

Effects of Hesperidin as a Radio-protector on Apoptosis in Rat Peripheral Blood Lymphocytes after Gamma Radiation

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ABSTRACT

Introduction: Hesperidin (HES), as the most abundant flavonoid existing in the citrus, is widely used by human daily. The radio-protective effects of Hesperidin have been confirmed in various measurement systems. This study aimed to evaluate the effects of Hesperidin on the changes in the apoptosis level and expression of apoptotic genes target (bax, bcl-2 and ration of bax/bcl-2) in the peripheral blood lymphocytes of male rats after gamma radiation.

Materials and Methods: 64 male rats were divided into eight groups: Control, HES (100 mg/kg b.w, orally, 7 days), whole body irradiation with 2 and 8Gy, pre-administrated with 50 and 100 mg/kg body weight of Hesperidin for 7 days before irradiation with 2 and 8 Gy. 24 hours after radiation, apoptotic lymphocytes were evaluated using PE Annexin V Apoptosis detection I kit and the levels of mRNA for bax and bcl-2 were evaluated by real time reverse transcription polymerase chain reaction.

Results: A significant reduction in apoptosis of the lymphocytes was demonstrated in group animals receiving 8 Gy compared to the group which received 2 Gy irradiation ($p < 0.0001$). However, apoptosis significantly increased in group of rats who received Hesp before irradiation ($p < 0.05$). The increase of apoptosis by Hesperidin administration can be attributed to the decreased expression of bax and significantly reduced expression of bcl-2 and finally increasing the ration of bax/bcl-2.

Conclusion: The results suggest that administration of 50 and 100 mg/kg of Hesperidin induces apoptotic effects by changing expression level of bax, bcl-2 and also the ratio of bax/bcl2.

Keywords

Hesperidin, Radio-protector, Apoptosis, Bax, bcl-2

Introduction

Today, widespread use of ionizing radiation in different fields such as medicine, industry, agriculture, nuclear weapons testing as well as the radiation arising from nature has made the exposure of human to the radiation inevitable [1-3].

It has been reported that apoptosis or cell death through damage of DNA by free radicals is the main mechanism of radiation injury, both in tumor tissues and normal tissues, so the molecules with the properties

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of free radical scavenger are important as radio-protectors [4, 5]. These compounds can be used before exposure in the event of planned exposures or radiation accidents (e.g. radio-therapy patients, radiation workers, astronauts and the public) [6, 7].

Initially, the synthetic thiol compounds were taken into consideration but the complications arising from them have restricted their use in medical field [8], so, nowadays the matter of identification of a non-toxic, effective, cheap, accessible and acceptable route of administration (preferably oral or alternatively intramuscular) [8-10] radio protective compound has prompted the researchers to conduct studies on the natural compounds [2, 11-13].

Hesperidin, as a flavanone glycoside with the molecular formula of C₂₈H₃₄O₁₅ and molecular weight of 610.57 Daltons, belongs to the flavonoids family which are found plentiful in the skin and membrane parts of citrus fruits [14-16].

In recent years, the anti-microbial, anti-fungal, anti-cancer properties, the effects of immune system stimulants and antioxidants have led to its introduction as a natural radio-protector [17].

The aim of this study was to assess the effect of Hesperidin on the changes in the apoptosis level and expression of apoptotic genes target (bax, bcl-2 and the ratio of bax/bcl-2) in the peripheral blood lymphocytes of rat as the result of gamma radiation.

Material and Methods

Chemicals and Preparation of Hesperidin

Hesperidin (CAS registry number: 520-26-3) was purchased from M/s. Sigma Chemical Co., St Louis, USA. Hesperidin was dissolved in phosphate buffered saline at two concentrations (50 and 100 mg/kg body weight) and administered to rats once daily for seven con-

secutive days by gavage. Two drug doses (50 and 100 mg/kg Hesperidin, orally for a week) were selected according to a study conducted by Kumar et al. [18]. Hesperidin was prepared fresh every day and administered simultaneously throughout the experiment period by a ball-tipped needle.

Animal Care and Maintenance

8-10-week-old male Sprague-Dawley rats (180-220 g) from the Center of Comparative & Experimental Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, were used for the experiments. The rats were housed in accordance with the guideline of the Ethics Committee of Shiraz University of Medical Sciences.

All Animals were acclimatized under the controlled standard conditions of 12 h light/12 h dark cycles, at the temperature 20-22° C, 50-70% relative humidity, and given standard pellet diets and water ad libitum for 1 week before the start of the experiments.

Irradiation of Animals

One hour after the last administration of Hesperidin on the seventh day, the animals were transfected to Cobalt 60-gamma irradiator (Theratron 780, Atomic energy of Canada limited, Canada) room. The rats were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazin (20 mg/kg), and then the rats in groups 3, 4, 5, 6, 7 and 8 were subjected to a whole-body gamma irradiation doses of 2 and 8Gy at a dose rate of 30 cGy/min; the source-to-skin distance was 80 cm by field size of 30×30 cm².

The doses of gamma-radiation were selected for this study based on the results of Shirazi et al., showing the radioprotective effects of Melatonin against irradiation-induced oxidative damage in the rat's peripheral blood [19-22]. Also, the Hesperidin concentrations and the dose of gamma radiation were selected

based on the experience from the studies performed by other investigators [30].

Experimental Group Design

After acclimatization for 2 weeks to laboratory conditions, they were randomly divided into eight groups with 8 animals in each (n=8 per cage). Eight groups are shown in Table 1.

The control and radiation groups (alone) did not receive Hesperidin but they received both oral dose of PBS (as much as Hesperidin) for 7 consecutive days and sham irradiation.

Twenty four hours after exposure to gamma irradiation, the animals were anesthetized with ether, and the blood samples (4ml of peripheral blood from each animal) were collected from the heart puncture in EDTA sterile tubes.

Each blood sample was divided into parts. One part was used for evaluation of flow cytometric analysis of apoptotic lymphocytes using PE Annexin V apoptosis kit I and the other for measurement of Bax and Bcl-2 expression levels using real-time reverse transcriptase polymerase chain reaction (RT²qPCR).

Flowcytometry Analysis

Lymphocyte isolation from each blood sample using ficoll Lymphodex (innotrain, Germany) was performed according to the standard protocol. Blood was diluted 1:3 with phosphate-buffered saline (PBS) and layered onto the ficoll in the ratio of 2:1(blood+PBS: Ficoll). The blood was centrifuged at 3000 RPM for 20 min at room temperature. The lymphocyte layer was separated and after 3 times washing with PBS and centrifuging at 1400 RPM for 10 min. the supernatant layer was removed, and we added 500 µl of PBS to the sediment (lymphocytes layer). Then, we continued according to the instructions of PE Annexin V Apoptois Kit I. The cells were solved in binding Buffer at a concentration of 1×10⁶ cell/ml; then we transfected 100 µl of the solution to a 2 ml tube. 5 µl of PE Annexin v and 5 µl 7-AAD were added and incubated for 15 min at RT(25°C) in the dark. Finally, we added 400 µl of 1X Binding Buffer to each tube. Lymphocyte samples were analyzed for the presence of apoptotic cells by flow cytom-

Table 1: Experimental groups

Groups	Criteria
Sham control	Rats received PBS as vehicle.
HES	Rats received oral dose of HES (100 mg/kg b.w, orally) for 7 consecutive days.
2Gy	Received whole body gamma irradiation of 2 Gy
8Gy	Received whole body gamma irradiation of 8 Gy
HES (50 mg/kg b.w) + 2Gy	Pre-administration with dose (50mg/kg b.w, 7 consecutive days ,orally) of HES before whole body gamma irradiation of 2Gy
HES (100 mg/kg b.w) +2Gy	Pre-administration with dose (100 mg/kg b.w, 7 consecutive days, orally) of HES before whole body gamma irradiation of 2Gy.
HES (50 mg/kg b.w) + 8Gy	Pre-administration with dose (50 mg/kg b.w , 7 consecutive days, orally) of HES before whole body gamma irradiation of 8Gy
HES (100mg/kg b.w) +8Gy	Pre-administration with dose (100 mg/kg b.w, 7 consecutive days, orally) of HES before whole body gamma irradiation of 8Gy.

etry on a FACS Calibur flowcytometer (Becton-Dickinson, San Jose, CA, USA).

Quantitative Real-time RT-PCR

As mentioned, the lymphocytes were separated from blood and then extraction of RNA was done based on the protocol of RNX-Plus kit (Cinnagen, Karaj, Iran). Before cDNA synthesis, to remove pollution of RNA with DNA, the DNase I kit (Thermo Science, USA) was used. The synthesis of cDNA was done by using Revert Aid First Strand cDNA Synthesis kit (Fermentase, Lithuania) and based on the manufacturer's instruction.

The used primers of Bax, Bcl-2 and Gapdh gene are shown in Table 2. Gapdh gene was used as the endogenous reference.

The Real-time quantitative RT-PCR was done by CYBR Green kit (Yekta Tajhiz, Iran) using the plates with 48 wells specialized for ABI Step one system.

The conditions of QPCR were 2 minutes at 95°C for initial denaturation, then 40 cycles of denaturation for 10 seconds in 95°C, annealing for 15 seconds in 58°C, extension for 20 seconds in 72°C, and at the end the final proliferation for 3 minutes and 72°C based on the protocol. Target genes were quantified relative to the reference gene using the mathematical model described by $\Delta\Delta CT$ [23].

Statistical Analysis

All values have been expressed as Mean \pm SEM for eight rats of each group. The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. The value of $p < 0.05$ was considered as the significant level.

Results

Apoptotic Lymphocytes Analysis

As shown in Figure 1, the percentage of total apoptotic lymphocytes on 24 hr post irradiation in the radiation group (8Gy) in comparison with the radiation (2 Gy) and control groups showed a significant decrease ($p < 0.0001$ and $p = 0.004$, respectively). Pre-administration of Hesperidin (50 mg/kg b.w, 7 consecutive days, orally) before whole body gamma irradiation of 8 Gy significantly increase the percentage of total apoptotic lymphocytes in comparison with whole body gamma irradiation of 8 Gy only ($p = 0.001$). Oral administration of Hesperidin (100 mg/kg b.w) before 8 Gy of gamma irradiation in comparison with pre administration of Hesperidin (100 mg/kg b.w) before 2Gy of gamma irradiation significantly decreased ($p = 0.04$). Rats exposed to 2 Gy of gamma irradiation alone and pre administration of Hesperidin only showed insignificant increase in the percentage of total apoptotic

Table 2: List of primers used in this study

Primer Name	Sequence(5'– 3')	Length(bp)
bax-F	ACG CAT CCA CCA AGA AGC	143bp
bax -R	GCC ACA CGG AAG AAG ACC	
bcl 2-F	GT GGT GGA GGA ACT CTT CA	153bp
bcl2 -R	ATG CCG GTT CAG GTA CTC AG	
GAPdH-F	GGCAAGTTCAATGGCACAGT	161
GAPdH-R	TGGTGAAGACGCCAGTAGACTC	

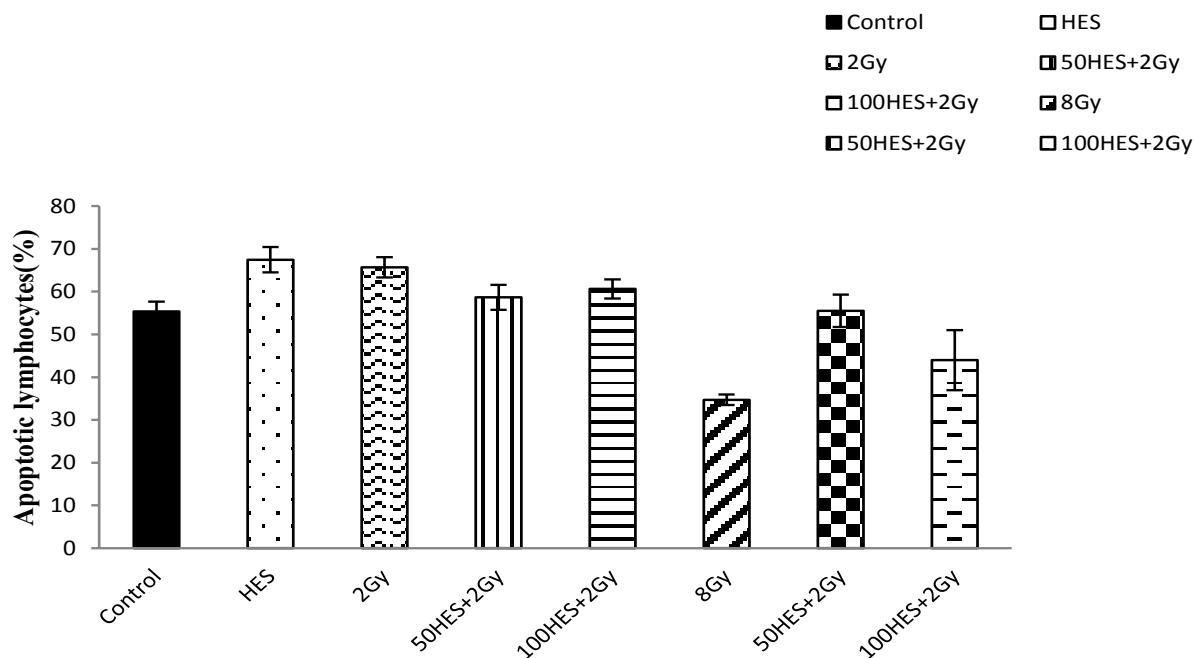


Figure 1: Effect of Hesperidin pre-administration on radiation-induced apoptosis in the rats' peripheral blood lymphocytes 24 h after exposure to 2Gy and 8Gy irradiation. Vertical bars represent mean \pm SEM, n= 8 for each group HES, Hesperidin only; 2Gy, 2Gy irradiation only; HES1+2Gy, 50 mg/kg body weight of Hesperidin+ 2Gy irradiation; HES2+2Gy, 100 mg/kg body weight of Hesperidin + 2Gy irradiation; 8Gy, 8Gy irradiation; HES1+8Gy, 50 mg/kg body weight of Hesperidin + 8Gy irradiation; HES2 + 8Gy, 100 mg/kg body weight of Hesperidin + 8Gy irradiation. ^ap < 0.05 when compared with the control group, ^bp < 0.05 when compared with the Hesperidin group, ^cp < 0.05 when compared with 2Gy, ^dp < 0.05 when compared with HES1+2Gy, ^ep < 0.05 when compared with HES2+2Gy, ^fp < 0.05 when compared with 8Gy group, ^gp < 0.05 when compared with 50HES+8Gy, ^hp < 0.05 when compared with 100HES+8Gy

lymphocytes in comparison with the control group (p=0.41, p=0.34). Pre-administration of Hesperidin (100 mg/kg b.w, 7 consecutive days, orally) before the whole body gamma irradiation of 8Gy significantly decreased the percentage of total apoptotic lymphocytes in comparison to pre-administration of Hesperidin alone (100 mg/kg b.w, 7 consecutive days, orally) (p=0.03).

Changes in Relative Expression Level of Bax and bcl-2 Genes

Figure 2 shows the relative expression level of Bax gene in eight experimental groups 24 h after the whole body gamma irradiation. The

relative expression level of Bax gene in pre-administration of Hesperidin (100 mg/kg b.w, 7 consecutive days, orally) only, 8Gy irradiation only, 50 mg/kg of body weight Hesperidin before 8Gy radiation, 50 mg/kg b.w before 8Gy irradiation, and 100 mg/kg b.w Hesperidin before 8Gy irradiation groups showed a significant reduction in comparison with the control group (p<0.05).

Pre-administration of Hesperidin at a dose of 100 mg/kg body weight before exposure to 2Gy in comparison with Hesperidin at dose of 50 mg/kg body weight before exposure to 2Gy, exposure to 2Gy only and Hesperidin at dose of 100 mg/kg body weight only showed

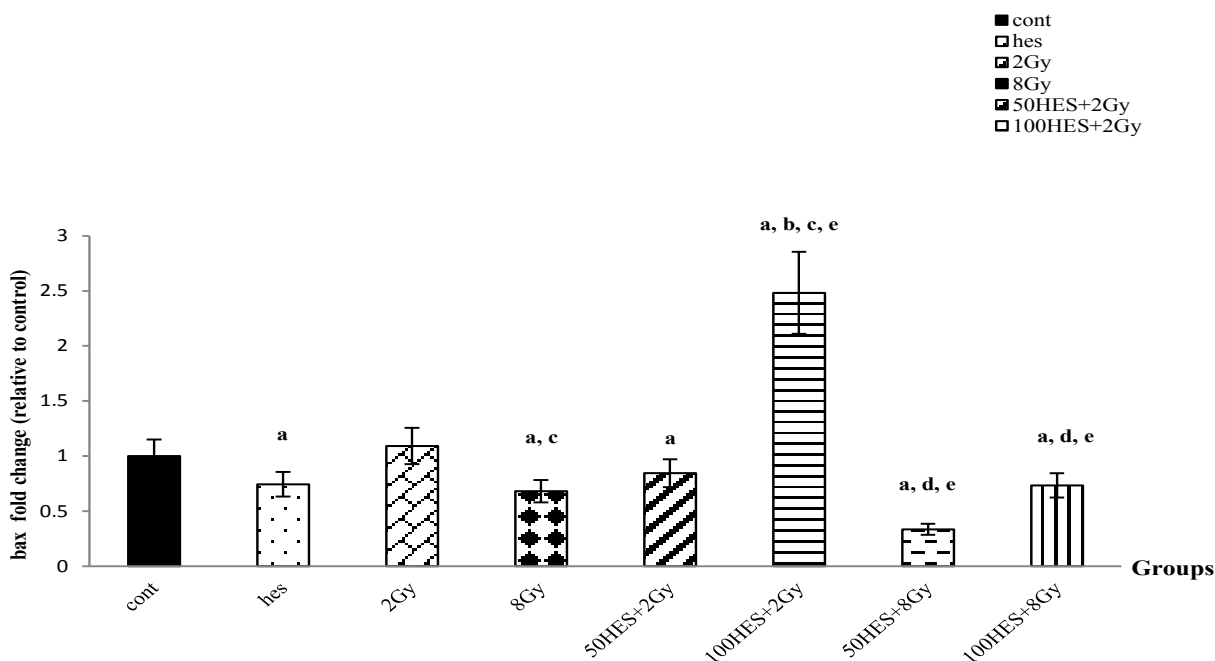


Figure 2: Effect of Hesperidin pre-administration on gene expression of Bax in the rats' peripheral blood lymphocytes 24 h after exposure to 2Gy and 8Gy irradiation. Vertical bars represent mean \pm SEM, n= 8 for each group.; HES, Hesperidin only; 2Gy, 2Gy irradiation only; HES1+2Gy, 50 mg/kg body weight of Hesperidin+ 2Gy irradiation; HES2+2Gy, 100 mg/kg body weight of Hesperidin + 2Gy irradiation; 8Gy, 8Gy irradiation; HES1+8Gy, 50 mg/kg body weight of Hesperidin + 8Gy irradiation; HES2 + 8Gy, 100 mg/kg body weight of Hesperidin + 8Gy irradiation. ^ap < 0.05 when compared with the control group, ^bp < 0.05 when compared with the Hesperidin group, ^cp < 0.05 when compared with 2Gy, ^dp < 0.05 when compared with HES1+2Gy, ^ep < 0.05 when compared with HES2+2Gy, ^fp < 0.05 when compared with 8Gy group, ^gp < 0.05 when compared with 50HES+8Gy, ^hp < 0.05 when compared with 100HES+8Gy

a significant increase in the relative expression level of Bax gene ($p < 0.0001$). Exposure to 8Gy only in comparison with 2Gy only revealed a significant reduction at relative expression level of Bax gene ($p < 0.0001$). Oral administration of Hesperidin at a dose of 50 mg/kg body weight before exposure to 8Gy in comparison with 8Gy only showed a significant decrease ($p < 0.0001$). In contrast, Hesperidin at a dose of 100 mg/kg body weight before exposure to 8Gy caused a significant increase in the expression level of Bax gene in comparison to the oral administration of Hesperidin at a dose of 50 mg/kg body weight before exposure to 8Gy ($p < 0.0001$). A signifi-

cant reduction was observed in the expression level of Bax gene in the group of Hesperidin at a dose of 50 mg/kg body weight before exposure to 8Gy in comparison to the group in which 50 mg/kg of Hesperidin was administered before exposure to 2Gy ($p < 0.0001$).

Figure 3 shows the relative expression level of Bcl-2 gene in eight experimental groups 24 h after whole body gamma irradiation. In all groups, we found a significant decrease in expression levels of Bcl-2 in comparison with the control group ($p < 0.0001$). Oral administration of Hesperidin at dose of 50 mg/kg body weight before exposure to 2 Gy has shown a significant decrease in in expression level of

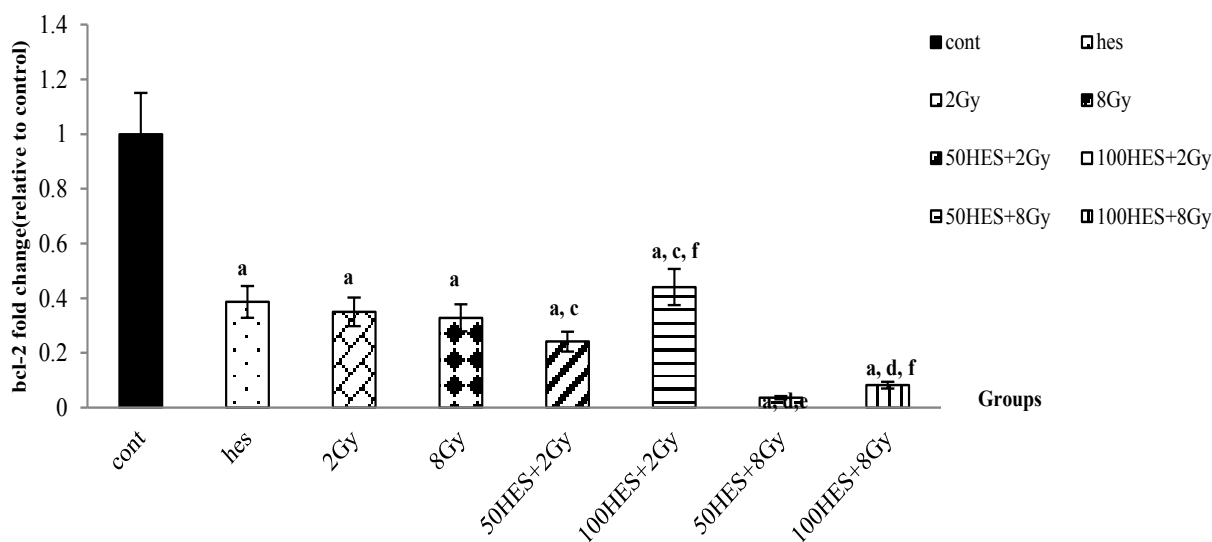


Figure 3: Effect of Hesperidin pre-administration on gene expression of Bcl-2 in the rats' peripheral blood lymphocytes 24 h after exposure to 2Gy and 8Gy irradiation. Vertical bars represent mean \pm SEM, n= 8 for each group.; HES, Hesperidin only; 2Gy, 2Gy irradiation only; HES1+2Gy, 50 mg/kg body weight of Hesperidin+ 2Gy irradiation; HES2+2Gy, 100 mg/kg body weight of Hesperidin + 2Gy irradiation; 8Gy, 8Gy irradiation; HES1+8Gy, 50 mg/kg body weight of Hesperidin + 8Gy irradiation; HES2 + 8Gy, 100 mg/kg body weight of Hesperidin + 8Gy irradiation. ^ap < 0.05 when compared with the control group, ^bp < 0.05 when compared with the Hesperidin group, ^cp < 0.05 when compared with 2Gy, ^dp < 0.05 when compared with HES1+2Gy, ^ep < 0.05 when compared with HES2+2Gy, ^fp < 0.05 when compared with 8Gy group, ^gp < 0.05 when compared with 50HES+8Gy, ^hp < 0.05 when compared with 100HES+8Gy.

bax gene in comparison with the dose of 2 Gy whole body gamma irradiation ($p=0.04$). In contrast, Hesperidin at dose of 100 mg/kg body weight before exposure to 2 Gy in comparison administration Hesperidin at dose of 50 mg/kg body weight before exposure to 2 Gy has shown a significant increase ($p=0/001$). Hesperidin at doses of 50 mg/kg and 100 mg/kg body caused significant decrease in relative expression levels of bcl-2 gene in comparison with exposure to 8 Gy only ($p<0.0001$). Our findings have shown that oral administration of Hesperidin at dose of 50 mg/kg before exposure to 8 Gy caused a significant decrease in expression level of bcl-2 in comparison with administration of Hesperidin at dose of 50 mg/kg before exposure to 2 Gy ($p=0.001$). Oral administration of Hesperidin at dose of

100 mg/kg body weight before exposure to 8 Gy caused a sharp reduction in expression level of bcl-2 in comparison with Hesperidin at dose of 100 mg/kg body weight before 2 Gy ($p<0.0001$).

Assessing the Ratio of Bax/bcl-2

As observed in Figure 4, in all groups we found a significant decrease in expression level of bcl-2 in comparison with control groups ($p<0.05$). Oral administration of 100 mg/kg body weight of Hesperidin before exposure to 8Gy in comparison with the controls, exposure to 8Gy (only) and Hesperidin only showed a significant increase in the ratio of Bax/Bcl-2 ($p=0.001$, $p>0.0001$, respectively). Oral administration of Hesperidin at a dose of 50 mg/kg body weight before exposure to 8Gy in

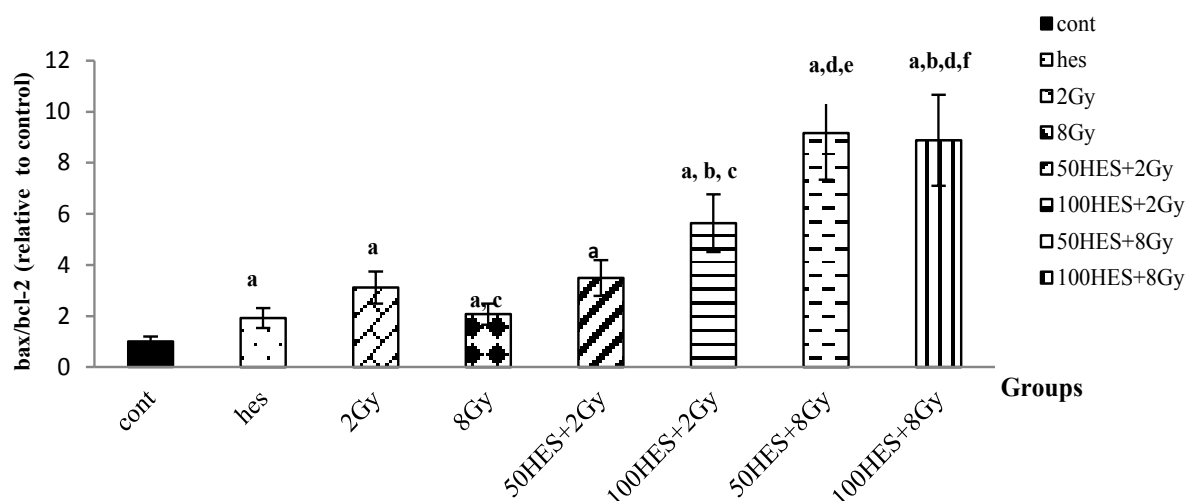


Figure 4: Effect of Hesperidin pre-administration on expression of Bax/Bcl-2 ratio in the rats' peripheral blood lymphocytes 24 h after exposure to 2Gy and 8Gy irradiation. Vertical bars represent mean \pm SEM, $n = 8$ for each group. HES, Hesperidin only; 2Gy, 2Gy irradiation only; 50HES+2Gy, 50 mg/kg body weight of Hesperidin+ 2Gy irradiation; 100HES+2Gy, 100 mg/kg body weight of Hesperidin + 2Gy irradiation; 8Gy, 8Gy irradiation; 50HES+8Gy, 50 mg/kg body weight of Hesperidin + 8Gy irradiation; 100HES + 8Gy, 100 mg/kg body weight of Hesperidin + 8Gy irradiation. ^a $p < 0.05$ when compared with the control group, ^b $p < 0.05$ when compared with the Hesperidin group, ^c $p < 0.05$ when compared with 2Gy, ^d $p < 0.05$ when compared with HES1+2Gy, ^e $p < 0.05$ when compared with HES2+2Gy, ^f $p < 0.05$ when compared with 8Gy group, ^h $p < 0.05$ when compared with 50HES+8Gy, ^g $p < 0.05$ when compared with 100HES+8Gy

comparison with the control and 8Gy groups caused a significant increase in the ratio of Bax/bcl2 ($p = 0.05$, $p = 0.02$, respectively).

Hesperidin administration at dose of 100 mg/kg before exposure to 2Gy irradiation in comparison with 2Gy only led to an increase in Bax/Bcl-2 ratio ($p = 0.04$) and compared with pre-administration Hesperidin at dose of 100 mg/kg before 8 Gy led to a decrease in bax / bcl-2 ratio ($p < 0.0001$). The ratio of bax/bcl-2 in radiation of 8Gy in comparison with 2 Gy has led to a significant reduction of bax/bcl-2 ratio (0.002).

Discussion

Ionizing radiation is not only an important component of modern medicine (both in the diagnosis and treatment field), but also in other fields of science, it has found a wide range of

applications [1]. Previous studies have shown that the lymphocytes, as the most sensitive cells in patient radiotherapy and public exposure, are easily affected by apoptosis [24-27]. With respect to the close relationship between formation of free radicals (reactive oxygen species) and apoptosis, molecules with free radical scavenging characteristics are important, especially as a radio-protector [28, 29]. Owing to the confirmed antioxidant and radio-protective properties of Hesperidin in various measurement systems [8, 30-36], the purpose of this study was to assess the role of Hesperidin in the changes of apoptosis levels and the expression of apoptotic gene targets.

Based on the obtained results of this study, the percentage of apoptotic lymphocytes 24 hours after 8Gy showed a significant reduction in comparison with 2Gy and receiving 100

mg/kg Hesperidin alone. This reduced level of apoptosis can be attributed to the reduction of Bax expression in the group of 8Gy alone and reducing the ratio of Bax/Bcl-2 as a key factor for the occurrence of apoptosis. Cui et al. have shown that 4 hours after gamma radiation in the dose of 2-8Gy whole body gamma irradiation, the percentage of apoptotic lymphocytes has reached their highest level; and up to 7 days after radiation it has remained in their most amounts. This result was in contrast with the results of our study [37]. On the other hand, as the process of apoptosis in a large number of cells and tissues has a short life, Takahashi and Jang have reported that the radiation of 2-8Gy for 8-12 hours after radiation reached its maximum amount, and then reduced. This was in the same line with the results of the present study [38, 39]. The results of the present study demonstrated that oral administration of Hesperidin reduced Bax expression and led to the significant reduction of Bcl-2 expression in comparison with the control group. The pro-apoptotic effects of Hesperidin have been reported in several studies [25-27, 40-42]. We found that administration of 50 and 100 mg/kg Hesperidin before 2Gy radiation in comparison with 2Gy radiation alone has no significant effects on the expression rate of apoptotic genes target of Bax and Bcl-2 and finally the ratio of Bax/Bcl-2. 100 mg/kg Hesperidin before 2Gy radiation in comparison with Hesperidin alone has shown a significant increase of Bax expression and finally revealed the ration of Bax/Bcl-2. Hesperidin in the doses of 50 and 100 mg/kg before 2Gy radiations in comparison with the control group showed a significant increase in the ratio of Bax/Bcl-2. However, in the groups receiving the doses of 50 and 100 mg/kg before 8Gy radiation, a significant reduction of Bax expression and so more reduction of Bcl-2 expression were seen. Finally, the ratio of Bax/Bcl-2 increased in comparison with the control group and 8Gy alone. Consequently,

the results of our study indicated the effects of pro-apoptotic effects in doses of 50 and 100 mg/kg of Hesperidin before 8Gy radiation.

Conclusion

Based on the results obtained in this study, it indicated that the oral administration of Hesperidin would reduce the apoptosis in irradiated rats, mainly attributed to its pro-apoptotic properties.

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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