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Original Article

The Effects of Royal Jelly on the Pro-Inflammatory Innate Immunity Cytokines in Patients Infected with Hepatitis B Virus and Its Antiviral Activity

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Abstract

Introduction: Innate immunity cytokines conduct significant functions in the stimulation and induction of liver complications in Hepatitis B infected patients. It has been reported that Royal Jelly (RJ) has important roles in decreasing the pro-inflammatory cytokines in both *in vitro* and *in vivo* conditions. This project aimed to investigate the impacts of 1-month RJ administration on interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6) serum levels in the Hepatitis B patients.

Materials and Methods: In this research, 30 Hepatitis B cases (patients) were entered in order to be under treatment of RJ for 1 month. Before and after the treatment with RJ, Hepatitis B Virus (HBV) copy numbers were evaluated using Real-Time PCR and IL-1β, TNF-α, and IL-6 serum levels evaluated using the ELISA technique.

Results: The RJ treatment significantly reduced the number of HBV-DNA copies and led to down-regulation of TNF-α and IL-6, which were not substantial. The IL-1β, TNF-α, and IL-6 serum levels were not changed after RJ treatment in both men and women.

Conclusions: Based on the findings of the present study, it seems that RJ plays anti-viral and anti-inflammatory roles in the *in vivo* conditions in infected patients with HBV.

Keywords: Royal Jelly, IL-1β, TNF-α, IL-6, Hepatitis B
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Introduction

Nowadays, HBV chronic infection has been commonly known as a maintained culprit of liver disorder and a hepatotropic DNA virus. Worldwide, assessments have demonstrated that about 360,000,000 people are infected regularly and are at expanding risk of chronic liver inflammation, which leads to liver cancer (hepatocellular carcinoma) and liver cirrhosis.[1] Innate immunity related cytokines play key roles against viral infections, such as hepatitis viruses, and also significantly participate in the viral infection related complication, such as tissue fibrosis.[1] Various physiological processes containing inflammatory responses and immune regulation reactions are crucially correlated with different proinflammatory cytokines, in particular, IL-6, IL-1, and TNF-α. For instance, TNF-α has an important function in the defense system of the host through restriction of pathogenic organisms' distribution in the blood circulation and activated macrophages produce TNF-α against pathogens in different situations. In addition, many findings demonstrated that the abovementioned proinflammatory cytokines participate in the pathophysiological phase of disorders through the upregulated expression of these cytokines. IL-6, TNF-α, and IL-1 have been indicated to be produced through the synovial membrane in rheumatoid arthritis and are implicated in the disease pathogenesis. Moreover, TNF-α displays its vital role in the rheumatoid arthritis symptoms, when the concentration of TNF-α is fitting at the peak of inflammatory and pro-inflammatory cascades. Antibodies of anti-TNF-α have a key role in the treatment of symptoms in patients with rheumatoid arthritis in decrement of disorder activity.[2]Among the innate immunity cytokines, TNF-α, IL-6 and IL-1β make the first and important barriers against viral infections.[3] However, the cytokine also participates in some infection related complications, including septic shock syndrome and tissue fibrosis.[4,5] Findings have shown that the cytokines have crucial roles in the stimulation and induction of Hepatitis B related complications, like Hepatocellular Carcinoma (HCC) and liver cirrhosis.[6] Accordingly, several investigations

revealed that Chronic Hepatitis B (CHB) patients suffer from chronic increased IL-1β, IL-6 and TNF-α serum levels.[6,7] Thus, the complementary nutrients, that are able to modulate production of the cytokines, can be considered as a complementary treatment to protect the patients from cirrhosis and HCC. One of the cephalic gland productions of worker bee secretion is RJ which has a significant role in caste differentiation due to its importance in honeybee larva's diet.[8] RJ, the reason for the long lives of the queen, is a particular food for the queen in her whole life, and also is the only food given to all of the larvae in their growth procedure for the first 2-3 days. RJ has different properties in both modern medicine and traditional medicine as beneficial and effective compounds for humans. Although RJ has a controversial effect as a food supplement, it has an important role in preserving tissues and DNA against oxidative damage.[9] Numerous findings have shown the chemical composition and biological activities of RJ. It has significant commercial attraction and is commonly used in different fields from cosmetic manufactures to food and pharmaceutical industries because of its biological features. This has led to a large number of imports in countries whose supply has not caught up with the demands. Therefore, studies capabilities should be strengthening to evaluate both the quality and quantity of the various components and to conduct analytical tests on existing commercial products.[10] RJ, as a natural compound, is made by worker bees (*Apis mellifera*), and is widely used as human dietary supplements.[10,11] The potential immunomodulatory, antimicrobial and antioxidant effects of RJ have been reported by several investigations.[12-15] Due to the immunomodulatory properties of RJ, it has been hypothesized that the component may be useful to reduce pro-inflammatory cytokines in the CHB patients. Thus, this clinical trial study was designed to assess the RJ treatment effects on the IL-1β, TNF-α, and IL-6 serum levels in CHB patients.

Materials and Methods

Patients

This clinical trial study was performed on 30 (13 men and 17 women) CHB patients who referred to Samenal-Hojaj hospital in Kerman, Iran with hepatitis B in 2020. The patients had liver enzymes in normal ranges and without anti-HBV therapy. The recent experimental and clinical records based on the "Guide of Prevention and Treatment in Viral Hepatitis" were used for CHB diagnosis.[16] The co-infected participants with other viruses, such as hepatitis E, D, C, and A viruses, HIV, and cytomegalovirus have been eliminated from this study. The exclusion criteria included: liver associated diseases, pregnancy or breastfeeding, age between 18 and 55 years; mental disorders, and receiving antiviral and immunosuppressive drugs. The project protocol was approved by Kerman University of Medical Sciences Ethical Committee (IR.kmu.REC.1398.092) and the Iranian Registry of Clinical Trials (IRCT20210329050786N1). All the patients gave informed consent in order to participate in the study. Blood samples in tubes without anti-coagulant agents were collected and serum was separated before one month after treatment with RJ (1 gr per day). RJ was purchased commercially from Pars Asal Company, Shiraz, Iran.

HBV DNA Extraction and Detection

Template DNA was extracted from 200 µl serum with a viral DNA kit (QIAamp Viralextraction, Qiagen) according to manufacturer's instructions. A commercial kit from the Karmania Pars Gene Company was utilized for HBV-DNA quantification using Taq-Man Real time PCR. The PCR program was: Initial denaturation: 95 ºC 15 min, First: 5 cycles: 95 ºC: 5 sec, 60 ºC: 20 sec, 72 ºC 15 sec. Fluorescent detection: in yellow (HEX) channel and in 60 ºC. The kit had 5 copy numbers/ml sensitivity and 5% intra-assay Coefficient of Variation (CV).

Quantification of Serum Levels of TNF-α, IL-6 and IL-1β Serum Levels

TNF-α, IL-6 and IL-1β serum levels were measured using ELISA kits (commercial kits from Karmania Pars Gene Company, Kerman, Iran) according to the instructions of the manufacturer. Standard curves were created by utilizing a standard sample.

Statistical Analysis

Details and data were carried out as means ± SD. General linear model, Mann–Whitney U test, or Student's t-test were used to compare variables. SPSS software (SPSS version 18) for Windows was also utilized for statistical analysis. Analyses of simple linear correlation were performed with Pearson's method to evaluate the association between the IL-1β, TNF-α, and IL-6 serum levels before and after therapy with RJ. The p value was examined meaningful at <0.05.

Results

Patients with Chronic HBV Infection and Serum Levels of TNF-α, IL-6 and IL-1β Serum Before and After Therapy with RJ

Treatment with RJ significantly decreased HBV-DNA copy number from 26800 ± 3360 to 15400 ± 2120 copy number/ml (p<0.001). The statistical analysis showed that TNF-α serum levels were 71.78 ± 11.70 before and 57.24 ± 7.03 after RJ treatments. The analysis revealed that the decrease in TNF-α serum levels was not significant (p = 0.253). Similar to TNF-α, one month of RJ treatment did not change IL-6 serum levels. Accordingly, IL-6 serum levels were 2.89 ± 0.85 and 1.78 ± 0.43 before and after RJ treatment. However, the difference was not significant (p = 0.223). The IL-1β serum levels did not change after RJ treatment (from 4.73 ± 1.85 to 5.03 ± 2.36, p = 0.922) (Figure 1).

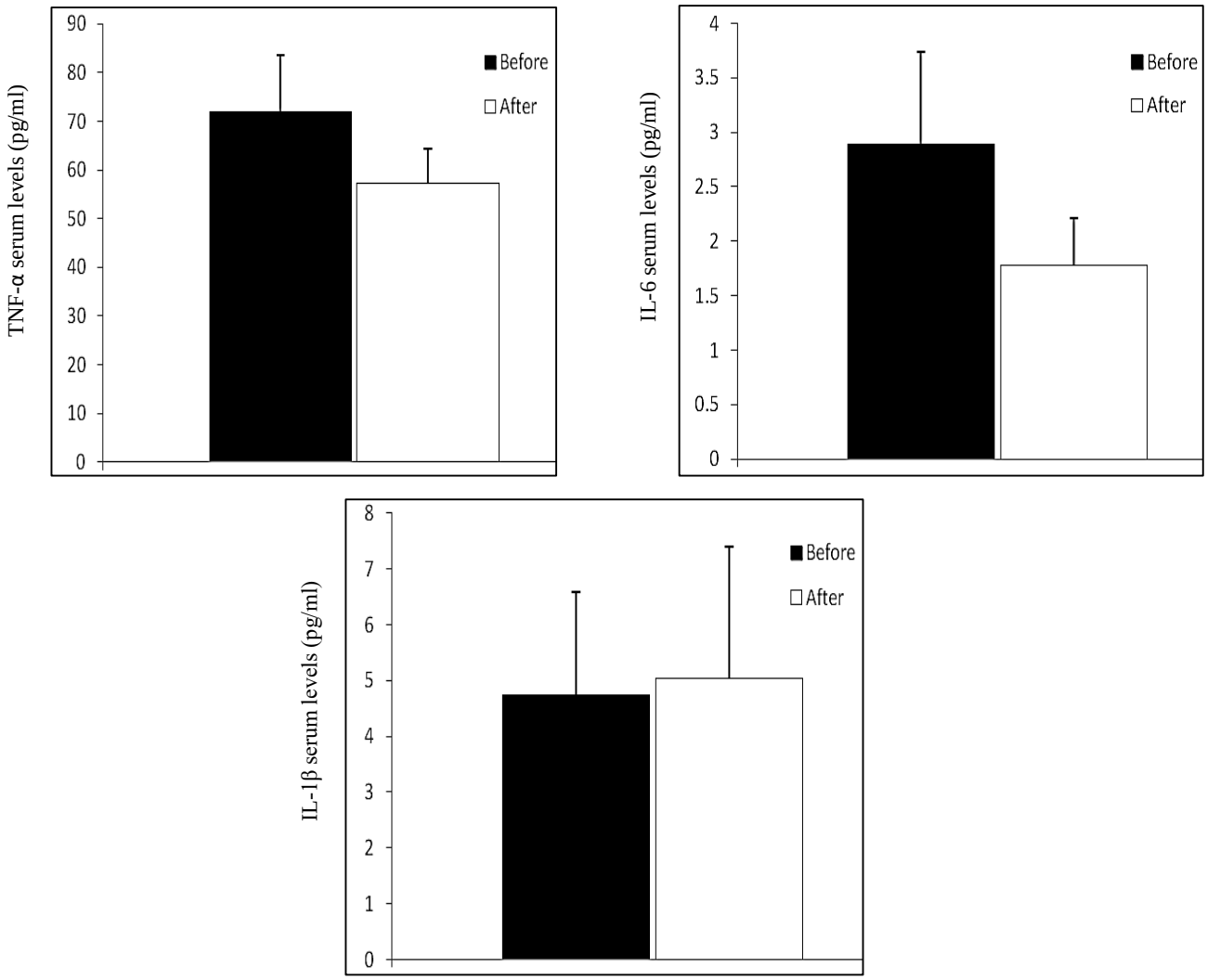

Figure 1. TNF-α, IL-6 and IL-1β Serum Levels in the Patients Suffering from Chronic Hepatitis Before and After Treatment with Royall Jelly (RJ). The statistical analysis revealed that TNF-α ($p = 0.253$), IL-6 ($p = 0.233$) and IL-1β ($p = 0.922$) serum levels were not changed after 1 month treatment with RJ.

Table 1. TNF-α, IL-6 and IL-1β Serum Levels in the Men and Women CHB Patients Before and After Royal Jelly Treatment

		TNF-α	IL-6	IL-1β
Men	Before	62.74 ± 19.16	4.36 ± 1.94	5.46 ± 4.68
	After	34.39 ± 5.70	0.96 ± 0.44	1.48 ± 0.55
	p value	0.321	0.141	0.456
Women	Before	76.94 ± 15.50	2.04 ± 0.70	4.31 ± 1.62
	After	70.30 ± 6.47	2.25 ± 0.59	7.05 ± 3.55
	p value	0.642	0.705	0.491

Data Analysis

Data analysis revealed that RJ treatment did not change IL-1β, TNF-α, and IL-6 serum levels, when the cytokine serum levels were compared in men and women separately. Table 1 illustrates the details of the IL-1β, TNF-α, and IL-6 serum levels in men and women before and after RJ treatment. Data analysis revealed that TNF-α, IL-6 and IL-1β serum levels did not change while using RJ in both men and women.

Discussion

In chronic HBV infection, prolonged unpredictable progression of liver disorder makes longitudinal sampling of immune-associated to liver injury difficult. In the background of chronic HBV, recurrent hepatic flares happened and prevailed the disease through a permitted intensive variant of immunopathogenic events correlated with expeditious changes in viral load and liver disorder.[1] The RJ is widely utilized as an ingredient in dietary supplements or cosmetic products, and has been considered one of the most appealing compounds in healthy food because of the point of view that it seems to enforce the same effects on humans and honeybees. RJ has several pharmacological functions including, hypotensive and vasodilative, antitumor, anti-infection, anti-inflammatory,

and anti-hypercholesterolemic effects. In addition, RJ has other properties such as antitumoral, antiaging, and hypoglycemic effects.[10] It also has crucial biological action in the regulation of immunity and inflammation, which are important for malignant invasiveness and carcinogenesis in several cancers. In addition, many pro-inflammatory cytokines involving transforming growth factor-β (TGF-β), TNF-α, and IL-6 are correlated with adverse effects of anti-cancer therapies in different types of cancers and malignant transformation. Recent studies have demonstrated that RJ manages pro-inflammatory cytokines synthesis, particularly TGF-β, TNF-α, and IL-6.[17] The results of this study demonstrated that HBV-DNA copy numbers significantly decreased in the Hepatitis B patients after one-month oral administration with RJ. Results demonstrated that RJ treatments for one month led to a decrease in TNF-α and IL-6 serum levels in CHB patients, but the differences were not significant. The results revealed that IL-6 was in a normal range in CHB patients, while TNF-α serum levels were up-regulated in CHB patients, when compared to normal ranges, which have been reported by researchers.[18]

RJ has some pharmacological features that have been demonstrated in various studies such as anti-inflammatory, antimicrobial, immunomodulatory, vasodilatory, antioxidant, and metabolic properties. The immunosuppressive and immunostimulatory effects of RJ have been shown in *in vivo* studies. These features might be the result of the compound possessing various activities of the immunomodulatory system. To date, most of the RJ immunomodulatory effects have been attributed to its protein components, particularly to apalabumin 1 and the major RJ protein 3. The major RJ protein 3 has been known as the immunosuppressive and anti-inflammatory components that encourages the anti-allergic response. On the contrary, glycoprotein, the major RJ, and apalbumin 1 utilizes proinflammatory and immune-stimulatory properties through upregulation of TNF-α production.[10] In stimulated macrophages, the production of TNF-α is inhibited by RJ through interferon-γ and lipopolysaccharide in an animal model. RJ and 10-HAD combination suppressed the TNF-α production of cells in colon cancer. Also, serum levels of TNF-α were dramatically higher in rats just treated with cyclophosphamide than those treated with RJ and cyclophosphamide.[17] Therefore, according to results it may be hypothesized that more RJ doses may be useful to modulate TNF-α serum levels. Additionally, due to the fact that, based on our understanding, this is the first research to evaluate RJ treatments effects on the IL-1β, TNF-α, and IL-6 serum levels in the CHB patients, we had to use a small sample size. Therefore, it appears that larger sample sizes may be associated with a more significant decrease in the TNF-α serum levels. Previous studies have indicated the role of RJ on the modulation of TNF-α expression.[9,17] Kohno et al. reported that RJ significantly decreased the production of TNF-α by activated macrophages in *in vitro* conditions.[2] However, as mentioned previously, there are no investigations in the search data bases regarding the RJ impacts on the cytokines serum levels in CHB patients. Therefore, it may be hypothesized that using more doses of RJ with a bigger sample size can be associated with down regulation of TNF-α in CHB patients, who suffer from increased TNF-α serum levels. Furthermore, the results revealed that IL-1β and IL-6 were in normal ranges in CHB patients. Hence, if the cytokines were increased in the patients, RJ may affect their expression, because previous studies confirmed the modulatory effects of the component on the cytokine productions.[8,19,20] The results showed that RJ can change the cytokine serum levels in different manner in the men and women. Although the differences were not significant, the patterns of the cytokines decreased in men, in comparison to women. Therefore, it might be hypothesized that sex may affect the RJ effects in the innate immune cell functions.

Conclusion

It seems that RJ had no side effects on the hepatocytes via increased serum liver enzymes. Thus, it can be considered as a safe complementary subject for patients suffering from hepatitis B. Collectively, due to the obtained results, it seems that RJ plays anti-viral and anti-inflammatory roles in the *in vivo* conditions. However, an investigation with more sample size and more RJ doses can be considered to complete clear roles played by RJ on the IL-1β, TNF-α, and IL-6 serum concentration in the *in vivo* condition.

Authors' Contributions

All authors have read and approved the manuscript. MN performed the laboratory tests and wrote the manuscript draft. AK and MKA designed the project, analyzed data and wrote the manuscript. FRZ helped to collect the samples before and after RJ treatment.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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