Immunomodulatory potential of Rutin and Quercetin in Swiss mice exposed to gamma radiation


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Abstract
Radioprotective mechanisms of Rutin (RUT) and Quercetin (QRT) against gamma radiation was studied by investigating recovery of endogenous spleen colony-forming units, haematological alterations in peripheral blood in Swiss albino mice. Radiation induced haematological alterations in peripheral blood was studied; mice were treated with RUT (10mg/kg.b.wt.) and QRT (20mg/kg.b.wt.) once daily for five consecutive days and exposed to 7.5 Gy of gamma radiation after the last administration. RUT and QRT treatment before exposure to 7.5 Gy of gamma radiation resulted in a significant (p<0.01) increase in cellular components of blood at various post-treatment time points when compared with the respective irradiated groups. Irradiation of mice reduced the spleen colony forming units in the spleen and the number of spleen nodules was inversely related with the increase in radiation dose. The potent antioxidant nature of RUT and QRT mitigate the oxidative stress induced by gamma radiation and thus protect the mice from hemopoietic damage.

Keywords: Rutin, Quercetin, irradiation, immunomodulatory.

1. Introduction
Radiation has diverse applications in medicine, agriculture, industry, biochemical research, and defence. It draws attention the evaluation of risks of exposure. Radiation therapy has been successfully used to treat malignant tumours of different histological origin and stages, (individually or in combination with chemotherapy and surgery, or both) for several decades. In clinics, the success of radiation treatment depends totally on its ability to selectively kill tumour cells while sparing the normal tissues that are in the vicinity of tumours. The response of mammalian cells to ionizing radiations at the cellular and molecular level is complex and is an active irreversible process that is dependent on both the radiation dose and the tissue-weighting factor [1]. Most of the tissue damage caused by ionizing radiation is mediated by the reactive oxygen species (ROS) generated from the interaction between radiation and water molecules in cells [2]. These ROS react with biological molecules including proteins, lipids, lipoproteins and DNA [3].

Many synthetic compounds have been studied for their ability to protect against adverse effects of radiation ever since the original observation of radioprotection by Patt and co-workers [4]. However, the practical applicability of the majority of these synthetic compounds remains limited, owing to high toxicity at their optimum protective dose [5]. Due to lack of effective protective agents, new compounds are currently under investigation as possible adjuvant in radiation treatment of cancer. Herbal medicines have only recently begun to receive some attention as possible modifiers of the radiation response [6-
9]. Naturally occurring dietary components also offer opportunities for development as effective radioprotective agents because of their potential low toxicity [8,10]. Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as radiation-induced oxidative damages [11]. Hence, the studies on natural antioxidants have gained increasingly greater importance.

Rutin and Quercetin are common dietary flavonoids that are present in fruits, vegetables and plant-derived beverages such as tea and wine. Recently, flavonoids have attracted attention because of their beneficial biological activities to human health. Rutin's anti-inflammatory potential has been demonstrated in a number of animal studies [12,14]. Quercetin also has demonstrated significant anti-inflammatory activity because of direct inhibition of several initial processes of inflammation. The intestinal mucosa is extremely sensitive to ROS [13]. Since there is limited information on the importance of rutin and quercetin as an antioxidant in vivo, we focused on the protective effect of rutin on intestinal oxidative injury.

A severe depression of hematopoietic function often takes place in patients undergoing radiotherapy due to the high sensitivity of these organs. Developing a strategy to protect and stimulate these cell pools is very important and desirable to counteract the adverse effects of radiation, and thus allow a more intensive and effective therapy [15-18]. Therefore in the present study we have attempted to evaluate the protective role of RUT and QRT against radiation-induced alterations in cells of peripheral blood of mice as experimental end point.

2. Materials and methods

2.1 Animals

Four to six weeks old inbred mice of Swiss albino strain of either sex weighing 25 to 30 g were selected, and kept in well ventilated polypropylene cages under standard conditions of temperature (23±2°C), humidity (50±5%) and light (10 and 14 hours of light and dark, respectively). Animals were allowed food and water ad libitum. The animal experiments were carried with the prior approval from the Institutional Animal Ethics Committee. Animal care and handling was done according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the Indian National Science Academy, New Delhi, India.

2.2 Chemicals

2.2.1 Drug preparation and mode of administration

Rutin and Quercetin was purchased from Himedia Laboratories Pvt. Ltd., Mumbai, India. Rutin (RUT) and Quercetin (QRT) powder was, suspended in water using 0.5% w/v Carboxy Methyl Cellulose (CMC) and was given once daily (5 ml/kg body weight), various doses of RUT and QRT 10-100 mg/kg body weight orally once a day for five consecutive days. Radiation exposure was performed 1 hour after the last dose of RUT and QRT administration.

2.2.2 Other chemicals

RUT and QRT, glutathione, chloro-2, 4-dinitrobenzene (CDNB), 5,5-dithiobis-2- nitrobenzoic acid (DTNB), trichloroacetic acid (TCA), thiobarbituric acid (TBA), ethidium bromide, normal melting agarose (NMA), low melting agarose (LMA) and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acridine orange (AO) was purchased from BDH Chemicals Ltd, Poole, England. The other chemicals such as absolute alcohol, dimethyl sulphoxide (DMSO), ethylene diamine tetraacetic acid (EDTA), sodium bicarbonate, sodium chloride, potassium hydrogen phosphate and hydrochloric acid were purchased from Qualigens Fine Chemicals (A Division of GlaxoSmithKline Pharmaceuticals), Mumbai, India.

2.2.3 Radiation exposure

Unanesthetized mice were restrained in a specially designed well-ventilated acrylic box and exposed to whole-body radiation from 60Co gamma tele-therapy facility (Theratron Atomic Energy Agency, Canada) at the Shirdi Sai Baba Cancer Hospital, Manipal, at a dose rate of 1.33 Gy/minute and source to surface distance (SSD) of 61 cm. Total radiation exposure time was 5 minutes 59 seconds.

2.3 Experimental design for haematological and endogenous colony assay

To study the effects of Rutin (RUT) and Quercetin (QRT) on radiation induced alterations in mouse spleen and peripheral hematological cell count, animals were divided into following groups with six animals per group:

1) Untreated Control group: These groups of animals were given 0.1ml/kg.b.wt. of CMC orally for five consecutive days.

2) Radiation alone group: These groups of animals were given 0.1ml /kg.b.wt. of CMC orally for five consecutive days. One hour after the last administration on the third day animals were exposed to 7.5 Gy gamma radiation.

3) RUT and QRT + Radiation group: These groups of animals were given an optimum dose of 10mg/kg.b.wt. RUT and 20 mg/kg.b.wt. QRT orally for five consecutive days. One hour after the last administration on the fifth day animals were exposed to 7.5 Gy gamma radiation.

2.3.1 Haematological study

In the present study radiation dose of 7.5 Gy was selected as it clearly gives a quantitative difference in the recovery effects by protective agents on the haemopoietic syndrome [19]. After various treatments, blood was
collected from the orbital sinus of each animal from each group in a vial containing 0.5M EDTA at 0, 1, 3, 5 and 7 days of post irradiation. The blood smear was examined microscopically. The density of white, red blood cells and platelets were estimated. The functional red blood cell mass was measured by three parameters: red blood cell count (RBC), hematocrit (Hct), and hemoglobin concentration (Hb). A differential white blood cell count was performed which enumerates the various individual white blood cell types found in peripheral blood. The predominant circulating leukocytes in mice are the lymphocytes, followed by neutrophils, monocytes, eosinophils, and lastly basophils were evaluated by using standard laboratory procedures after staining the smears with Leishman’s stain.

2.3.2 Endogenous spleen colony forming units (CFU-S) assay

Endogenous spleen colony forming units (CFU-S) assay was performed according to the method of Till and McCulloch [20]. The mortality by bone marrow syndrome usually seen in irradiated animals when exposed to more than 6.0 Gy. Therefore, radiation dose of 7.5 Gy was selected in the present investigation for the CFU-assay ensuring the formation of sufficient number endogenous haemopoietic spleen colonies. After various treatments, all the animals from above groups were euthanized on day 14 of post-treatment and spleens were removed, weighed and fixed in Bouin’s fixative. The macroscopic nodules on the surface of the spleen were counted using stereomicroscope (Wild M3Z, Heerbrugg, Switzerland).

2.4 Statistical analysis

All the values are expressed as mean ± SEM and the statistical analyses were performed using one way analysis of variance (ANOVA) followed by Bonferroni’s Post-hoc test to determine the significance between the various groups. The differences between the groups were compared and p<0.05 was considered significant.

3. Results

3.1 Effect of RUT and QRT on radiation induced hematological alterations

The effect of RUT and QRT on peripheral blood, hematological parameters such as Hb, RBC, WBC and Hct were evaluated at different post-irradiation intervals. RUT and QRT treatment alone did not alter these hematological indicators when compared to that of the untreated control. However, a significant (P<0.01) decrease in WBC count was observed in irradiated animals, while treatment of mice with 10mg/kg.b.wt. RUT and 20 mg/kg. b.wt. QRT one hour before exposure to 7.5 Gy of gamma radiation resulted in a marginal increase in WBC count at 0, 1, 7, 14, 21 and 28 days compared with respective irradiated groups (Figure 1 and Table 1). RBC count and Hct in irradiated animals were 51% and 73% respectively, while animals pretreated with RUT and QRT before exposure to 7.5 Gy of gamma radiation resulted in 69.72% and 81.26% when compared with non-irradiated control animals (Figure 1 and Table 1).

Pre-treatment with RUT and QRT resulted in a significant (P<0.01) increase in the RBC count and Hct, and this increase was 1.35 and 1.1 folds higher than that of irradiated control group. Hb levels of control group were considerably decreased after 7.5 Gy gamma irradiation, however, pre-treatment of mice with RUT and QRT significantly (P<0.01) increased hemoglobin at different post-irradiation intervals when compared with those treated with radiation alone (Figure 1 and Table 1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Hb (gm%)</th>
<th>RBC (million/mm³)</th>
<th>WBC (10³ cells/mm³)</th>
<th>Hct(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>11.26 ± 0.40</td>
<td>3.78 ± 0.13</td>
<td>8.94 ± 0.05</td>
<td>31.47 ± 0.15</td>
</tr>
<tr>
<td>QRT alone</td>
<td>11.93 ± 0.17</td>
<td>3.98 ± 0.11</td>
<td>10.77 ± 0.09</td>
<td>37.23 ± 0.17</td>
</tr>
<tr>
<td>IR Alone</td>
<td>6.41 ± 0.17*</td>
<td>3.15 ± 0.10**</td>
<td>18.37 ± 0.06**</td>
<td>21.81 ± 0.45**</td>
</tr>
<tr>
<td>QRT + IR</td>
<td>11.77 ± 0.11*</td>
<td>3.79 ± 0.17**</td>
<td>12.23 ± 0.04**</td>
<td>32.86 ± 0.61**</td>
</tr>
</tbody>
</table>

Data represent the mean ± SEM from six mice per group. Further data was analyzed by one way ANOVA followed by Dunnet’s‘t’ test, n=6 **P<0.01, *P<0.05 (Data also represent the average value of 0, 1, 7, 14, 21 and 28 days)
Figure 1: Alterations in the peripheral blood cell counts, hemoglobin and hematocrit content in mice treated with 10 mg/kg.b.wt. RUT before exposure to 7.5 Gy gamma radiations

Data represent the mean ± SEM from six mice per group. Data is statistically significant p<0.05. Day zero indicates control values. Figure in clock-wise direction (a) RBC, (b) WBC, (c) Hemoglobin (Hb), (d) Hematocrit (Hct).

3.2 Effect of RUT on radiation induced hematopoiesis

Mean spleen weight of untreated mouse was 69.40 ± 0.45mg. Whole body exposure to 7.5 Gy gamma radiation significantly (P<0.01) decreased the spleen weight (Table 2). Treatment with RUT before exposure to 7.5 Gy significantly (P<0.01) increased the spleen weight. The spleen colony counts in the both groups also showed a similar pattern to that of spleen weight. Irradiation with 7.5 Gy resulted in 0.29 ± 0.23 colonies/spleen, while pretreatment with RUT (P<0.01) increased the number of spleen colonies to 8.06 ± 0.6 at 7.5 Gy on day 14 of post-irradiation (Table 2).

Table 2: Survival of spleen colony forming units in irradiated mice (7.5 Gy) with or without RUT (10mg/kg.b.wt.)

<table>
<thead>
<tr>
<th>Group</th>
<th>Spleen weight (mg) ± SEM</th>
<th>No. of macroscopic colonies ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>69.40 ± 0.45</td>
<td>-</td>
</tr>
<tr>
<td>RUT alone</td>
<td>67.25 ± 2.05</td>
<td>-</td>
</tr>
<tr>
<td>IR Alone</td>
<td>35.92 ± 1.34</td>
<td>0.29 ± 0.23</td>
</tr>
<tr>
<td>RUT + IR</td>
<td>47.20 ± 1.66*</td>
<td>8.06 ± 0.6*</td>
</tr>
</tbody>
</table>

Data represent the mean ± SEM from six mice per group. The significant level *p<0.001 compared to other groups. No symbol = Non-significant.
3.3 QRT enhances haematopoietic recovery

CFU-S assay was performed to study the protective effect of QRT against radiation injury to haemopoietic tissue. Animals exposed to 4.5 and 7.5 Gy gamma radiation resulted in a significant (p<0.001) increase in number of macroscopic spleen colonies in QRT (20 mg/kg.b.wt.) pretreated groups when compared with concurrent radiation alone groups (Table 3). Pre-treatment of mice with optimal dose of QRT (20 mg/kg.b.wt.) indicated a significant (p<0.001) gain in the spleen weight when compared with respective radiation alone groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Spleen weight (mg) ± SEM</th>
<th>No. of macroscopic colonies ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>63.45 ± 0.69</td>
<td>-</td>
</tr>
<tr>
<td>QRT alone</td>
<td>66.31 ± 1.41</td>
<td>-</td>
</tr>
<tr>
<td>IR Alone</td>
<td>33.27 ± 1.21</td>
<td>1.69 ± 0.37</td>
</tr>
<tr>
<td>QRT + IR</td>
<td>45.08 ± 1.88*</td>
<td>12.02 ± 1.66*</td>
</tr>
</tbody>
</table>

Animals were administered with Quercetin (20 mg/kg.b.wt.) for five consecutive days. On the fifth day, mice were exposed to 4.5 and 7.5 Gy of gamma radiation. Visible colonies on spleen surface were counted on day 14 post irradiation. Values were mean ± SEM (standard error of mean) from six spleens per group. QRT treated groups compared with respective radiation alone groups * p<0.001; No symbol = Non-significant.

4. Discussion

Radiotherapy either alone (non-metastatic solid tumours) or in combination with surgery or chemotherapy or both (for locally advanced malignancies) play an important role in the treatment of cancer. In clinics, the current therapeutic approaches favour treatment intensification, with the supposition that higher radiation therapy doses or novel fractionation schemes will result in increased patient survival [21]. However, the major challenge is to optimize the therapeutic ratio by minimizing treatment-related morbidity, while maintaining or improving local control and survival by selectively protecting the normal cells from the radiation effects [22,23]. Toxicity to normal organs not only limits use and achievement of complete therapeutic potential of radiotherapy but also cost in terms of patient morbidity and mortality [23,24]. In radiation therapy, one may mitigate the toxic effects of ionizing radiation on normal tissues by decreasing the dose of radiation and frequency of treatment. However, the major shortcoming of this strategy is insufficient control of the tumors [23]. One of the approaches to minimize the damaging effect of radiotherapy is the use of radioprotective agents.

Various radioprotective chemicals and agents have been explored, including thiol compounds, natural antioxidants, cytokines, immunomodulators, lipopolysaccharide and prostaglandins [6,7]. However, most of the synthetic drugs used are observed to possess inherent drug-induced toxicity at their optimum useful concentrations and need constant medical supervision [5]. In view of this bleak situation, the need of the time is to screen new radioprotectors that are devoid of cumulative or irreversible toxicity, offers long-term selective protection to normal tissues, possesses a long shelf life and economically cheap. In considerations of these, the investigation is on for novel less toxic and more effective radioprotective drugs. Recent reports have shown that dietary phytochemicals act as excellent free radical scavengers in different experimental systems [25]. The use of polyphenols as potential radioprotectors is of increasing interest because of their adaptogenic ability and abundance in the diet. Present study demonstrates potential of RUT and QRT, dietary polyphenols in ameliorating radiation induced toxicity under in vivo condition. Various tissues and organ systems of an individual differ in their response to radiation and as a rule the systems with proliferating cells are most sensitive [26-30].

In clinical studies, the acute and chronic bone marrow toxicities are the major limiting factors in the treatment of cancer as sublethal doses of radiations are used [31,32]. The bone marrow progenitor cells and the gastrointestinal epithelium are crucial for the maintenance of life and any damage to these cells will impair the normal physiological processes drastically, causing an undesirable impact on survival. It is generally agreed that radiation death in the sublethal dose range is due to impairment of bone marrow haemopoietic function and that the leucopenia, erythropenia and thrombocytopenia which ultimately predisposes to infection, haemorrhage and death [10]. In the present study, it is important to note that RUT and QRT could check the fall of total hematological parameters in irradiated animals contributing to the increased survival.

Most bone marrow progenitors are susceptible to dose-dependent decrements in viability, subpopulations of selectively radioresistant stem cells and/or accessory cells do exist. These cells may play an important role in recovery.
of hematopoiesis after exposure to high doses, albeit with a reduced capacity for self-renewal [32]. Since its inception by Till and McCulloch [20], CFU-S assay is a reliable time tested method for detecting and counting such pluripotent stem cells of the blood-forming system [33]. The method is based on the ability of stem cells, to multiply and differentiate to form localized colonies of descendants in the spleen of an irradiated mouse. The colonies originate from single cells and often found to contain more than one kind of differentiated cell [34,35]. CFU are capable of self-renewal, and the colonies to which they give rise contain significant numbers of new CFU. These properties for self-renewal and to give rise to several types of differentiated descendants are the essential properties of pluripotent stem cells [36]. There is general agreement that endogenous hematopoietic spleen colony formation is a good indication of stem cell viability and/or the stimulation, proliferation and survival of cells in animals recovering from exposure to radiation [32]. The potential of RUT and QRT in protecting the haemopoietic system was substantiated by enhanced number endogenous spleen colony forming units (CFU) compared to the radiation alone. Similar results have been reported for other plants like Acanthopanax senticosus, Hippophae rhamnoides, Tinospora cordifolia, Ocimum sanctum [38-41].

Radiation causes destruction of the marrow and haematopoietic cells, but may not cause their complete disintegration. The haematopoietic system is damaged and the levels of circulating mature blood cells, maturing lineage specific cells in haematopoietic tissues and stem cells are also reduced [42-45]. By the term haematopoietic recovery as used herein meant the process of normalization of the haematopoietic system after such damage. Leukocytic haematopoietic cells are important in maintaining the body's defence against disease. Circulating and tissue neutrophils have a short half-life of about 2 hours. An important consequence during radiotherapy is that neutrophil numbers drop very rapidly when the bone marrow is damaged. Such treatments destroy dividing cells within the tumour, but also devastate other highly proliferative cell populations such as the bone marrow and gut epithelial cells. The bone marrow toxicity kills haematopoietic precursors indiscriminately, but the major impact on mature cell numbers is seen with neutrophils and to lesser extent platelets because of the short half-life of these cells. A drastic reduction in the leukocyte count after irradiation is in agreement with earlier findings [45-48]. Since, the contribution of neutrophil to the total leukocyte (WBC) count is more (40-70%); a reduction in the neutrophil count affects the total WBC count. RUT and QRT pretreatment increased the RBC as well as hemoglobin levels non-significantly when compared with the control groups.

The present study indicates that RUT and QRT pretreatment with radiation normalized the haematological parameters, protected the radiation-induced hematopoietic stem cells, gastrointestinal stem cells and intestinal mucosa of Swiss albino mice.

References


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