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Protection of Radiation induced Somatic Damage by the Reduction of Oxidative Stress at Critical Organs of Rat with Naringenin Administration

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Abstract

Free radicals originate due to the radiolysis of cytoplasmic water with low "Linear Energy Transfer" (LET) radiations. Naringenin (Ng) is a natural antioxidative compound found in citrus fruits. This study revealed that Naringenin (Ng) reduced the radiation damage of critical organs by scavenging oxidative free radicals. In the study, Ng was orally administrated to rats daily for 7 consecutive days, prior to whole body exposure to gamma-rays. The scavenging efficacy was evaluated biochemically by measuring the concentration of cytotoxic byproducts and the activity of enzymes relevant to oxidative free radicals, after extracting the organs from the exposed rat. We observed increased levels of malondialdehyde (MDA) concentration, and decrease in the activities of superoxide dismutase (SOD) and catalase (CAT) in the exposed control group. However, pretreatment with Ng significantly reduced the MDA concentration, and increased the activities of SOD and CAT, as compared to the control group, due to the free radical scavenging by Ng. The results indicate that Ng administration prior to irradiation could protect critical organs from radiation damage.

Key words: naringenin, low linear energy transfer radiation, oxidative, free radicals, scavenging

Introduction

Radiation has been applied to various fields such as industry, medicine, agriculture, and aerospace (Zhao et al. 2012). However, the absorption of ionizing radiation can induce chemical and biological changes caused by the aberration of atomic structure of molecules (Azzam et al. 2012). When the aberration was generated by the direct interaction of secondary electrons, it is called as the "direct effect" (Citrin et al. 2010). It can also be induced by the indirect ways of physicochemical reactions of the reactive oxygen species (ROS) and/or the free radicals originated by the radiolysis of cytoplasmic water (Citrin et al. 2010; Li et al. 2015). Cytotoxic radicals from the radiolysis of water are e^-_{aq}, $^{\cdot}OH$, $H^{\cdot}$ (Dixit et al. 2012). In the presence of oxygen, those free radicals are rapidly converted to superoxide ($O_2^{-\cdot}$), perhydroxyl($HO_2^{\cdot}$) radical, and hydrogen peroxide H_2O_2. H_2O_2 is converted to hydroxyl radical($^{\cdot}OH$) by Fentonreaction(Azzam et al. 2012). The successive chemical reactions of ROS can result in damages of the nucleotides themselves, single strand break, or double strand break (Theriot et al. 2010). The damages are called as oxidative damage, and it affect to most of the biological molecules such as protein, lipid, and DNA (Azzam et al. 2012; Dixit et al. 2012). Those express acute radiation syndrome (ARS) such as the dysfunction/malfunction of hematopoietic, or chronic radiation syndrome followed by genetic mutations and immune dysfunction (Kumar & Tiku 2016). A whole body exposure to radiation of mammals to dose of over than 5 Gy can cause radiation sickness, hematopoietic syndrome, and gastrointestinal syndrome (Hall & Giaccia 2006, Koukourakis 2014). The hematopoietic system and lymphatic system are both high radiosensitive systems (Hall & Giaccia 2006). Also, digestive system is also a high radiosensitive organ because both the epithelial cells of mucous membrane and precursor cells at the basis of the villi are high radiosensitive (Hall & Giaccia 2006).

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Radioprotector is a modification agent used to protect biological systems from hazardous indirect effect induced from the radiation exposure. It could be required to protect a person who is potentially exposed to high dose of radiation either planned operation or an accident. In other case, radioprotector could be used in cancer radiotherapy to protect normal tissues of patient from radiation injury. Amifostine (WR-2721) is a U.S. FDA approved effective radioprotector which is a phosphorothioate (Hall & Giaccia 2006). However, the clinical use of Amifostine is limited due to the severe side effects such as nausea, vomiting, hypotension, nephron- and neuro-toxicity (Kamran et al. 2016). Therefore, the studies on new naturally occurring compounds from plants or fruits have been fulfilled for the development of safe radioprotector which is less toxic and more effective. Ng is member of the flavonoid family, and it is found from citrus fruits and tomatoes (Erlund 2004). These compounds have health benefits expected by free radical scavenging effect, antioxidant activity and anti-inflammatory mechanisms (Cavia Saiz et al. 2010). They protect cells against the damaging effect of ROS and free radicals by the hydrogen/electron transfer at the position of 4-hydroxyl group in the B ring of Ng (Benkovic et al. 2009, Di Meo et al. 2013, Kumar & Tiku 2016). (Fig. 1)

This study was accomplished to evaluate the radioprotective efficacy of Ng against low LET radiation by using biochemical methodology and histological examination. We focused on the damages on critical organs of rat exposed to gamma-ray.

Materials and Method

1. Reagent

Naringenin (95% purity), β-Mercaptoethanol (β-ME), Tris base, Tween X-100, Phenylmethylsulfonyl-fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

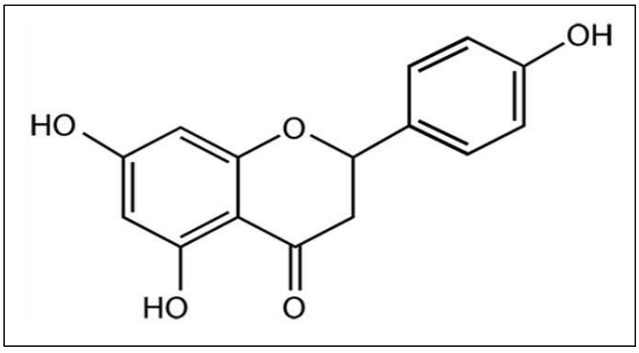

Fig. 1. Chemical structure of naringenin.

2. Animals

Eleven-week-old male Sprague Dawley (SD) rats were obtained from the experimental animal center of Konyang University (Daejeon, South Korea). All of 15 rats used in the present study were housed in an air conditioned room (23 ℃) with humidity control (50 ~ 60 %) under 12 hr light/12 hr dark cycles with free access to food and tap water. All experimental processer were confirmed by the Institutional Animal Care and Use Committee (IACUC; approved number: P-16-20-A-01) of the Konyang University (Daejeon, South Korea).

3. Irradiation

Prior to the irradiation, rats were anesthetized with intraperitoneal injection of ketamine (40 mg/kg) and xylazine (5 mg/kg); both purchased from Daihan Pharmaceutical Co., Ltd., Seoul, Korea. The absorbed dose of every rat by the whole-body gamma-irradiation was 5 Gy at a dose rate of 30 Gy/hr. Radiation exposure was carried out using a Gamma cell 40 Exactor (MDS Nordion, Ottawa, Canada) at Advanced Radiation Technology Institute (ARTI), Korea Atomic Energy Research Institute (KAERI).

4. Experimental design

A total of fifteen SD rats were randomly divided into three groups with five animals each.

1) Control group (CG): rats were treated with saline orally for 7 consecutive days.
2) Exposed group without pretreatment (EG): rats were treated with saline orally for 7 consecutive days, 2 hr after the last administration, and exposed to 5 Gy of irradiation
3) Exposed group with naringenin pretreatment (EG-Ng): rats were treated with 50 mg/kg Ng orally for 7 consecutive days, 2 hr after the last administration, and exposed to 5 Gy of irradiation

Before oral administration, body weight of rats was measured each day, and sacrificed 24 hours after the exposure. The critical organs (spleen, lung, stomach, and colon) were extracted from the rat after perfusion.

5. Determination of lipid peroxidation

Lipid peroxidation in tissues was evaluated using Thiobarbituric Acid Reactive substances (TBARS) assay kit. The malondialdehyde (MDA)-TBA adduct formed from the reaction of MDA in samples with TBA can be measured colorimetrically and TBARS levels are determined from a MDA equivalence standard. The tissues

were perfused with PBS and homogenized in phosphate buffer pH 7.4 containing 1X BHT solution to prevent further oxidation. The supernatant were transferred to a tube containing SDS lysis solution and TBA reagent and then incubated for 60 min at 95 ℃. The absorbance was measured at 532 nm using a micro plate reader.

6. Superoxide dismutase (SOD) activity assay

The tissues were perfused with PBS to remove red blood cells and homogenized in ice-cold 0.1 M Tris/HCl, pH 7.4 containing 0.5 % triton X-100, 5 mM β-ME and 01 mg/mL PMSF. The crude homogenate were centrifuged at 14,000 g for 5 min at 4℃ and the supernatants were harvested and added well. The SOD activity was evaluated using a SOD activity assay kit (BioVision Inc. Mountain View, CA, USA) in accordance with the manufacturer's instruction. The kit utilizes WST-1 that produces a water-soluble formazan dye upon reduction with superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. The inhibition activity of SOD was determined by a colorimetric method and the absorbance was read at 450 nm using a microplate reader.

7. Catalase activity assay

CAT activity was determined by using 'Catalase Activity Colorimetric assay kit' (BioVision). The tissues were homogenized in cold assay buffer and centrifuged at 10,000 g for 15 min at 4℃. The supernatant collected were reacted with H_2O_2. In the assay, the activity of CAT was measured at 570 nm using a microplate reader.

8. Evaluation of radioprotective factor (DRF)

The radioprotective factor (dose reduction factor, DRF) of radioprotective material is defined as shown below,

$$\mathrm{DRF} = \frac{\left(\frac{\text{Effect}}{\text{Dose}}\right)_{absence\ of\ radioprotector}}{\left(\frac{\text{Effect}}{\text{Dose}}\right)_{presence\ of\ radioprotector}}$$

If the DRF of a certain material is larger than 1, the material is regarded as a radioprotective.

9. Statistical analysis

All measured data were expressed as mean±S.D., Statistical analysis of the measured data was accomplished by using the one-way analysis of variance (ANOVA), and the differences were considered significant with the condition of $p<0.05$.

Results

1. Effect of naringenin on body weight of rats

Body weight of all group increased gradually in experimental period, and there was no significant difference between Ng pre-treatment groups and control groups. It was assumed by the observation that Ng was nontoxic and not affective to the body weight. The results are shown in Fig. 2.

2. Effect of naringenin on lipid peroxidation status

To examine whether treatment with Ng inhibited the oxidative stress of the tissues, *in vivo* TBARS assay was performed. Effect of treatment with Ng on lipid peroxidation statues of rats was shown in Fig. 3. MDA contents were significantly increased in organ tissues of EG compared to CG; lung: 245.0 ± 66.8 %, $p<0.001$, stomach: 470.9 ± 162.8 %, $p<0.001$, spleen: 135.9 ± 26.0 %, $p<0.05$, colon: 157.0 ± 42.9 %, $p<0.05$. However, the concentration of MDA in EG-Ng was decreased compared to EG; lung: 43.6 ± 12.1 %, $p<0.001$, stomach: 36.2 ± 10.3 %, $p<0.001$, spleen: 56.2 ± 29.2 %, $p<0.05$, colon: 48.8 ± 11.6 %, $p<0.05$.

DRF of Ng with MDA for critical organs was calculated as 2.29 for lung, 2.76 for stomach, 1.78 for spleen, and 2.05 for colon.

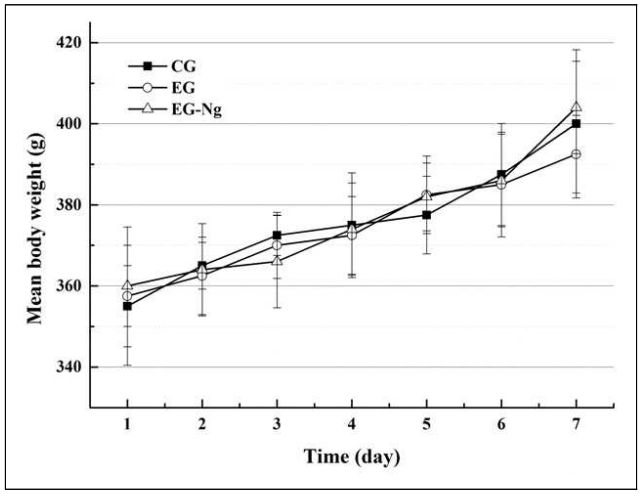

Fig. 2. Effect of naringenin on body weight of rats in experimental period. NG (50 mg/kg) was administrated orally to EG-Ng of rats, and Saline was administrated orally to CG and EG or rats per day for 7 consecutive days.

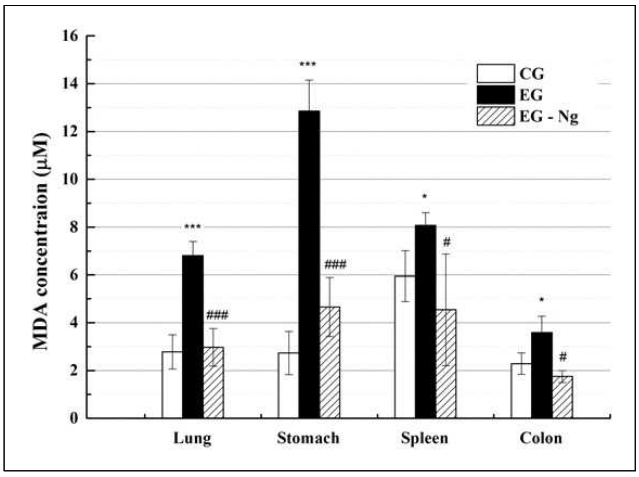

Fig. 3. Measured MDA concentration in critical organs of control group (CG), exposed groups without and with Ng pretreatment (EG and EG-Ng respectively). MDA concentration reflects the effect of naringenin on lipid peroxidation in critical organs. The data are shown as mean ± S.D. P-values represent the comparison of the CG vs. EG and EG vs. EG-Ng. $^{*}p<0.05$ and $^{***}p<0.001$ represent significant differences compared with CG. $^{\#}p<0.05$ and $^{\#\#\#}p<0.001$ represent significant differences compared with EG.

3. Effect of naringenin on the status of superoxide dismutase

It is shown in Fig. 4 that the effects of Ng administration on the activity of superoxide dismutase (SOD) in tissues. Radiation exposure decreased the activity of SOD in tissues of EG in a significant ratio compared to CG; lung: 46.7 ± 14.4 %, $p<0.001$, stomach: 56.5 ± 17.2 %, $p<0.05$, spleen: 67.9 ± 8.5 %, $p<0.05$, colon: 68.5 ± 12.9 %, $p<0.05$. In case of EG-Ng, the activity of SOD was increased compared to EG; lung: 206.7 ± 67.8 %, $p<0.01$, stomach: 189.1 ± 53.6 %, $p<0.01$, spleen: 159.9 ± 43.3 %, $p<0.5$, colon: 143.5 ± 19.5 %, $p<0.01$.

Calculated DRF of Ng with SOD activity for critical organs was 2.07 for lung, 1.89 for stomach, 1.60 for spleen, and 1.43 for colon.

4. Effect of naringenin on the activity of catalase

The effect of naringenin treatment on the regulation of CAT activity was investigated. The results were showed in Fig. 5. The activity of CAT decreased in EG compared to CG; lung: 77.9 ± 4.0 %, $p<0.01$, stomach: 70.3 ± 8.8 %, $p<0.05$, spleen: 85.9 ± 2.1 %, $p<0.001$, colon: 66.1 ± 12.0 %, $p=0.118$. However, the activity of CAT was increased compared to EG; lung: 124.3 ± 10.1 %, $p<0.05$, stomach: 130.4 ± 21.1 %, $p=0.065$, spleen: 119.8 ± 7.7 %, $p<0.05$, colon: 110.1 ± 24.4 %, $p=0.142$

Disccusion

Ionizing radiation produces a number of free radicals and ROS in the exposed tissue. Free radical and ROS in the tissue further lead to oxidative stress on essential macromolecules such as DNA, protein, and cell membrane consisted of lipid (Azzam et al. 2012, Matés & Sánchez-Jiménez 2000). On the contrary to ionizing radiation, most of the radioprotective materials protect cellular molecules by scavenging free radicals, reducing ROS, decreasing oxygen concentration, and composing disulfide on a specific protein. The power of reducing ROS could be a criterion of radioprotective material. Nevertheless, it is difficult to measure ROS concentration directly because of the extremely short half-life. Therefore indirect methods are commonly used in the measurement of ROS concentration.

The aim of the present study was to reveal the radioprotective effect of Ng against the gamma-ray induced organ damage. We measured oxidative stress markers to evaluate the radioprotective effect of Ng; MDA, SOD, and CAT induced by ROS in cells.

Our experimental results based on biochemical markers revealed that the oral pretreatment of Ng was effective in reducing oxidative stress caused by gamma irradiation. As shown in Fig. 3, MDA level is significantly increased in the critical organs of EG compared to CG. However, measured MDA concentration of EG-Ng was remarkably lower than those of EG. Statistical analysis showed that there was significant difference in measured MDA concentration between EG and CG. Also, there was significant differences between EG and EG-Ng. It could be claimed that Ng quenches ROS which is induced by radiation exposure and reduced MDA in result. MDA is highly reactive end-product of lipid peroxidation induced by oxidative degradation of polyunsaturated fatty acid. It could form multiple deleterious adducts by reaction with protein and nuclide acid bases, and give rise to mutagenic potentiality (Ayala et al 2014, Marnett 1999). In other words, the concentration of MDA could be the index of conclusive radiation damage. The diminution of MDA level could substantiate the antioxidant property of Ng as an effective radioprotective agent.

Fig. 4 and 5 showed the influences of radiation exposure and Ng administration on the level of antioxidant enzyme activity. The activity of SOD and CAT significantly decreased in tissues of EG compared with it of CG as the result of radiation exposure.

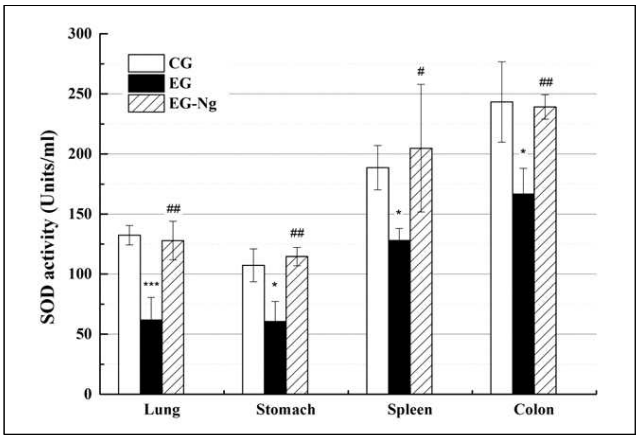

Fig. 4. Effect of naringenin on the status of SOD activity in critical organs. The data are shown as mean ± S.D., *P*-values represent the comparison of CG vs. EG and EG vs. EG-Ng. $^{*}p<0.05$ and $^{***}p<0.001$ represent significant differences compared with CG. $^{\#}p<0.05$ and $^{\#\#}p<0.001$ represent significant differences compared with EG.

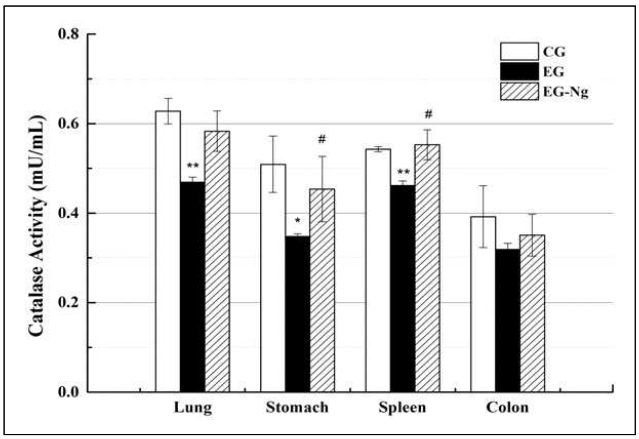

Fig. 5. Effect of naringenin on the activity of CAT. The data are shown as mean ± S.D., *P*-values represent the comparison of the CG vs. EG and EG vs. EG-Ng. $^{*}p<0.05$ and $^{**}p<0.01$ represent significant differences compared with CG. $^{\#}p<0.05$ represents significant differences compared with EG.

Pretreatment with Ng increased the activity of antioxidant enzymes compared to EG. Less impact in activity of SOD and CAT on radiation exposure in EG-Ng indicated that the free radicals formed by radiation exposure were eliminated by scavenging capability of Ng. It is claimed that the free radical scavenging capability of Ng seemed to prevail over reaction of free radicals and the enzymes. These experimental measurements proved that Ng could be used as a radioprotective agent mitigating radiation induced oxidative stress and damage in tissues. Oxidative stress caused by the imbalance between excessive formation of ROS and limited anti-oxidative defense is related to many pathologies including age-related disorders, cancer, cardiovascular, inflammatory, and neurodegenerative disease (Krumova & Cosa 2016). SOD, the first line of defense against oxidative stress, catalyses the decomposition of toxic $O_2^{-\cdot}$, and converts it into one of two less toxic species; hydrogen peroxide (H_2O_2) and oxygen gas (O_2) (Hosseinimehr 2007). CAT subsequently detoxifies by decomposing of H_2O_2 into H_2O (Apel & Hirt 2004).

The radiosensitive organs used in the present study had high perils of occurrence disease by radiation exposure. Therefore, the protection of the organs is important to gain reducing effect of radiation induced damage at large. Consequentially, experimental results revealed that Ng could protect the critical organs from the radiation damages with its free radical scavenging activities.

Conclusion

In this study, we revealed experimentally that Ng protected organs by the successive process of scavenging radiation induced free radicals and mitigating radiation induced oxidative stress. For the evaluation of the radioprotective efficacy of Ng, cytotoxic byproduct (MDA) and anti-oxidant enzymes (SOD, CAT) were measured. The calculated DRF using measured MDA and SOD at the critical organs were larger than 1, and there were significances in measured values of MDA and SOD between EG and EG-Ng. The measured data of antioxidant enzymes SOD and CAT indicated that Ng is prevailing to those enzymes in scavenging free radicals and ROS, and consequently Ng lessens the consumption of the antioxidant enzymes such as SOD and CAT. In conclusion, Ng was claimed to be a promising candidate as radioprotective agent without side effects.

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