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# The efficacy of royal jelly in the restoration of stress-induced disturbance of lymphocytes and granulocytes

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## Abstract

**Restraint stress has been reported to suppress the conventional T and B lymphocyte responses, while inversely activating the granulocyte response in the peripheral blood. Since royal jelly (RJ) helps the body to cope with stress and fatigue, we asked whether RJ could restore the stress-induced dysregulatory numbers of lymphocytes and granulocytes in the peripheral blood and different organs such as the spleen, liver and thymus. In unstressed mice, we did not observe any significant change in the number of these immune cells in the peripheral blood and organs examined, regardless of the administration of RJ and PBS as a control. On the other hand, the mice which were subjected to the restraint stress without RJ administration showed significantly decreased numbers of total lymphocytes, including both T and B lymphocytes, but a significantly higher granulocyte number in the peripheral blood. The stress also affected a decrease in the total MNCs in the spleen and thymus. The RJ administration to these stressed mice showed a significant effect to restore the dysregulatory number of total lymphocytes, T and B lymphocytes, and granulocytes in the peripheral blood. The RJ administration to the stressed mice also partially restored the absolute numbers of total MNCs in the thymus, which was prone to the development of severe atrophy. These findings provide interesting evidence that RJ did not affect the immune function in the normal situation but exhibits an anti-stress effect through the well-balanced restoration of the dysregulation of immune cells under the stressed condition.**

## Introduction

It remains a widely accepted view that excessive or prolonged stress has a negative impact on health. Stress has been reported to be associated with human cardiovascular diseases, gastrointestinal disorders and mental illness [1-3]. Stress has also been shown to have various deleterious effects on the immune system. An ongoing period of stress has been found to cause disruption of both cell-mediated and humoral immunity [4]. It has also been reported that glucocorticoid (GC)-mediated immunosuppression induced by stress might be linked, not only to marked thymic atrophy, but also to the arrest of the formation of new lymphocytes, and to the inhibition of the release of antibodies and cytokines [4-7]. Restraint stress is also known to be implicated in the development of lymphocytopenia, granulocytemia, thymic atrophy and many other changes related to the immune functions in mice [8,9]. Restraint stress causes the reduction of $CD4^+CD8^+$ double-positive thymocytes through the apoptic DNA fragmentation of thymocytes [6,7], thus resulting in a decrease in peripheral lymphocytes through the induction of thymic atrophy coupled with apoptosis of mature lymphocytes. The stress-induced thymic involution has been demonstrated to be a direct consequence of elevated GC levels [6,7,10].

Royal jelly (RJ) is the milky-white gelatinous substance secreted from the cephalic glands of nurse worker bees (*Apis mellifera*) for the sole purpose of stimulating the growth and development of the queen bee. When fed RJ, the queen bee can develop superior characteristics in size, strength, stamina, and longevity. The queen bee can live 5-7 years, while the worker bees live only about 35-40 days.

RJ has been known to show many pleiotropic functions [11-14] in humans, and as a function of them, RJ has been reported to be associated with the potential of immunomodulation in mice by stimulating antibody production and immunocompetent cell proliferation [15-17]. Therefore, we asked whether RJ could restore the stress-induced dysregulation of immune cell homeostasis in mice.

To address the above mentioned issue, RJ was given orally to mice under restraint stress, and it was found that RJ administration restored significantly the disturbed distribution of peripheral lymphocytes and granulocytes, accompanied with recovery from thymic atrophy due to restraint stress. The present observation provides new evidence that RJ possesses an immunomodulatory function in favor of the host to give protection against substantial types of stress.

## Materials and Methods

### *Animals*

Male C57BL/6 (B6) mice at the age of 6-12 weeks were used. The mice were maintained under specific pathogen-free conditions throughout the experiment. All experiments were conducted according to the ethical principles and guidelines established by the University of the Ryukyus for the care and use of experimental animals.

### *Royal jelly and administration regimens*

Native RJ produced by Yamaguchi's organic bee-culture [18] was provided by the Japan Royal Jelly Co. Ltd., Tokyo, Japan. The RJ was diluted in PBS and 30 μl of diluted RJ solution containing 2.0 mg of proteins was orally administrated to each mouse with an administration regimen of once every 2 days during the experimental period. The control mice received the same regimen with RJ-free PBS.

***Restraint stress***

The mice subjected to restraint stress were kept between stainless steel meshes so that they were unable to turn around for 12 hours. The restraint stress was given twice per week for 6 weeks [19-21].

***Analysis of peripheral blood mononuclear cells (PBMC).***

Tail snip blood samples were collected from each mouse into heparinized capillary tubes. The total white blood cells (WBCs), lymphocytes, including their subsets, and granulocytes were counted for each sample. Thin blood smears were prepared on a microslide glass and were stained for the differential counts of the PBMCs. The differential counts were determined by oil immersion microscopic examination of May-Grunwald Giemsa-stained samples according to the cell morphology. The PBMCs were further analyzed for lymphocyte subsets by staining them with fluorescent-labeled monoclonal antibodies against their surface phenotypes followed by a flow cytometric analysis.

***Cell preparation from organs.***

The mice were sacrificed at day 42 following the RJ administration through total bleeding by cardiac puncture, and the thymus, liver and spleen were removed for the isolation of mononuclear cells (MNCs). The single cell suspension of thymocytes was harvested into Eagle's Minimal Essential Medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5.0 mM HEPES and 2% heat-inactivated newborn calf serum, by disintegrating the thymus tissues and passing them through a 200-gauge stainless steel mesh. The splenocytes were prepared as follows: The spleen was minced and the splenocytes were harvested into Eagle's MEM supplemented with 5.0 mM HEPES and 2% heat-inactivated newborn calf serum by forcing minced pieces of spleen through a 200-gauge stainless steel mesh.

The single-cell suspension was then centrifuged at 1500 rpm and the subsequent cell pellet was allowed to undergo erythrocyte lysis with 3 ml hemolyzing solution, consisting of 155 mM NH4Cl, 10 mM KHCO3 and 170 mM Tris for 3 min at 4˚C. Finally the pure white blood cells were obtained by centrifugation and washing with Eagle's MEM.

The liver MNCs were prepared by a previously described method [22]. Briefly, the livers were minced with scissors, passed through a stainless steel mesh, and suspended in the Eagle's medium. After washing with medium, the MNCs were fractionated in 15 ml of 35% Percoll solution (Amersham Pharmacia Biotech, Piscataway, NJ) by centrifugation for 15 min at 2,000 rpm. The pellet obtained was lysed by resuspension in 5.0 ml of erythrocyte lysis solution (155 mM NH4Cl, 10 mM KHCO3, 1 mM EDTA-Na and 170 mM Tris) followed by incubation for 10 min at 4˚C and washed twice for use. The MNCs were ready for immunofluorescence staining with labeled monoclonal antibodies as follows.

***Flow cytometry***

A flow cytometric analysis was performed with the labeled monoclonal antibodies (mAb) to the phenotypic antigens of mouse. The FITC-labeled anti-CD8 (53-6.7), anti-CD3 (145-2C11), anti-CD45/ B220 (104) and PE-labeled anti-CD4(RM4-5) were purchased from Pharmingen. The PerCP-labeled anti- CD45 (clone 2D1; Becton Deckinson, San Jose, CA) was also used in the experiments. To prevent nonspecific binding with mAbs, the cells were pre-incubated with anti-CD32/CD16 mAb (Pharmingen) before staining with the labeled mAbs. The analysis of the peripheral blood lymphocytes was performed on the $CD45^+$ gated cells with a FACS Caliber flow cytometer (Becton Dickinson Co.). The dead cells were excluded by forward scatter, side scatter, and propidium iodide gating.

### *Statistical analysis*

The statistical significance of the data was determined by Student's *t*-test using a computer software program (GraphPad Software). A $p$ value of $< 0.05$ was considered to be significant.

## Results

### *Influence of RJ on the number of total WBC, lymphocytes and granulocytes in the peripheral blood of unstressed mice*

In order to estimate the effect of RJ administration on the immune cell compartments of mice under unstressed condition, we estimated the kinetics of the absolute number of total WBC, lymphocytes and granulocytes in the pe

ripheral blood of unstressed mice, orally administrated RJ or PBS. As shown in Figure 1A-C, we did not observe any differences in the circulating WBCs, lymphocytes and granulocyte counts between the RJ-fed mice and the control mice administered PBS.

Subsequently, we further asked whether there was any modification of the numbers of T and B lymphocytes by RJ. As shown in Figure 2A and 2B, the absolute numbers of these T and B lymphocytes also showed no significant differences between the mice administered RJ and PBS.

### *Influence of RJ on the number of mononuclear cells (MNC) and their subsets in the thymus, spleen and liver of unstressed mice*

To assess whether RJ causes a change in the homeostasis of MNCs in the thymus, spleen and liver of healthy mice, the total numbers of MNCs (cells/organ) were calculated
on day 42 after the RJ administration. The results are represented in Figure 3. The absolute numbers of MNCs in these organs of RJ-treated mice remained unchanged in comparison to the PBS-treated control mice.

As for the MNCs recovered from the liver and spleen, the lymphocyte subsets were similarly estimated for $CD3^+$, $B220^+$, $CD4^+$ and $CD8^+$ cells. As shown in Figure 4, we could not identify any significant differences in the absolute numbers of these cell subsets between the RJ-treated and the PBS-treated mice, although the $B220^+$ B cells showed an insignificant decrease in the liver of the RJ--treated mice. For both the $CD4^+$ and $CD8^+$ T cell subsets in the thymus, the absolute numbers were compared between the mice, RJ- or PBS-administered, and they were almost the same between the RJ-treated and PBS-treated mice (Fig. 5).

### *Influence of RJ on the number of total WBC, lymphocytes and granulocytes in the peripheral blood of stressed mice.*

When mice were subjected to restraint stress, the number of total WBCs and lymphocytes in the peripheral blood were shown to decrease significantly under the stressed condition (Fig. 6A and Fig. 6B). On the other hand, the number of peripheral granulocytes increased significantly during the stress exposure (Fig. 6C). When RJ was administered to the stressed mice, it was found that the absolute number of peripheral blood lymphocytes was restored significantly at days 14 ($p < 0.05$ for RJ vs PBS groups) and 28 ($p < 0.0005$ for RJ vs PBS groups) (Fig. 6B). Conversely, the number of peripheral granulocytes which increased significantly under the restraint stress were decreased on the same days ($p < 0.05$ at day 14 and $p < 0.0005$ at day 28) after the RJ administration (Fig. 6C). With a concomitant increase in the absolute number of lymphocytes and a decrease in the granulocytes, the total number of circulating blood WBCs in the RJ-treated mice did not differ at different time points from those of the in PBS-treated mice under restraint stress (Fig. 6A). As reflected from the above results, the stress-induced increase in the ratio of granulocytes to total lymphocytes, 7:1 at day 14 and 3:1 at day 28, were restored significantly by RJ administration to the ratios of 2.5:1 at day 14 and

1:1.2 at day 28, and the ratio was almost normal at day 28. However, RJ was not able to exhibit its effect when the stress was continued over a time period of 4 weeks, indicating almost the same numbers in the RJ-treated and PBS- treated mice (Fig. 6B and Fig 6C). These results suggest that RJ is significantly effective in the maintenance of different peripheral blood WBC compartments unde rstressed conditions, which are not continued for a very long time.

***Influence of RJ on the absolute numbers of T and B lymphocytes in the peripheral blood of stressed mice***

The findings that RJ could restore a stress-mediated decrease in peripheral blood lymphocytes prompted us to investigate whether RJ administration is effective to restore the absolute numbers of both T and B lymphocytes. We therefore analyzed and calculated the numbers of circulating T and B lymphocytes, and observed that the absolute numbers of both populations of lymphocytes were equally restored (Fig 7A, B) by RJ administration on days 14 and 28, consistent with the increase of the total number of lymphocytes. As a result, no statistically significant difference in the ratios of T and B lymphocytes between the unstressed and RJ-fed stressed mice was found.

***Influence of RJ on the number of mononuclear cells (MNC) and their subsets in the thymus, spleen and liver of restraint stressed mice.***

To assess whether RJ could cause any modification in the distribution of MNCs in the liver, spleen and thymus of stressed mice, the total numbers of cells/organ were calculated from the mice on day 42 after the RJ and PBS administration. The numbers of MNCs in the spleen and thymus decreased markedly in the stressed mice. The decrease in the number of thymocytes recovered in the mice administered RJ, although the splenocyte numbers did not show any recovery by the RJ administration. The difference in the number of thymus cells, however, was not statistically significant (Fig.8). When the CD3, B220, CD4, and CD8 subsets of lymphocytes were compared for MNCs from the liver and spleen, we could not identify any significant differences in the absolute numbers of the lymphocyte subsets between the RJ-treated and non- treated stressed mice (data not shown).

On the other hand**,** although it did not reach a statistically significant difference, there was a good trend toward an increase in the absolute numbers of total MNCs, $CD4^+CD8^+$ (DP) cells, and $CD4^+$ and $CD8^+$ T cells in the stressed thymus of the RJ administered mice (Fig. 9).

## Discussion

Stress is a part of our daily life. Stress has been demonstrated to be associated with immunosuppression [23,24] and disease susceptibility [1-3]. Therefore, finding ways to lessen the impact of stress is vital to physical, mental and physiological health. It has been reported that royal jelly is effective against oxidative stress in rats [25]. However, there are, as yet, no reports regarding a modulation effect of royal jelly on the immune function of mice exposed to restraint stress, which is known as an animal model of psychological stress. In the present study, we investigated whether RJ could protect mice from the severity of restraint stress-induced immune dysfunction, because of its potent immunomodulatory function through the stimulation of antibody production and immunocompetent cell proliferation [11,12,16]. The experiments were designed to observe the immunomodulatory effects of RJ on the restraint stress-induced immune cell dysregulation.

The mice which were subjected to chronic 12-h restraint stress, twice per week for 6 weeks, exhibited a significant decrease in the absolute numbers of thymocytes, splenocytes, and hepatic and peripheral blood MNCs. Along with the tendency of the total MNCs in the peripheral blood to decrease, granulocytemia and lymphocytopenia were markedly apparent in the stressed mice. When RJ was administered to these mice, it was found that the RJ administration markedly ameliorated the restraint

stress-induced lymphocytopenia and granulocytemia by an up-regulation of the peripheral blood lymphocyte counts with a concomitant down-regulation of the granulocyte counts. It is likely that RJ might contain mitogenic components for lymphocyte proliferation and negative regulators of the granulocytes. The present findings, that RJ could cause up-regulation of peripheral blood lymphocyte production in the mice under restraint stress, are supported by a report which demonstrated that RJ might have a potential component for immunocompetent cell proliferation in mice [15]. The down-regulation of the stress-induced granulocytemia might be explained by several findings, whereby RJ showed anti-inflammatory actions through an inhibition of proinflammatory cytokine production by macrophages [26-28]. These results suggest that RJ administration can withstand a restraint stress-induced dysregulatory pattern of immune cell distribution in the peripheral blood by the maintenance of a partial, but significant, normalization of the circulatory lymphocytes and granulocytes. On the other hand, a possible explanation for the maintenance of homeostasis of the circulatory immune cells by RJ under restraint stress might be because of the continuous supply of T cells from the thymus to replenish the stress-induced loss of lymphocytes. This view is broadly consistent with our data demonstrating the partial restoration of thymic total MNCs, $CD4^+$ and $CD8^+$ T cells by RJ under restraint stress. The boost in the supply of $CD4^+$ and $CD8^+$ T cells from the thymus and B cells from the bone marrow to the peripheral circulation might have assisted in the maintenance of normalization of the peripheral blood T and B cell populations. Our data are consistent with the reports demonstrating that RJ is protective against the hematopoietic dysfunction observed in X-irradiated mice through the induction of macrophage activity and hematopoietic stem cell proliferation [29,30]. The importance of RJ in the up-regulatory development of T cells in the thymus to provide T cells to the periphery and in the induction of the systemic immune response has also been reported [24].

Paradoxical to this effect of RJ on the peripheral blood, RJ did not show a potential for the abolition of the stress-mediated disturbance of the MNCs and their subsets in the liver and spleen. However, the effect of RJ on the systemic organs, especially the liver and spleen, was only evaluated on day 42, when there had been no observed effect of RJ on the maintenance of the circulating MNCs homeostasis. As a result, we cannot rule out the possibility that we could observe the effects of RJ in these organs similar to that produced in the peripheral blood on days 14 and 28, if we would have investigated the parameters between days 14 and 28 following RJ administration. On the other hand**,** although not in a statistically significant direction, there was a good trend for the RJ-administered thymus to show an increase in the absolute numbers of total MNCs, $CD4^+CD8^+$ (DP) cells, and $CD4^+$ and $CD8^+$ T cells which were decreased significantly by restraint stress. Based on our findings on the peripheral blood parameters at days 14 and 28, and on the thymus at day 42, it may be speculated that the thymus was more potent in the generation of T cells between days 14 and 28. Therefore, another likely reason for the failure of RJ to show its effect in the maintenance of homeostasis of hepatic and splenic cellularity is that there might be a disturbance in the trafficking and homing of lymphocytes in the stressed liver and spleen, and because of such disturbance in lymphocyte homing, the RJ could not replenish the stress-induced loss of lymphocytes in these organs. Further studies are thus considered to be necessary to clarify these speculations.

The present study was unique in the sense that RJ administration to stressed mice caused a simultaneous appearance of both proinflammatory and anti-inflammatory actions, as manifested by the RJ-mediated restoration of peripheral blood lymphocytes during stress-induced lymphocytopenia and the inhibition of stress-induced granulocytemia. Although RJ has been reported to be associated with both proinflammatory and anti-inflammatory actions, there have been no reports to demonstrate the simultaneous appearance of these phenomena together in a single experimental system. The present results that RJ played a contrary role to promote proinflammatory action and anti-inflammatory action are easy to consider as homeostatic roles of the systemic immune system, rather than the respective role of RJ. In any event, the significant decrease of the circulating lymphocyte numbers may cause depressed immunocompetition, and granulocytemia may be responsible for the onset of systemic inflammation and tissue damage in certain disease states and stressed conditions. The present study in which RJ was found to significantly suppress stress-stimulated granulocytemia,

thus raises speculation that RJ may have a beneficial effect in the treatment of granulocyte-associated inflammation and tissue damage. Based on the data of previously published reports and our findings in this study, we hypothesize that the partial abolition of the impact of restraint stress by RJ may be a balancing action of different RJ ingredients favoring the host.

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