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The Effects of Royal Jelly on Autoimmunity in Graves' Disease

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***Objective*. Graves' disease is an organ-specific autoimmune disease with unknown etiology. TSHR Ab plays the most important role for the pathogenesis of Graves' disease. Recently, the role of cytokines for the pathogenesis of Graves' disease has been studied extensively. Royal jelly (RJ) is a creamy product secreted by young nurse worker bees (*Apis mellifera*), and it is synthesized in the hypopharyngeal and mandibular glands. RJ has been reported to have such pharmacological characteristics as antitumor, antibacterial, antihypercholesterolemic, antiallergic, antiinflammatory, and immunomodulatory properties. The major aim of the present study is to evaluate the effect of RJ on autoimmunity in peripheral lymphocyte culture and to establish the therapeutic doses.**

***Research Design and Methods*. In the first phase, lymphocyte cell isolation from four voluntary healthy subjects was performed to find the effective concentration of RJ on immunity. Serial dilutions of the RJ were prepared (0–5 mg/mL). All isolated lymphocyte cells were treated with the above diluted samples. MTT test was carried out after incubation of 72 h. In the second phase, six patients with Graves' disease, newly diagnosed by clinical and laboratory methods and admitted to my hospital and untreated were identified. RJ samples of 0 and 4 mg/mL were incubated in a culture medium for 72 h with isolated lymphocytes obtained from the patients. After incubation, MTT test in lymphocyte cell culture, Th1 cytokines IFN-γ, TNF-α, and IL-12, and Th2 cytokines IL-4 and IL-10 levels by the enzyme amplified sensitivity immunoassay (EASIA) method and TSHR Ab by the radioreceptor method were determined.**

***Results*. The concentration causing lymphocytes to proliferate was found to be 4 mg/mL by MTT test after incubation of 72 h in cell culture medium. Of the cytokines produced and secreted from lymphocytes, IFN-γ increased, whereas, other cytokines decreased in RJ concentration of 4 mg/mL. Significant differences were found only for IFN-γ and TNF-α. IL-4 concentrations were kept near the level of significancy. Of Th1/Th2 ratios, IFN-γ/IL-4 and IFN-γ/IL-10 ratios also exhibited significant differences between 0 and 4 mg/mL. RJ treatment in lymphocytes from patients with Graves' disease shifted the Th1/Th2 cytokine ratio to the side of Th1 cytokine. Therefore, RJ using the treatment and establishing a remission of Graves' disease may be effective as an antithyroid drug treatment. TSHR Ab levels of lymphocyte cell culture supernatants treated with RJ showed significant decreases. Also, the result may suggest that RJ may exert an effect similar to an antithyroid drug for decreasing TSHR Ab levels.**

***Conclusions*. RJ may be effective as an immunomodulatory agent in Graves' disease.**

Key Words: Autoimmunity; Graves' disease; lymphocyte cell culture; royal jelly; cytokines.

Introduction

Hyperthyroidism affects approx 2% of women and 0.2% of men *(1)*. Among autoimmune diseases, Graves' disease (GD) is the most frequent cause seen in hyperthyroidism. GD is an organ-specific autoimmune disease with unknown etiology *(2)*.

Humoral and cellular immune responses are involved in the pathogenesis of GD, as demonstrated by the presence of autoantibodies to the thyrotropin (TSH) receptor (TSHR Ab) responsible for Graves' hyperthyroidism, as well as by the finding of activated T cell and B cell infiltration in thyroid tissue from patients with GD *(3)*. TSHR Abs are responsible for hyperthyroidism and goiter by overstimulating the TSHR.

Cytokines are a group of polypeptides produced mainly by inflammatory cells, and have a key role in triggering and coordinating inflammatory and immune reactions *(4)*. Because they have a pivotal role in the generation and perpetuation of immune and inflammatory responses, it has been suggested that the polypeptide mediators may also be involved in the development and perpetuation of autoimmune diseases *(5)*. Mosmann et al. reported that mouse helper cells could be divided into two subpopulations, Th1 and Th2 cells, according to differences in their cytokine expression profiles *(6)*. Th1 cells, predominantly secrete interferon-

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γ (IFN-γ), produce tumor necrosis alpha (TNF-α), TNF-β, IL-1, IL-2, IL-8, IL-12, and IL-18, and promote cellular immune responses. In contrast, Th2 cells produce mainly IL-4 including IL-3, IL-5, IL-6, IL-10, IL-13, and TGF-β, and are responsible for B-cell differentiation and antibody production *(2,4,7)*. IFN-γ is known to induce differentiation of Th0 to Th1 cells and to inhibit the proliferation of Th cells. On the other hand, IL-4 and IL-10, secreted from Th2 cells, have been known to induce the differentiation of Th0 to Th2 cells and to inhibit the function of Th1 cells *(8)*.

Thyroid is a major site of TSHR Ab synthesis. Peripheral blood and thyroidal lymphocytes obtained from patients with GD may produce in vitro TSHR Abs. Thionamide drugs, such as propylthiouracil, carbimazole, and methimazole, control the hyperthyroidism of GD primarily by blocking iodine organification *(9)*. Thionamide therapy is also associated with reduction in circulating levels of thyroid autoantibodies *(10,11)*, including the TSHR Abs that appear to be responsible for the hyperthyroidism *(2)*. Because methimazole inhibits synthesis of thyroid autoantibodies by lymphocytes in vitro *(11,12)*, the fall in titers of thyroid autoantibodies in Graves' patients treated with antithyroid drugs (ATDs) may be due to the immunosuppressive action of the drug on thyroid lymphocytes.

Royal jelly (RJ), which is secreted from the hypopharyngeal and mandibular glands of worker honeybees (*Apis mellifera*), is the exclusive principal food source of the queen honeybee and larvae. It directs the development of honeybee larvae into queen bees *(13–15)*. RJ is composed of proteins (12–15%), sugars (10–16%), lipids (3–6%), vitamins, and free amino acids, and has been used for medical and nutritional purpose in folk medicine *(13)*. In in vitro and in vivo studies, RJ has been reported to have such pharmacological characteristics as antimicrobial and antioxidative activities *(16,17)*, insulin-like effect *(18)*, antitumor activity *(19)*, vasodilatotary activity *(20)*, antihypercholesterolemic *(21)*, antihypertensive *(22)*, antiallergic *(23)*, antifatigue *(24)*, wound-healing properties *(25)*, and protective activity against hematopoietic dysfunction in X-irradiated mice *(26)* and endogenous sepsis in X-irradiated mice, through activation of macrophages and hematopoietic stem cells *(26, 27)*. Furthermore, RJ was reported to enhance Th1 responses in aged mice *(28)*. Sver et al. first reported that RJ exhibited immunomodulatory properties by stimulating antibody production and immunocompetent cell proliferation in mice or depressing humoral immune functions in rats *(14)*. Oka et al. studied the immunomodulatory effects of RJ in immunized mice, and they reported that RJ suppressed antigen-specific IgE production and histamine release from mast cells in association with the restoration of macrophage function and improvement of Th1/Th2 cell responses in immunized mice *(15)*. Majtan et al. *(29)* showed that immunostimulatory effect of RJ on TNF-α release is explained by a protein named apalbumin-1.

Table 1
Findings of the MTT Test Performed in Lymphocyte Cell Cultures Belonging to Healthy Individuals Following 72 h Incubation with Royal Jelly (Absorbance)

RJ concentration (mg/mL)	Absorbance [$n = 4$, X (±SD)]*
0	0.182 (0.05)
0.025	0.177 (0.06)
0.050	0.170 (0.04)
0.10	0.164 (0.06)
0.25	0.166 (0.02)
0.50	0.162 (0.02)
1.0	0.188 (0.03)
2.0	0.215 (0.05)
4.0	0.270 (0.02)
5.0	0.300 (0.03)

*Arithmetic mean (±standard deviation).

Despite these investigations, which did not completely establish a biological desirable (protective but not toxic) activity for RJ, it is certain that the immunopharmacological *(30)* or therapeutic *(31)* effects can be ascribed to 10-hydroxy-2-decenoic acid (10-HDA) or to RJ, respectively. In particular, several substances contained in RJ, including 10-HDA, royalisin, and apisin, have been found to exhibit these pharmacological activities. In recent studies, RJ has been confirmed to have antiallergic, anti-inflammatory and immunumodulatory effects *(32–35)*.

In the literature, there is no experimental and clinical study regarding to use of RJ in GD. The major aim of the present study is to evaluate the effect of RJ on autoimmunity in peripheral lymphocyte culture and to establish the therapeutic doses in patients with GD.

Results

Determination of Effective Concentration in the Establishment of the Effect (Stimulation, Inhibition, or Immunomodulation) of Royal Jelly on Immunity

The results of the MTT test performed in lymphocyte cell cultures belonging to healthy individuals incubated with royal jelly in 0–5 mg/mL concentrations prepared as described previously with the aim of determining the effective concentration of royal jelly to be used in our experiments are given in Table 1. As shown in Table 1, the 0, 0.025, 0.05, and 1 mg/mL results are close to one another. There is a decreasing shift in absorbance values of 0.1, 0.25, and 0.5 mg/mL concentrations according to the lowest concentration. The maximum absorbances were obtained between 2 and 5 mg/mL. However, because cell viability was partially lost at a concentration of 5 mg/mL, it was

Table 2
MTT Test Findings with Graves' Disease
(Absorbance × 10^3) [n = 10, X (±SD)]

Patient	Control*	0	4
C.K.	96.5 (10.6)	114.9 (13.6)	174.4 (9.4)
S.T.	119.8 (10.5)	104.1 (9.5)	222.9 (20.2)
S.H.	126.5 (16.6)	121.8 (17.0)	222.8 (10.5)
E.T.	138.2 (10.6)	106 (12.3)	170 (17.5)
S.K.	89.5 (4.1)	72.1 (8.8)	178.9 (11.8)
Y.A.	88.7 (6.6)	81.4 (7.6)	183.2 (15.8)
X (±SD)	109.9 (21.1)	100.1 (19.4)	192.0 (24.3)**

*RPMI was used instead of PBS in control specimens.
**Post-hoc Wilcoxon test by Friedman test (w = 21.0, p = 0.004).

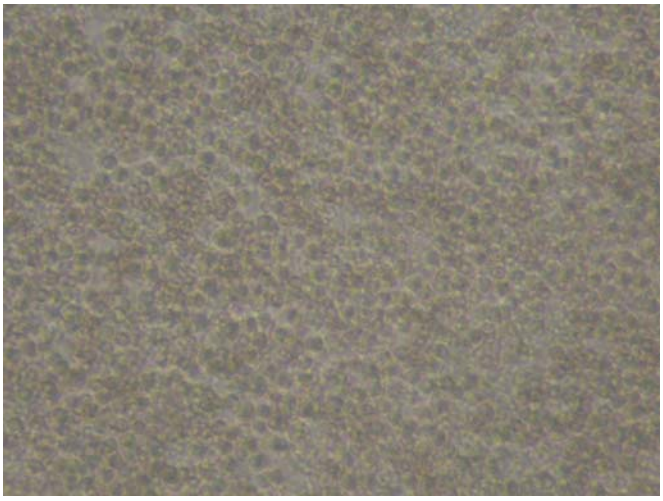

Fig. 1. Microscopic image following 72 h of incubation of lymphocytes treated with a 4 mg/mL concentration of royal jelly (×100).

Table 3
Cytokine Levels Obtained from Cell Culture Supernatants [n = 2, X (±SD)]

		Th1 cytokines			Th2 cytokines	
Patient	Concentration	IFN-γ (IU/mL)	TNF-α (pg/mL)	IL-12 (pg/mL)	IL-4 (pg/mL)	IL-10 (pg/mL)
C.K.	0	2.45 (0.44)	40.0 (2.83)	15.8 (0.84)	13.0 (1.02)	10.0 (2.82)
	4	2.85 (0.51)	8.84 (2.08)	11.6 (0.36)	0.10 (0.02)	8.5 (1.16)
S.T.	0	2.23 (0.65)	9.4 (0.29)	12.7 (0.64)	28.2 (1.08)	15.4 (1.10)
	4	3.62 (0.46)	3.2 (0.18)	22.4 (1.02)	0.12 (0.02)	9.5 (0.84)
S.H.	0	1.81 (0.50)	10.8 (1.20)	22.6 (0.36)	30.4 (0.84)	9.5 (0.80)
	4	2.88 (0.35)	0.10 (0.03)	8.9 (0.22)	14.8 (0.32)	3.7 (0.74)
E.T.	0	1.85 (0.34)	31.5 (0.80)	19.3 (0.24)	1.8 (0.16)	12.5 (2.16)
	4	2.16 (0.74)	19.4 (0.96)	18.5 (0.21)	0.12 (0.02)	10.4 (1.32)
S.K.	0	0.95 (0.13)	26.9 (1.32)	3.16 (0.24)	11.1 (1.20)	5.9 (0.96)
	4	5.14 (0.74)	8.3 (0.26)	2.43 (0.33)	2.9 (0.45)	0.7 (0.19)
Y.A.	0	2.08 (0.55)	32.2 (0.94)	13.9 (0.66)	13.7 (0.82)	10.2 (2.64)
	4	3.85 (0.24)	12.4 (0.38)	2.43 (0.20)	5.6 (0.44)	6.1 (0.94)

decided to use 0 and 4 mg/mL concentrations in subsequent experiments.

The Tests in Cell Culture and Its Supernatants

MTT Test

The MTT test results obtained as a result of 72 h of incubation with 0 and 4 mg/mL of royal jelly in lymphocyte cell cultures isolated from Graves' disease patient specimens are given in Table 2. A significant difference at each concentration was found in the general means given in the table below and obtained using Friedman test with post hoc Wilcoxon test. The 4 mg/mL concentration was found to be significantly different from 0 concentration (Wilcoxon w = 21.0, p = 0.004). It was therefore determined that RJ of 4 mg/mL affected the proliferation ability of lymphocytes. A microscopic image obtained as a result of 72 h of incubation of 4 mg/mL of royal jelly with lymphocytes is given in Fig. 1.

Cytokine Levels

The Th1 marker cytokines (IFN-γ, TNF-α, IL-12) and Th2 marker cytokines (IL-4, IL-10) levels determined in supernatants obtained as a result of 72 h lymphocyte incubation with royal jelly are shown in Table 3. The results obtained and statistical comparisons when the statistics in Table 3 for each concentration and each patient are combined in groups are given in Table 4. As shown in Table 4, IFN-γ concentrations showed a tendency to increase and all the other cytokine concentrations displayed a tendency to decrease between concentrations of 0 and 4 mg/mL. Among the cytokines, significant differences were determined in IFN-γ and TNF-α. IL-4 concentrations decreased and remained at the significance threshold (p = 0.05).

Table 4
Group Means of Cytokines on Table 3 [$n = 6$, X (±SD)]

Parameter	0	4 mg/mL
IFN-γ (IU/mL)	1.90 (0.52)	3.42 (1.04)*
TNF-α (pg/mL)	25.13 (12.39)	8.70 (6.83)**
IL-12 (pg/mL)	14.58 (6.67)	11.04 (8.22)
IL-4 (pg/mL)	16.36 (10.92)	3.89 (5.80)
IL-10 (pg/mL)	10.58 (3.18)	6.49 (3.74)

*Wilcoxon $w = 25$, $p = 0.010$.
**$w = 25$, $p = 0.025$.

Table 5
Group Means of Th1/Th2 Cytokine Ratios
Obtained from Cell Culture Supernatants [$n = 6$, X (±SD)]

Parameter	0	4
IFN-γ/IL-4	0.27 (0.38)	13.22 (14.15)*
IFN-γ/IL-10	0.18 (0.04)	1.61 (2.82)**
TNF-α/IL-4	4.34 (6.55)	46.97 (65.48)
TNF-α/IL-10	2.67 (1.56)	2.86 (4.48)
IL-12/IL-4	2.40 (4.09)	76.45 (86.01)
IL-12/IL-10	1.37 (0.65)	1.96 (1.05)

*$w = 23.5$, $p = 0.013$; **$w = 26.5$, $p = 0.045$.

Th1/Th2 cytokine level ratios were also investigated in our study. These ratios are shown in Table 5. A significant group difference was determined for IFN-γ/IL-4 and IFN-γ/IL-10 from the Th1/Th2 ratios.

TSHR Ab Levels

The TSHR Ab levels measured in supernatants obtained as a result of 72 h of incubation of lymphocyte cell culture with royal jelly are shown in Table 6. When patient results were evaluated individually, the lowest Ab levels for each patient were found with treatment with royal jelly in a 4 mg/mL concentration.

Discussion

It is known that the MTT test is used to define proliferation ability and number of living cells with continuing mitochondrial activity *(37)*. We also determined that royal jelly increased absorbance significantly in all Graves' disease patients at a concentration of 4 mg/mL as a result of 72 h of incubation with royal jelly in lymphocyte cell culture isolated from Graves' disease patient specimens. To put it another way, royal jelly at a concentration of 4 mg/mL increases living lymphocyte numbers and proliferation capability. The antigenic property of royal jelly and therefore its increasing lymphocyte proliferation as a foreign protein is an expected finding. Kamakura et al. reported that a 57-kDa protein (now called as apalbumin-1) in RJ enhances proliferation of primary cultured rat hepatocytes *(38)*.

In different studies, it was reported that serum IFN-γ levels in patients with GD increased *(39)*, decreased *(5)*, and/or did not change *(40,41)*. IFN-γ, IL-2, and TNF-α all

Table 6
TSHR Ab Levels
Obtained from Cell Culture Supernatants

	TSHR Ab (U/L)	
Patient	0	4
C.K.	29	16
S.T.	34	19
S.H.	28	14
E.T.	36.5	17.5
S.K.	31	16
Y.A.	33	15.0
X (±SD)	31.9 ± 3.2	16.6 ± 1.8*

*$w = 21$, $p = 0.004$.

suppressed the production of antithyroid autoantibodies by thyroid B cells in vitro *(42)*. Although the evidence in somewhat conflicting, the majority opinion is that GD is promoted by type 2 cytokines and regulated by type 1 cytokines. It has been reported that serum IFN-γ levels were increased after ATD or RAI treatments, whereas Th1/Th2 ratios were lower than those of healthy subjects, i.e., were in favor of Th2 *(1,43)*.

There are a few studies that evaluate the effect of RJ on IFN-γ. Oka et al. studied the immunomodulatory effects of RJ *(15)*. They reported that, in immunized mice, IFN-γ production from T4 cells was suppressed and IL-4 production from T4 cells was increased as compared to normal mice. On the other hand, RJ (1 g/kg, po) improved the balance of Th1/Th2 cell responses from Th2-dominant to Th1-dominant (an increase in IFN-γ and a decrease in IL-4). Taniguchi et al. reported that, in experimental mice model–induced atopic dermatitis-like skin lesions, oral administration of RJ suppresses the development of these skin lesions in immunized mice *(44)*. They suggested that the mechanism of this result is a decreased IFN-γ production by spleen cells and increased inducible nitric oxide (NO) synthase (iNOS) expression in the dorsal skin lesions of immunized mice *(44)*. Thus, IFN-γ may play pivotal roles in the accumulation of inflammatory cells in lesional skin of chronic atopic dermatitis. Moreover, Okamoto et al. reported that major royal jelly protein 3 (MRJP3), purified 70-kDa glycoprotein, markedly inhibited IFN-γ, IL-12, and IL-4 production by stimulated purified splenic T cells *(35)*. IFN-γ and TNF-α inhibit thyroid follicular cell (TFC) growth and proliferation *(45)*.

In the present study, IFN-γ levels of cell culture supernatants obtained after incubation of peripheral blood lymphocyte cell culture with RJ of 4 mg/mL for 72 h increased. The result shows that RJ for GD may change Th1/Th2 ratio in favor of Th1. In view of the above positive effects (growth and differentiation of supressor T cells, inhibition of IgE response, and in vitro supression of antithyroidal Ab production by thyroid B cells), IFN-γ increasing effect of RJ may be evaluated as a beneficial effect.

The TNF-α system might be a role in the regulation of the pituitary–thyroid axis. TNF-α receptors have been demonsrated on human thyroid cells *(46)*.

In vivo production of TNF-α has been demonstrated by both intrathyroidal lymphocytes and TFCs in patients with GD *(8)*. In different studies performed in patients with GD, serum levels of TNF-α have been reported to be elevated *(47–49)* or in normal ranges *(50)*. ATDs exert an immunosuppressive effect by blocking production of some inflammatory mediators such as TNF-α, IL-1, and IL-6 *(4,9)*. Diez et al. showed that TNF-α concentrations were increased in relation to controls, and these levels normalized by ATD, radioactive therapy, or surgical treatment *(47)*. However, plasma TNF-α has a short half-life and tissue levels of TNF-α are more closely related to pathophysiological conditions.

There are a few studies that evaluate the effect of RJ on TNF-α. Kohno et al. have examined the inflammatory actions of RJ at a cytokine level, and when supernatants of RJ suspensions were added to a culture of mouse peritoneal macrophages stimulated with polysaccharide and IFN-γ, the production of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1, was efficiently inhibited in a dose-dependent manner without having cytotoxic effects of macrophages *(32)*. They have suggested that RJ has anti-inflammatory actions through inhibiting proinflammatory cytokine production by activated macrophages, and it is an effective dietary supplement for the improvement of quality of life in the autoimmune diseases. Simuth et al. reported that apalbumin-1 (monomeric form, 55 kDa), the most abundant protein of RJ, and apalbumin-2 (49 kDa), stimulate mouse macrophages to release TNF *(33)*. They suggested that TNF-α might play a role in cytokine-induced activation of genes important for immune response of honeybees and humans, and it could play a pivotal role as the factor participating on regulation of important cellular processes such as cell proliferation and inflammation.

In our study we found that TNF-α levels we measured in cell culture supernatants obtained after 72 h of incubation of peripheral blood lymphocyte cell cultures with RJ of 4 mg/mL decreased. Reduction in TNF-α in Graves' disease patients may show remission of the disease or, as stated above, a reduction in disease activity. For that reason, this effect of royal jelly in peripheral blood lymphocyte cell culture in our patients may be considered one that is therapeutic and provides remission.

In various studies, it was reported that serum IL-12 levels in patients with GD increased *(51,52)* and did not change *(41)*. Kocjan et al. reported that the mononuclear cell (MNC) cultures from the peripheral blood of patients with newly diagnosed GD before treatment produced significantly less IL-12 and significantly more IL-10 and IL-4 than normal lymphocytes from healthy donors *(40)*. Also, all calculated ratios Th1 against Th2 cytokines in MNC cultures from patients with GD were significantly lower than in MNC cultures from healthy controls. They showed a systemic shift of cytokine production in patients with GD toward the Th2 cytokine response, thus confirming the key role of TSHR Abs and humoral immunity in the pathogenesis of GD. Jones et al. reported that peripheral blood MNC cultures from patients with GD before treatment with RAI are produced significantly less IFN-γ and IL-4 compared with healthy controls *(1)*. IL-12 production was normal. Tamaru et al. reported that the serum IL-12 levels in GD were significantly increased in the hyperthyroid state, and were decreased during treatment with methimazole or propylthiouracil (in euthyroid state) *(53)*.

There is an only one study that evaluates the effect of RJ on IL-12. In the study, Oka et al. reported that RJ suppressed antigen-specific IgE production and histamine release from mast cells in association with the restoration of macrophage function (increased IL-12 p40 mRNA expression and NO production, and decreased PGE_2 production) and improvement of Th1/Th2 cell responses in immunized mice *(15)*. But, in this study, IL-12 production in lymphocytes as Th1 cytokine was not investigated.

In our study we found that IL-12 levels decreased as RJ concentrations increased. But, this decrease was not statistically significant.

In different studies, it was found that serum IL-4 levels in patients with GD increased *(1,39,43)*. Kocjan et al. reported that IL-4 levels in the MNC culture supernatants in patients with newly diagnosed GD were higher than those of the normal lymphocytes from healthy subjects *(40)*. Mysliwiec et al. found that IL-4 and IL-12 levels are elevated in patients with GD, and that there is an increase in the ratios of IL-4/IFN-γ, IL-4/TNF-α, IL-10/ IFN-γ, and IL-10/TNF-α after steroid therapy in patients with Graves' ophthalmopathy. They suggested that these cytokines may have a role in disease remission. In that study, the cytokine levels did not correlate with severity, remission and recurrence of the disease *(54)*.

The effects of RJ on Th1/Th2 cell responses have first been investigated by Oka et al. *(15)*. They reported that IFN-γ production from Th cells in immunized mice increased as compared to normal mice. Kataoka et al. found that intraperitoneal administration of RJ into immunized mice resulted in the inhibition of both antigen-specific IgG1 and IgE production, and IL-4, IL-5, and IL-10 production by antigen-stimulated spleen cells *(23)*. Recently, Okamoto et al., using a series of column chromatographies, purified a 70-kDa glycoprotein, MRJP3, that suppresses IL-4 production. In this study, MRJP3 suppressed the production of not only IL-4 but also that of IL-2 and IFN-γ by T cells concomitant with inhibition of proliferation *(35)*. Interestingly, in spite of the antigenicity, MRJP3 inhibited serum antigen-specific IgE and IgG1 levels in immunized mice.

In our study we found that IL-4 levels decreased with RJ of 4 mg/mL. Although not statistically significant, this decrease was nevertheless very close to significance thresholds ($p = 0.06$). This result is probably due to our patient

numbers being small ($n = 6$). Royal jelly suppression of manufacture of Th2 cytokine IL-4 from lymphocytes may be regarded as a beneficial and therapeutic effect. That is because in relapsed Graves' patients Th2 cytokines such as IL-6, IL-10, and IL-13 increase in direct relation to disease activity *(55,56)*.

IL-10 is likely to have a major influence on autoantibody production in GD. In various studies, it was reported that serum IL-10 levels in patients with GD increased *(39,54,57)*. Kocjan et al. reported that IL-10 levels in the MNC culture supernatants are elevated in patients with GD compared with controls. The ratios IFN-γ/IL-10 and IL-12/IL-10 in MNC cultures from patients with GD were significantly lower than in MNC cultures from healthy controls *(40)*. Takeoka et al. found that serum IL-10 levels were significantly higher in patients with seriously intractable GD than in patients with GD in remission, although serum IL-4 levels did not differ significantly between these two groups *(58)*. They suggested that IL-10, but not IL-4, may play a major role in GD intractability. Mysliwiec at al. reported that serum IL-10 was elevated significantly in patients with GD in comparison to the control group. In this study, serum IL-10 levels increased significantly after glucocorticoids *(56)*.

In the literature, there is an only one study that evaluated the effects of RJ on IL-10 *(23)*. In this study, Kataoka et al. reported that intraperitoneal administration of RJ into immunized mice resulted in the inhibition of IL-10 production by antigen-stimulated spleen cells *(23)*. But, in this study, IL-10 production as Th2 cytokine was not investigated.

In our study we found that IL-10 levels decreased as royal jelly concentrations increased. But, this decrease was not statistically significant.

As already stated, Th cell cytokine response in Graves' disease has been found to be different in various studies performed. In this autoimmune disease Th2 cytokine response generally increases, and Th1 cytokine response decreases, increases, or remains unchanged *(40,41,43)*. In these patients, however, the Th1/Th2 ratio may generally be regarded as decreasing (shifting toward the Th2 cytokine), and following ATD therapy this ratio is regarded as either falling *(55)* or remaining unchanged *(43)*. Very recently, Kocjan et al. evaluated the balance shift in Th1/Th2 cytokines in the PBMC culture supernatants from patients with GD after 1 yr of methimazole treatment, when compared to the same balance in patients with newly diagnosed GD before treatment and in healthy controls *(43)*. They reported that PBMC from patients with GD after treatment produced significantly more IFN-γ and IL-4 than PBMC from patients with GD before treatment, but there were no significant differences in calculated ratios of Th1 against Th2 cytokines between these two groups. When compared to PBMC from healthy controls, PBMC from patients with GD after treatment produced significantly more IL-4 and significantly less IL-2. The calculated IL-12/IL-4 ratio after treatment was significantly lower than the same ratio from healthy controls. In conclusion, they reported that there is no significant change in the ratio between Th1 and Th2 cytokines produced by PBMC from patients with GD after 1 yr of methimazole treatment, when compared to the ratio before treatment.

In our study we also evaluated the effect of royal jelly on the Th1/Th2 ratio. As the concentration increased from 0 mg/mL toward 4 mg/mL we observed a change in the Th1/Th2 ratio in favor of Th1. This change was found to be statistically significant in IFN-γ/IL-4 and IFN-γ/IL-10 ratios. This finding with royal jelly is compatible with that in Graves' disease patients entering remission based on ATD therapy.

The stimulatory effect of the Th2 cytokines (especially IL-4, IL-10, and IL-13) on thyroid B lymphocytes, in such a way that causes synthesis and secretion of TSHR Ab, is very important. TSHR Abs are responsible for hyperthyroidism and goiter by overstimulating the TFCs *(59,60)*. There is a positive correlation between serum TSHR Ab concentrations and disease activity. In patients who have highly increased antibody levels, clinical progression is more serious and response to treatment is delayed, therefore, relapse is seen more frequently *(2,10,11)*. Serum TSHR Ab concentrations decline in most patients after long-term ATD therapy. Propylthiouracil decreases release of Ig from B lymphocytes and increases a number of suppressor cells *(61)*. Methimazole blocks the increase in serum TSHR Ab concentrations that occurs in patients with GD treated with RAI, suggesting that an organ-specific effect, rather than generalized immunosuppression, is of primary importance *(2)*. In another study, patients treated with either PTU or carbimazole had identical decrements in serum thyroid hormone concentrations, but the carbimazole-treated patients had greater decreases in serum TSHR Ab concentrations and increases in the number of suppressor T cells, suggesting, indirectly, an effect on the immune system independent of thyroid function *(62)*. Serum TSHR Ab concentrations tend to decrease during ATD therapy because of an immunosuppressive effect of the drug, amelioration of thyrotoxicosis, spontanenous remission, or a combination of these factors. The failure of serum TSH Ab to become undetectable during ATD therapy signifies almost certain relapse after discontinuation of therapy *(10)*. If serum TSHR Abs do disappear, there is still a 30–50% change of relapse *(63)*. Thus, detectable serum TSHR Ab activity, but not its absence, has prognostic value *(9)*.

Decreasing TSHR Ab level is most probably linked to direct inhibition of antibody production in B lymphocytes by royal jelly or else to a decrease in the stimulant effect of IL-4 and IL-10 on B lymphocytes under the effect of royal jelly in a cell culture environment. In addition, it has been reported that increasing Th1 cytokine IFN-γ under the effect of royal jelly suppresses the production of thyroid antibodies by in vitro thyroid B cells *(64)*. This result is most important because imbalance in the Th1/Th2 cytokine ratio

leads to defects seen in Graves' disease by way of humoral immunity. The most important role in humoral immunity belongs to TSHR Ab.

Another of the striking results from our study is that the levels of cytokine released from lymphocyte cell cultures at a 0 and 4 mg/mL concentrations show a wide range of variation among patients. When we formed groups of patients with the same royal jelly concentration, this had a negative effect on significance levels among the groups. For example, although the IL-4 level decreased as royal jelly concentration increased, statistical significance remained within the threshold only because the initial levels among patients were very different (IL-4 level at 0 mg/mL concentration was 30.4 pg/mL in patient S.H., but 1.8 pg/mL in patient E.T.). How is this to be explained? It is known that analysis of the peripheral blood T cell population, particularly after mitogen stimulation, will clearly be biased by the inclusion of the majority of lymphocytes that do not have specificity for thyroid autoantigens and even the intrathyroidal population will not be free from such biases. In vitro culture after cell fractionation, with measurement of cytokine release into culture supernatant, has clear advantages over RT-PCR methods in terms of directly quantifying cytokine-as-protein but requires large numbers of cells for purification of population such as the $CD4^+$ cells, and may not be free from the possible artefacts of any in vitro system *(7)*.

One of the interesting findings in our study is that TSHR Ab increased in only half the Graves' disease patients, and that while antibody levels were normal in patients 2, 3, and 6, the TSHR Ab levels we measured in these patients' PBLCs were high. This finding shows that TSHR Ab levels measured in lymphocyte culture supernatants are much more important, reliable, and valuable in the diagnosis of Graves' disease than serum THSR Ab levels. In practice, however, TSHR Ab levels may still be prepared given their ease of use.

In conclusion, RJ in lymphocyte cell culture obtained from GD patients decreased TNF-α, Th1 cytokine, and increased IFN-γ, Th1 cytokine, changed Th1/Th2 ratio in favor of Th1; therefore, RJ may be effective as an immunomodulatory agent in Graves' disease.

Materials and Methods

In the first phase of the present study, lymphocyte cell isolation from four voluntary healthy subjects without any known autoimmune, allergic, or infectious disease was performed to find effective concentration of RJ on immunity (stimulation, inhibition, or immunomodulation). The peripheral blood lymphocyte cells (PBLC) were isolated from the peripheral venous blood samples with centrifugation on Ficoll-Paque (Pharmacia, Sweden) density gradient *(36)*.

The sample of RJ used in the study were collected fresh from Trabzon in the Turkey was provided by Trabzon Agricultural Development Cooperative. It was kept frozen at –85°C until used.

RJ was suspended in sterile phosphate-buffered saline (PBS) at a concentration of 500 mg/mL. The supernatant of the RJ suspension was collected by centrifugation at 10,000*g* for 10 min. From the samples serial dilutions of 500, 400, 200, 100, 50, 25, 10, 5, and 2.5 mg/mL were prepared. PBS solution was used for zero concentration. RJ samples were passed through 0.2 μm filter unit in a laminar airflow to sterilize them; 240 μL of lymphocyte sample isolated from each healthy subjects and 60 μL of RJ sample at various concentrations (1:5 final dilution) were added to cell culture wells. Final volume of wells was completed to 3 mL by RPMI-1640. Therefore, final RJ concentrations were 5, 4, 2, 1, 0.5, 0.25, 0.10, 0.05, and 0.0025 mg/mL, respectively. The PBLC cultures were incubated in the CO_2 incubator with 5% CO_2 and 95% humidity for 72 h.

MTT Test (Tetrazolium Dye-Reduction Assay) (37)

PBLCs were seeded in 96-well plates (100 μL/well at density of 1×10^5/mL) and exposed to different concentrations of RJ for 72 h. The cell-survival fraction was determined with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction test assay. In brief, after incubation with RJ, 10 μL MTT solution (2 mg/mL) was added to the well plates and further incubated for 4 h at 37°C. The formazan crystals formed were dissolved by addition of 200 μL isopropanol /well. Absorbtion was measured by ELISA reader (Bio-Tek Instruments, USA) at 540 nm, reference filter 620 nm. The experiments were performed four times on all concentrations and the means of the results were used for final analysis. An increase in absorbance indicated a greater proliferating activity.

Selection of the Patients with GD

In the second phase, patients with GD who untreated and newly diagnosed by clinical and laboratory methods admitted to Endocrinology and Metabolic Diseases Clinic of Medical Faculty, Karadeniz Technical University. Table 7 shows demographic and laboratory characterictics of patients with GD. Each patient was clinically and biochemically hyperthyroid, defined as having increased serum thyroid hormone levels, a suppressed TSH concentration (<0.1 μU/mL). The diagnosis of GD was defined as the presence of biochemical hyperthyroidism (raised serum total T_4, total T_3, free T_4, and free T_3 concentrations and suppressed TSH) together with the presence of two of the following: a palpable diffuse goiter, a significant titer of thyroid peroxidase, Tg autoantibodies and/or TSH receptor antibodies, and/or the presence of ophthalmopathy. At the time of the study, patients were neither taking drugs nor had diseases known to affect immunity.

Blood was drawn in the morning 0:800/09:00 h after an overnight fast. Serum total and free triiodothyronine (TT_3 and FT_3), total and free thyroxine (TT_4 and FT_4), and TSH concentrations were measured by automated chemiluminescence system (Roche, E-170, Switzerland). Normal ranges

Table 7
Demographic and Laboratory Characteristics of Patients with Graves' Disease

Patient no	1	2	3	4	5	6
Age (yr)	26	31	26	40	45	51
Gender	M	M	F	F	F	M
TT_3 (normal: 0.8–2.0 ng/mL)	6.5	6.4	4.1	6.5	5.4	4.7
TT_4 (normal: 5.1–14.1 μg/dL)	22.8	18.6	17.5	24.9	16.9	21.2
FT_3 (normal: 1.8–4.6 pg/mL)	23.1	21.2	13.7	32.6	24.5	22.6
FT_4 (normal: 0.9–1.7 ng/dL)	7.8	4.3	4.3	6.4	4.1	5.4
TSH (normal: 0.27–4.2 μU/mL)	0.01	0.01	0.01	0.01	0.01	0.01
Anti-TPO (normal: <34 IU/mL)	643	235	864	16.0	16	44.1
Anti-Tg (normal: <115 IU/mL)	41.8	238	46.1	294	<20	938
TSH R Ab (normal: 0–10 U/L)	25.5	8.8	9.30	14.0	58	2.5

are 0.8–2.0 ng/mL for TT_3, 5.1–14.1 μg/dL for TT_4, 1.8–4.6 pg/mL for FT_3, 0.9–1.7 ng/dL for FT_4, and 0.27–4.2 μU/mL for TSH.

PBLC isolation from patients with GD were performed as explained previously.

RJ samples of 0 and 4 mg/mL were incubated in a culture medium for 72 h with isolated lymphocytes obtained from the patient such as explained above. After incubation, the MTT test in lymphocytic cell culture was performed. For each patient and control samples, 10 experiments were performed.

After the incubation period, the culture supernatants were removed for the measurement of cytokines and TSHR Abs. Th1 cytokines IFN-γ (cat no. KAC1231), TNF-α (cat no. KAC1751), and IL-12 (cat no. KAC1561), and Th2 cytokines IL-4 (cat no. KAC1281) and IL-10 (cat no. KAC1321) levels were measured by an immunoenzymometric assay (EASIA) using commercially available kits (Biosource, Belgium) in the culture supernatants. Two experiments was performed for each concentration. The ratios of Th1 against Th2 cytokines were calculated. TSHR Ab levels by radioreceptor method were determined in the culture supernatants using commercially available kits (Brahms, USA).

Statistical Analysis

All statistical analyses were performed using SPSS/PC statistical program (version 11.0 for Windows; SPSS, Inc., Chicago, USA). Nonparametric Friedman test with a signed Wilcoxon post-hoc test was used to find the differences in the groups. Results were shown as chi-square and p value on the tables. Wilcoxon test was used to compare the concentrations of groups. Results were calculated as w and p values on the tables. $p < 0.05$ was considered statistically significant.

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