Royal Jelly Supplementation Ameliorated Immune Impairment via Inhibition of Oxidative Stress in Low Micronutrient-induced Immunodeficient Mice

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Abstract The effect of royal jelly (RJ) on the survival and immune dysfunctions of C57BL/6 mice induced by low micronutrient supplementation was investigated. Female C57BL/6 mice were supplemented with 0.2% or 1% royal jelly following induction of immune deficiency by 7.5% low micronutrient intake. The administration was maintained for 16 weeks for mortality assay and 10 weeks for the immunological analysis. Supplementation of 1% RJ was found to extend the median survival time in immune deficient mice. Although dysfunction of T- and B-cell mitogenesis was observed in primary cultured splenocytes during micronutrient deficiency, 1% RJ supplementation significantly increased T- and B-cell response against mitogens. In addition, 1% RJ supplementation partially recovered the abnormal alteration in cytokine secretion, indicated by the decreased secretion of T-helper 1 cytokines and increased secretion of T-helper 2 cytokines resulting from low micronutrient levels. The hepatic vitamin E level was significantly decreased (p<0.05) following micronutrient deficiency, in accordance with the increased hepatic lipid peroxidation level. However, 1% RJ supplementation reduced hepatic lipid peroxidation, which may result from the restoration of the hepatic vitamin E level. Therefore, the present study indicated that 1% RJ supplementation may ameliorate the premature mortality in mice through the restoration of immune dysfunctions, which may result from antioxidative ability of RJ in immune deficient mice caused by low micronutrient intake.

Keywords: malnutrition, royal jelly, antioxidant, immunodeficiency, cytokine

1. Introduction

Malnutrition is the underlying cause of childhood mortality in 45% of cases in children under the age of five in developing countries [1]. A diet consistently low in micronutrients result in cells that lack the nutrients essential for normal metabolic functions. Micronutrient deficiency is a common cause of secondary immune dysfunction and has been found to be associated with increased susceptibility to infection in humans [2]. In a previous study, 89% of drug users have been demonstrated to have an inadequate nutritional status with respect to at least one nutrient, while 41% presented multiple abnormalities, with the most predominant one being inadequate plasma levels of micronutrients, including vitamin A, C and E, as well as zinc and selenium [2]. A further study indicated that 46% of subjects exhibited abnormal levels of zinc and iron, while 21% of subjects presented abnormally low albumin levels. Although the micronutrient status is not a predominant etiological determinant, micronutrient levels are known to be able to alter immune function to affect both morbidity and mortality [3].

Royal jelly (RJ) is produced by the hypopharingeal and mandibular glands of worker honey bees (*Apis mellifera*) and is an important food source of the queen honey bee [4]. The physical and chemical properties of RJ, as well as its constituents, which include proteins (12~15%), sugars (10~16%), lipids (3~6%), vitamins, minerals and free amino acids, have been well-established in a number of studies [5,6,7,8,9]. However, to the best of our knowledge, no studies exist on the effect of RJ on immune restoration in micronutrient-induced immune dysfunction. In the present study, immune restoration by RJ supplementation was investigated in an immune-deficient animal model.

2. Materials and Methods

2.1. Materials and Animal Care

RPMI 1640, fetal bovine serum, glutamine, penicillin and streptomycin were purchased by Hyclone, Waltham, MA, USA. Concanavalin A, lipopolysaccharide and

diethyl ether were obtained from Sigma-Aldrich, St. Louis, Mo, USA. Elisa kits to detect cytokines were purchased from R&D Systems, Minneapolis, MN, USA. C18 column (3.9×150 mm) was bought at NovaPak, Bedford, MA, USA. A *K-Assay*™ LPO-CC Assay Kit obtained from Kamiya Biomedical Company, Seattle, WA, USA. The animal diets were manufactured by Unifaith Inc, Seoul, Korea and its compositions were stated in the Table 1.

Female C57BL/6 mice (4 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan) and housed at the animal facilities of Kyung Hee University (Yongin, Republic of Korea). The study was approved by the Institutional Animal Care and Use Committee of Kyung Hee University (KHUASP(SU)12-14). After one week of acclimatization, the mice were randomly assigned into four groups (6 mice per group); Control group, with AIN-93M rodent diet, micronutrient deficient group, with AIN-93M diet containing only 7.5% of the recommended amount of micronutrients; and two groups receiving low micronutrient AIN93M diet along with RJ supplementation (0.2% or 1% RJ; a % of total weight of the diet). Oral administration of RJ was initiated two days prior to commencement of the AIN-93M diet containing 7.5% of the recommended micronutrient amount and continued for 16 weeks for mortality assay (10 mice per group) and 10 weeks for the immunological analysis (6 mice per group). RJ was obtained from nutritional biochemistry laboratory in Kyung Hee University in Korea. When lymph nodes were found to be enlarged, all mice were sacrificed by diethyl ether anesthesia. Spleens were dissected and primarily cultured to measure lymphocytes mitogenesis and cytokine release. Livers were collected for vitamin E and lipid peroxidation (LPO) analysis and stored at -70°C until assayed.

Table 1. Diet composition of experimental groups (g/kg)

Ingredients	Control	7.5% micronutrient	0.2% RJ	1% RJ
Casein[1]	140	140	140	140
L-cystine	1.8	1.8	1.8	1.8
Corn oil	40	40	40	40
Cellulose	50	50	50	50
Vitamin mix[2]	35	0.75	0.75	0.75
Mineral mix[3]	10	2.625	2.625	2.625
Sucrose	100	100	100	100
Dextrin	155	155	155	155
Corn starch	465.7	465.7	463.7	455.7
t-Butylhydroquinone	0.008	0.008	0.008	0.008
Royal jelly			2	10

[1]Casein (nitrogen x 6.25), 870 g/kg.
[2]Vitamin mix (AIN-93-VX)(g/kg mixture): niacin 3.00, calcium pantothenate 1.60, pyridoxine HCl 0.70, thiamine HCl 0.60, riboflavin 0.60, folic acid 0.20, biotin 0.02, vitamin E acetate (500 IU/g) 15.00, vitamin B12 (0.1%) 2.50, vitamin A palmitate (500,000 IU/g) 0.80, Vitamin D3 (400,000 IU/g) 0.25, Vitamin K1/Dextrose Mix (10 mg/g) 7.50 and sucrose 967.23
[3] Mineral mix (AIN-93M-MIX)(g/kg mixture): calcium carbonate 357.00, potassium phosphate monobasic 250.00, potassium citrate H_2O 28.00, sodium chloride 74.00, potassium sulfate 46.60, magnesium oxide 24.00, ferric citrate U.S.P. 6.06, zinc carbonate 1.65, manganous carbonate 0.63, cupric carbonate 0.30, potassium iodate 0.01, sodium selenate 0.01025, ammonium paramolybdate 4 H2O 0.00795, sodium metasilicate 9H2O 1.45, chromium potassium sulfate 12H2O 0.275, lithium chloride 0.0174, boric acid, 0.0815 sodium fluoride 0.0635, nickel carbonate 0.0318, ammonium vanadate 0.0066 and sucrose finely powered 209.806.

2.2. Survival during Low Micronutrient and RJ Supplementation

Female C57BL/6 mice (4 weeks old), were randomly assigned to one of the groups and fed low micronutrient-AIN93M rodent diet for 16 weeks in order to assess the effect of RJ supplementation on survival rate [12]. The mouse survival and behavior were monitored daily, while water supply and specified diet was changed every three days. The median survival time represented the day at which 50% mortality was observed in the treatment group.

2.3. ELISA for Cytokines

Production of Th1- and Th2-type cytokines from mitogen-stimulated splenocytes was measured as described in a previous study [9]. Briefly, spleens were minced, subsequently passed through 40-μm mesh and collected into 50 ml conical tubes containing culture medium. The cell concentration was adjusted to $1×10^7$ cells/ml after cell count with trypan blue dye. The primary splenocytes stimulated with concanavalin A (Con A; $1×10^{-2}$ g/l, 0.1 ml/well) to determine the production of interleukin (IL)-2 and IL-4 following incubation for 24 h and interferon (IFN)-γ following incubation for 72 h in a 37°C, 5% CO_2 incubator. In addition, splenocytes were incubated for 24 h following the addition of lipopolysaccharide (LPS; $1×10^{-2}$ g/l, 0.1 ml/well) to induce IL-6 and tumor necrosis factor (TNF)-α production. Following incubation, the supernatants were collected by centrifugation at 800 g for 10 min (Eppendorf model 5810R, Hamburg, Germany) and stored at -70°C for further analysis. The cytokine levels were determined using a sandwich ELISA kits as mentioned by a manufacture procedure.

2.4. Mitogenesis of Splenocytes

To determine T- and B-cell proliferation, splenocytes were cultured in 0.1 ml culture medium ($1×10^7$ cells/ml) in 96-well flat-bottom culture plates with 10 μg/ml Con A and 10 μg/ml LPS [6]. The samples were incubated at 37°C, 5% CO_2 incubator for 24 h and then pulsed with ^{3}H-thymidine (0.5 μCi/well; Perkin Elmer Inc., Waltham, MA, USA). After 24 h, the samples were harvested using a cell harvester (Cambridge Technology Inc., model 200A, Cambridge, MA, USA). Radioactivity was determined using a liquid scintillation counter (Tri-Carb, 2200 CA; Packard, Laguna Hills, CA, USA). Data were collected as counts per minute.

2.5. Measurement of Lipid Peroxidation (LPO)

To determine total hepatic LPO, approximately, 0.2 g liver tissue was minced in 3 ml $CHCl_3$/methanol (2:1, v/v) and 0.6 ml 0.9 % NaCl, and the mixture was centrifuged at 3,000 g for 10 min [10]. The supernatant was discarded, while the bottom layer that included $CHCl_3$ was evaporated under N_2 gas. A total of 100 μl isopropanol was added to dissolve lipid residue and 20 μl of the sample was used to measure lipid peroxides with the LPO kit. Methylene blue, final product, was colorimetrically measured at 675 nm to quantitate the lipid peroxides. LPO

values were calculated according to the manufacturer's instructions and converted to percentages for illustrative purposes.

2.6. Determination of Vitamin E Levels

Vitamin E levels in liver tissue were determined using Burton's method, as described previously with slight modification [11]. Approximately 0.2 g tissue was minced in 1 ml distilled water, and pentane and ethanol in sodium dodecyl sulfate were used to extract α-tocopherol from the homogenate. The extracts were evaporated under a steady flow of N_2 gas at 20°C and then dissolved in 0.5 ml methanol injected into a C18 column. A mobile phase composed of methanol and 1 mol/l sodium acetate (98:2, v/v) was used at a flow rate of 1.5 ml/min. The levels of α-tocopherol with a retention time of ~5 min was monitored using a fluorescence detector (2475 FLR detector, Waters, Milford, MA, USA) at 290 nm excitation and 320 nm emission wavelength.

2.7. Statistical Analysis

The experimental results are presented as the mean ± standard deviation (SD). Data was analyzed by ANOVA followed by the Bonferroni test for multiple comparisons and Student's *t*-test using SAS software. Differences were considered statistically significant at p<0.05.

3. Results

3.1. Body Weight, Dietary Intake, and Spleen Weight

No statistically significant differences were identified in dietary intake among the groups during supplementation. However, a decrease in food consumption was observed in the 7.5% micronutrient group till the end of supplementation. In addition, a statistically significant difference was observed between the body weight of mice receiving a normal AIN93M diet (28.3±1.21 g/mouse) and 7.5% micronutrient diet (21.9±1.27 g/mouse; *p*<0.05). The reduction in the body weight of the mice receiving the 7.5% micronutrient diet was partially ameliorated by 1% RJ supplementation (24.9±1.73 g/mouse), revealing a significant difference between the 7.5% micronutrient and 1% RJ supplementation groups. Furthermore, spleen weights were found to be significantly reduced in the 7.5% micronutrient group (*p*<0.05; data not shown) compared with the control group. Notably, the spleen size was found to be same in 1% RJ supplementation groups compared to control group, indicating that treatment with 1% RJ, but not 0.2% RJ, may be sufficient to compensate for the spleen size reduction resulting from a 7.5% micronutrient diet.

3.2. Survival during Reduced Dietary Micronutrient Intake

The median survival time was found to be 78 days in the mice receiving the 7.5% micronutrient diet, which is consistent with the median survival time of 81 days. However, the median survival time of mice receiving 0.2% and 1% RJ was found to be 81 and 97 days, respectively (Table 2).

3.3. Mitogenesis and Cytokine Production of Primary Splenocytes

Mice in the 7.5% micronutrient group presented a significantly impaired B-cell proliferation ability when compared with the normal control mice (Figure 1). Similar results were obtained regarding T-cell proliferation in mice in the 7.5% micronutrient group (Figure 1). The T- and B-cell response against mitogens significantly increased upon 1% RJ supplementation; however, no statistically significant difference was identified between mice in the 0.2% RJ group compared with mice in the 7.5% micronutrient group. The production of IL-2 and IFN-γ by Con A-stimulated splenocytes was found to be significantly reduced (*p*<0.05) in the 7.5% micronutrient group (Table 3). In addition, the release of TNF-α, IL-4 and IL-6 by LPS-stimulated primary splenocytes was found to be significantly increased (*p*<0.05) in the 7.5% micronutrient group (Table 4). Furthermore, intake of 1% RJ enhanced IL-2 and IFN-γ production, but reduced IL-4, IL-6 and TNF-α production.

Table 2. Median survival time of mice in the various study groups

Groups	Median Survival Time (Days) [*]
Control	> 112 [a]
7.5% micronutrient	78 [c]
0.2% RJ	81 [c]
1% RJ	97 [b]

[*] Median survival time represents the day at which 50% mortality is observed in the treatment group. RJ, royal jelly.

Table 3. Effect of RJ supplementation on T-helper cytokine production by primary cultured splenocytes

Groups	Cytokines (pg/ml)	
	IL-2	IFN-γ
Control	112.8±10.4 [a]	184.3±29.1 [a]
7.5% micronutrient	64.7±7.2 [c]	81.9±14.6 [c]
0.2% RJ	66.9±6.5 [c]	76.3±8.1 [c]
1% RJ	83.5±5.1 [b]	120.6±12.5 [b]

Data are presented as the mean±standard deviation of triplicate experiments. RJ, royal jelly; IL, interleukin; IFN, interferon.

Table 4. Effect of RJ supplementation on T-helper 2 cytokine production by primary cultured splenocytes

Groups	Cytokines (pg/ml)		
	IL-4	IL-6	TNF-α
Control	144.9±23.8 [c]	114.7±10.1 [c]	134.8±21.3 [c]
7.5% micronutrient	291.7±14.9 [a]	198.5±15.5 [a]	423.3±38.7 [a]
0.2% RJ	274.4±28.4 [a]	214.0±22.7 [a]	384.6±28.1 [a]
1% RJ	204.7±27.6 [b]	168.3±21.9 [b]	310.2±20.4 [b]

Data are presented as the mean±standard deviation of triplicate experiments. RJ, royal jelly; IL, interleukin; TNF, tumor necrosis factor.
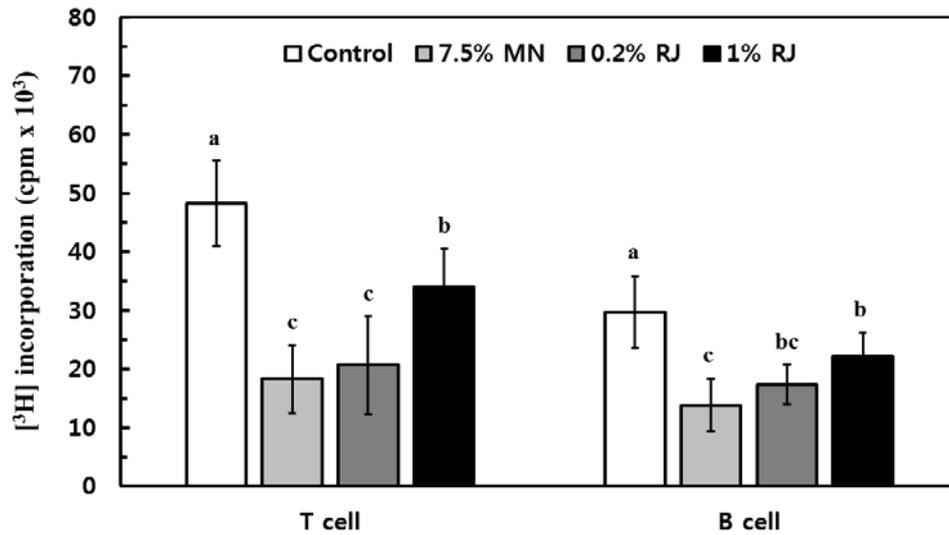
Figure 1. Effect of RJ supplementation on T- and B-cell mitogenesis *in vitro*. Data are presented as the mean ± standard deviation of triplicate experiments. CPM, count per minute; MN, micronutrient; RJ, royal jelly
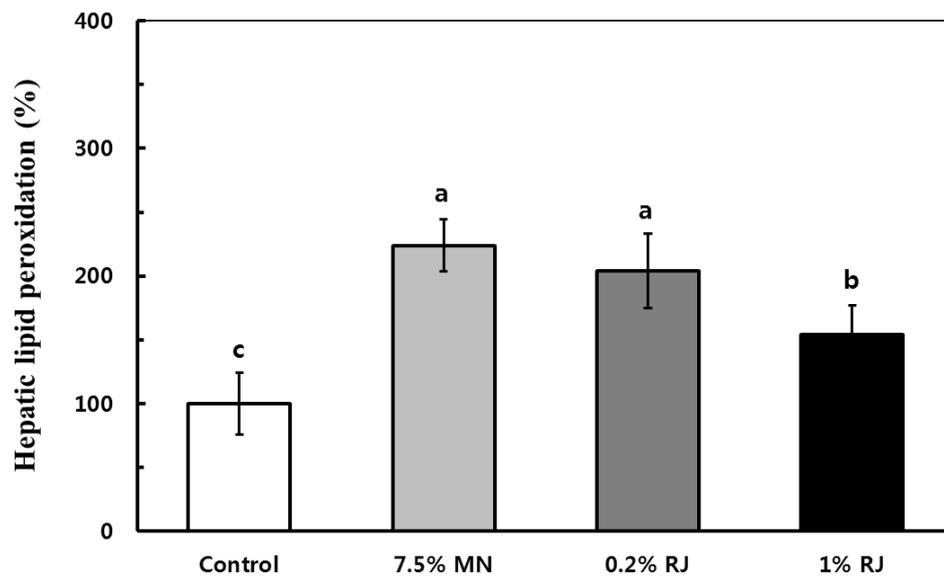

Figure 2. Effect of RJ supplementation on hepatic lipid peroxidation. Data was converted to percent unit (% = value of treatment group/value of control group x 100) and are presented as the mean ± standard deviation of triplicate experiments. MN, micronutrient; RJ, royal jelly
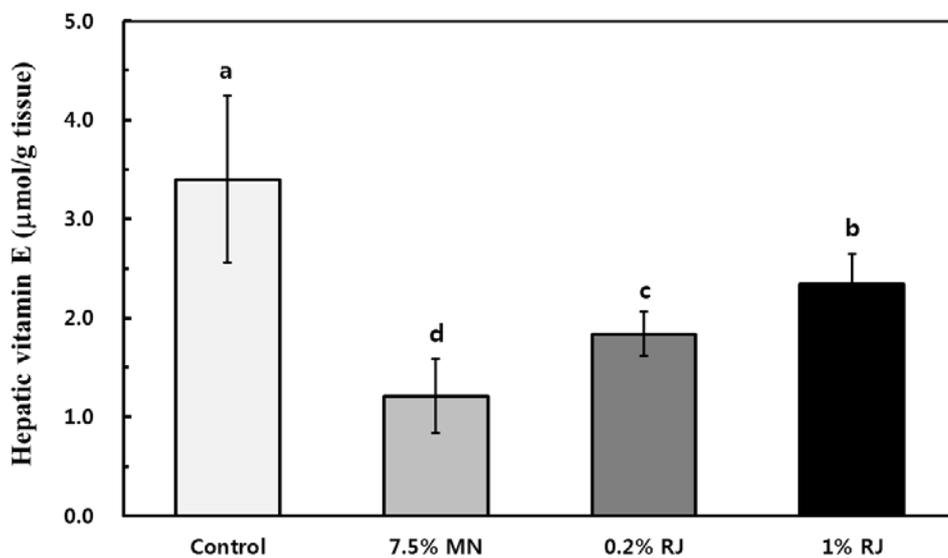

Figure 3. Effect of RJ supplementation on vitamin E levels in liver. Data was presented as the mean ± standard deviation of triplicate experiments. MN, micronutrient; RJ, royal jelly

3.4. Hepatic Lipid Peroxidation and Vitamin E Levels

Intake of the 7.5% micronutrients enhanced the hepatic LPO by 224%. Consumption of 1% RJ significantly reduced the total hepatic lipid peroxidation; however, intake of 0.2% RJ reduced the hepatic LPO only partially (Figure 2). Hepatic vitamin E level decreases during low levels of antioxidant and/or micronutrient intake due to the increase in free radicals, resulting in increased tissue LPO. To investigate the effect of RJ supplementation on the production of oxidative stress, hepatic vitamin E levels were measured. The 7.5% micronutrient diet significantly decreased the hepatic vitamin E levels. However, 1% RJ supplementation restored the hepatic vitamin E levels in a dose-dependent manner (Figure 3).

4. Discussion

Nutritional deficiency and immune dysfunction are known to markedly affect life span. In a study on the population of Zambia, nutritional impairment was found to be significantly associated with impairment of immune system, resulting in increased morbidity and premature mortality [13]. Certain micronutrients act as antioxidants and are particularly important regarding malnutrition, a status in which increased oxidative stress and antioxidant deficiencies are observed [14]. Therefore, the results of the present animal survival study suggested that RJ may partially delay premature mortality by compensating the cellular antioxidative status resulting from the lack of micronutrients.

Two possible mechanisms may explain the reduced life span in animals with low micronutrient intake, including immune dysregulation and excessive free radical production. The reduced consumption of micronutrient is hypothesized to result in cytokine dysfunction as micronutrient deficiencies may alter the cytokine production by shifting from balanced Th1/Th2-cell secretion of cytokines into increased Th2- and decreased Th1-cell cytokine production [15,16]. A previous study revealed that intake of 50% of the recommended diet led to an altered cytokine expression profile, resulting in a change of the Th1/Th2 cytokine balance, which is associated with lymphocyte proliferation [17]. These observations are consistent with the findings of the present study, which revealed a decreased lymphocyte number under conditions of micronutrient deficient dietary intake. Royal Jelly (RJ) has been demonstrated to have various immunopotentiating activities, including inhibition of tumor cells and protection against hemopoietic dysfunction through activation of macrophages and hematopoietic stem cells [18,19]. However, in a systemic allergy study, the suppression of allergic response in mice treated with RJ was found to be a consequence of the down-regulation of Th2 cytokine production [20]. Specifically, major royal jelly protein 3 has been demonstrated to be responsible for the decreased production of IL-1, IL-6 and TNF-α, reducing the inflammatory response, and may be associated with life span extension [21]. In the present study, 1% RJ supplementation was able to restore T- and B-cell function associated with micronutrient deficiency and partially normalize the imbalance of Th1/Th2 cytokine production by increasing Th1 (IL-2 and IFN-γ) and Th2 (IL-4, IL-6, and TNF-α) cytokines.

Excessive free radical generation is another possible mechanism of premature mortality, which may be the result of a micronutrient deficient diet, evidenced by increased LPO (Figure 2). Previously, Azab *et al* observed that RJ is able to alleviate tissue injury by reducing oxidative stress. In addition, the depletion of trace elements, including vitamins and minerals, was found to be associated with antioxidative enzyme activity in immune cells, which was recovered by RJ supplementation [22,23]. Therefore, RJ may, at least partially, substitute for low micronutrient levels and restore cellular antioxidant status. In accordance with results from LPO, increased free radical production under reduced micronutrient intake may stimulate utilization of antioxidants, including membranous vitamin E and cellular vitamin C. For instance, during low selenium and zinc intake, the majority of cytokine alterations resulted from deficiency of antioxidant nutrients [24]. In addition, pro-inflammatory cytokines (IL-1, IL-6, and TNF-α) may stimulate the production of reactive oxygen species [25]. This process involves activation of nuclear factor-κB (NF-κB), which evokes a systemic inflammatory reaction, resulting in premature mortality. Similarly, the present results demonstrated that LPO was markedly increased by low micronutrient intake and restored by RJ supplementation (Figure 2). A previous study suggested that the RJ acid, 10-hydroxy-trans-2-decenoic acid, inhibits LPS- and IFN-β-induced nitric oxide production via inhibition of NF-κB activation [26,27]. Furthermore, oxidative stress due to low antioxidant levels may induce DNA damage in micronutrient deficient cells, resulting in the long-term consequences of immunosuppression [28]. In a further study, RJ was found to possess the ability to regulate the cellular secretion of cytokines by inhibiting NF-κB and was hypothesized to inhibit non-enzymatic and enzymatic oxidation associated with DNA damage [29]. Thus, based on the present study, the restoration of the immune system by RJ supplementation is hypothesized to be associated with the antioxidative ability of RJ to prolong the life span of T- and B-lymphocytes by suppressing DNA damage.

In conclusion, the present results indicated that RJ supplementation may help increase the reduced life span in the mice, associated with micronutrient deficiency, by restoring immune dysfunction and cellular antioxidant levels. Future studies should investigate the effect of major chemical components and/or enzymatic-hydrolyzed products of RJ on immune restoration.

Acknowledgments

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