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Radiation injury to cardiac arteries and myocardium is reduced by soy isoflavones

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Abstract

Objective—The negative effects of incidental radiation on the heart and its vessels, particularly in the treatment of locally advanced non-small cell lung cancer, esophageal cancer, left-sided breast cancer, and lymphoma, are known. Late cardiac events induced by radiotherapy including coronary artery disease, ischemia, congestive heart failure, and myocardial infarction can manifest months to years after radiotherapy. We have previously demonstrated that soy isoflavones mitigate inflammatory responses induced in lungs by thoracic irradiation resulting in decreased vascular damage, inflammation, and fibrosis. In the current study, we investigate the use of soy isoflavones to protect cardiac vessels and myocardium from radiation injury.

Methods—Mice received a single dose of 10-Gy thoracic irradiation and daily oral treatment with soy isoflavones. At different time points, hearts were processed for histopathology studies to evaluate the effect of soy isoflavones on radiation-induced damage to cardiac vessels and myocardium.

Results—Radiation damage to arteries and myocardium was detected by 16 weeks after radiation. Soy isoflavones given in conjunction with thoracic irradiation were found to reduce damage to the artery walls and radiation-induced fibrosis in the myocardium.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and the use of animals were followed. The animal protocol was approved by Wayne State University Animal Investigation Committee. Mice were housed and handled in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed consent Statement of informed consent was not applicable since the manuscript does not contain any patient data.

Conclusion—Our histopathological findings suggest a radioprotective role of soy isoflavones to prevent cardiac injury. This approach could translate to the use of soy isoflavones as a safe complement to thoracic radiotherapy with the goal of improving the overall survival in patients whose cancer has been successfully controlled by the radiotherapy but who otherwise succumb to heart toxicity.

Keywords

Radiation; Soy isoflavones; Heart arteries; Myocardium; Radioprotection

Introduction

Cardiotoxicity has been clearly demonstrated in analyses of thoracic radiation studies for common cancers, including locally advanced non-small cell lung cancer (NSCLC), esophageal cancer, left-sided breast cancer, and lymphoma [1-5]. Despite technical advances allowing for treatment with high precision under image guidance, when targets are in close proximity to the heart, incidental radiation to this organ is unavoidable and can result in clinically relevant sequelae. Subacute and chronic cardiac complications are observed beginning several months after radiotherapy (RT) and extending to years after RT. Potential late cardiac events induced by RT injury include coronary artery disease, ischemia, congestive heart failure, and myocardial infarction and can manifest several months to years after RT [1, 2]. Cardiotoxicity is related to both RT dose and the volume of heart receiving RT, which is defined, for example, as V30 for the percentage of heart volume receiving at least 30 Gy [1, 4]. Recent advances in the treatment of unresectable stage IIIA or stage IIIB NSCLC, with improved RT techniques combined with concurrent chemotherapy, have resulted in an increase in median survival of 29 months as documented in RTOG 0617 [4] compared with historic controls. That study, however, reported an association between heart dose and overall survival, specifically heart V5 and V30 as predictors of patient death. A conventional fractionated regimen of 2 Gy per fraction for a total of 60 Gy over a period of 6 weeks is estimated to deliver no more than 45 Gy to two thirds of the heart volume. In breast cancer and lymphoma patients with good prognosis after RT and longer survival, the incidental radiation dose to heart tissue can result in heart complications detected within years after RT [1, 5-7]. In left-sided breast cancer patients, a recent study found that the rates of major coronary events increased linearly with increase in mean heart dose by 7.4% per Gy within the first 5 years after RT and continued for at least 20 years [5]. Fractionated RT at doses of 2 Gy per fraction for breast cancer patients is delivered to a total dose of 46–66 Gy while typically restricting the mean heart dose to less than 4 Gy. However, it is estimated that the coronary vessels, specifically the left anterior descending artery (LAD), might receive 25–35 Gy despite techniques used to decrease incidental mean dose to <4 Gy. As we continue to improve our treatments for cancers that typically develop in close proximity to the heart, we must consider the subacute and chronic effects of radiation dose to heart and find new ways to improve the therapeutic ratio and mitigate the negative long-term effects of our treatments.

Preclinical animal studies have documented the radiation effects in heart and the mechanisms of radiation injury [3]. Radiation-induced endothelial damage and recruitment

of inflammatory cells, both in the coronary arteries and in the cardiac microvasculature, can lead to atherosclerosis and myocardial infarct [3]. Radiation-induced interstitial fibrosis, inflammatory and thrombotic changes, capillary loss, and ischemia in the myocardium itself can result in congestive heart failure [3]. Such radiation-induced fibrosis in myocardium has been documented in animal and human studies [3, 8-11]. Finally, pericarditis may present as edematous thickening of the pericardium with inflammatory infiltrates as seen in animal studies using high-dose RT [3, 8-10]. These studies on heart toxicity suggest a mechanistic role for radiation-induced inflammatory responses triggered by vascular and tissue damage [2]. Approaches including anti-inflammatory and anti-thrombotic drugs have shown limited efficacy in preventing coronary artery disease [3]. It is therefore imperative that we develop new approaches to prevent and decrease radiation injury to the heart.

In the study reported here, we have investigated the use of soy isoflavones (SIF) to protect heart structures from radiation toxicity, based on our previous findings documenting radioprotection of thoracic organs including lungs and esophagus by SIF [12-14]. SIF are naturally occurring phytoestrogens found in soybeans and consist of genistein, daidzein, and glycitein. SIF have a chemical structure similar to estrogen; however, they have weak estrogenic activity and act as chemopreventive and anti-cancer agents [15-18]. We have demonstrated that SIF given in conjunction with thoracic RT augmented the destruction of lung tumor nodules in xenografted lung tumor-bearing mice but also simultaneously mitigated radiation-induced damage to lung tissues [12, 13, 19]. SIF decreased the extent of vascular damage, pneumonitis, and fibrosis caused by RT in the lungs [12, 13, 19]. SIF radioprotective effects were associated with decreased inflammatory cytokines and reduced activation and infiltration of macrophages and neutrophils in lung tissue [20]. SIF also mediated a significant reduction in radiation-induced damage to esophageal tissues in mice [14]. Furthermore, SIF reduce radiation-induced skin injury and restore pulmonary function in naive mice, confirming its role as a radioprotector of normal tissue [13]. In a clinical trial for prostate cancer patients treated with SIF tablets in conjunction with RT, we observed reduced radiation toxicity resulting in improved urinary, sexual, and gastrointestinal functions [21]. Collectively, both our pre-clinical and clinical studies indicate that SIF can alter the detrimental effect of the radiation-induced inflammatory response and could potentially mitigate radiation-induced damage in coronary arteries and myocardium.

In the current preclinical study, we have investigated the time course for the detection of heart injury by a high dose of RT, evaluating histopathological changes to arteries and heart tissues and the effect of SIF on these alterations. Damage to arteries and myocardium was detected at a late time point of 16 weeks after RT. SIF given in conjunction with thoracic radiation were found to reduce damage to the artery walls and decrease the process of radiation-induced fibrosis in the myocardium, suggesting a radioprotective role of SIF to reduce cardiac injury.

Methods

Mice

Female C57BL/6 NHsd mice of 5–6 weeks old (Harlan, Indianapolis, IN) were housed and handled in animal facilities accredited by the Association for Assessment and Accreditation

of Laboratory Animal Care (AAALAC). All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC).

Soy isoflavones

The natural soy isoflavone (SIF) 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). SIF 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.

Thoracic irradiation

A radiation dose of 10 Gy in a single fraction was chosen on the basis of expected equivalence in late effect on the coronary vasculature. In the linear-quadratic framework, describing dose fractionation equivalence, using average $\alpha/\beta = 3$ Gy for late normal tissue endpoints, including heart, a single dose of 10 Gy corresponds to equivalent total dose in 2 Gy fractions $(EQD2)_3 = 26$ Gy. This EQD2 of 26 Gy is under but close to the 30 Gy defining the V30, which predicts for heart toxicity in 2 Gy per fraction clinical RT [4], and is therefore a realistic radiation dose to test. Radiation was delivered to the mouse thorax including bilateral lungs and mediastinum. Three anesthetized mice in jigs were positioned under a 6.4-mm lead shield with three cutouts in an aluminum frame mounted on the X-ray machine to permit selective thorax irradiation in three mice at a time, as previously described [19]. 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 back plate of the jig was thinned to 1.6-mm 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 at a dose of 10 Gy 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 pretreated with oral SIF each day for 3 days at a dose of 5 mg/day (equivalent to 250 mg/kg). On day 4, the thorax was selectively irradiated with a dose of 10 Gy. Soy treatment was continued on a daily basis for five more days at 5 mg/day. Then, the mice were treated with a lower SIF dose of 1 mg/day (equivalent to 50 mg/kg), given daily 5 days a week for up to 16 weeks. The rationale for giving a higher dose of SIF for pretreatment and just after radiation is to optimize the expected effect of SIF based on our previous studies [12-14]. Mice in the control and radiation-alone groups received DMSO-sesame seed oil vehicle alone.

Heart tissue preparation for histological examination

At time points of 4, 10, and 16 weeks after thoracic RT, mice were sacrificed and hearts were extracted, bisected in the craniocaudally direction, fixed in 10% buffered formalin, and processed for paraffin embedding and sectioning. Sections were stained with hematoxylin-

eosin (H&E). Perivascular and myocardial fibrosis was evaluated with Masson's trichrome (MT) staining (NovaUltra Kit, IHCWORLD, Woodstock, MD) [12, 19]. Smooth muscle cells were stained by immunohistochemistry with purified primary monoclonal antibodies directed against alpha-smooth muscle actin (α SMA) (Sigma-Aldrich, St. Louis, MO), followed by biotinylated secondary antibodies (Vector Labs, Burlingame, CA) [14, 20]. Staining was amplified with the avidin-biotin system immunoperoxidase technique. Heart sections were examined on a Nikon E800 microscope. Four mice per group were used for data analysis in two identical experiments.

Immunofluorescence of heart arteries

Heart tissue sections were immunostained with fluorescent antibodies to visualize the wall of the arteries, as previously detailed [12, 22]. Endothelial cells were identified with rat anti-mouse CD31 Ab (BD Pharmingen, San Diego, CA) followed by Alexa Fluor 488-labeled secondary goat antirat Ab (Invitrogen, Carlsbad, CA). Smooth muscle cells were identified with mouse anti- α SMA (Sigma-Aldrich, St. Louis, MO) followed by Alexa Fluor 350-conjugated secondary goat anti-mouse Ab (Invitrogen, Carlsbad, CA). The vessel basement membrane was stained with rabbit anti-collagen type IVAb (Millipore, Billerica, MA) followed by Alexa Fluor 594-conjugated secondary goat antirabbit Ab (Invitrogen, Carlsbad, CA). All slides were examined using a Nikon E-800 fluorescent microscope. Digital images were taken separately with each fluorescent dye, including green for endothelial cells, blue for smooth muscle cells, and orange/red for collagen, and were then processed to create composite images with the three colors using Image-ProPlus version 6.2 software (Media Cybernetics).

Statistical analysis

To compare the percentage of damaged arteries between each treatment group, pair-wise group differences were tested using ANOVA and the Tukey-Kramer method for multiple testing adjustment. All adjusted *p* values are two-sided with a significance level of 0.05. All calculations were performed with R version 3.0.2.

Results

Histological evidence of SIF inhibition of radiation-induced injury to cardiac arteries

To evaluate the effects of thoracic irradiation and SIF on cardiac structures, the heart was resected at 4, 10, and 16 weeks after RT. Heart tissues were processed for histological examination. Representative images of stained sections are shown in Fig. 1. Insets 1 and 2 show a cross section of a cardiac artery and its wall components including the tunica externa consisting of connective tissue, collagen fibrils and fibroblasts, the tunica media which contains smooth muscle cells and collagen fibrils, and the tunica intima supporting the endothelium (Fig. 1). Aberrations in the artery walls caused by radiation were detected at a late time point of 16 weeks after radiation. Whereas arteries from untreated control mice had an integral and well-defined wall (Fig. 1 (A1), arrows), radiation caused distortion of the artery walls with extensive vacuolation in smooth muscle cells observed by H&E staining (Fig. 1 (B1), arrows). The morphology of the arteries in mice treated with SIF and radiation

showed less aberrations and an integral wall structure akin to control mice (Fig. 1 (C1), arrows).

To further assess the radiation-induced changes caused in the smooth muscle cells of the tunica media, heart tissue sections were immunostained with α SMA. The tunica media in control arteries presented as a dense smooth muscle cell layer (Fig. 1 (A2)). Radiation-treated arteries showed cytoplasmic vacuolation in smooth muscle cells, which is indicative of cell damage (Fig. 1 (B2), arrows). Cardiac arteries from mice treated with combined SIF and radiation had reduced smooth muscle cell damage (Fig. 1 (C2)).

Detection of collagen fibers, by MT staining, also revealed changes induced by radiation in collagen fibrils in the tunica externa with diffuse and increased perivascular collagen fibers (Fig. 1 (B3), arrows), in contrast to the thin layer of collagen fibrils lining the tunica externa in control arteries (Fig. 1 (A3)). Arteries from SIF- and radiation-treated mice showed perivascular thin collagen fibrils and reduced diffusion of fibrils (Fig. 1 (C3)). These findings were consistent and reproduced in a second identical experiment.

Quantitation of the number of damaged arteries was performed on heart tissue sections stained with anti- α SMA by IHC. Damaged arteries were identified based on aberrations including distortion of arteries, irregularities in arterial wall, and vacuolation in smooth muscle cells of tunica media. The percentage of damaged arteries in 23–26 fields at $\times 40$ magnification for each treatment group, with three to four mice/group, showed a significant decrease of 23% damaged arteries with SIF combined with radiation compared to 65% with radiation alone (Tukey-Kramer adjusted $p = 0.011$), 12% for SIF alone, and 14% in control (Table 1).

SIF prevention of artery wall injury caused by radiation

To further study the effect of RT on the components of artery wall, heart sections were immunostained with fluorescent dyes for three main components of the wall as endothelial cells (green for anti-CD31) and smooth muscle cells (blue for anti- α SMA) and for collagen (orange/red for anti-collagen IV). Two representative images of arteries are presented in Fig. 2. Control arteries show an intact vessel wall with the three stained components and dominant blue and orange corresponding to the main structural support of smooth muscle cells and collagen fibers (Fig. 2 (A1, A2)). Radiation caused interruptions, disruptions, and thinning in the walls of arteries (Fig. 2 (B1, B2)). The arteries of the hearts treated with SIF and radiation showed full intact arterial walls with staining comparable to control arteries (Fig. 2 (C1, C2)). These stainings were reproduced in four mice per treatment group showing consistency in our findings.

SIF prevention of collagen band formation caused by radiation in myocardium

To visualize myocardial fibrosis, heart tissue sections were stained with MT at 4, 10, and 16 weeks after radiation. No fibrosis was observed in the myocardium tissue of control mice at each time point (Fig. 3 (A1–A3)). A few discrete collagen bands were detected at 10 weeks after radiation (Fig. 3 (B2)) but were increased and more prominent at 16 weeks presenting as diffuse bands of collagen between myocytes (Fig. 3 (B3), arrows). These collagen bands

were not observed in myocardium of mice treated with SIF and radiation at early or late time points (Fig. 3 (C1–C3)). These data were consistent in four mice per treatment group.

Discussion

Awareness of cardiotoxicity associated with thoracic RT of cancers has increased with retrospective clinical studies documenting acute and late effects of RT in surviving patients [1-5]. Such complications can be severe and include coronary artery disease, ischemia, congestive heart failure, and myocardial infarction [1, 2]. The large variation in risk reported in different studies suggests that volume effects are important [1, 4], but also that the heart auricles and the proximal parts of the coronary arteries could be particularly sensitive to radiation damage, as reported in animal studies [23]. To develop a safe approach to protect the heart vessels and myocardium from radiation-induced injury, the goals of this study were to explore the use of non-toxic, natural SIF. This approach derives from our preclinical and clinical observations showing the ability of SIF to prevent radiation-induced toxicity to thoracic organs in lung cancer [12-14] and pelvic organs in prostate cancer [21]. We have shown that SIF can have the rare dual capability of enhancing radiation damage in a malignancy while simultaneously protecting normal lung from radiation injury due to a differential effect on cancer cells versus normal cells [12]. SIF mitigated vascular damage, inflammation, and fibrosis caused by high-dose radiation injury to normal lung tissue both in naïve mice and in lung tumor-bearing mice [12]. SIF radioprotective effects have been associated with reduced activation and infiltration of macrophages and neutrophils in lung tissue, suggesting that SIF can alter the detrimental radiation-induced inflammatory response [20]. Based on these studies in thoracic organs, we hypothesized that SIF could decrease the severity of high-dose radiation injury to myocardium and heart vessels by reducing vascular damage and inflammatory responses in these normal tissues.

Previous studies in rats reported histopathological changes caused by radiation-induced damage to coronary arteries including endothelial cell loss, smooth muscle cell loss, and thickening of the tunica media, with fibrosis extending to the outer tunica adventitia by 3 months after 20 Gy RT [24]. In our study, comparable late injuries to artery wall structures were observed at 4 months after a clinically relevant dose of 10 Gy thoracic RT. Cardiac arteries showed disruptions and distortion of the artery wall and extensive vacuolation in smooth muscle cells. Specific staining of smooth muscle cells with anti- α SMA confirmed cytoplasmic vacuolation of these cells in the tunica media. Collagen fibrils in the tunica externa were also disrupted by radiation showing diffuse and increased perivascular collagen fibers, as observed in other animal studies [8, 11, 24]. These radiation-induced aberrations in the structure of the arteries were mitigated by daily oral treatment of mice with SIF administered prior to RT and following RT for up to 4 months. Arteries from mice treated with SIF and RT showed integral wall structure, reduced smooth muscle cell vacuolation, and normal distribution of fibrils in the artery walls. Quantitation of damaged arteries versus healthy arteries showed a significant decrease to 23% damaged arteries in heart tissues from mice treated with SIF and RT compared to 65% with RT alone. These data confirm that injuries caused by RT in artery walls are decreased by continuous treatment with SIF after RT. These findings in cardiac arteries are in agreement with our previous observations of radiation damage in the smooth muscle cells of the muscularis mucosa of irradiated

esophagus and SIF-mediated mitigation of injury to smooth muscle cell layers [14]. Further evaluation of arterial injury by fluorescent staining of the three main components of artery walls including endothelial cells in the tunica intima, smooth muscle cells in the tunica media, and collagen fibrils in the tunica externa confirmed clear damage with interruptions and thinning of arterial walls caused by radiation. In contrast, arteries of mice treated with SIF and RT showed integral wall structure, suggesting radioprotection of artery walls by SIF. Damage to heart vessels by radiation and its alleviation by SIF supplemental treatment corroborate our previous findings on SIF radioprotection of irradiated lung vessels using a similar immunofluorescent technique to detect vessel abnormalities [12]. Taken together, these studies suggest that SIF given in conjunction with RT could protect the integrity of vessels. RT-induced coronary artery damage could lead to myocardial infarct [9]; therefore, SIF radioprotective effects on arteries may be relevant for preventing long-term heart disease observed in cancer patients treated with RT.

Radiation-induced injury to myocardium includes microvasculature damage, pericarditis, damage to the endocardium, and interstitial fibrosis [3, 8, 9, 24]. As a result of RT damage to blood capillaries, interstitial fibrosis in myocardium is progressive and consists of diffuse proliferation of bands of collagen crisscrossing the tissue to separate or replace myocytes [8, 9, 11]. In the current study, discrete bands of collagen were observed in the myocardium by 10 weeks after RT but were increased by 16 weeks after RT presenting as diffuse bands between myocytes. This interstitial fibrosis was minimal in the myocardium of SIF- and RT-treated hearts. Radiation-induced microvascular damage in myocardium leads to inflammatory, pro-thrombotic changes and progressive fibrosis that could result in congestive heart failure [2, 3]. Radioprotection of myocardium by SIF could have clinical implications to prevent cardiomyopathy.

A few drugs were tested in animal models to reduce radiation-induced heart damage including the ACE inhibitor captopril, amifostine, statins, pentoxifylline, and alpha-tocopherol, but they showed limited efficacy [3, 25]. The use of SIF to supplement RT as radioprotectors is attractive because they are non-toxic natural extracts from soybeans, which were proven to be safe in controlled human clinical trials [26, 27]. High soy intake among Asians is associated with about one third reduction in the risk of both pre- and postmenopausal breast cancer [28]. Regarding the application of SIF supplement to RT for breast cancer patients, recent results from epidemiologic studies conducted in Asian and Western populations show a decrease in risk of breast cancer and reduced mortality or recurrence in association with high intake of soy of 10–20 mg/day [29]. Previous preclinical controversial studies raised concern over the potential for deleterious effects of SIF as an estrogen agonist/antagonist for breast cancer patients [28]. However, since 2005, three clinical studies from the USA, one study from Korea, and four from China showed in a meta-analysis that high soy intake was significantly inversely associated both with recurrence (HR = 0.71) and mortality (HR = 0.76) in postmenopausal women whereas the findings in premenopausal women were non-significant (HR = 0.86, HR = 0.94) [28, 29]. These clinical studies suggest that SIF could be safely given to breast cancer patients undergoing RT. SIF can be given orally in tablets to cancer patients as previously done in our prostate cancer clinical trial of RT supplemented with SIF [21].

SIF supplementation to RT could also be applied to NSCLC patients receiving fractionated RT combined with concurrent chemotherapy to alleviate the increased toxicity caused by this protocol [30, 31]. In preliminary preclinical mouse studies, thoracic fractionated RT combined with cisplatin caused tissue injury in lungs and esophagus, which were more severe than by RT alone, as observed in patients. Still, supplementation with SIF to RT and cisplatin treatments demonstrated the same protective effects in thoracic organs (Hillman et al., personal communications). As a clinical biomarker of cardiotoxicity, serum high-sensitive cardiac troponin T (hs-cTnT) could be used to monitor the effect of SIF on cardiac function [32].

Recent novel approaches combining RT and immunotherapy are under extensive investigation [33]. Whether SIF could be used in conjunction with RT and immunotherapy to protect against RT injury remains to be elucidated. Our studies showed that SIF decreased RT-induced lung injury by inhibiting inflammatory processes [20], which are different from the anti-tumor immune pathways triggered by RT and immunotherapy, as recently published [34]. The sequence and timing of SIF, RT, and immunotherapy will be critical to improve therapeutic efficacy and minimize toxicity.

Conclusion

This approach could translate to the use of SIF as a safe complement to thoracic RT with the goal of improving the overall survival in patients whose cancer has been successfully controlled by RT but who otherwise succumb to heart toxicity.

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Fig. 1.

Histology of heart tissue sections treated with SIF and radiation. Heart tissue sections were obtained from control (Con) mice and mice treated with radiation (RT) or SIF and radiation (SIF + RT) at 16 weeks after radiation. Tissue sections were stained by H&E or with anti- α SMA by IHC or Masson's trichrome for detection of collagen fibers (MT). *Inset 1* shows a cross section of an SMA-stained artery, and *Inset 2* shows the enlargement of an area of the artery wall depicted in *Inset 1*. As detailed in *Inset 2*, the artery wall components include tunica externa (connective tissue, collagen fibrils, and fibroblasts), tunica media (smooth muscle cells, collagen fibrils), and the tunica intima (endothelium). **a** Arteries from control mice showing normal morphology of artery walls (*A1*), dense smooth muscle layer (*A2*), and thin collagen fibril layer (*A3*). **b** Radiation caused distortion and aberrations including vacuolation in tunica media smooth muscle layer in artery walls (*B1*), disruption and vacuolation in smooth muscle cells as seen by SMA staining (*B2*), and diffuse perivascular collagen staining (*B3*). **c** Arteries from mice treated with SIF + RT showed less aberrations with integral artery wall (*C1*), dense smooth muscle layer (*C2*), and perivascular thin collagen fibrils (*C3*) (all magnifications $\times 40$)

Fig. 2.

Fluorescent staining of artery walls in heart tissue sections. Heart tissue sections obtained from control-, RT-, and SIF + RT-treated mice at 16 weeks after radiation were immunostained with fluorescent dyes, including *green* for endothelial cells (anti-CD31), *blue* for smooth muscle cells (anti-SMA), and *orange/red* for collagen (anti-collagen IV). Representative images of arteries are presented. *A1* Arteries with integral vessel wall from control mice stained with the three dyes of artery components (*red arrows*). *B1* Arteries from radiation-treated mice showing interruptions in the wall of arteries and minimal SMA staining (*red arrows, B1*) with some arteries showing much thinner and disrupted wall (*red arrows, B2*). *C1* Arteries from SIF- and radiation-treated mice show staining comparable to control arteries and full thickness in artery walls (*red arrows*) (all magnifications $\times 40$)

Fig. 3.

Collagen staining of myocardium at 4, 10, and 16 weeks. Heart tissue sections obtained from control (Con) mice and mice treated with radiation (RT) or SIF + radiation (SIF + RT) at 4, 8, and 16 weeks after radiation were stained with Masson's trichrome. No collagen staining was seen in control (*A1–A3*). The myocardium of mice treated with radiation showed minimal staining at 10 weeks (*B2*), but increased diffuse bands of collagen were observed at 16 weeks between myocytes (*B3*). Minimal and reduced collagen staining was seen in heart tissues treated with SIF + radiation at all time points (*C1–C3*) (all magnifications $\times 40$)

Table 1

Quantitation of damaged arteries in heart treated with radiation and soy

Treatment	Proportion of damaged arteries	
	Damaged/total arteries	Percent damaged arteries (%)
Control	7/50	14
Soy	5/41	12
Radiation	45/69	65
Radiation + soy	15/66	23

Heart tissue sections stained with anti- α SMA by IHC were used to quantify the number of damaged arteries based on aberrations including distortion of arteries, irregularities in arterial wall, and vacuolation in smooth cells of tunica media. The total number of arteries and the total number of damaged arteries were counted in 23–26 fields of $\times 40$ for each treatment group ($n = 3$ –4 mice/group)