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ORIGINAL ARTICLE

EFFECTS OF GENISTEIN SUPPLEMENTED BEFORE OR AFTER IRRADIATION ON DNA INJURY IN HUMAN LYMPHOCYTES *IN VITRO*

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ABSTRACT

Background. Ionizing radiation (IR) carry adequate energy to ionize or remove electrons from an atom. Particles interact with water to produce reactive oxygen species (ROS). Genistein (GEN) is a naturally occurring phytoestrogen and the basic isoflavonoid in soybeans and soybean-enriched products and is believed to have the strongest antioxidant activity.

Objective. The study aimed at the investigation if application of GEN at different time prior or past irradiation may ameliorate or reduce injury of DNA in human lymphocytes.

Material and Methods. The isolated lymphocytes were exposed to X-irradiation (0.5; 1 Gy). GEN (1 μM/ml; 10 μM/ml) was appended to attempts at various times prior or past irradiation (1 h prior, immediately prior, immediately past, 1 h past). We joined each X-rays dose with each GEN dose. After 1h of incubation DNA damages were examined using Comet assay.

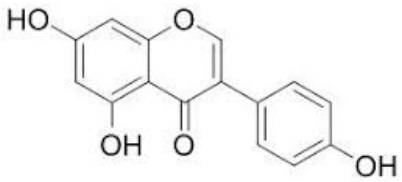
Results. Combination of 1 μM/ml of GEN given 1 h before irradiation with low or high dose markedly decreased induced by irradiation DNA injury. Higher dose of GEN applied immediately before or after irradiation markedly extended the frequency of DNA injury generated by irradiation. The result of application 1 μM/ml GEN 1 h after irradiation was not significantly different compared to control. The effect of 1 Gy + 10 μM/ml GEN was not significantly lower compared to each agent alone.

Conclusions. Only a very low concentration of GEN applied before irradiation, may be considered as a potential radiomitigator/radioprotector. High doses of GEN work as a radiosentitizer and may potent the effects of radiotherapy.

Key words: *radioprotection, radiomitigation, DNA damage, genistein, irradiation*

INTRODUCTION

Genistein (4',5.7-trihydroxyisoflavone, GEN) is a naturally occurring phytoestrogen and the basic isoflavonoid in soybeans and soybean-enriched products [26]. As the most rich in genistein is indicated psoralea (*Psoralea corylifolia*). Miscellaneous legumes like soybean (*Glycine max* L.), green bean (*Phaseolus vulgaris* L.), alfalfa sprout (*Medicago sativa* L.), mung bean sprout (*Vigna radiata* L.), cowpea (*Vigna unguiculata* L.), kudzu root (*Pueraria lobata* L.), red clover blossom and red clover sprout (*Trifolium pratense* L.) are also the source of genistein. They have been investigated for their estrogenic activity [6]. Since GEN is structurally similar to 17β-estradiol, it can compete with this estrogen and bind to estrogen receptors. GEN shows a much higher relationship toward the estrogen β (ER-beta) than toward estrogen α (ER-alfa) receptor [22]. At physiological concentrations, GEN activates both nuclear estrogen α and β receptors ER and influences TGF-beta signaling pathways [8, 18, 21, 22].



The chemical structure of genistein

Isoflavones, which belong to the family of naturally occurring isoflavonoids, may protect oxidative damage by direct influence on free radicals or antioxidant scavenger enzymes. Among them GEN is believed to have the strongest antioxidant activity [19]. GEN is a component of the human diet, especially Asian soy-based foods, infant formulas, and dietary supplements. It is believed to be an anticancer, antiproliferative,

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cardioprotective, and/or chemopreventive agent due to its ability to act as an inhibitor of tyrosine kinase, histidine kinase, and topoisomerase [1, 4]. Moreover, GEN showed beneficial effects on hypercholesterolemia and osteoporosis [46].

Ionizing radiation (IR) is described as electromagnetic waves and particles that carry adequate energy to ionize or remove electrons from an atom. Two principal mechanisms of IR interactions with tissues are known: direct and indirect. In the direct action, radiation simply disrupts the molecular structure. Indirect action occurs when noncharged particles interact with water to produce reactive oxygen species (ROS) [3, 34]. Ionization generated by radiation induces several chemical reactions, which are the reason of aberrations in atoms and molecules. Above changes may lead to cell damage, induction of mutation, carcinogenesis, or lethality.

Two kinds of electromagnetic waves can ionize atoms, X-rays and γ-rays. Among the damages induced by them, 60% is caused by indirect action. The total effect of IR depends on the physical attributes of radiation type, dose, and whether the exposure is acute, fractionated, or chronic. Biological responses to irradiation are addicted to the age, the kind of tissue, genetic background, and physiological status of the exposed individual [3, 10, 47].

IR induced cellular injuries are caused mainly by free radicals, so molecules with direct free radical scavenging features are likely to act as so-called radiation modifiers/protectors, factors which apply prior to or shortly after irradiation modify the response of tissues to radiation. Similarly, agents which may be used to reduce toxicity even applied after radiation are usually called mitigators [9]. During the last years, numerous compounds, including these coming from plants, have been discovered as radioprotective agents. Due to their antioxidant properties, they may serve as radioprotective agents to protect from irradiation damage [23]. Agents acting as radioprotectors/radiomitigators should be nontoxic, cheap, and easy to use. Such factors might be very helpful in health prevention, mainly when used after irradiation when damages already exist.

The aim of the study was the investigation if application of genistein at different times before or after irradiation may prevent or reduce radiation-induced injury of DNA in human white blood cells.

MATERIAL AND METHODS

Insulation of lymphocytes

The lymphocytes were isolated from samples of human peripheral blood. For this aim, blood was aseptically collected in heparinized sterile tubes from a nonsmoking, healthy individual (female, 35 years) according to the procedure of *Anderson* et al. [2]. Whole blood was then mixed 1:1 with phosphate buffered saline (PBS). From this mixture was taken 5 ml and cautiously placed on top of 2.5 ml of lympho separation medium (MP Biomedicals) and centrifuged at 918 × g for 20 min at room temperature. After isolating the lymphocyte layer, they were mixed with 10 ml PBS and centrifuged at 450 x g rpm for 10 min. The supernatant was then removed. The precipitated cell pellet was transferred to Eppendorf tubes (50 µl of cell suspension for each tube).

Preparation of genistein

Genistein (GEN) was prepared as follows: 2.7 mg of GEN (purity ≥ 98 %, M = 270, 24 g/mol; ROTH GmbH, Germany; item number: 0716.1) was dissolved in 1 ml of ethanol (EtOH). From this solution, two different concentrations (1 and 10 µM/ml) of GEN were taken and added to the cells in Eppendorf tubes. The choice of doses for this study was based on a previous study [2]. Then the RPMI 1640 medium was added to the lymphocytes in such an amount that each Eppendorf tube contained 1 ml of solution. As a control, 0.1% concentration of EtOH in the test tubes was used, which corresponds to the maximum concentration of alcohol in the test samples. In a similar way were made up the controls for each dose of GEN and for the indication of vitality analysis of lymphocytes with trypan blue.

Treatment of the cells

The secluded lymphocytes (past indication of their vitality) were X-irradiated at doses of 0.5 and 1 Gy. Control cells were not irradiated. A therapeutic Roentgen unit, Medicor type THX-250, served as the X-rays origin. It was served with the succeeding factors: 155 kV, 18 mA, additional filtration, 0.25 mm Cu, and HVL 2 mm Al. Lymphocytes were irradiated at the dose rate of 0.2 Gy/min. GEN dissolved in EtOH at diverse doses, was inserted to test attempts at various times prior or past irradiation (1 h prior, just prior, just past, and 1 h past). The time points were selected based on scientific literature and our own earlier investigation. We have introduced a scheme to join every X-ray dose (0.5 and 1 Gy) with every GEN dose (1 µM/ml and 10 µM/ml). Afterwards, the cells were kept for 1h in a water bath at 37°C. Simultaneously, control cells (negative control), cells subjected single to GEN and single to X-rays. Three irrespective (n = 3) experiences were executed. The blood from the donor was donated at 3 various days in one month. The frequency of DNA injury was assessed by alkaline comet assay.

Comet assay

To assess the effect of GEN on X-ray induced DNA damage in lymphocytes, single cell gel electrophoresis (comet assay) was used according to the procedures of *Singh* et al. [38] and *Anderson* et al. [2].

At first, every lymphocyte specimen was swirled at 1778 x g for 3 min. The supernatant was then eliminated and 75 µl of 0.5% low melting point agarose (LMPA) at 37 ° C was inserted into the pellet remaining in the Eppendorf tube. The resulting solutions were mixed and mounted on microscope slides that had earlier been coated with 1% normal melting point agarose (NMPA).The slides were then coated with cover slips and stored in fridge (4 °C) to concentrate the agarose. Coverslips were removed after concentration and the next layer of LMPA was inserted and the slides were again coated with coverslips and permitted to solidify again at 4 ° C. Post removing the coverslips, the slides were submerged in a lysis solution (2.5 M sodium chloride – NaCl, 100 mM ethylenediaminetetraacetic acid – EDTA, 10 mM Tris, 1% sodium lauryl sarcosinate, pH 10, plus 1% Triton-X and 10% dimethyl sulfoxide - DMSO) until morning at 4 °C. The slides were then kept in the electrophoresis solution (10 N NaOH, 200 mM EDTA – pH 10 in distilled water at 4 °C) for 20 min to permit DNA to develop. Alkaline electrophoresis was carried out for 20 min at 4 °C, 0.6 V/cm, and 300 mA. The level of the electrophoresis buffer was around 0.25 cm above the slides. Past alkaline electrophoresis, the slides were neutralized and dyed with ethidium bromide (EtBr).

The DNA damages in lymphocytes were examined using a fluorescence microscope. To this aim, images of 100 randomly selected lymphocytes from each sample were recorded and analyzed with the CASP image analysis software [20]. As the parameters for analysis Tail Moment and Percentage of DNA in Comet Tail (Tail DNA %) were chosen.

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine any significant differences between the results from different groups. The *Fisher's* test was used as a *post hoc* test. The values $p<0.05$ were considered statistically significant.

RESULTS

DNA injury in human lymphocytes treated with genistein 1 h before irradiation is shown in Figure 1. The cell viability varied between 62 in the group of 1 Gy + 10 µM/ml GEN and 92 % in controls. The Tail Moment and % Tail DNA for low dose of GEN and both doses of irradiation as well as for solvent control were not markedly varied like to negative control, however, the impacts after irradiation to 1 Gy were several times higher than control values. Combination of 1 µM/ml of GEN with low as well as high doses of irradiation markedly but not significantly decreased induced by irradiation DNA injury. The dose of 10 µM/ml GEN significantly enhanced the DNA injury in human lymphocytes compared to controls. Similarly, application of 10 µM/ml GEN 1 h before irradiation enhanced the DNA damages compared to those noted after irradiation with 0.5 or 1 Gy.

DNA injury in human lymphocytes treated with genistein immediately before irradiation is presented in Figure 2. The cell viability ranged from 62 in 0.5 Gy + 10 µM/ml GEN group to 92 % in the control. Results of solvent control and low dose of GEN were

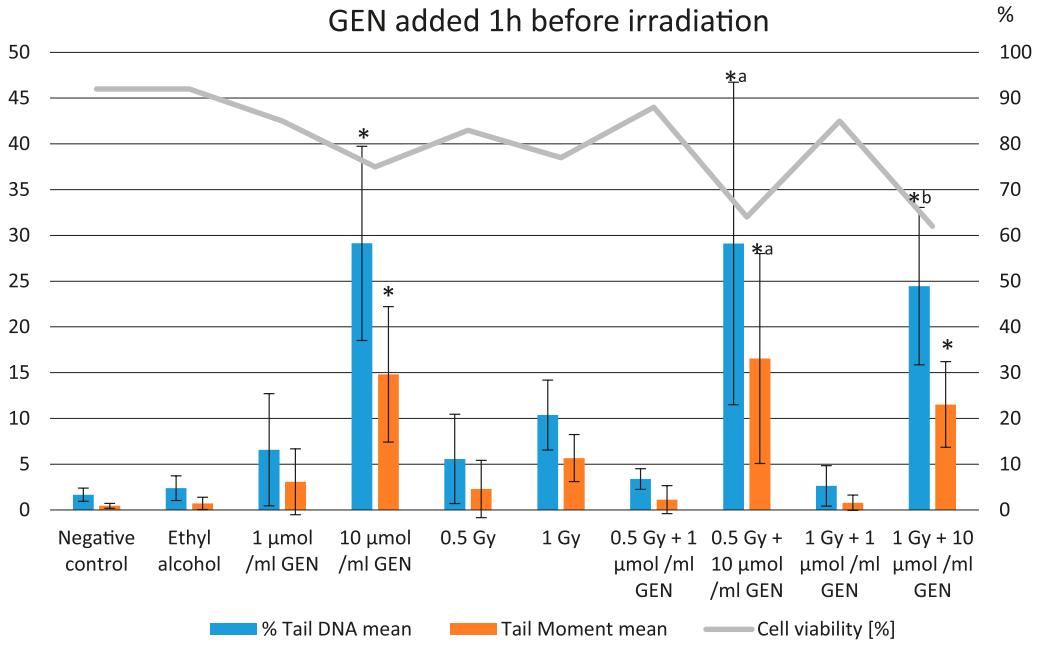

Figure 1. The effect of genistein supplementation 1 h before irradiation on the DNA damage of human lymphocytes

not significantly different compared to the negative control. Results of irradiation alone, especially at high dose were markedly higher than the negative controls but not markedly varied. Higher dose of genistein significantly enhanced the frequency of DNA injury acting alone as well as in combination with irradiation. The results after exposure to 0.5 Gy + 10 µM/ml GEN were significantly higher compared to 0.5 Gy or 1 Gy alone, whereas 1 Gy +10 µM/ml GEN in comparison to 1 Gy alone only. Treatment with low dose of GEN and each dose of irradiation induced a similar levels of DNA injury that a single exposure to irradiation or GEN.

DNA damage in human lymphocytes treated with genistein immediately after irradiation is shown in Figure 3. The cell viability ranged from 70 in the group 1 Gy + 10 µM/ml GEN to 92 % in the control group. Results of solvent control and low dose of GEN were not significantly differed from the negative control. The DNA damage (% Tail DNA and Tail Moment) after irradiation alone, especially at high dose were markedly higher than in negative control but not markedly varied. Genistein at the dose of 10 µM/ml acting alone as well as in a combination with irradiation significantly enhanced the level of DNA injury. The results after exposure to 0.5 Gy + 1 µM/ml GEN and to 1 Gy +1 µM/ml were slightly higher but not markedly varied as compared to negative control, 0.5 Gy or 1 Gy alone.

DNA injury in human lymphocytes treated with genistein 1h after irradiation is presented in Figure 4. The cell viability varied between 68 in 0.5 Gy + 10 µM/ml GEN group and 92 % in the control group. Results of solvent control and low dose of GEN were not significantly different like to the negative control. DNA damage after a higher dose of irradiation was almost twice higher compared to that after the lower dose, but both were not markedly varied compared to control. Higher dose of genistein significantly enhanced the frequency of DNA injury. The results of application of GEN alone 1 h after irradiation 0.5 Gy + 1 µM/ml GEN and 1 Gy + 1 µM/ml GEN were similar and not markedly varied compared to control. Treatment with high dose of GEN and low dose of irradiation enhanced the level of DNA injury. The effect of 1 Gy + 10 µM/ml GEN was not significantly lower compared to each agent acting alone.

DISCUSSION

People may be exposed to ionizing radiation constantly due to their occupation or accidentally due to the breakdown of nuclear power plants or terroristic attacks, which may cause a temporally enhanced levels of radiation leading to harmful health effects. A number of jobs may be connected with an enhanced levels of exposure to man-made sources of radiation by employees. There are, for example, medical personnel, uranium miners, nuclear plant workers, and other employees using radiation for industrial and scientific purposes [45]. Moreover, patients ongoing radiotherapy may be exposed to an overdose of radiation.

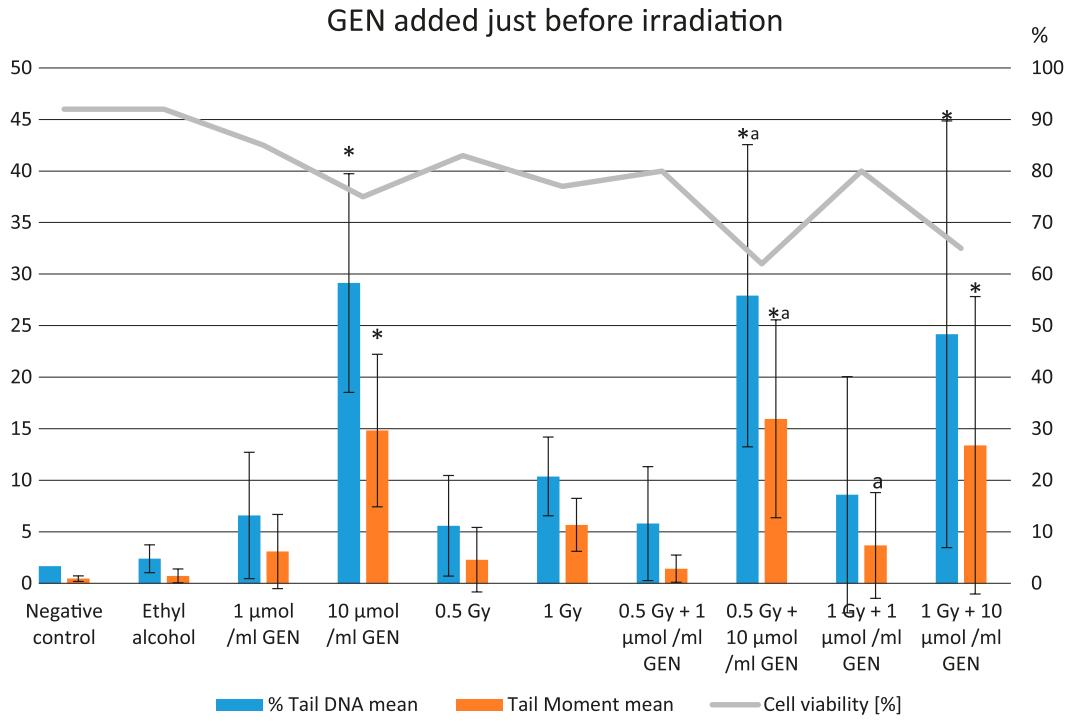

Figure 2. The effect of genistein supplementation immediately before irradiation on the DNA damage of human lymphocytes

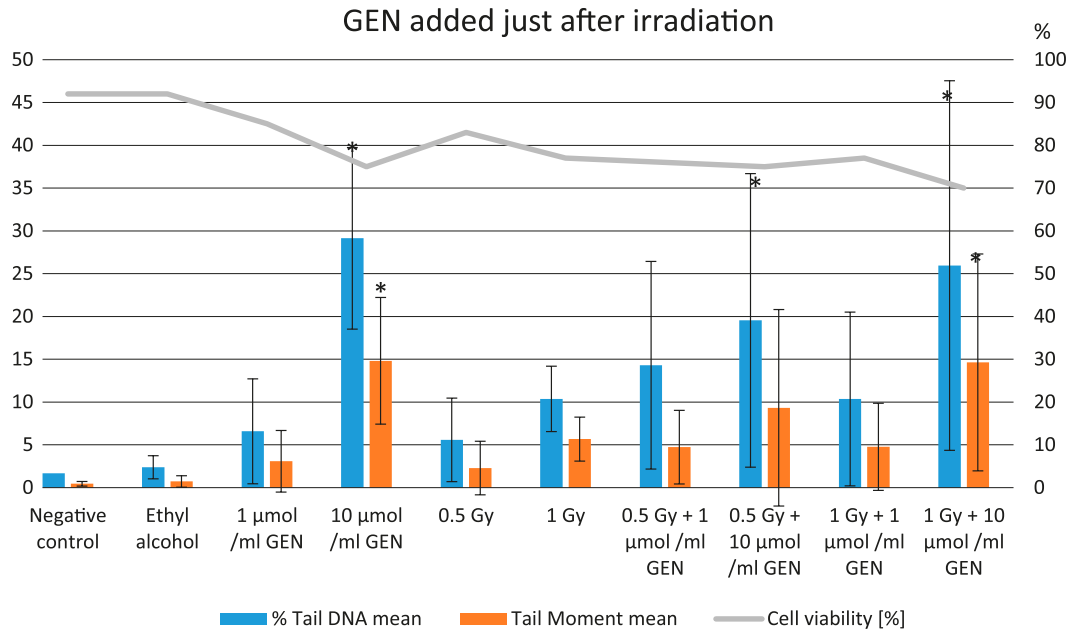

Figure 3. The effect of genistein supplementation immediately after irradiation on the DNA damage of human lymphocytes

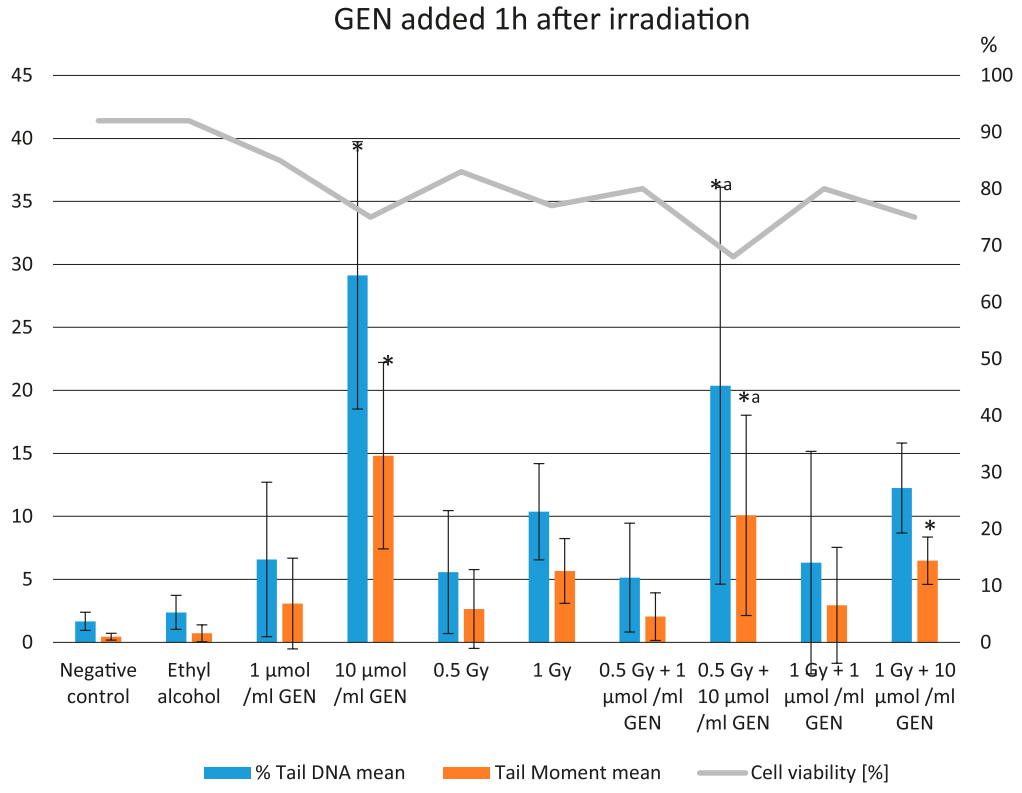

Figure 4. The effect of genistein supplementation 1 h after irradiation on the DNA damage of human lymphocytes

Ionizing radiation may leads to damage of living tissue, and later to induction of mutations, cancer, or cell death. Potentially dangerous cells are eliminated from the organism by lethal mutations. The impact of miss-repaired damage may be chromosomal damage or mutations. The above modifications may be transmitted to further generations of cells and may eventually lead to cancer [13, 40, 41].

Increased production of reactive oxygen (ROS) and nitrogen (RNS) species may be a reason of oxidative stress. ROS might oxidize cellular biomolecules, like carbohydrates, proteins, lipids, and DNA. Approximately 60 % of the damage induced by ionizing radiation is caused by reactive oxygen species (ROS). The widespread ROS are superoxide anion (O2−), hydrogen peroxide (H_2O_2), and hydroxyl

radicals (OH−) [25]. ROS may also have the beneficial effect on the use of normal circular metabolism at low or moderate concentration when regulating the physiological function of cells [14, 44]. At higher concentrations, radicals become toxic and disrupt the antioxidant defense system leading to oxidative stress [32]. Major ROS mediated reactions include lipid peroxidation, removal of thiol groups from cellular and membrane proteins, strand break and base alterations leading to DNA damage [37]. Oxidative modification of important functional groups in essential membrane proteins (ion channels) leads to changes in purine and pyrimidine bases, single- and double-strand breaks, removal of bases, and cross-linking of DNA with DNA or adjacent proteins [42, 48].

Irradiated cells that avoid death may undergo mutations, which induce defects in the DNA scheme leading to altered gene expression and protein modifications, like peptide bond cleavage and cross-linking. It may influence protein localization, interactions and change enzyme activity [23]. Double-strand breaks (DSBs) are more difficult to repair than single-strand breaks and cause frequently mutagenesis or cell death [29]. Radiation also generates point mutations and deletions [28]. ROS-mediated DNA injury can finally evolve into cancer, particularly if the regulation of tumor suppressor genes is impaired [49].

In the present study, the effect of lower dose, i.e. 1 μM/ml GEN was not significantly different compared to negative and solvent control. The higher dose 10 μM/ml GEN induced significant damage to DNA of human lymphocytes. In the previous study, both doses of GEN did not induce DNA injury in human lymphocytes, however, the higher dose induced damage to DNA of human sperm [2]. In a hamster lung cell line (V79), GEN was found to induce micronuclei at concentrations ranging from 5 to 25 μM, which declined at higher doses, whereas the comet assay showed the induction of DNA damage only at high concentrations [12].

The connection between genetic modifications by isofavones and oxidative damage was described by *Cantanhêde* et al. [7] The study showed that soy isoflavones are nontoxic and efficient in reducing genomic lesions and the frequency of micronuclei induced by meglumine antimoniate. *Sarkar* and *Li* [36] showed a protective role of GEN against the genotoxic effects of cancer-induced agents. According to other papers, GEN has strong antioxidant activity against radiation-induced oxidative stress through upregulating endogenous glutathione levels and glutathione peroxidase activity [15]. Due to the recognized antioxidant capacity, GEN is a deliberated as an important agent in cancer prevention [27, 31, 33, 35].

GEN works by enhancing the expression of genes that engages in the detoxification of ROS, such as superoxide dismutase, glutathione peroxidase, and catalase [11]. Similarly, there was noted that GEN was able to induce a significant increase of expression of the gene *GPx-1* in human prostate cancer cell lines (LNCaP and PC-3), which help in antioxidant defense and inhibition of proliferation of cancer cells [43].

GEN administration prior to irradiation protects mice against liver injury [46]. An alternative likely mechanism to clarify the antimutagenic impact of genistein is connected with its ability to stimulate the expression of genes involved in DNA repair, such as *BCRA1* and *ATM* and *p53* [5, 50]. *Song* et al. [39] stated that GEN was capable to increase expression of genes involved in DNA repair, such as *hHR23A*, *HUS1*, *RAD1*, and *RAD9* to prevent and repair radiation-induced DNA damage in HL-7702 cells. The study of *Hillman* [16] showed that soy isoflavones radiosensitized cancer cells. The mechanisms of radiosensitization, studied both in vitro and in vivo in showed that isoflavones targeted signaling survival pathways upregulated by radiation, including DNA repair and transcription factors, causing death of cancer cells. On the contrary, radioprotection of normal tissues and organs was mediated by SIF supplementation to radiation and was noted in the renal cell carcinoma model and lung preclinical models, and in a prostate cancer clinical trial.

The glucosides GEN administered singly 2 h before irradiation with 60 mJ/cm^2 of UVB, did not significantly prevent UVB-induced DNA injury [17]. The outcomes of the current study showed that the dose of 10 μM/ml of GEN applied both prior as well as after irradiation enhanced damage to DNA of human peripheral lymphocytes induced by irradiation alone. In contrast, the low dose of GEN not significantly decreased the level of induced by irradiation DNA injury when applied before irradiation, especially 1 h prior to exposure.

GEN was effective against hematopoietic acute radiation syndrome when administered 48 to 12 h before irradiation with the maximal effect when applied 24 h prior radiation exposure [24]. Similarly to our current results, the study of *Song* et al. [39] showed that low concentration of GEN (1.5 μM) protected L-02 cells against radiation damage via inhibition of apoptosis, alleviation of DNA damage and chromosome aberration, down-regulation of GRP78 and up-regulation of HERP, HUS1 and hHR23A, whereas high concentration of GEN (20 μM) induced radiosensitization through the promotion of apoptosis and chromosome aberration, impairment of DNA repair, up-regulation of GRP78, and down-regulation of HUS1, SIRT1, RAD17, RAD51 and RNF8. Soy phytoestrogen was not mutagenic and reduced cyclophosphamide-induced DNA damage. The results

from the comet assay revealed a reduction of DNA damage; however, phytoestrogen did induce genotoxic damage during the 24-h treatment [30].

CONCLUSIONS

Taking together the above previous and the current study, there is confirmed that GEN may under special conditions act both as a radioprotector and radiosentitizer. Only very low concentration GEN applied before irradiation, which may be considered as a potential candidate for radiomitigator/radioprotector. Contrary, high doses of GEN work as a radiosentitizer and may be useful to the potent effects of radiotherapy. Further investigations are necessary to clarify the above findings.

Conflict of interest

The authors declared that they had no conflicts of interest.

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