Due to the sample size of the present study, the increased sample size and evaluation of the molecules at the protein levels may be associated with diverse factors.

Acknowledgments

The authors are grateful to the staff of Samenal-Hojaj hospital of Kerman, Iran, who helped collect samples for this study.

Conflict of Interests

The authors declare that they have no known competing financial interests or personal relationships that could apparently influence the work reported in this study.

Ethical Approval

The Ethical Committee of Kerman University of Medical Sciences (IR.IAU.Kerman.REC.1400.010) and the Iranian Registry of Clinical Trials approved the project protocol (IRCT20210620051640N1).

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