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Anti-Apoptosis Effects of Green Tea in Diagnostic and Therapeutic Doses of Ionizing Radiation

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ABSTRACT

Introduction: Patients who receive ionizing radiation for diagnosis or treatment may suffer side effects in normal tissues. Radioprotective agents have the potential to decrease DNA damage and free radicals produced by ionizing radiation. Green tea is a natural product from the Camellia sinensis plant and have been shown to have antioxidant and radioprotective properties. The aim of the present study is assessing the protective effect of green tea on lymphocytes affected by radiation injury.

Material and Methods: Blood samples was obtained from four adults healthy human and lymphocytes were extracted. Afterward, lymphocytes were exposed to X-radiation by OPG, CBCT, CT and radiotherapy instruments. The protective effects of the green tea polyphenol on Bcl-2 and Bax genes expression levels were evaluated in cultured lymphocytes obtained from treated cells (24 h and 1 h before and 1 h after exposure to ionizing radiation) and non-treated cells (Receiving X-radiation and non-receiving green tea).

Results: The results of this investigation demonstrated Bax/Bcl-2 ratio, as an index of the radiation sensitivity, was significantly decreased in groups which had been received dose of 2 Gy in treated cells groups of 1 h before and 1 h after X-radiation ($p<0.05$).

Conclusion: The results indicated different effects of green tea on low and high doses of X-radiation. Most protective effects of green tea as an apoptosis inhibitor were observed in high doses of ionizing radiation. Consequently, green tea may be useful as an adjunct to radiotherapy and medical imaging to diminish apoptosis and side effects in normal tissues.

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Introduction

Ionizing radiation, specifically X-radiation has a critical role both in diagnosis and therapy nowadays. Radiotherapy is one of the most important therapeutic methods in most malignancies so that it is used in different cancers treatment in addition to surgery [1]. Ionizing radiation has different effects such as DNA damages, chemical changes in DNA structure and genes expression level changes [2-4]. When mammalian cells are exposed to various stresses, complicated molecular, responses were shown that most of them induced by gene expression level changes [5]. So principal cell harms after irradiating by ionizing radiation can be attributed to the DNA damages [6]. Cell's response to DNA damage includes cell cycle arrest [7], repair of DNA [8] and apoptosis [9]. Double Strand Breaks (DSBs) induced by ionizing radiation lead to activation of DNA damage sensors and cause cell cycle arrest, thereby the cell is avoided the transformation of abnormal DNA structures into inheritable mutations, and minimize irreparable cell survival occur [10]. Apoptosis involves the B-cell lymphoma [11] family and it's intervened through intrinsic and extrinsic pathways. Bcl-2 family proteins are the most critical elements of apoptotic pathway [12]. Pro-apoptotic members of Bcl-2 family proteins, such as Bcl-2 associated X protein (Bax) induce mitochondrial outer membrane permeabilization to cause cytochrome-c release, while anti-apoptotic members like Bcl-2 act as guardians of the outer membrane and avert its integrity by conflicting Bax function [13].

Recent researches show some of the herbs and foods contain antioxidants that responsible for protectors against Ionizing radiation induced dangers such as x-ray and thereby reduce risk of radiation induced cancers [14]. Green tea with the scientific name of Camellia sinesis (C.sinesis) is native to eastern Asia such as China and Japon. This plant

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contains compounds such as polyphenols, Tinin 32, Caffein and carotenoids [15, 16]. C.sinesis contains four principle polyphenols: epigallocatechin, epigallocatechin 3 gallate (EGCG), epicatechin 3 gallate and epicatechin (EC) which EGCG has most therapeutic effect against radiation carcinogenic action [17] and it has a synergic effect with anticancer drugs [18]. Various therapeutic effects were reported for C.sinesis including effect on atherosclerosis such as reduction of lipidic oxidation and aortic ulcers and anti- atherosclerosis effects [19]. The gene expression of Bax in human blood lymphocytes after receiving gamma-ray low doses was significantly reduced, while Bcl-2 gene expression and Bcl-2 / Bax ratio were increased [20].

In this study, the protective effects of C.sinesis polyphenol against X-ray induced damage was examined on cultured lymphocytes by Bax and Bcl-2 genes expression after radiation.

Materials and Methods

Plant and extract

For preparation of polyphenol product, 3.75 mg of green tea powder was added to 1.5 mg distilled water. The mixture was then passed through syring filter, the solvent was removed under reduced pressure and the extract was kept in refrigerator. The yield extract was 50 µg/ml and the nontoxic chosen concentrate and dose were based on the previous studies [21, 22].

Preparation of lymphocytes culture and groups

Lymphocytes were extracted from four adult human peripheral blood (25-35 years old) and suspended in complete RPMI 1640 with 10% FBS at a density of 5.10^6/ml. Lymphocytes isolation was carried out using gradient Ficoll. The study was carried out in the following groups:

(A) Lymphocytes treated with polyphenol 1 h before radiation (1 hBR).

(B) Non radiated Lymphocytes treated with polyphenol at the same time of A group.

(C) Lymphocytes treated with polyphenol 1 h after radiation (1 hAR).

(D) Non radiated Lymphocytes treated with polyphenol at the same time of C group.

(E) Radiated Lymphocytes non-treated with treatment by polyphenol (WT).

(F) Non radiated and non-treated Lymphocytes (W R).

In all of these groups 4 h after radiation Lymphocytes were separated and 1 ml Tripure was added to each microtube. In this study irradiation were carried out to radiation groups

20 25 ml-flasks were used and were divided to five groups: four flasks for CBCT doses, four flasks for CT doses, four flasks for OPG doses, four flasks for radiotherapy doses and four flasks were cultured at the same condition but were not exposed to radiation.

Irradiation

So as to the irradiation dose measurements in OPG, CBCT, CT, and radiotherapy, 20-40 pieces of TLDs (for each measurement) were used. As for OPG, irradiation was done by a panoramic SAMSUNG device with kVp=74 kV and I=110 mAs. Also, for CBCT these factors were 84 kV and 156 mAs respectively in a Planmeca Promax. For CT, a 16-Slice Siemens within kVp=110 kV and I=350 mAs was utilized and finally we used a 6 MV Primus linear accelerator in order to radiotherapy dose delivery. Also, in all techniques the flasks were localized in the middle of irradiation fields.

RNA extraction

Using Tripure isolation reagents (Roche Applied Science, Germany), total RNA was isolated from Lymphocytes as described by the manufacturer's instructions. Samples were incubated for 10 min at room temperature. Then, 200 µl of chloroform solution was added to the microtubes and the mixture was vortexed for 15 s. Then, the mixture was incubated for 15 min at 4°C in dim light samples were centrifuged at 12000 rpm at 4°C for 15 min. A clear supernatant was carefully removed and transferred to other microtubes. Afterwards, 500 µl of cold isopropanol was added and after incubation in room temperature for 15 min, samples were centrifuged for 10 min at 4°C in dim light and centrifuged at 12000 rpm at 4°C for 10 min. At the end of this stage, sediment RNA was visible as a tiny white pellet. The supernatant was removed and washed, the RNA precipitated by 96% Ethanol (Merck, Darmstadt, Germany), and then supernatant was carefully and completely emptied and the remaining ethanol in the microtubes was removed by air flow. DEPC water was added to microtubes containing RNA, and for the duration of 10 min at 56°C was placed on a dry-block device; eventually moved to -20°C and held until cDNA synthesis.

Synthesis of cDNA

Due to the low half-life of extracted mRNA, it must be transcribed to cDNA, which was performed by the enzyme reverse transcriptase. This DNA sequence was then used as a template in PCR and second strand DNA synthesis by gene-specific primers.

A fixed volume of input RNA (1 µL) was used for each cDNA reaction. Reverse transcription reaction was carried out with Revert Aid™H Minus M-MulV First Strand according to manufacturer's instructions (RevertAidTM First Strand cDNA Synthesis Kit, Fermentas). Also, all samples were amplified by control polymerase chain reaction (PCR) using GAPDH according to the manufacturer protocol (Prime Taq DNA polymerase, Genet Bio, South Korea).

Gene expression analyses by real time PCR

All of real-time RT-PCR reactions were done doubly in MicroAmp™ Fast Optical 48-well reaction plate, and Optical Adhesive Film (Applied Biosystems) was performed. Each sample was run in a total volume of 15

µl, including 300 nM of forward and reverse primers, 1.5 µl of cDNA, 7.5 µl of SYBR® Premix Ex TaqTM and ROX_{TM} 0.3 µl of Reference Dye II (Takara, Japan) and 5.1 µl of dH_2O and all of evaluations were carried out using a StepOne (48-well) Real-Time PCR system (Applied Biosystems). Thermal cycles were applied according to the following conditions: 60 seconds at 95°C, 10 seconds at 95°C (for 40 cycles) and finally 30 seconds at 60°C. Then, the results were analyzed by Step One software v. 2.1 (Applied Biosystems) automatically. Afterward, cDNA quantification was done using the relative standard curve method in which quantity of an unidentified sample is obtained from interpolation of the standard curves. The quantity of Beta-2 Microglobulin (β2M) as the housekeeping gene or "stably expressed" gene was performed for quantity normalization of the target genes. Finally, Relative Quantity (RQ) was calculated for each sample by dividing the normalized quantity of the treated samples by the normalized quantity of the control sample. Primer sequences (Metabion-Martinsried, Germany) are shown in the table 1:

Table 1- β2M, Bcl-2 and BAX forward primers sequences

β2M forward	5'-GTA TGC CTG CCG TGT GAA C-3', reverse 5'-AAC CTC CAT GAT GCT GCT TAC-3'
Bcl-2 forward	5'-TAC TTA AAA AAT ACA ACA TCA CAG-3', reverse 5'-GGA ACA CTT GAT TCT GGT G-3'
BAX forward	5'-GCT TCA GGG TTT CAT CCA G-3', reverse 5'-GGC GGC AAT CAT CCT CTG-3'

Statistical analysis

The statistical analysis was carried out using non parametric tests for comparison of dependent groups. The results were considered statistically significant if P-value<0.05 as *, P-value <0.01 as ** and P-value<0.001 as ***. GraphPad Prism software, version 9.3.1 and 2way ANOVA test was used for data analysis.

Results

Dosimetry

In order to measure the radiation dose in CT, CBCT and OPG techniques, TLD (20-40 pieces for each measurement) was used and the absorbed dose values were equal to 10.002, 6.7 and 0.1 mGy, respectively. Also, as for radiotherapy simulation a dose equal to 2 Gy was irradiated.

Gene expression changes

Relative quantitative Real Time PCR method was employed to compare gene expression levels of treated and control groups. In any plate, Bax, Bcl-2 and β2M in four groups: 2GY, OPG, CT, and CBCT were studied. Inasmuch as the dose values measured in CT, CCBT and OPG are all in the range of very low doses of ionizing radiation and also because there was no significant difference between the results of these three groups, their results combined together and was compared with another groups (2 Gy and control) as low dose group.

Effects of different radiation doses on BAX gene expression in different polyphenol doses

The results indicate that Bax gene expression in different doses in receiving tea groups significantly decrease ($p<0.05$). There is a significant difference in Bax gene expression between 1 hBR and WT (without treatment) in groups that received low doses of ionizing radiation ($p<0.05$). It means that when tea isadded to culture environment, Bax gene expression significantly lower than WT group (non-treated). In high dose groups that treated 1 hBR and 1 hAR, Bax gene expression in comparison to WT group was decreased. Whereas if polyphenol 1 h after radiation was added to culture environment, Bax gene expression is increased. This subject show that polyphenol can affect and prevent apoptosis (Figure1).

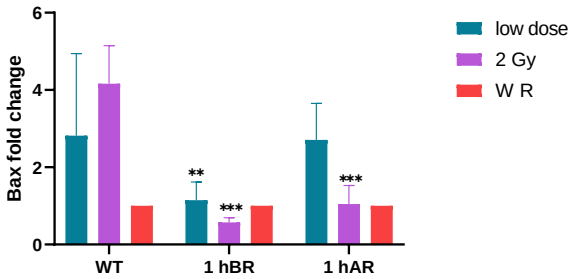

Figure 1-Bax gene expression levels (Mean with SEM) in the groups without tea (WT), received tea 1 hour before irradiation (1 hBR) and received tea 1 hour after irradiation (1 hAR). Significance of changes is indicated by * (P-value < 0.05), ** (P-value<0.001)

Effects of different radiation doses on Bcl-2 gene expression in different polyphenol doses

In this study, the effect of green tea on the Bcl-2 gene expression level following exposure was also examined. There is no significant difference in Bcl-2 gene expression in all of the groups. Nevertheless, the highest increase in Bcl-2 gene expression was observed in group that received polyphenol 1 h before radiation (1 hBR). Also, a reduction in Bcl-2 gene expression was observed in WT groups in comparison to group that received polyphenol (1 hBR and 1hAR).

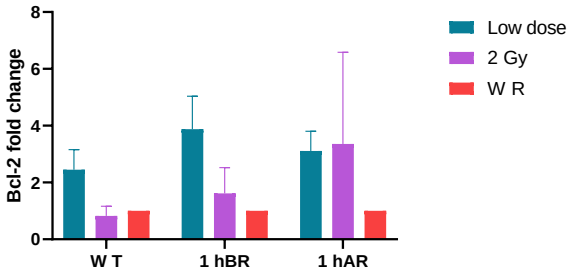

Figure 2. Bcl-2 gene expression levels (Mean with SEM) in the groups without tea (WT), received tea 1 hour before irradiation (1 hBR) and received tea 1 hour after irradiation (1 hAR). Significance of changes is indicated by * (P-value < 0.05), ** (P-value<0.001)

Effects of different radiation doses on Bax/Bcl-2 gene expression in different polyphenol doses

The Bax/Bcl-2 ratio has been introduced as a marker for radio-sensitivity [23, 24]. Particularly, the high ratio is considered as an enhancement in lymphocyte's radio-resistance. In 2 Gy radiation dose, Bax/Bcl-2 gene expression ratio was decreased in groups that received tea 1 h before radiation ($p<0.01$) and 1 h after radiation ($p<0.01$).

Also, as expected, 2 Gy irradiation without polyphenol caused to an enhancement in the Bax/Bcl-2 ratio, albeit this increase was not significant.

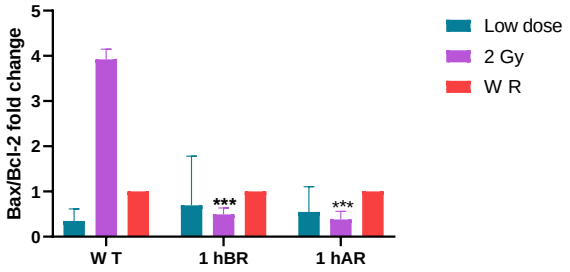

Figure 3. Bax/Bcl-2 ratio (Mean with SEM) in the groups without tea (WT), received tea 1 hour before irradiation (1 hBR) and received tea 1 hour after irradiation (1 hAR). Significance of changes is indicated by * (P-value < 0.05), ** (P-value<0.001)

Discussion

Although many plant-based products and compounds have been identified as radiation-protecting agents in animals, very few studies have examined their efficacy in human volunteers. In this study, the effects of green tea extract on human lymphocytes that exposed to ionizing radiation was studied. In the present investigation to deliberation of polyphenol consequences after irradiation and compare the effects of low and high radiation doses on cells.

Generally, the responses of mammalian cells to ionizing radiation involves three main processes: cell cycle arrest, damage repair, and apoptosis. The apoptosis induced by ionizing radiation is an important phenomenon in normal and tumor cells. Because after radiotherapy treatment, apoptosis is the most important pathway that leads to the tumor cells death. In low dose range of ionizing radiation, that unrepaired damages can potentially cause carcinogenesis, apoptosis of the cell that has silent damage is a beneficial process.

Hereupon, in the present study, the effect of apoptosis as an important and practical route was investigated.

Based on Figures 1, 2 and 3, the effects of 2 GY (therapeutic doses) and low doses (diagnostic doses) of radiation was investigated separately. According to Figure 1, Bax expression increased after receiving selected radiation doses. Also, based on Figure 2, the Bcl-2 gene expression level has increased in the group receiving low-dose radiation compared to the group without radiation WR = 1, which can be attributed to the protective effects of radiation at low doses, while its expression decreased at 2 GY. However, these changes were not significant. In Figure 3, in WT (without treatment) groups, the Bax / Bcl-2 ratio decreased following low doses irradiation, while in the group irradiated by 2 GY, it increased and these results indicates a decrease in apoptosis in the low dose groups. Nonetheless these results were not significant, other studies conform them.

Azimian et al. Have obtained similar results in the investigation of the low doses of gamma rays effects, and in fact these results confirm the adaptive responses of low doses of ionizing radiation [25].

In a study of 10 patients undergoing breast cancer radiotherapy, it was found that at the doses of 1 and 2 Gy, the BAX relative expression (proapoptotic) increased and the expression of Bcl-2 gene (anti-apoptotic) decreased, which means activation of the apoptosis pathways [26]. These changes are similar to the results obtained in the present study, too.

In Liu et al. study, a significant decreasing in apoptosis was observed in the doses less than 200 mGy. Also, increasing in apoptosis in proportion to the dose in doses greater than 500 mGy was observed [27]. Hence, the results of their study corroborate present investigation's results.

In a study conducted by Sudprasert et al. that was performed on peripheral blood lymphocytes, in addition to determining the effects of gamma irradiation on DNA, it was concluded that radiation at doses of 50 mSv leads to chromosomal aberrations such as deletion. This important finding underscores the fact that the genotoxic effects of gamma rays can be observed even at doses of 50 mSv [27]. These results conflict the present investigation's consequences and this contradiction can be due to differences in irradiation dose (our maximum low dose (CT) was 10 mGy, which is less than 50 mSv in the Sudprasert study).

The effect of polyphenol on Bax and Bcl-2 genes expression levels and Bax / Bcl-2 ratio

In accordance with figure 1, Bax fold changes occurred in the irradiated groups (low doses and 2 Gy) that received polyphenol 1 hour before radiation and the gene expression levels reduced in comparison to WT groups. Moreover, a down-regulation was observed in the group 1 hAR that received 2 Gy radiation. These results suggest that polyphenol can influence on apoptosis reduction, though definitive effect depends on Bax/Bcl-2 ratio changes.

According to Figure 2, the greatest effect of inhibiting polyphenol apoptosis in low dose radiation is one hour before irradiation on the samples. Of course, this amount one hour after radiation is also higher than the control group and in high dose, the use of polyphenol one hour after Irradiation results in the highest expression of Bcl-2, however these changes were not significant. As can be seen the use of polyphenols leads to an increase in the rate of apoptosis compared to the control group. This means that the use of polyphenol one hour before and after radiation in the

high dose group causes a protective effect and reduces the amount of apoptosis.

Given that the Bax / Bcl-2 ratio is presented as an indicator of radiation sensitivity and the large Bax / Bcl-2 ratio indicates a sensitive cell and the small Bax / Bcl-2 ratio indicates cellular resistance [28, 29] It can be concluded that receiving tea (in this study even one hour after irradiation) increases Bcl-2 gene expression and decreases Bax / Bcl-2 ratio.

In the study by Matsuno et al., It was shown that the amount of H2ax Foci, which indicates the amount of DNA double-strand damage, was significantly reduced by using polyphenols [30], which this results from polyphenols addition before irradiation(Pretreatment) is consistent with the present study.

In a study by Davari et al., It was shown that drinking green tea 3 hours before irradiation reduces cell damages [31], which emphasizes the importance of the protective effect of green tea against radiation at a specific time.

In H Kondo et al. study, polyphenols were given to the cells and 16 hours later irradiation by dose of 20 and 80 Gy was performed, which show significant increasing in reduction in apoptosis in comparison to pretreated groups [32].

Jin Ho Chung's study showed that green tea has the ability to have anti-proliferative and apoptotic functions specifically on cancer cells, as well as proliferative and anti-apoptotic functions on normal cells. This increase in cell proliferation was dose-dependent and showed a significant increase in Bcl-2 gene expression [33].

The exact mechanism of action of green tea is not known, but the oxygen produced (ROS) due to radiation and carcinogenic chemicals play a key role in carcinogenetic function, and green tea may act as a reducing agent of ROS [34, 35].

Conclusion

As a conclusion of this study, it can be said that green tea can make a reduction in apoptosis, and Bax/Bcl-2 ratio alterations induced by ionizing radiation even in high doses in human peripheral blood lymphocytes as normal radiosensitive cells. As regards common doses used in radiotherapy, his results can be considered for normal tissues radiation protection.

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