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Radiation-Induced Esophagitis Is Mitigated By Soy Isoflavones

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RADIATION-INDUCED ESOPHAGITIS IS MITIGATED BY SOY ISOFLAVONES

by

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THESIS

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CHAPTER 1: HYPOTHESIS, SPECIFIC AIMS & INTRODUCTION

Soy isoflavones are natural compounds extracted from soybeans and have demonstrated chemoprotective effects and anti-cancer properties (1). In addition, Dr. Hillman's lab has recently shown that soy isoflavones act as radioprotectants of normal lung tissues when given in conjunction with radiation therapy. In a mouse model of lung tumors and in naïve mice, soy isoflavones reduced the extent of pneumonitis and fibrosis caused by radiation damage to lung tissue (2,3,4).

Hypothesis: Based on previous studies showing mitigation of radiation-induced pneumonitis and fibrosis by soy isoflavones, we hypothesized that soy isoflavones could provide radioprotection of normal esophageal tissues from radiation-induced tissue damage. To address this hypothesis, we have examined esophageal tissues from naïve mice treated with radiation and soy isoflavones using histological and immunohistochemistry methods.

The **specific aims** of my research project were:

- 1) To characterize the histopathological changes of radiation-induced damage in esophagus.
- 2) To investigate the effect of soy isoflavones on reducing tissue damage in esophagus.

Clinical Significance: Prevalence and Clinical Presentation of Radiation Esophagitis

Radiation-induced toxicity to normal esophageal tissue is a dose-limiting side effect of radiotherapy for cancers of the thorax as well as head and neck. Based on Alevronta et al., the esophagus has dose-dependent levels of toxicity (5), and this involves tissue responses to different radiation strategies – single high dose, hyperfractionated, and

hypofractionated. Additionally, radiation toxicity in the esophagus can be exacerbated by the addition of one or more chemotherapeutic drugs to the radiation treatment. (6). In a clinical report conducted by Werner-Wasik et al. (7), radiation esophagitis is a highly common ailment experienced by most patients undergoing cancer therapy. Werner-Wasik identified that 33% of non-small cell lung cancer patients (NSCLC) receiving chemoradiation developed grade 3 or higher radiation esophagitis, but 95% of patients developed some level of radiation esophagitis (grade 1-4) (7) (see table 1 below).

Table 1. RTOG Grading Scale with Esophagus Radiation Toxicity & Werner-Wasik Study Results

<i>RTOG Grade</i>	<i>Associated Clinical Symptoms of Radiation Esophagitis</i>	<i>From Werner-Wasik Study: % of Total Patients Experiencing Symptoms*</i>
<u>Grade 1</u>	mild dysphagia or odynophagia/may require topical anesthetic or non-narcotic analgesic/may require soft diet	20%
<u>Grade 2</u>	moderate dysphagia/odynophagia/may require narcotic analgesics/may require puree or liquid diet	41%
<u>Grade 3</u>	severe dysphagia/odynophagia with dehydration or weight loss (more than 15% from pretreatment baseline) requiring N-G feeding tube, i.v. fluids or hyperalimentation	31%
<u>Grade 4</u>	complete obstruction, ulceration, perforation, fistula	2%

**Findings from Werner-Wasik et al. study found only 5% of patients did not experience any level of radiation esophagitis (7).*

As described in Table 1., radiation-induced esophagitis presents with acute and/or chronic clinical symptoms, which include odynophagia (irritation and burning sensation upon swallowing) and dysphagia (difficulties in the function of swallowing).

Odynophagia symptoms usually have a rapid onset, and can ultimately be dose-limiting, presenting a serious challenge for radiation oncologists to follow through with radiation regimens and commonly results in 'premature' (early) termination of radiation treatment (7,18). Odynophagia can be attributed to one or many of the following histological changes from radiation insult: rapid mucosal epithelium depletion, limited basal cell proliferation for epithelial repopulation, and tissue ulceration. (8,9,10,11)

Alternatively, dysphagia results from an acute or chronic tissue response to radiation injury and produces a mechanical restriction (and subsequent discomfort) in the action of swallowing. First, in an early response to radiation as seen in many tissues, the esophagus undergoes extensive tissue edema, which is a hallmark of radiation induced tissue damage (8,9,12,20,21). Radiation produces a cyclic, chronic inflammatory response, which includes the deposition of collagen and reconstitution of extracellular matrix compartments (13). Accumulation of collagen and extracellular matrix components over a long period of time explains long-term development of esophageal strictures, an ailment that emerges months to years after radiation treatment (5,6,8,13). Ultimately, whether it's sudden tissue swelling from an edematous response, or late-forming regions of inappropriate collagen deposition within submucosal tissue layers, radiation-induced tissue damage has multiple events contributing to dysphagia, and limiting the esophagus's essential role as an organ.

In summary, esophagitis is an early side effect of radiation or chemoradiation, causing pain, difficulties in swallowing and eating, and dehydration, and in the chronic phase, strictures may develop. These symptoms could lead to early interruption of radiation treatment, limiting the efficacy of radiation therapy. Therefore, it is imperative

to design strategies to alleviate the clinical symptoms of radiation-induced toxicity to the normal esophagus.

Soy Mitigates Radiation Injury to Normal Lung

Our lab has observed the effect of soy isoflavones to act as an anticancer agent and enhance radiation-induced cancer cell killing in studies in vitro and in vivo in tumor models of prostate cancer, renal cancer, and lung cancer (14,15,16). In addition to radiosensitizing tumor cells, studies in lung tumor models demonstrated that soy isoflavones mitigate radiation-induced pneumonitis and fibrosis in normal lung tissue. This differential effect of soy isoflavones on cancer cells versus normal tissue was shown in an orthotopic murine model of lung cancer, which involves soy increasing radiation-induced destruction of lung tumor nodules while mitigating vascular damage, inflammation, and fibrosis caused by radiation in normal lung tissue. (2,3).

To further investigate the radioprotective effects of soy isoflavones on lungs from naïve mice, thoracic irradiation at high doses of 10 Gy or 12 Gy administered alone or in combination with soy isoflavones, given prior and after irradiation, protected the lungs against adverse effects of radiation. Soy isoflavones provided many radioprotective effects, including reduced hair loss, restored respiratory function (breathing rates comparable to control), reduced pneumonitis (i.e. thickening of alveolar septa), decreased inflammatory infiltrates, and decreased lung fibrosis (4). These findings within normal lung tissue are indicative of early and late effects of radiation-induced tissue injury and subsequent inflammatory response, and support a radioprotective role for soy isoflavones in the context of pneumonitis and pulmonary fibrosis, and potentially in overall radiation-induced toxicity.

The radiation damage and inflammatory response seen in these lung studies is one example of the radiation-induced tissue injury and associated organ dysfunction, either as sudden thickening of alveolar septa and progressive accumulation of fibrotic collagen in the tissue microenvironment. However, as the lung studies have suggested, soy isoflavones have the capacity to affect multiple aspects of the inflammatory-mediated outcome to radiation injury, and could potentially play a role in alleviating tissue injury in other thoracic organs.

Goals of Study and Overview of Findings

Given the severity of esophagitis caused by radiation in lung cancer patients, we have now investigated whether soy isoflavones could reduce the tissue damage in normal esophagus. These studies were conducted in naïve immunocompetent mice (non tumor-bearing), using conditions of high-dose radiation at 10 Gy or 25 Gy to detect significant tissue damage, which have been shown to result in greater toxicity in clinical studies. (17). Soy isoflavones were administered to mice in a controlled dose given by gavage prior to and after radiation for up to 4 months. Esophageal tissues were obtained at different time points after radiation from untreated mice and mice treated with soy isoflavones alone, radiation alone, and combined radiation and soy to evaluate the effect of combined therapy compared to single therapy.

Using immunostaining techniques, we have defined the histopathological changes induced by radiation in multiple tissue layers of the esophagus. This included disruptions to the mucosal epithelium, lamina propria, muscularis mucosa and submucosa tissue layers. Our studies showed that soy isoflavones reduced the extent of damage to the various esophageal tissue layers and confirmed a role for soy

isoflavones as a complementary and safe approach to improve the efficacy of radiotherapy for lung cancer.

CHAPTER 2: MATERIALS AND METHODS

Mice

Female C57BL/6 mice of 5-6 week old (Harlan, Indianapolis, IN) were housed and handled in animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

Soy Isoflavones

The soy isoflavones mixture G-4660 used is a pure extract of 98.16% isoflavones from soybeans consisting of 83.3% genistein, 14.6% daidzein and 0.26% glycitein (manufactured by Organic Technologies and obtained from NIH). The soy isoflavone mixture was dissolved in dimethyl sulfoxide (DMSO) and diluted 1:20 in sesame seed oil just prior to gavage to avoid irritation of the esophagus by DMSO. Mice were orally treated with soy isoflavones by gavage. Mice in the control and radiation-alone groups received vehicle alone.

Thoracic Irradiation

Radiation was delivered at a dose of 10 Gy to the mouse thorax including esophagus, bilateral lungs and mediastinum. Three anesthetized mice, in jigs, were positioned under a 6.4 mm lead shield with 3 cut-outs in an aluminum frame mounted on the X-ray machine to permit selective irradiation of the lung in 3 mice at a time, as previously described (2,3,4). Radiation dose to the thorax and scattered dose to areas of the mouse outside of the radiation field were carefully monitored. To minimize backscattering of radiation, the bottom of the aluminum frame that holds the jigs was hollowed and the backplate of the jig was thinned to 1.6mm thickness. Dose to shielded regions was reduced to 1% of the dose in the irradiated field. Dose rate was 117.5

cGy/min and HVL was 2 mm Cu. Photon irradiation was administered using a Siemens Stabilipan X-ray set (Siemens Medical Systems, Inc) operating at 250 kV, 15 mA, with 1 mm copper beam filtration at a distance of 47.5 cm from the target.

Experimental protocol

Mice were pre-treated with oral soy isoflavones each day for 3 days at a dose of 5mg/day (equivalent to 250mg/kg). On day 4, the thorax was selectively irradiated with a dose of 10 Gy. Soy treatment was continued on a daily basis for 5 more days at 5mg/day. Then, mice were treated with a lower dose of 1mg/day (equivalent to 50mg/kg), given daily 5 days a week for up to 16 weeks. The rationale for giving a higher dose of soy isoflavones for pre-treatment and just after radiation is to optimize the effect of soy based on our previous studies (3,4).

Esophagus tissue preparation for histology

Mice were sacrificed at different time points between 1 to 16 weeks after irradiation. Esophagi were resected from the proximal end (near the pharynx) to the distal end (esophageal-gastric junction) within the field of radiation. Distal ends of the esophagus were marked with blue dye (Davidson Marking System, Bloomington, MN) to ensure that sections which were irradiated were analyzed. Resected esophagi were fixed and curved into a U shape for embedding in vertical position in paraffin and transverse tissue sections were cut. Esophagus sections were stained with hematoxylin-eosin (H&E) and examined using a Nikon E-800 microscope. Morphometric measurements of inner esophageal tissue layers were performed using Image-ProPlus version 6.2 software (Media-Cybernetics) (ref). The effect of radiation and soy on esophageal layers was evaluated using Masson Trichrome (MT) stain for detecting collagen fibers

(NovaUltra Kit, IHCWORLD, Woodstock, MD). Histological findings were quantified using a scoring system on a scale from weak (\pm), moderate (+), strong (++) , and heavy (+++).

Immunohistochemistry (α -SMA, CD45)

Sections of esophagi were stained by immunohistochemistry. Sections were blocked with IHC Tek Antibody Diluent, and then incubated with primary purified monoclonal antibodies directed against α -SMA (Sigma-Aldrich, St Louis, MO), CD45 (eBioscience, San Diego, CA) and Ki-67 (LifeSpan, Seattle, WA) overnight at 4° C. Sections were then incubated with biotinylated secondary antibodies (Vector Labs, Burlingame, CA) at 1:300 for 1 hour at room temperature. Staining was amplified with the avidin-biotin system Vectastain ABC Reagent Kit (Vector Labs) and visualized with the Vector DAB Substrate Kit for peroxidase (Vector Labs). Sections were counterstained with IHC-Tek Mayer's Hematoxylin (IHC World) and mounted with Permount mounting media (Electron Microscopy Sciences, Hatfield, PA). Esophageal layers were examined on a Nikon E800 microscope (Nikon Inc., Melville, NY). Histological findings were quantified using a scoring system on a scale from weak (\pm), moderate (+), strong (++) , and heavy (+++).

Statistical Analysis

For histological data, differences in the thickness of inner esophageal tissue layers among the various treatment groups were analyzed by two-tailed unpaired Student's t test. A value of $p < 0.05$ was considered statistically significant and results were summarized as found in table 1.

CHAPTER 3: RESULTS

Soy isoflavones reduce radiation damage to esophageal layers.

To assess the effects of irradiation and soy isoflavones on normal esophagus, the esophagus was resected at 4, 10, and 16 weeks after treatment with either soy alone, radiation alone, or both radiation and soy isoflavones, and esophagus tissues were processed for histology studies. A transverse section of the esophagus shows tissue layers lining the lumen and extending into the 2 skeletal muscle layers of the muscularis externa (inner circular layer and outer longitudinal layer)(ME, Fig. 1A). Esophageal tissue layers include the mucosal epithelium (EPI), lamina propria (LP), muscularis mucosae (MM), submucosa (SM) lining the muscularis externa (ME) (Fig. 1B).

Because alterations induced by radiation were observed in LP, MM, and SM, these three layers were grouped as inner esophageal layer (IEL) and to evaluate the differences in IEL between treatment groups, morphometric measurements of IEL were performed (Table 2). At all time points, esophagus from control mice showed normal esophageal tissue layer structure and integrity, including a healthy mucosal epithelium, a thin, dense and continuous LP, and small smooth muscle cells within the MM (Fig. 1C1,2,3, Table 2). Treatment with soy isoflavones did not show alterations in mucosal epithelium nor IEL, which looked normal compared to untreated esophagi (Fig. 1D1,2,3). In contrast, mouse esophagus treated with radiation caused significant tissue damage that was observed at 4, 10, and 16 weeks after radiation. Mucosal epithelium showed disruption in the basal cell layer alignment (circle in Fig. 1E1). Smooth muscle cells in the MM became enlarged in response to radiation (arrows in Fig. 1E2) and the IEL became thickened starting at 4 weeks, and increased at 10 and 16 week time points

(bracket in Fig. 1E3, Table 2). Esophagus from mice treated with soy isoflavones before and after radiation showed minimal tissue damage with a mucosal epithelium and IEL comparable to that in normal esophagus (Fig. 1F1,2,3). Soy isoflavones decreased IEL tissue thickening that was seen in mice treated with radiation alone (Fig. 1F3) and differences in IEL tissue thickness were statistically significant ($P < 0.05$) at all time points compared to radiation alone. (Table 2).

Soy isoflavones reduce disruptions of lamina propria connective tissues caused by radiation.

Previous studies in our lab showed that soy isoflavones decrease the pulmonary fibrosis induced by radiation (2,3,4). In order to evaluate the effect of radiation and/or soy isoflavones on collagen fibers within the lamina propria (LP), esophagus tissue sections were stained with Masson's Trichrome stain. Esophagi from control mice showed a dense blue, collagen-rich, lamina propria (LP) tissue layer at all time points (circles in Fig. 2A1,2,3). Similar findings were shown in mice treated with soy isoflavones alone (circles Fig. 2B1,2,3). Irradiated esophagus revealed that density of collagen fibers was reduced in the LP (circle in Fig. 2C1) and collagen fibers were seen infiltrating into muscularis mucosae (MM) layer (arrows in Fig. 2C2,3). Esophagi from mice treated with irradiation and soy isoflavones showed increased collagen stain density in LP, and limited positive collagen staining within the MM. These findings were comparable to that of control and soy isoflavone treated mice, predominately at 4 and 10 weeks after radiation (Fig. 2D1,2,3, Table 3).

Soy isoflavones decreases smooth muscle cell destruction induced by radiation.

H&E staining and Masson's Trichrome stain of esophagus tissues showed hypertrophy and vacuolation of smooth muscle cells in muscularis mucosae (MM). In order to evaluate smooth muscle cell hypertrophy within the MM tissue layer, tissue sections were stained by immunohistochemistry using anti- α smooth muscle actin (α SMA). Untreated esophagus showed normal dense staining of smooth muscle cells and an overall thin and highly integral MM at all time points (Fig. 3A1,2,3). Findings were similar in mice treated with soy isoflavones (Fig. 3B1,2,3). Esophagi from mice treated with radiation showed smooth muscle cells that had become hypertrophied, significantly increasing cell size, and cytoplasmic vacuolization at 4 weeks and persisted through 16 weeks (circles in Fig. 3C1, 3C3). Additionally, irradiated esophagi showed fragmentation in the continuity of the densely packed smooth muscle cells within the MM layer at all time points (arrows in Fig. 3C2). Esophagi from mice treated with combined radiation and soy isoflavones showed reduced smooth muscle cell hypertrophy, with cell size comparable to control, and limited fragmentation of MM smooth muscle throughout all time points after radiation treatment (Fig. 3D1,2,3, Table 3).

Soy isoflavones reduce leukocytic infiltration within esophageal tissue layers after radiation.

In response to radiation-induced tissue injury, infiltration of immune cells to the site of injury can occur, contributing to the overall inflammatory response. This effect has been well documented, including previous studies by our lab on radiation-induced lung injury, resulting in pneumonitis. These studies found that supplementation of soy

isoflavones to radiation therapy reduced inflammatory infiltrates within the normal lungs (2,3,4). In order to evaluate inflammatory infiltration induced by radiation and the effect of soy isoflavones, esophagus tissue sections were obtained at 4 weeks after treatment and stained by immunohistochemistry using the pan-leukocyte CD45 marker. Esophagi from untreated mice and mice treated with soy isoflavones showed no CD45+ cells within tissue sections (Fig. 4A,B). Irradiated esophagus tissues contained several focal areas of CD45+ cells at the edge of the mucosal epithelium lining the lamina propria (LP) (arrows Fig. 4C) and these clusters were found in multiple sites throughout tissue sections. Esophagi treated with radiation and soy isoflavones showed minimal infiltration of CD45+ cells (Fig. 4D).

Soy isoflavones mitigates early effects of radiation-induced damage on the mucosal epithelium.

We have discussed the histopathological changes occurring in response to radiation, and mitigation by soy isoflavones at 4 to 16 weeks after radiation treatment and at a single dose of 10 Gy radiation. However, it is known that early tissue responses to radiation-induced injury are highly implicated in the development of acute symptoms and side effects to radiation treatment (7,8,9,10,11,18). Additionally, a single dose of 10 Gy irradiation is not fully representative of what is used in the clinic, and a fair proportion of patients develop radiation-induced esophagitis as a result of high doses of radiation (5,7,8,11). Therefore, it is of high interest to investigate and evaluate the effects of soy isoflavones at an early time point after radiation and at a higher dose of radiation.

In the following experiment, esophageal tissue sections were evaluated at 1 week after radiation from mice receiving either 10 Gy or 25 Gy irradiation alone or 10 Gy / 25

Gy radiation in combination with soy isoflavones. Untreated esophagi showed normal tissue layers at 1 week after radiation (Fig. 5A1, 5A2), and were comparable to esophagi analyzed in initial studies at later time points (Fig. 1C). Esophagi from mice treated with soy isoflavones showed normal, undisturbed tissue layers, similar to control (Fig. 5B1, 5B2). Esophagus tissue from mice treated with 10 Gy radiation or 25 Gy irradiation showed multiple alterations to the mucosal epithelium basal cell layer. Basal cells showed morphological disruptions that were more pronounced in mice treated with 25 Gy radiation. Some basal cells appeared karyorrhexic with a fragmented nucleus (arrow in Fig. 5D1) and formed giant cells (arrows in Fig. 5D1,2). Other basal cells appeared to have become hyperplastic, showing active mitosis in the mucosal epithelium (circles in Fig. 5C1, 5D1). Additionally, in esophagi from 10 Gy or 25 Gy radiation treatments, inner esophageal layers (IEL) showed increased thickening (bracket in 5C2) and disruptions in the continuity of mucosal basal cells (dotted lines in 5C2). Esophagi from mice treated with radiation and soy isoflavones showed limited karyorrhexic basal cells with no giant cells present, a reduced proportion of basal cells undergoing active proliferation, reduced thickening of IEL, and no disruption in the alignment of basal cells along the basement membrane (Fig. 5E1,2, 5F1,2).

CHAPTER 4: DISCUSSION AND CONCLUSION

For NSCLC patients, radiation-induced esophagitis presents a major radiation dose-limiting challenge to radiation oncologists and disrupts efficacy of radiation regimens (5). Multiple studies by Gomez et al. has associated radiation-induced esophagitis inflammatory cytokine profiles, inflammatory markers, chemoradiation-induced neutropenia, and gene polymorphisms as a result of inflammatory responses (19). Nearly all patients experience some level of esophageal toxicity within the field of their radiation treatment, and present with varying severity of odynophagia and dysphagia as a result of tissue injury and inflammation (7), necessitating the need for intervention and effective therapeutics.

Our lab has previously demonstrated the radioprotective effect of soy isoflavones in lung tumor bearing mice and in naïve bearing mice, mitigating radiation-induced pneumonitis and pulmonary fibrosis in normal lung tissue (2,3,4). Based on these findings, the goals of my master's research project were to identify and characterize the histopathological changes that occur in normal esophagus tissue in response to radiation injury, and to investigate the effect of soy isoflavones on reducing tissue damage in the esophagus. These aims are based on the hypothesis that soy isoflavones may provide radioprotection of normal esophageal tissues from radiation-induced tissue damage.

In order to assess histopathology of normal tissue, studies were performed in naïve, non-tumor bearing mice. Mice were treated with a high dose of 10 Gy thoracic irradiation and an effective dose of soy isoflavones (1mg/day)(2,3,4), given prior and after radiation. In separate experiments, tissues were evaluated at 4, 10, and 16 weeks

after radiation to address late effects of radiation or at 1 week after radiation to address early effects of radiation.

Soy isoflavones reduce esophageal tissue edema in response to radiation injury.

We have identified significant alterations in esophageal tissue layers caused by radiation injury. In particular, thickening of the inner esophageal layers (IEL) was observed from 4 to 16 weeks after radiation. These findings were also found already at 1 week after radiation in separate experiments and could be the result of tissue edema. A hallmark of radiation-induced tissue damage in multiple organ systems includes tissue edema, or the subcompartmental accumulation of fluids, ultimately causing a swelling of one or more tissue compartments (20,21). The edematous response of esophageal tissues is a highly documented phenomenon and esophageal swelling can be considered a contributor to dysphagia caused by radiation treatment (8,9,12). Our observation in irradiated mouse esophagus also reflected an edematous response. Interestingly, this thickening of IEL was significantly decreased by complementing radiation with soy isoflavones, suggesting that soy isoflavones influence one or many events associated with fluid accumulation from radiation-induced tissue damage.

Soy isoflavones decrease collagen disintegration in lamina propria and limit collagen infiltration into muscularis mucosae.

Reduced density of Masson's trichrome staining of collagen in lamina propria at different time points after radiation suggested that radiation causes collagen disruption and subsequent remodeling of the fibrous lamina propria compartment. Additionally, radiation-induced tissue damage cause the infiltration of collagen fibers between hypertrophied smooth muscle cells in muscularis mucosae. These effects are normal

responses to radiation-induced tissue injury, and were reduced when radiation treatment was combined with soy isoflavones. Bentzen et al. describes the remodeling process a result of radiation tissue damage to include compartmentalized fibrosis (13). Fibrosis is a process to repair tissue damage and is a result of limited replenishment by existing stem cells. This process involves a cytokine cascade that generates excessive collagen and extra cellular matrix deposition into sites of dead cells (12,13). The process of fibrosis is necessary to maintain structural integrity, but at a serious cost as it replaces functional cells with non-functional tissue, therefore limiting the physiological capabilities of a damaged organ. Fibrotic accumulation of collagen over long periods of time—months to years—can lead to esophageal stricture formation and can present as dysphagia, a chronic symptom from radiation (5,6,8,13). Therefore, soy isoflavone supplementation to radiation therapy could be applied to reduce the progression of long-term stricture formation as a result of radiation-induced fibrosis.

Soy isoflavones mitigate radiation-induced smooth muscle cell hypertrophy in muscularis mucosae.

By immunohistochemical staining of smooth muscle cells within the muscularis mucosae, we observed changes caused by radiation, including increased cellular size, or hypertrophy, of smooth muscle cells with fragmentation in the continuity of smooth muscle layer. This effect was seen as early as 1 week after radiation and persisted up to 16 weeks after radiation. However, smooth muscle cell disruption was reduced in tissues with combined treatment of radiation and soy isoflavones at all time points. Smooth muscle cell hypertrophy in response to radiation has been documented and observed in multiple organs containing smooth muscle (20,21). But specific studies

investigating the pathological events related to dysfunctional smooth muscle cells from radiation injury remain limited. Preservation of smooth muscle cell structure by soy isoflavones, both at the cellular level by reducing hypertrophy, and at the tissue level by reducing fragmentation, could provide functional conservation of muscularis mucosae smooth muscle, limiting the development and progression of radiation-induced dysphagia.

Soy isoflavones abrogate leukocyte infiltration at the edge of mucosal epithelium.

Assessment of radiation-induced damage to esophagus demonstrated significant infiltration of leukocytes in lamina propria at the edge of the mucosal epithelium, whereas tissue treated with radiation and soy isoflavones showed minimal presence of leukocytes. Inflammatory infiltration is known to be a normal response by various tissues to radiation injury (12,13,22) and was reported to occur in our previous studies on radiation-induced toxicity to the lungs (2,3,4). The initial insult of radiation initiates an inflammatory phase with chemokine and cytokine (TNF- α , IL-6, IL-1 β) production to attract inflammatory cells to site of injury. This initial response occurs within hours and may extend over months (12,13,22) in conjunction with tissue edema, cellular swelling, lymphocytic infiltration, and apoptotic cell induction followed by tissue regeneration by stem cells (12). Based on our findings that soy isoflavones mitigate the majority of these events, it is worth investigating if and how soy isoflavones may alter aspects of the immune system in the context of normal tissue inflammatory responses to radiation.

Additionally, it was reported by McBride et al. that radiation elicits a range of tissue responses that include a cytokine 'breeze', rather than cytokine storm (22). It is shown that the strength of the inflammatory response is proportional to the intensity of radiation

treatment (22), and considering the radioprotective effects of soy isoflavones in limiting leukocyte infiltration shown in our data, future studies may want to evaluate the effects of soy isoflavones on inflammatory response at higher, clinically relevant, doses of radiation.

Soy isoflavones limit the disruption of basal cell alignment and continuity.

The regular arrangement of basal cells depend on intracellular tight junctions, which hold cells together and are the basis for maintaining mucosal tissue barrier functions (23). Radiation-induced tissue damage included disruption in the alignment of basal cells within mucosal epithelium, and this trend was seen at both early and late time points after radiation. In esophagus tissues from mice treated with radiation and soy isoflavones, the disruption of basal cell alignment was diminished, and resembled the basal cell continuity found in control. A review examining the role of flavonoids in intestinal tight junction regulation showed that genistein, the primary isoflavone found in soy, protected intestinal tight junction barrier function by counteracting disassembly of occludins induced by oxidative stress and acetaldehyde (24). These findings could explain observations that soy isoflavones could reduce radiation-induced disruptions to mucosal basal cells in normal esophagus, providing maintenance of mucosal barrier function.

Soy isoflavones reduce the number of radiation-induced abnormal basal cells.

The mucosal epithelial response to radiation injury included cellular changes in basal cells that were eliminated by combined radiation and soy isoflavone treatment. In response to radiation, some basal cells became karyorrhexic, where the nucleus becomes fragmented and precedes the potential cellular apoptosis induction.

Abnormally dividing basal cells form into giant cells that will ultimately die (12,13,22,28). Basal cells that have not undergone significant radiation damage, and subsequent induction of apoptotic cell death, will remain as actively dividing cells. As a function of actively replenishing the mucosal epithelium, these remaining basal cells may become hyperplastic in order to compensate for neighboring basal cells that have died and no longer contributed to replenishing mucosal epithelial cells. (12,25,28). Drastic changes in basal cell morphology reflecting radiation damage were pronounced with a higher dose of 25 Gy irradiation, and were seen already at 1 week after radiation.

Soy isoflavones showed reduced presence of both karyorrhexic cells and actively dividing cells. This effect was also observed with a higher dose of 25 Gy, confirming soy isoflavone's radioprotective role in mucosal epithelium. This could be explained by two previous studies identifying the role of genistein in reducing apoptosis induction in normal testicular mucosal tissues (28) and intestinal mucosal tissues (29). Limiting the overwhelming induction of basal cell apoptosis in response to radiation by soy isoflavones could be of great benefit in maintaining the integrity of mucosal epithelium and reducing rapid and prolonged epithelial depletion implicated in radiation-induced odynophagia, and warrant further investigation.

Summary and Conclusions

Taken together, these studies show that soy isoflavones provide a radioprotective role in numerous aspects of radiation-induced tissue damage and subsequent inflammatory responses. Multiple radiation-induced histopathological alterations of the normal esophagus were identified, including tissue edema, collagen remodeling and disintegration, smooth muscle cell hypertrophy and fragmentation, inflammatory

leukocyte infiltration, and disruption to mucosal basal cell morphology and function. By treating mice with combined therapy of radiation and soy isoflavones, esophageal tissues were comparable to that of control esophagi, showing limited tissue edema, dense collagen fibers that make up a thin lamina propria, reduced smooth muscle cell hypertrophy and continuous smooth muscle throughout the muscularis mucosae, minimal inflammatory leukocyte infiltration at the edge of the mucosal epithelium, and maintenance of alignment with decreased numbers of both karyorrhexic and hyperplastic mucosal basal cells. While many of these changes are mediated by an inflammatory response that varies from patient to patient upon radiation injury, nearly all patients receiving thoracic radiation experience some level of radiation-induced esophagitis. These findings can translate into the potential application of soy isoflavone supplementation during radiation therapy with the goal to mitigate the harmful side effects of radiation-induced tissue damage to normal esophagus. Further investigation of the various radioprotective properties of soy isoflavones in preserving normal esophagus tissue and organ function is warranted.

CHAPTER 5: FIGURES, TABLES, AND LEGENDS

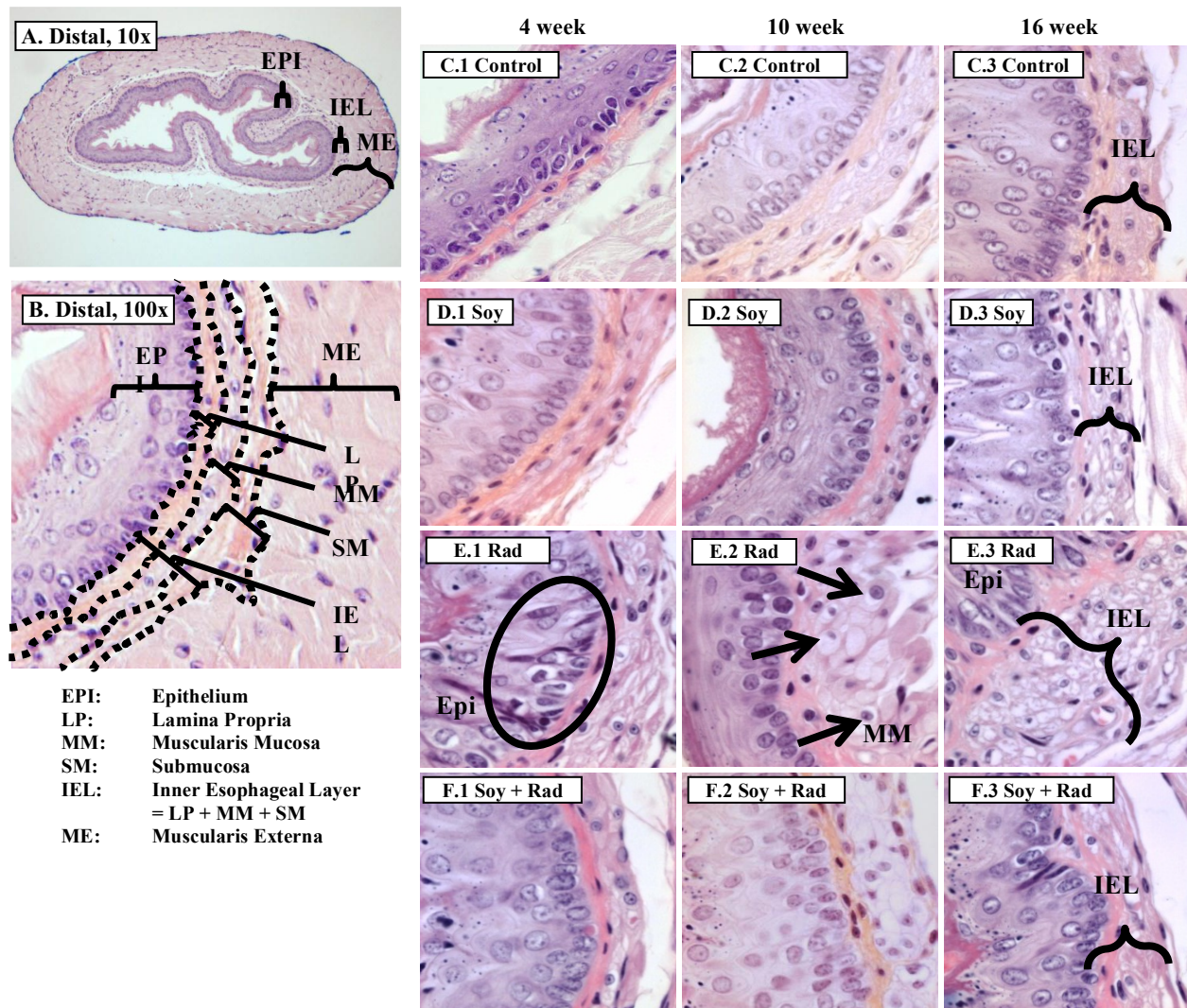


FIGURE 1. H&E staining of esophagus tissue sections treated with soy, radiation, and radiation + soy. Esophagi tissue sections were obtained from control mice and mice treated with soy or radiation (Rad) or radiation + soy (Soy + Rad) at 4, 10, and 16 weeks after radiation. Sections were stained with H&E. **(A).** Transverse tissue section of the esophagus at low magnification [10x]. **(B).** Esophageal tissue layers include the mucosal epithelium (EPI), lamina propria (LP), muscularis mucosae (MM), submucosa (SM) lining the muscularis externa (ME) [x100]. Because alterations induced by radiation were observed in LP, MM, and SM, these three layers were grouped as inner esophageal layer (IEL). **(C).** Sections from control mice showing normal morphology of the different tissue layers. **(D).** Sections from mice treated with soy are showing mild changes in IEL and EPI. **(E).** Sections from mice treated with radiation showing alterations in the EPI with disruptions in the alignment of epithelial cells in the basal cell layer (circle in E.1). In the MM layer, consisting of smooth muscle cells, hypertrophy and cytoplasmic vacuolation were observed (arrows in E.2). Overall thickening of IEL and extensive vacuolation were observed at 4, 10, and 16 weeks after radiation (brackets in E.3). **(F)** Sections from mice treated with radiation + soy showing less alterations than in radiation alone, mostly in LP and MM layers. [All magnifications x100].

Treatment	Inner Esophageal Layer Thickness (Mean $\mu\text{m} \pm \text{SEM}$)		
	Day 28	Day 70	Day 112
<i>Control</i>	15.77 \pm 0.40	22.74 \pm 0.58*	25.13 \pm 0.54
<i>Soy alone</i>	20.58 \pm 0.59	21.80 \pm 0.64*	30.24 \pm 1.08**
<i>RT alone</i>	30.66 \pm 0.67	41.54 \pm 1.18	40.43 \pm 0.91
<i>RT+Soy</i>	25.54 \pm 0.67	27.03 \pm 0.74	33.28 \pm 0.80**

TABLE 2. Morphometric measurements of inner esophageal tissue layers. Using Image Pro Plus software analysis of H&E slides, the thickness of the IEL was quantified in 5 fields of 100x for each mouse esophagus. Data computer on mouse esophagus was from 4 representative mice from 2 separate experiments, yielding 100 measurements per treatment. Student's T-test revealed significant differences between all treatment groups except those denoted by * and **.

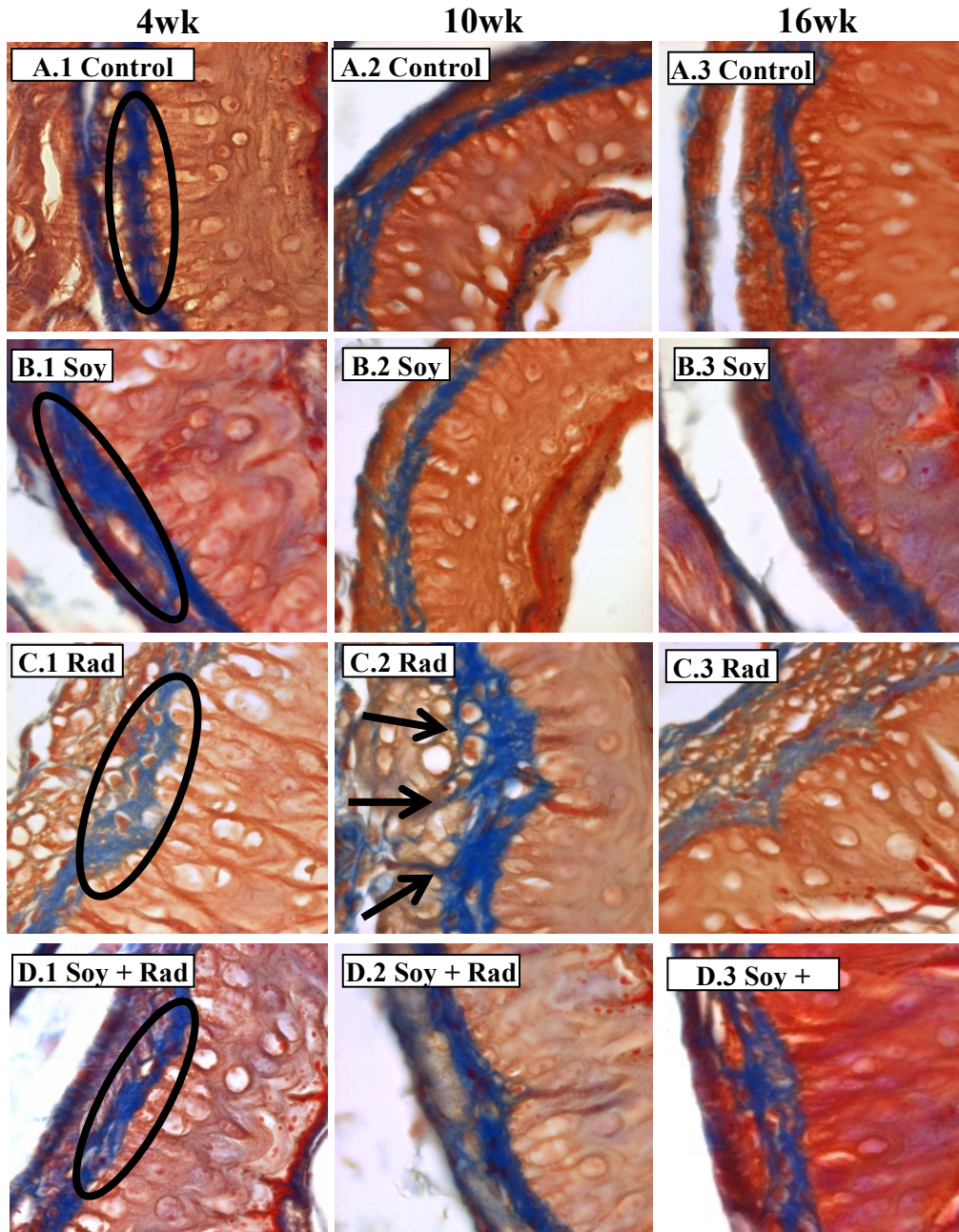


FIGURE 2. Effect of soy and radiation on fibrotic connective tissue layers of esophagus.

Esophagus tissue sections obtained from the experiments described in figure 1 were stained with Masson's Trichrome for detection of collagen fibers within the LP and SM connective tissues. **(A)** Sections from control mice showed normal, dense and well organized layers of collagen fibers within the LP at all time points [x100]. **(B)** Sections from mice treated with soy showed LP and SM fibrous layers similar to that of control. **(C)** Sections from mice treated with radiation showed alterations in LP and SM with disruptions in the integrity of connective collagen fibers including thickening of LP and decreased density of collagen fibers as well as focal infiltration of fibers around smooth muscle cells within the MM. **(D)** Sections from mice treated with radiation + soy showed reduced collagen infiltration into MM smooth muscle compared to radiation and a higher density of fibers that is comparable to LP of control and soy-treated esophagus. [All magnifications x100].

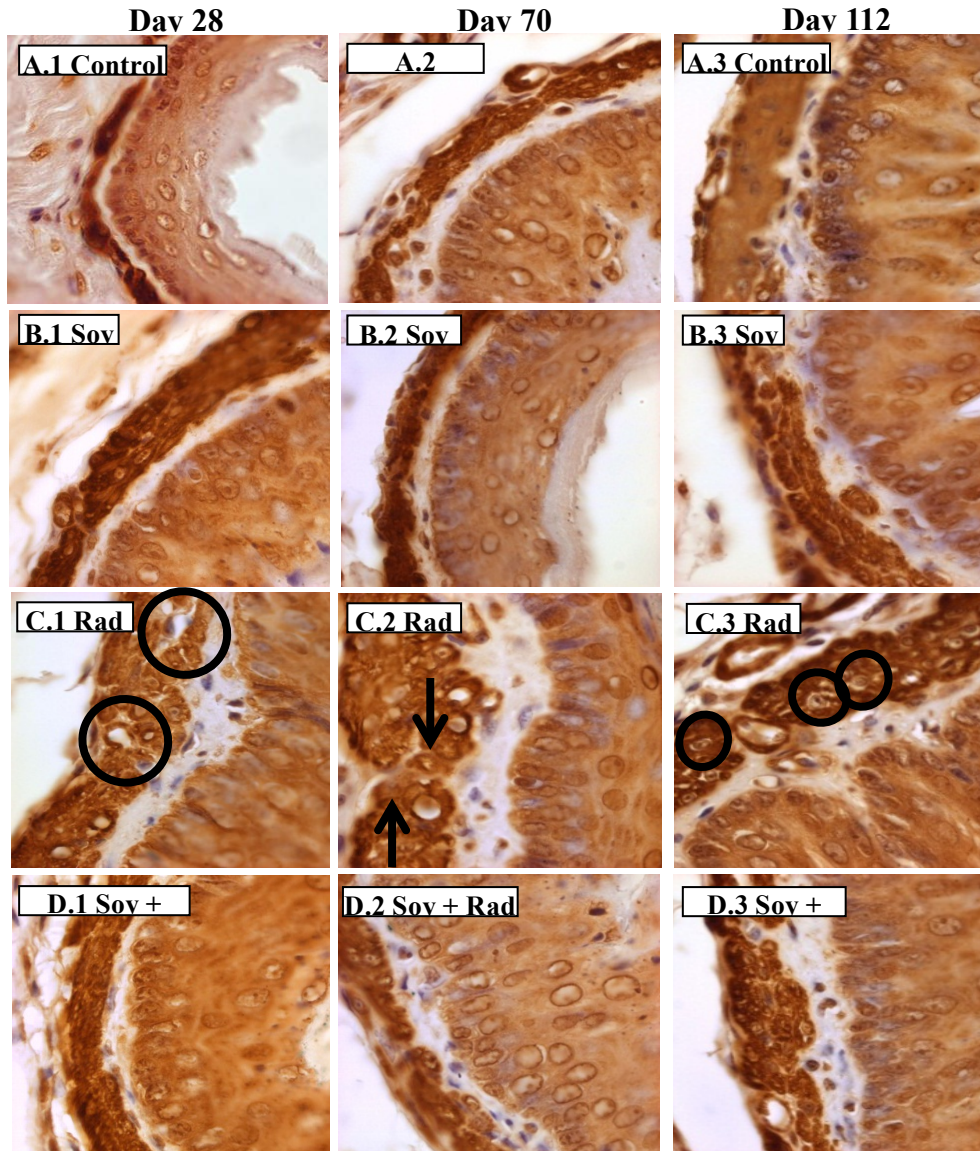


FIGURE 3. Effect of soy and radiation on smooth muscle cells in muscularis mucosae and epithelial layer of esophagus. Esophagus tissue sections obtained from the experiments described in figure 1 were stained with anti- α SMA by IHC for detection of smooth muscle cells in MM. **(A)** Sections from control mice showed normal thin MM layer with dense staining of smooth muscle cells separating the LP and SM. **(B)** Sections from mice treated with soy showed normal MM similar to control. **(C)** Radiation caused obvious thickening of LP and MM seen by MT. Smooth muscle cell hypertrophy (circles in C.1), large empty vacuoles, and both rounding up and condensation of smooth muscle cell nuclei were evident in MM. Interruptions in the integrity of MM layer were also observed (arrows in C.2). **(D)** Sections from mice treated with radiation + soy showed reduced smooth muscle cell hypertrophy and limited disintegration of MM compared to radiation alone. [All magnifications x100].

	<u>Fibrosis Collagen</u> <u>Density</u> (Masson Trichrome - Lamina Propria)			<u>Smooth Muscle Cell</u> <u>Hypertrophy</u> (α-SMA - Muscularis Mucosa)		
	D28	D70	D112	D28	D70	D112
Control	++	++	++	±	±	±
Soy	++	++	++	±	±	+
Radiation	+	+	+	++	+++	+++
Rad + Soy	++	++	++	±	+	++

Table 3. Summary and Quantification of Histological Observations. Histological findings from experiments presented in Figures 2 and 3 were quantified. The extent of fibrosis (collagen density) and MM damage (smooth muscle cell hypertrophy) were scaled from weak (\pm), moderate (+), strong (++), to heavy (+++).

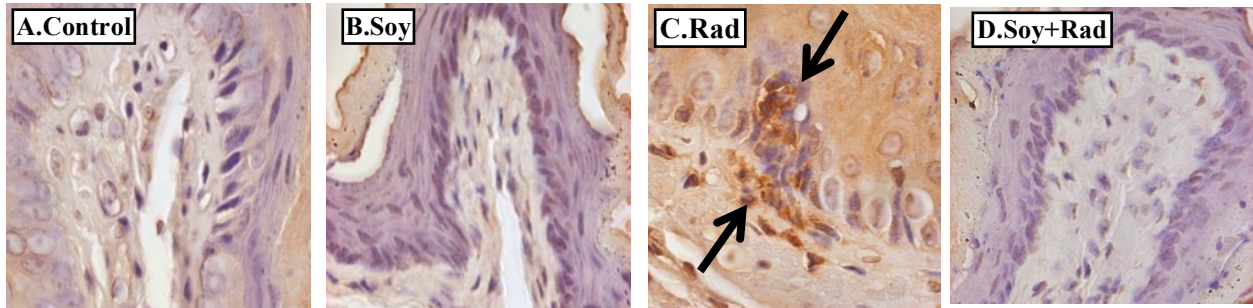


FIGURE 4. Leukocyte infiltration in esophageal tissues treated with soy and radiation. Esophagi tissue sections were obtained from control mice and mice treated with soy or radiation (Rad) or radiation + soy (Soy + Rad) at 4 weeks after radiation. Esophagus tissues were stained with anti-CD45 by IHC for detection of leukocytes at the border between LP and EPI. **(A)** Sections from control mice showed no staining of CD45+ cells within the LP nor EPI tissue layers, and **(B)** similar results were shown in mice treated with soy. **(C)** Sections from mice treated with radiation showed focal CD45+ leukocyte infiltration in LP and forming clusters at the edge of EPI basal layer (arrows in C). **(D)** Sections from mice treated with radiation + soy showed minimal CD45+ cell infiltrates comparable to control and soy-treated esophagus. [All magnifications x100].

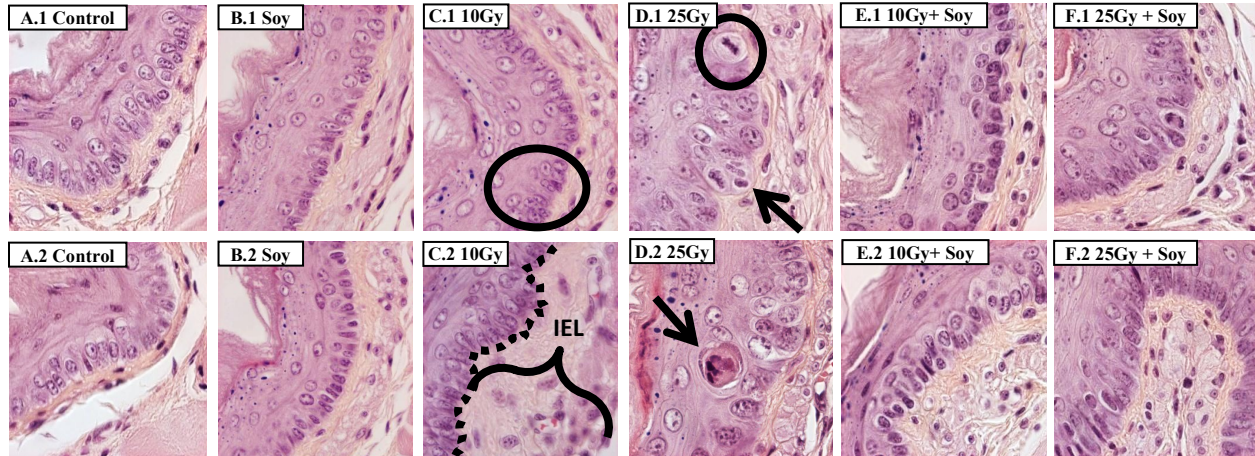


FIGURE 5. Early effects of soy on esophageal tissues treated with 25 Gy radiation compared to 10 Gy radiation. Esophagi tissue sections were obtained from control mice and mice treated with soy or radiation (Rad) or radiation + soy (Soy + Rad) at 1 week after a radiation dose of 10Gy or 25Gy. Sections were stained with H&E and each treatment includes two representative images. **(A)** Sections from control mice show healthy esophagus tissue layers, including normal mucosal epithelium, including normal basal cell shape, size and morphology. **(B)** Sections from mice treated with soy showed normal mucosal epithelium comparable to control mice. **(C)** Sections from mice treated with 10Gy radiation showed several changes in the mucosal epithelium, and **(D)** a higher dose of 25Gy radiation caused greater alterations in the EPI mucosal layer, including basal cell hyperplasia showing increased mitosis (circles in C.1 and D.1), karyorrhexic basal cells and formation into giant cells (arrows in D.1 and D.2), and disruptions to basal cell basement membrane continuity (dotted lines in C.2). Additionally, tissue sections showed large regions of increased thickness of IEL, including LP and MM (brackets in C.2). **(E, F)** Sections from mice receiving combined treatment of 10Gy or 25Gy radiation with soy showed decreased alterations in mucosal basal cell morphology, including reduced basal cell hyperplasia, few to no giant cell formation, and fewer regions of disrupted basal cell basement membrane continuity, similar to control and soy treated mice. Additionally, sections from mice having soy treatment with both radiation doses showed decreased thickening of the IEL.

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ABSTRACT**RADIATION-INDUCED ESOPHAGITIS IS MITIGATED BY SOY ISOFLAVONES**

by

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Lung cancer patients receiving radiotherapy present with acute and chronic esophagitis, resulting in pain and difficulties swallowing. These effects are due to radiation injury to the normal tissues in the esophagus. Our previous studies in pre-clinical models of lung cancer and naïve mice have shown that soy isoflavones alleviates radiation-induced toxicity to normal lung, including a decrease in pneumonitis and fibrosis. In this study, we have investigated whether radiation-induced esophagitis can be reduced by soy isoflavones. C57BL/6 mice were treated with 10 Gy or 25 Gy for thoracic irradiation and soy isoflavones given daily at 1mg/mouse up to 16 weeks. Esophagi were resected at various time points (1,4,10,16 weeks post-radiation). Damage to esophageal tissues was assessed by H&E staining and Masson's Trichrome. The effects on smooth muscle cells and leukocyte infiltration were determined by immunohistochemistry using anti- α SMA and anti-CD45. Histological analysis revealed esophagus tissue edema and inflammatory leukocyte infiltration. Specific findings included alterations in the mucosal epithelium, connective tissue disruption in the lamina propria, and smooth muscle cell hypertrophy in the muscularis mucosa. Radiation-induced tissue injuries were reduced by combining soy isoflavones

with radiation treatments. Quantitative evaluation of thickened, edematous tissue layers was performed by morphometric measurements. Analysis of 100 measurements per treatment group revealed a significant decrease in the thickness of esophageal layers in irradiated mice treated with soy ($25.54\mu\text{m}\pm 0.67\text{SEM}$) to radiation alone ($30.66\mu\text{m}\pm 0.67\text{SEM}$) ($p < 0.0001$). Radiation-induced damage presented within 1 week and progressively worsened with time, being more pronounced with 25 Gy compared to 10 Gy, but was also decreased by soy supplementation. Thoracic irradiation caused injury to multiple layers of the esophagus, and these effects were mitigated by soy isoflavones. These findings suggest that soy isoflavones have a radioprotective effect on the esophagus, mitigating the early and late effects seen in radiation-induced esophagitis.

AUTOBIOGRAPHICAL STATEMENT

I have planned for graduation in May 2015 with a Masters of Science in Immunology/Microbiology from Wayne State University School of Medicine. I am continuing with this research project as well as other current projects in the Hillman laboratory as the basis for my PhD studies. Upon completion of my PhD, I intend to continue on to post-doctoral studies in order to pursue a career in teaching and translational research in academia, government, and scientific industry.