Title: Protective effect of genistein on radiation-induced intestinal injury in tumor bearing mice

Authors:

Tae Gen Son (tgson@dirams.re.kr)
Eun Ji Gong (eunji226@dirams.re.kr)
Min Ji Bae (wlalsgo@dirams.re.kr)
Sung Dae Kim (sdkim@dirams.re.kr)
Kyu Heo (kyuh33@dirams.re.kr)
Kwangmo Yang (knyang@dirams.re.kr)
Changjong Moon (moonc@chonnam.ac.kr)
Joong Sun Kim (jskim@dirams.re.kr)

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Author's response to reviews: see over
Responses to the Reviewers’ Comments:

<Editor’s comments>

Comment 1. In cancer radiotherapy usually uses fractioned radiation doses and not a single therapy. However a single dose affects on apoptosis in normal cells such as intestines, it is not significantly affects on tumor growth. In figure 5A, on 7 day, 5 Gy was not affected on tumor size as compare to shame, only high dose 10 Gy was affected.

Response: We partially agreed with your view on the effect of cancer radiotherapy. In this study, we want to know the radioprotective effect of genistein on the intestinal injury induced by radiation rather than its anti-cancer effect. To confirm this purpose, we used single radiation to induce intestinal injury because it is difficult to induce intestinal injury by fractioned radiation.

Comment 2. Actually, authors should be presenting these data in a table and statistically compare these data. It is important that authors compared 10 Gy (without genistein) group with Gen+10 Gy for showing a radiosensitizer effects of genistein, while authors compared Gen+10 Gy with shame.

Response: Fig. 5. was transformed Table 2. We have revised the description about the effect of genistein in CT26 cells in the results.

“Although genistein alone and 5 Gy irradiation alone decreased the size of the tumors compared with that in the sham group, the difference was not significant. 10 Gy irradiation alone and combination therapy resulted in a significant decrease in tumor size compared with that in controls after 7 d ($P < 0.05$ vs. sham group 7 d after 10 Gy). In the combined treatment (10 Gy + genistein) produced the best tumor regression and growth inhibition among the 6 groups, with no significant difference between the vehicle and genistein treated groups (Table 2. $P = 0.5$, 10 Gy group vs. 10 Gy +genistein group).”

Comment 3. For more judgment, authors are better to determine size of tumor up to at least 21 days.

Response: Xenograft lesion shows the necrosis and overgrowth 16 days after injection of CT26 cancer cells in sham group (7 days after irradiation). Furthermore, previous studies have shown that death occurs 5–10 days after exposure in mice, which was most likely caused by gastrointestinal damage (Zhang et al., 2009; Jia et al., 2010; Kim et al., 2012). With these points of views, we decided that a later time point was at 7 days after irradiation.

<Reviewer (Regina Day) #1>

Comment 1. Please indicate the location(s) of tumors in mice-are they all localized to the site of injection?

Response: CT26 colon cancer cells were injected subcutaneously into the right flanks of mice (Fig. 1). We changed the Fig 1, and indicated the location of cancer injection.
Comment 2. In the Methods, please indicate the numbers of mice that were used to evaluate tumor sizes after irradiation (mice not euthanized to evaluate intestinal cell apoptosis).

Response: We have revised the description about the group in the methods.

“After 7 d of xenograft implantation, when the tumors had reached ~5 mm in diameter, Ninety mice were randomly divided into 6 groups as follows: (1) vehicle + sham irradiation group (n = 15), (2) genistein + sham irradiation group (n = 15), (3) vehicle + 5 Gy irradiation group (n = 15), (4) genistein + 5 Gy irradiation (n = 15), (5) vehicle + 10 Gy irradiation (n = 15), and (6) genistein + 10 Gy irradiation (n = 15), according to the treatment schedule (Fig. 1A). The five mice were euthanized randomly in each group 12 h after irradiation, mice tumors were weighed and evaluated the intestinal apoptotic change. For histopathological examination, the five mice were euthanized in each group 3.5 d after irradiation, mice tumors were weighed and evaluated intestine histological change. After 7 d after irradiation, mice were euthanized in each group and mice tumors were weighed.”

Comment 3. Also, throughout the manuscript, please substitute the word "euthanized" for 'sacrificed'.

Response: We have changed the statement “sacrificed” with “euthanized” in the revised manuscript.

Comment 4. In the Methods, please provide the location of genistein injections.

Response: Genistein was administered into the dorsal subcutaneous space. We have changed the statement in the revised manuscript.

“Genistein was administered into the dorsal subcutaneous space at a dose of 200 mg/kg body weight at 24 h prior to irradiation; the dose and dosage were considered optimum for radioprotection, as reported previously.”

Comment 5. In the last section of the Results (Effect of genistein on sensitivity to radiation in CT26 cell xenografted mice), please provide the numbers of mice from which tumors were taken (in each treatment group).

Response: Mice were divided into 5 groups (each of 5 animals).

“After 12 hr, 3.5 and 7 d, mice (5 mice in each group) with tumors were sacrificed, and the tumors were weighed.”

Comment 6. In the Discussion, the authors state that genistein has "strong" antioxidant properties, however the use of genistein 24 h prior to irradiation suggests that genistein is having a biological effect rather than an antioxidant effect. The authors should check to ensure that genistein is a "strong" antioxidant (compared to many other radioprotective compounds, genistein is actually a weak antioxidant). The authors should consider the possibility that genistein (injected 24 h prior to irradiation) is not likely present in significant
levels in the tissues at the time of radiation. Many other radiation protective agents that do act via antioxidant properties (e.g. Tempol) must be administered immediately prior to radiation exposure. In their previous study of the testis, what was the time of administration and the time of irradiation prior to measurement of oxidative radicals?

Response: We sincerely appreciate the reviewer’s comment. We partially agreed with your view on the antioxidant effect of genistein. We deleted the “strong” in the discussion in the revised manuscript. However, we are still confident with the antioxidant property of genistein. Because many reports shows the antioxidant properties of genistein in the various damage model (Liang et al., 2008; Kruk et al., 2005; Filipe et al., 2005). Further, previous our study shows that ROS were evaluated using DCFDA method 12h and 21 days after irradiation. Irradiation elevated ROS levels in the testis and genistein treatment significantly attenuated the levels. Then, the genistein was also administered by subcutaneous injection 24 h prior to irradiation.

Liang et al., Genistein attenuates oxidative stress and neuronal damage following transient global cerebral ischemia in rat hippocampus. Neuroscience Letters 2008, 438:116–120.


Comment 7. The authors cite the regulation of GCSF by genistein. Have the authors measured GCSF in their system in response to genistein? Finally, the authors should mention cell cycle regulation and gene regulation activities (especially DNA repair genes) by genistein. These activities have been confirmed in multiple publications.

Response: We had a mistake the cited reference about the regulation of G-CSF by genistein. We have changed the reference. Although we did not measure the serum levels of G-CSF, the cited reference showed the genistein increases serum levels of G-CSF in irradiated and non-irradiated mice after exposure irradiation (Singh et al., 2009). Recent study showed the radioprotective effect of some agents is mediated the through G-CSF induction (Grace et al., 2012; Sing et al., 2011). Further previous our study also showed that G-CSF attenuated intestinal damage after radiation exposure. We have revised the description in the discussion.

“Genistein administration stimulated serum granulocyte-colony stimulating factor (G-CSF) after sham irradiation or gamma-irradiation [32]. Recent study showed the radioprotective effect of some agents is mediated the through G-CSF induction [33, 34]. Further previous our study also showed that G-CSF attenuated intestinal damage after radiation exposure [7]. G-CSF activates several signaling pathways to promote survival and proliferation, and it also protects against apoptosis after irradiation exposure [7, 35, 36]. The induction of G-CSF by genistein is also responsible for protection against radiation injury.”


<Comment 1.> Abstract: Method section second line- creates a misunderstanding regarding genistein administration, it appears that genistein was administered daily, but text shows it was administered only once before irradiation. Sentence needs to be reframed. Introduction: Last paragraph second sentence needs to be reframed.

Response: We sincerely appreciate the reviewer’s comment. We had a mistake, which have been corrected in the revised manuscript. “Abstract: The tumor-bearing mice were treated with abdominal radiation at 5 and 10 Gy, and with genistein at 200 mg/kg body weight for 1 d before radiation.”

<Comment 2.> Introduction: Last paragraph second sentence needs to be reframed.

Response: We have revised the last paragraph in the introduction in the cleaned manuscript. “Understanding the mechanisms are involved in the radiosensitization effect of genistein will acts as a radiosensitizer in various cancers [15, 19-21].”

<Comment 3.> Results: Anti-apoptotic effect of genistein in the jejunal crypts: First paragraph last two sentences are somewhat contradictory. Do the authors mean genistein had no significant anti-apoptotic action against 10 Gy (which is more likely)? But this is contradicted by the previous sentence since it does not mention the radiation dose.

Response: As recommended, we have revised the description of results about anti-apoptotic effect of genistein. “The number of apoptotic cells decreased significantly in the genistein-treated group compared with the vehicle-treated 5 Gy irradiated group (Fig. 2C and D) (P < 0.05 vs. irradiation group at 12 hours after 5 Gy). Although the administration of genistein also decreased the average number of apoptotic cells in the crypts in 10 Gy irradiated group, there was no significant difference between the vehicle- and genistein-treated groups exposed to 10 Gy irradiation (Fig. 2D).”

<Comment 4.> Discussion: Paragraph-Last but one: suitable references may be included.

Response: We have revised in the discussion section and added the reference of the revised manuscript.
Although the cancer patients might benefit from radiotherapy, it is not devoid of side effects. Among patients receiving abdominal radiotherapy more than 70% develop gastrointestinal symptoms during treatment [40]. Our findings show that genistein reduces various parameters related to intestinal injury caused by radiation exposure, but has not an effect on the tumor growth in cancer-bearing mice. Therefore, genistein may be clinically useful for colon cancer patients who require radiotherapy.


Comment 5. Fig 1A: The treatment scheme to be corrected so as to depict the 3.5 and 7d time points.

Response: We have corrected the Fig. 1A.

Comment 6. Fig 5b: really does not replicate the histogram representation above (Fig 5A) as it shows genistein to be much more radiosensitizing, authors may consider giving another representative image.

Response: As the editor recommended, Fig 5. was transformed Table 2.

Comment 7. Discussion part may be made based more on the observations of the current study rather than based more on other published work.

Response: We have added the current reference in the discussion section of the revised manuscript.


Comment 8. Discretionary Revisions: The study though bring the beneficial effects of genistein pretreatment in protecting against radiation-induced intestinal injury without compromising the tumor control aspect within the study time period, it does not describe/justify or comment on the quality of life, fate of the tumor and overall survival of the animals over a prolonged time. Even there is no follow up study of the intestinal health at a later time point (say on Day 6) and observation of the tumor volume, animal
health and survival over a longer period of time. Inclusion of those observations would improve the 
importance of the work done.

**Response:** Recovery from radiation induced intestinal damage is dependent on clonogenic stem cell survival 
in the intestinal crypt. If too many crypts are sterilized (no surviving clonogenic cells), ulceration of the 
intestine will denudation, and if extensive ulceration occurs the animal will die. Crypts that contain one or 
more surviving clonogenic stem cells regenerate to form a crypt structure at 3–4 days post-irradiation. We 
have focused on the crypt structure, and studied on day 3.5 after irradiation exposure. Previous studies have 
shown that death occurs 5–10 days after exposure in mice, which was most likely caused by gastrointestinal 
damage (Zhang et al., 2009; Jia et al., 2010; Kim et al., 2012). As a result, we do not prolong the study a later 
time point the 7 days after irradiation exposure.