**In vivo radioprotective activity analysis by light microscopy:**

**Methodological approaches**

S.L. Jothy¹, S. Gothai¹, Y. Chen² and S. Sasidharan¹

¹Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
²Dental Research & Training Unit, and Oral Cancer Research and Coordinating Centre (OCRCC), Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia

The world research community is showing an increasing interest in exploring the natural products such as medicinal plants as a radioprotective agent and understanding the healing processes involved may open new avenues for exploring novel radioprotective agent. This chapter provides a detailed description of the methodological approaches of radioprotective activity analysis of *in vivo* model of plant extract pretreated and non-treated mice prior to lethal irradiation. The preparation of plant extract, oral administration of the extract to mice and the evaluation of the *in vivo* radioprotective activity by using light microscopic method applied in combination with histopathological technique are discussed. The usage of light microscopic method will help to understand the *in vivo* histoarchitecture of radiation sensitive organs such as intestine, liver and spleen as well as the radioprotective effects.

**Keywords:** light microscopic; radioprotective; histoarchitecture

1. **Introduction**

Radioprotective agents are chemical or molecular pharmacological agents that could be effective in protection and treatment of radiation damage to surrounding normal tissues. Radiotherapy is the most important treatment modalities for human cancer. The use of ionizing radiation to treat cancer cells during radiotherapy unavoidably brings adverse effects to normal cells. As results, patients may experience symptoms and inconvenient after the radiotherapy. Therefore, the world research community is showing an increasing interest in exploring the natural products such as medicinal plants as a radioprotective agents and understanding the healing processes involved may open new avenues for exploring novel radioprotective agent. As mentioned earlier, ionizing radiation is an important modality in cancer treatment and almost 80% of cancer patients required radiotherapy treatment [1]. However, during the radiotherapy treatment the overproduction of free radicals which are toxic can interact with the critical bio-macromolecules and induce cell damage eventually leads to cell death [2]. For this reason, the use of radioprotectors represents an obvious strategy to improve the therapeutic index in as radiotherapy [3]. Plant extracts eliciting radioprotective efficacy contain an excess number of phytochemicals including antioxidants, immunostimulants, and cell proliferation stimulators, antiinflammatory and antimicrobial agents [4]. Hence, such phenomenon diverts many researches attention towards the medicinal plants and natural products focus on new drug discovery for the development of radioprotectors [4]. Research in the development of radioprotectors worldwide has focused on various methodology and approaches. However, this chapter provides a detailed description of usage of light microscopic method applied in combination with histopathological technique to study the radioprotective activity.

From olden times, scientist has wanted view the area that is beyond our vision with the naked eye. However, the invention of the microscope gives humans the ability to view and study the realm that is beyond our vision. Moreover, the invention of the microscope has allowed science to jump forward in many fields includes radioprotective activity analysis in an animal model. The usage of microscopic method such as light microscopic enables the understanding of how new drugs or herbal products work as a radioprotective agent in *in vivo* animal models studies. Light microscopic method offers several advantages including the effectiveness, simplicity, and low cost, and has been widely adopted in histopathological technique. However, there are some limitations of this method such as usage of outdated instruments and equipment, microscopic characters were simply described by words or coupled with pictures drawn by hands, and none of them provide detailed illustrations or photos of transverse sections of animal organs. On the other hand, the advancement in the microscopy technique and the development of digital imaging techniques, much detailed and accurate information about microscopic features can be obtained [5].

Thus, in this chapter we analyzed and discussed the usage of light microscopy technique for the radioprotective activity analysis of *in vivo* model of plant extract pretreated and non-treated mice prior to lethal irradiation.

The preparation of plant extract, oral administration of the extract to mice and the evaluation of the *in vivo* radioprotective activity by using light microscopic method applied in combination with histopathological technical are also discussed. The usage of light microscopic method will help to understand the *in vivo* histoarchitecture of radiation sensitive organs such as intestine, liver and spleen as well as the radioprotective effects. The methods presented in this chapter are illustrated on the model of *Polyalthia longifolia* leaf which is a local herbal plant.
2. Microscopy technique in radioprotective activity analysis

Figure 1 show the various steps involved in the usage of microscopy technique in radioprotective activity analysis of natural products such as medicinal plants.

2.1 Plant Sample Collection

The investigation of medicinal plant properties on radioprotection mechanism begins with extraction process with plant collection. All plant materials should be properly authenticated. Then the plant material will be washed with water and oven dried at room temperature for approximately 7 days. After this period, plants will be grinded into powder by conventional grinder. The powders will be preserved in a clean plastic container away from light, heat and moisture until further use.
2.2 Preparation of Plant Extracts

Next steps are extraction process in which the soluble materials (active compounds) are removed from an insoluble residue by treatment with a liquid solvent. The choice of extraction technique depends on the nature and components to be isolated. The powdered plant material (100 g) will be soaked in a container with solvent (400 ml) and allowed to stand at room temperature for a period of 7 days with frequent agitation until the soluble matter dissolves. The mixture then will be filtered through clean muslin cloth. Finally the solvent will be recovered via rotary evaporator and the extracts will be dried at 37°C.

2.3 Animals

Albino mice (male) weighing between 30 and 40 g will be employed in this study. They will be housed in well ventilated acrylic box at 24±2°C in hygienic provision under natural light and dark schedule and will be fed on food and water. They will be allowed to acclimatize for two weeks prior to experiment. Mice will be divided into three groups of 20 mice.

2.4 Administration of plant extract

The plant extract is dissolved in Tween 20 (10% v/v) and dose rate of 500 mg/kg body weight (will be based on the calculated LD$_{50}$ in a toxicity study) will be orally administered daily of the plant extract which continues for 30 days prior to irradiation.

2.5 Experimental Induction for Organ Damage

After the oral administration of plant extract, the mice will be subjected to whole body irradiation (1.33 Gy/min of dose rate). The mice will be inspected for 30 days post irradiation for body weight and behavioral changes. The various experimental groups will be grouped as shown in the Table 1.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Various experimental groups for radioprotective study</th>
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<tbody>
<tr>
<td>Group</td>
<td>Normal food</td>
</tr>
<tr>
<td>1 (control)</td>
<td>✓</td>
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<td>2</td>
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At the end of the experiment, all animals will be anesthetized. Blood sample will be collected by cardiac puncture and stored in heparin anticoagulant tube. Animals will be sacrificed. Intestine will be promptly excised and stored in Bouin’s solution for at least 1 day before further analysis.

2.6 Histopathological Examination of Intestinal Tissue

Small pieces of tissue (intestine) will be transferred to 10% formalin for proper fixation and will be processed and embedded in paraffin wax. Tissue of 5 to 6 µm in thickness will be sectioned and stained with haematoxylin and eosin (H&E). Finally the tissue is microscopically examined to assess the severity of lesions.

2.7 In vivo radioprotective activity analysis by light microscopy method

The usage of light microscopy technique for the radioprotective activity analysis of in vivo model of plant extracts pretreated and non-treated mice prior to lethal irradiation can be done by observing various changes in the histoarchitecture of radiation sensitive organs such as intestine. The small intestine is a specialized tubular structure within the abdominal cavity in continuity with the stomach proximally and the colon distally. The wall of the small intestine is composed of four layers namely mucosa, submucosa, muscularis and adventitia. The mucosa is the innermost layer formed by glandular epithelium, lamina propria, and muscularis mucosae. The glandular epithelium forms cylindrical structures, called crypts (Fig. 2b) and composed of various cell types includes goblet cells (Fig. 2c). Moreover, the mucosa of the small intestine is characterized by mucosal folds and villi (Fig. 2a). Villi are mucosal folds that decrease in size from the proximal to distal small intestine and are of different shapes in the various segments of the small intestine. As one index of radiation injury, intestinal crypts will be counted by the method of Withers and Elkind [6]. Data will be expressed as crypts per circumference. Moreover, the villus heights and villus goblet cell density in each site also will be calculated and recorded as the number of goblet cells/µm$^2$ and the heights of villus in µm. Various methods have been suggested to determine the villus height. However, the advancement in the light microscopy technique and the development of digital imaging techniques are enables the determination of accurate villus height as...
shown in Fig. 2a. Finally, the data from each group of mice will be compared to the control and extract treated group to study the radioprotective study.

**Fig. 2** Photomicrograph of the small intestine demonstrating the (a) villus: V, (b) crypts: C and (c) goblet cells: G using statistical test for the crypt, goblet cells count and villus height to study the radioprotective activity of the medicinal plant. For additional estimation of injury and radioprotective activity, the histologic damage of intestine will be observed by using light microscopic method.
3. Materials and Methods

The methods presented in Section two of this chapter are illustrated on the model of *Polyalthia longifolia* leaf extract which is a local herbal plant for *in vivo* radioprotective study.

3.1 Plant Sample Collection

The leaves of *P. longifolia* were collected from various areas in Universiti Sains Malaysia, Penang, in May 2013, and authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia, where a sample was deposited (Voucher specimen: USM/HERBARIUM/11306). The leaves were separated and cut into small pieces, which were first washed with tap water and then with distilled water. The leaves were then dried in an oven at 60°C for 7 days, after which the dried leaves were ground into a fine powder using a grinder and stored in clean, labeled airtight bottles.

3.2 Preparation of *P. longifolia* leaf extract

The leaf sample was sequentially extracted with methanol by adding approximately 100 g of the dried sample into 400 mL of methanol. The extraction was carried out at room temperature by soaking for 7 days with intermittent stirring during the first day. The extracts were filtered through clean muslin cloth and the extraction process was repeated a second time by adding another 400 mL of methanol to the sample residue. The filtrate from each extraction was combined and concentrated under vacuum on a rotary evaporator (Buchi, Switzerland) at 40°C to 50°C in order to evaporate the excess methanol solvent and until a dark green methanol extract was produced. The concentrated extract was poured into Petri dishes and brought to dryness at 60°C in the oven until a paste-like mass was obtained. Then, a paste form extract was sealed in the Petri plates and stored at room temperature (RT).

3.3 Administration of plant extract

The *P. longifolia* leaf extract was dissolved in Tween 20 (10 % v/v) and given at the dose rate of 500 mg/kg body weight for 15 consecutive days before irradiation. The dose of the extract was selected on the basis of experiments conducted to determine the LD₅₀ value of *P. longifolia* leaf extract in an acute oral toxicity study [7]. The control animals received Tween 20 (10 % v/v) alone.

3.4 Animals

Specific pathogen-free and age-matched (8 to 10 weeks old) Swiss albino mice weighing 30 g to 35 g were used to study the radioprotective activity of the *P. longifolia* leaf extract. The Institution Animal Ethics Committee of Universiti Sains Malaysia, Penang, Malaysia, approved the animal study (USM/Animal Ethics Approval/2011/(74) (365)) for this project. The animals were kept at 27 ± 2°C, relative humidity 44–56% and light and dark cycles of 10 and 14 h, respectively, for a week before and during the experiments. The animals were provided with a standard diet (Lipton, India) and water ad libitum. The animals were allowed to acclimatize to the laboratory conditions for a week before starting the experiment. All experiments were performed in the morning according to current guidelines for the care of the laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals [8]. For separate experiments, the animals were killed humanely after irradiation and tissues were collected for various assays. At the selected times after total-body irradiation, the mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Fifteen minutes after the intraperitoneal injection of ketamine and xylazine, the mice were tested for righting, toe-pinch, and palpebral reflexes. Immediately after cervical dislocation the intestine of the mice was excised and immersed in Bouin's solution for at least 1 day or further used for other assays.

3.5 Irradiation

Unanesthetized mice were restrained in a specially designed well-ventilated acrylic box (14.5 cm x 23.5 cm) and exposed to whole-body radiation from 6 Mv, X-ray teletherapy facility (VARIAN 3100 Hansen Way, Palo Alto, CA 94304, USA) at the Radiotherapy and Oncology Department, Low Guanlye Hospital, Penang, Malaysia, at a dose 10 Gy with a source-to-surface distance (SSD) of 100 cm (1.33 Gy/min of dose rate).

3.6 Experimental design

To determine the radioprotective activity of *P. longifolia* leaf extract against lethal whole body irradiation, the experiment was conducted for 30 days. The mice were distributed into three groups of 20 animals each and randomly selected before being marked on the tail for individual identification, as follows:

Group I: Control (administered with distilled water alone or tween 20 (10 % v/v)
Group II: X-ray irradiation alone (10 Gy)
Group III: 500 mg/kg *P. longifolia* leaf extract plus X-ray irradiation (10 Gy)
3.7 Histopathology

The animals were sacrificed by cervical dislocation on the 10th post irradiation day for studying the histopathology of the small intestine. The organ was examined macroscopically for the post irradiation adverse effects before being preserved in a fixation medium of 10% solution of buffered formalin for histopathological study. The sections were embedded in paraffin, and 5µm thick sections were cut with a rotary microtome and stained with hematoxylin and eosin. The total number of crypts, goblet cells, dead cells in the small intestine were observed and compared with the experimental and control groups. The status of the mucosal erosion, basement membrane and villus height was also assessed [9, 10].

4. Results and discussion

Small intestinal epithelial damage is significant component of the radiation gastrointestinal syndrome. It appears that the gastrointestinal tract has a significant role in patient outcome following high dose radiation exposure that leads to major damage to this system [11]. The histology view of the intestine of mice exposed to X-ray irradiations in the presence or absence of treatment with P. longifolia leaf extract are shown in Fig. 3 to Fig. 5. Fig. 3 shows normal mouse intestine architecture, and mucus cells are seen in the full length of villi with normal number of goblet cell. There were no remarkable histologic changes and damages were observed in the control group (Figs. 3). On the other hand, remarkable histologic changes and damages were observed in the 10 Gy radiation group (Figs. 4). In the 10 Gy radiation group mouse crypts are nonuniform, distorted, and depopulated in cells. Villi are shortened, fused, and eroded. Inflammatory infiltration and focal hemorrhage are noted in the lamina propria (Fig. 4). In addition, in the 10 Gy radiation regimes also produced a decrease in the number of goblet cell profiles. Conversely, after pretreatment of P. longifolia leaf extract before 10 Gy irradiation, crypts are increased in number and villi are longer and less distorted compared with those in the radiation alone group (Fig. 5). In the combination group of 10 Gy irradiation and P. longifolia leaf extract, there is nearly complete regeneration of crypts and villi (Fig. 5). Furthermore, in the P. longifolia leaf extract-treated group, the number of goblet cells was greater than in the only radiation treated groups. Finally, based on the data of this study we suggest P. longifolia leaf extract provides radioprotection from acute radiation damage to the mice intestine within the limit of tolerable radiation doses.

The results of the current study revealed that P. longifolia leaf extract could be used as a potential radioprotective agent. The mechanism of P. longifolia leaf extract therapeutic action is through free radical scavenging activity. Our previous study, which showed that P. longifolia leaf extract possessed significant antioxidant and hepatoprotective activity against various free radicals with a high content of polyphenol and flavonoid compounds, supports the above statement [12]. Being a known antioxidant, it was quite possible that P. longifolia leaf extract might have scavenged these various free radicals produced during irradiation and inactivated them by electron donation/hydrogen transfer. On scavenging by P. longifolia leaf extract, the availability of free radicals would decrease considerably resulting into lowering of damage and in turn the extent of regeneration in the intestine as exhibited by light microscopic photographs of the small intestine in this study. As a result, light microscopic method offers several advantages to study the radioprotective activity of P. longifolia leaf extract in this study. In summary, the present results shows that the P. longifolia leaf extract pretreatment provides a significant protection against radiation-induced damages in the intestinal tissue of mice as shown by light microscopy analysis. In addition, P. longifolia leaf extract may be used as a radioprotective agent in known dosages, especially in rural communities where conventional drugs are unaffordable or unavailable and the health facilities inaccessible particularly in developing country.
Fig. 3  Histologic microscopic photographs of the small intestine. Photomicrographs of small intestine section stained with H& E staining (Magnification X10, X20). Control group
Fig. 4  Histopathological demonstration of X-ray radiation effect on the small intestine of irradiated mice. Photomicrographs of small intestine section stained with H& E staining (Magnification X10, X20). 10 Gy whole body X-ray irradiated group.
Fig. 5  Histopathological demonstration of radioprotective effect of Polyalthia longifolia on small intestine of irradiated mice. Photomicrographs of small intestine section stained with H& E staining (Magnification X10, X20). 500 mg/kg of P. longifolia b.w. treated +10 Gy whole body X-ray irradiated group

5. Conclusion

Light microscopy method is an important tool for in vivo radioprotective activity analysis by medicinal plant in order to understand how a new drugs or herbal products work as a radioprotective agent. The above mentions methods demonstrated the great important of light microscopy method in the development of anti-irradiation agents. The main advantages of the presented methods are the following: cheap; simple; accurate and reliable. Our discussion also
demonstrates that the use of light microscopy is vital to disclose the tissue injury caused by irradiation and the radioprotective effects of plants extract. In conclusion, microscopic method plays an important role in the evaluation of in vivo radioprotective activity of medicinal plants.

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References


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