The Effect of Vitamin C on Apoptosis and Bax/Bcl-2 Proteins Ratio in Peripheral Blood Lymphocytes of Patients during Cardiac Interventional Procedures

Nematollahi H.[1], Haddadi Gh.[2,3]*, Jorat M. V.[4]

[1]MSc, Department of Radiology, Paramedical School, Shiraz University of Medical Sciences, Shiraz, Iran
[2]PhD, Associate Professor, Department of Radiology, Paramedical School, Shiraz University of Medical Sciences, Shiraz, Iran
[3]PhD, Associate Professor, Ionizing and Non-ionizing Radiation Protection Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
[4]MD, Associate Professor, Cardiovascular Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: There is a close relationship between the effects of free radicals and apoptosis, and vitamin C is known as a potent scavenger of free radicals.

Objective: The aim of this study was to evaluate the effect of vitamin C against the radiation-induced apoptosis and the ratio of Bax/Bcl-2 proteins in peripheral blood lymphocytes in patients undergoing cardiac procedures in vivo condition.

Material and Methods: In this clinical intervention study, blood samples from 6 patients in the first group were taken to assess the effect of radiation on the apoptosis and Bax/Bcl-2 proteins ratio, and 5 patients as the second group to evaluate the effect of vitamin C on the apoptosis and Bax/Bcl-2 proteins ratio before and 24 hours after the examination. Flow cytometry was used to analyze the apoptosis and ELISA method to assess Bax and Bcl-2 proteins amount.

Results: In the second group receiving 25 mg/kg vitamin C and a mean skin dose of 1001 mGy in the chest area, there was no significant difference ($P < 0.05$) in the percentage of early apoptosis in 24 hours after the examination than before it. This significant increase in the percentage of apoptosis in the first group (385.6 mGy) was associated with a significant increase in the Bax/Bcl-2 ratio ($P < 0.05$), while in the second group, it was not associated with a significant decrease in the Bax/Bcl-2 ratio in 24 hours after the examination than before it.

Conclusion: Our results suggest that vitamin C may modulate Bax and Bcl-2 proteins expression, in maintaining peripheral blood lymphocytes in patients undergoing cardiology in radiation-induced apoptosis.

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Keywords

Apoptosis; Bax; Bcl-2; Vitamin C; PCXMC; Enzyme-Linked Immunosorbent Assay; Flow cytometry; Radiation

Introduction

In developed countries, heart disease is one of the most important causes of death. The formation of plaque in the coronary arteries reduces blood supply to the heart and can result in symptoms such as chest pain or fatal heart attack. Abnormal changes in these arteries can be shown by coronary angiography. In the presence of blockage or vascular injury, percutaneous transluminal coronary angiography (PTCA) is used to re-establish cardiac blood flow. A balloon-tipped catheter is placed in the blocked area and then inflated to re-establish cardiac blood

*Corresponding author:
Gh. Haddadi
Associate Professor, Ionizing and Non-ionizing Radiation Protection Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
E-mail: pira_radio4@sums.ac.ir

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flow. Routinely, a wire mesh tube called stent is located in the blocked area [1].

Interventional angiography utilizes X-ray to guide the catheter through the blood arteries [2]. Prolonged imaging time and multiple exposures result in increased patient dose and subsequent risks [3, 4].

In the cell, DNA is the main target for biological effects of ionizing radiation with high and low LET. Damage can be caused directly through energy transmission that leads to the DNA strands breakage or indirectly through radiolysis of peripheral water molecules. This process involves reactive species such as free radicals, which react with the DNA molecules [5, 6]. In most DNA damage, double-strand breaks (DSBs) are important, and if they are not repaired or repaired incorrectly [7], it can lead to chromosomal abnormalities, mitotic cell death and apoptosis [8].

Previous research has shown that radiation can damage DNA through apoptosis. P53 is one of the most important genes of this process [9-11]. Over expression of the bax gene and its translocation from the cytoplasm to the mitochondria trigger p53 activation. On the other hand, the Bcl-2 gene family such as bcl-2 act as anti- apoptosis gene at the mitochondria level. Increased Bax/Bcl-2 ratio results in breakdown permeability of mitochondrial membrane and subsequently releases cytochrome c into the cytoplasm [9-11]. This causes caspase cascade that leads to generation of an apoptotic phenotype [12]. Nevertheless, the upregulation of pro- apoptotic gene and under regulation of anti-apoptotic gene are the main factors for cell death initiation [13].

Reactive oxygen species (ROS) are considered as moderators of apoptosis. One of the most damaging species is radical OH• [14]. Thus, every molecule that would competently scavenge the radical OH• can be considered as a significant antioxidant. After discovering the cysteine radiation protection feature in 1949 [15], a large number of compounds, mainly aminothiols, were studied for their radioprotective properties. The major mechanism of radiation protection of these compounds is the radical scavenging. They are effective at high doses and cause different side effects. Due to this fact, researches on more effective radioprotectors and less toxicity have been considered [16]. Compounds of antioxidant properties, especially those stemmed from a natural source, believe in having non-toxic and effective radio protectors. Dietary agents are often good candidates to protect against radiation [17].

Some researchers have shown that natural antioxidants, such as vitamin C, E and beta-carotene can act as radio protectors. Vitamin C, which is soluble in water and presents in the blood, plasma, intracellular fluid and tissue, is a very powerful antioxidant and considered as a dietary radioprotector [18, 19].

In this study, we aimed to evaluate the radio protective effect of vitamin C on radiation inducing apoptosis and changes in bax, Bcl-2 protein expression, the most important factor in apoptotic process of peripheral blood lymphocyte of patients during cardiac interventional procedures. Furthermore, in this study, the effective dose and organ dose of patients were calculated using the PCXMC computer program.

PCXMC is a software that calculates the effective dose and organ dose in different fluoroscopic and radiographic procedures. patient;s risk of death because of radiation-induced cancer can be estimated by this software [20].

Material and Methods

Blood sampling and isolation of lymphocytes

In this clinical intervention study, peripheral blood samples were obtained from six volunteers, including four men (mean age 63±5.6 years) and two women (mean age 53.5±9.2 years) as the first group, and five patients, including two men (mean age 63.5±4 years) and three women (mean age 64.3±2.9 years) as the

second group, before performing angiography and 24 hours after it. In the second group after sampling, about 3 hours before the examination, a vitamin C effervescent tablet, the amount about 25mg/kg, was dissolved in 100 ml water (C-Hedenkamp factory in Germany), and then administered to patients (3-4 hours after administration of vitamin C, its amount in lymphocytes is maximal [21]). These patients were all non-smokers without any infectious diseases, antibiotic consumption, and X-rays 24 hours prior to the sampling. Above all, patients in the second group were examined for stomach and acute or chronic kidney diseases. All patients were NPO from 12 hours before angiography and they did not receive any antioxidant substance for up to 24 hours after angiography.

Fresh blood samples (5-6ml) were transferred to EDTA sterile tubes. Blood was diluted 1:2 with phosphate-buffered saline (PBS) and carefully layered onto the Ficoll-Histopaque density gradients (Inno-Train, Germany) in Falcon tube with the ratio of 2:1 (blood + PBS: Histopaque). The blood was centrifuged at 1800 × rpm for 20 minutes at room temperature. The lymphocyte layer was removed and washed twice in PBS at 1800 × rpm for 10 minutes. Then the counted lymphocytes were divided into three parts .One part was used to evaluate flow cytometric analysis of apoptotic lymphocytes using Annexin V/7-AAD double staining, and other parts were used to measure bax and Bcl-2 proteins expression levels using ELISA method.

Flow cytometric analysis of apoptotic lymphocytes

Apoptosis was evaluated using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, USA) according to the instructions of the manufacturer. To stain the cells, first lymphocytes were washed twice with cold PBS, and then were resuspend in 1X Binding Buffer at a concentration of 1×10^6 cells/ml, and 100 µl of the solution was transferred (1×10^5 cells) to a 5 ml culture tube, and 5 µl of PE Annexin V and 5 µl 7-AAD was added. The cells were gently vortexed and incubated for 15 minutes at RT (25°C) in the dark. Ultimately, 400 µl of 1X binding buffer was added to each tube. Morever, for each patient, the sample before the examination was considered as the control for the sample after it. Lymphocyte samples were analyzed for the presence of apoptotic cells by flowing cytometry over a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA). In each sample, a minimum of 10,000 events was counted and analyzed.

ELISA method

ELISA method was used to measure the expression of pro-apoptotic bax and anti-apoptotic Bcl-2 proteins. Abcam's Bax Human in vitro SimpleStep ELISA™ (Enzyme-Linked Immunosorbent Assay) kit and Abcam's Bcl-2 in vitro SimpleStep ELISA® kit were used for the quantitative measurement of Bax and Bcl-2 in human lymphocytes. According to the kits's instructions, after washing the lymphocytes twice in PBS, pellet was solubilized at 2×10^7 cell/mL in chilled 1X Cell Extraction Buffer PTR. On ice for 20 minutes was incubated and centrifuged at 18000 × g for 20 minutes at 4°C. The supernatants were transferred into clean tubes and discarded the pellets. 50 µL of all samples and standards were added to appropriate wells, then 50 µL of the Antibody Cocktail (A mixture of 5µl 10X Capture Antibody, 5µl 10X Detector Antibody and 40µl Antibody Diluent CPI) were added to each well. The plate was sealed and incubated for 1 hour at room temperature on a plate shaker set to 400 × rpm. Each well was washed with 3 × 350 µL 1X Wash Buffer PT and after the last wash inverted the plate against clean paper towels to remove excess liquid. 100 µL of TMB Substrate was added to each well and incubated for 10 minutes in the dark on a plate shaker set to 400 × rpm. 100 µL of Stop Solution was added to each well, on a plate shaker for 1 minute to mix, and record the OD at

450 nm with ELISA reader. Finally, according to the standard curve and the OD value, the amount of protein concentration was read.

Calculating patient's effective dose with PCXMC program

We performed dose calculations in 24 patients, including 14 CA (56.86 ± 9.75 years old, 10 males, 4 females) and 10 PTCA (58.8 ± 7.3 years old, 6 males,4 females). Procedures were performed by resident cardiologists or by an expert cardiologist. During CA, cardiologists usually use a typical set of projection, while during PTCA; they use two or three projections to achieve a good view on the damaged artery. Patient's information was noted for every examination, including age, height, weight and gender. Projection angles, mean X-ray tube voltage, the dose area product (DAP, recorded on device), the total fluoroscopy time and the total skin dose were recorded for every projection or examination. The device used in this study was Siemens-Artis zee. The required information about the device includes tube potential (40-125 KV), the total filtration (2.5 mm Al (+ 0.1 mm Cu)), anode angle (8◦), the flat detector (diagonal) (25cm), Focal spot-to-Detector distance (usually 120cm). Finally, using the PCXMC program (PC-based Monte Carlo program, version 2.0) and the necessary information mentioned calculated patients' organ doses, the effective dose and the risk of death for radiation-induced cancers.

Statistical analysis

Data were statistically analyzed and all values were expressed as Mean ± SD for the two groups. The significant difference in each group (before and after the examination) was evaluated by Wilcoxon signed-rank test. P-value of less than 0.05 was considered as significant. In order to investigate the correlation between variables, the bivariate correlation coefficient (r) test was used in a combination with independent and dependent variables.

Results

The apoptotic lymphocytes analysis, bax and Bcl-2 proteins expression and Bax/Bcl-2 ratio of two groups are shown in Figures 1 and 2.

Apoptotic lymphocytes analysis

As shown in Figure 1, in the first group, at the 24 hours post-irradiation (examination), with a skin dose of 385.6 ± 450.52 mGy in the chest area the percentage of early apoptosis (4.88 ± 1.76% vs. 11.24 ± 7.24%, $P<0.05$) and late apoptosis (0.39 ± 0.07% vs. 0.49 ± 0.08%, $P<0.05$) of the lymphocytes increased. The difference between the percentage of early apoptosis before the examination and 24 hours after it was significant. Furthermore, the percentage of survived cells significantly decreased (94.05 ± 1.66% vs. 87.57 ± 6.9%, $P<0.05$).

In the second group (Figure 1), the administration of 25 mg/kg of vitamin C in 3 hours prior to examination and skin dose of 1001 ± 1134.1 mGy in the chest area resulted in controlling the percentage of early apoptosis, late apoptosis and survived lymphocyte cells. This means that in the 24 hours after examination, the percentage of early apoptosis (1.1 ± 0.4 vs. 5.63 ± 4.95, $P<0.05$), and late apoptosis (0.19 ± 0.02% vs. 0.26 ± 0.05%, $P<0.05$) of the lymphocytes increased and the percentage of survived cells (98.04 ± 0.49 vs. 93.48 ± 4.67, $P<0.05$) of the lymphocytes decreased, but these differences were not significant.

Bax and Bcl-2 proteins expression and Bax/Bcl-2 ratio

As shown in Figure 2, in the first group, 24 hours after examination, Bax protein expression significantly increased (25.7 ± 6.04 vs. 19.89 ± 2.9, $P <0.05$) and Bcl-2 protein expression significantly decreased (8.11 ± 2.6 vs. 10.31 ± 3.5, $P <0.05$). This increase in Bax expression and Bcl-2 reduction caused a significant increase in Bax/Bcl-2 ratio in 24 hours after the examination than before it. (3.4 ± 1.03 vs. 2.14 ± 0.8, $P <0.05$). In the second

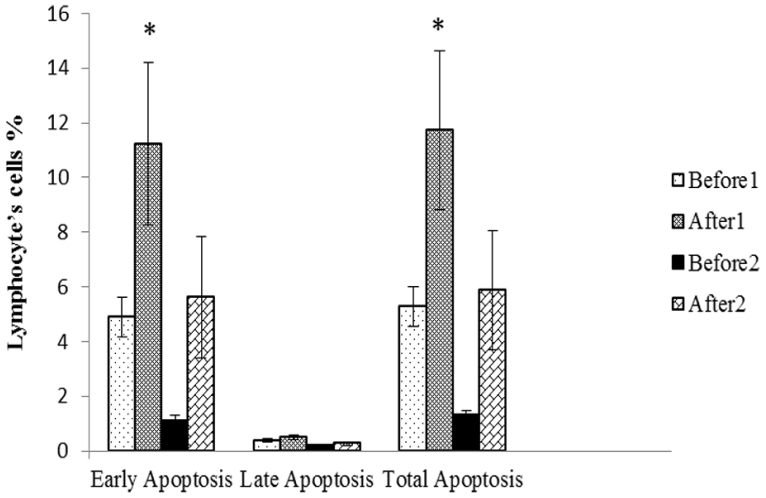

Figure 1: The effect of radiation (first group) and prescription of 25 mg/kg of vitamin C (second group) on peripheral blood lymphocyte apoptosis in patients undergoing cardiac interventional procedures. Patients of first group and second group received skin dose 385.6 ± 450.52 mGy and 1001 ± 1134.1 mGy in the chest area respectively. The percentage of apoptotic lymphocytes was analyzed before and 24 hours after radiation (examination) by flow cytometry. The values are shown as Mean ± SEM. P <0.05 was considered to show a significant difference between before and 24 hours after radiation.

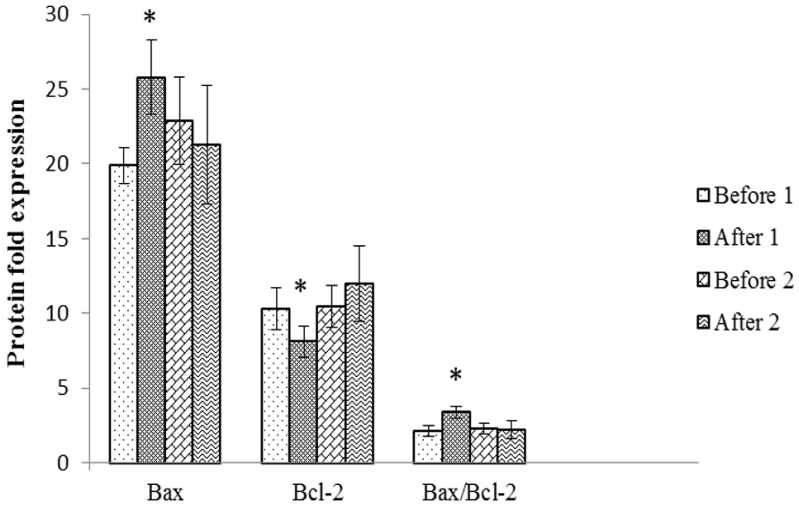

Figure 2: The effect of radiation (first group) and prescription of 25 mg/kg of vitamin C (second group) on Bax, Bcl-2 proteins expression and Bax/Bcl-2 ratio in peripheral blood lymphocytes in patients undergoing cardiac interventional procedures. Patients of first group and second group received skin dose 385.6 ± 450.52 mGy and 1001 ± 1134.1 mGy in the chest area respectively. The expression levels of Bax, Bcl-2 proteins and Bax/Bcl-2 ratio were measured by ELISA in before and 24 hours after radiation (examination). The values are shown as Mean ± SEM. P <0.05 was considered to show a significant difference between before and 24 hours after radiation.

group, administration of 25 mg/kg of vitamin C in 3 hours before examination and skin dose 1001 ± 1134.1 mGy in the chest area reduced the Bax protein (21.27 ± 8.8 vs. 22.85 ± 6.5) and increased the Bcl-2 protein (12 ± 5.6 vs.10.46 ± 3.2) in 24 hours after examination than before it. As well as, the Bax/Bcl-2 ratio decreased in 24 hours after examination than before it (0.75 ± 2.3 vs. 1.27 ± 2.21), however, these differences were not significant.

Results obtained from PCXMC program

The ranges of DAP, the mean DAP, and the ranges of fluoroscopy time per procedure CA and PTCA were obtained for CA procedure (1.71-21.83 Gy.cm^2, 10.94 Gy.cm^2, 1.07-8 min) and PTCA procedure (38.26-191.05 Gy.cm^2, 100.53 Gy.cm^2, 8.15-45.40 min), respectively. Amounts of dose in organs, including active bone marrow (ABM), lung, heart, liver, and skin for the CA and PTCA procedures are shown in Figure 3. As the chart shows, the maximum amount of equivalent dose for the lung and the lowest amount for the liver are obtained. For four patients with PTCA, the amount of air kerma was higher than 1 Gy. The ranges of effective dose and the mean effective dose were obtained for CA procedure (0.27-3.77 mSv, 1.92 mSv) and PTCA procedure (7.8-31.4 mSv, 17.65 mSv), respectively. The correlation between DAP and effective dose was evaluated, the result indicate that the correlation was positive and very strong ($r = 0.990$, $P<0.001$). Therefore, each DAP unit increase will add 0.002 to the effective dose;

Effective dose = 0.002 DAP + 0.099

Moreover, the risk of exposure induced cancer death (REID) was calculated using PCXMC computer program, shown in Figure 4 for the CA and PTCA procedures. As the Figure 4. Shows, the risk of exposure induced cancer death for lungs than other organs is higher in the CA and PTCA procedures. The correlation between DAP and REID was also evaluated, which was positive and strong ($r = 0.740$, $P<0.001$), the formula is as follow:

REID = 1.305×10^{-5} DAP + 0.005

Discussion

Humans are exposed to ionizing radiation

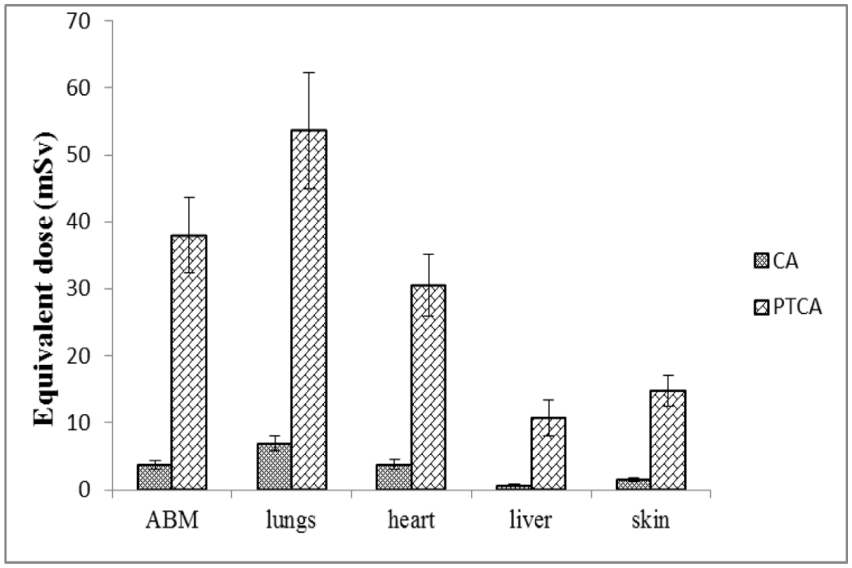

Figure 3: Organ doses calculation of the active bone marrow (ABM), lung, heart, liver, and skin for the CA and PTCA procedures with the PCXMC computer program. The values are shown as Mean ± SEM.

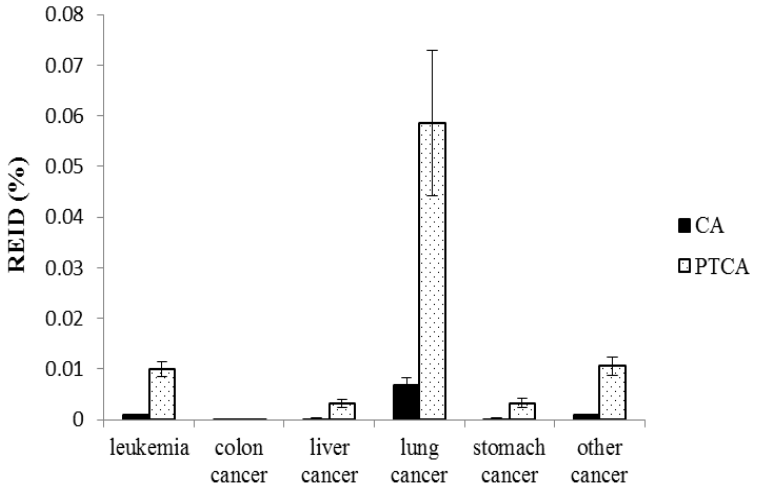

Figure 4: The risk assessment of exposure induced cancer death (REID) for the CA and PTCA procedures with the PCXMC computer program (the risk assessment is based on the equivalent doses). The values are shown as Mean ± SEM.

from natural and man-made resources [22]. Patients' medical exposure represents the most important contribution of man-made exposure [23]. Amongst all medical exposure, interventional radiology is on the top of the list of the more expansive radiological practice in terms of the effective dose per examination with a mean value of 20 mSv [24]. Many cardiac diagnostic and therapeutic procedures such as cardiac catheterization, CT and nuclear medicine scans (including >50% of all imaging methods) are significantly exposed to ionizing radiation [25, 26]. Ionizing radiation can cause various forms of DNA damage [27]. Free radicals produced by ionizing radiation are significant as apoptotic inducer mediators [14, 28]. On the other hand, in most DNA damage, double-strand breaks (DSBs) are considered [7], which play an important role in radiation-induced apoptosis [29].

In most studies, high values of apoptosis were observed at 24 and 72 hours after exposure to high doses to the cell in vitro conditions or to mice in vivo conditions. A study by Mozdarani et al., revealed that the number of apoptosis in the leukocytes increased at 42 and 72 hours after irradiation (4, 8, 12 Gy) that might be due to radiation-induced DNA damage [30]. Cui et al. have shown that 4 hours after 2-8 Gy whole body gamma exposure of mice, increased apoptosis in blood lymphocyte can be observed. Furthermore, over expression of bax and under expression of Bcl-2 are correlated with apoptosis exposed peripheral blood lymphocyte [31]. Due to close relationship between the formation of free radicals, especially reactive oxygen species (ROS) and apoptosis, molecules with free radicals scavenging properties are very important as radiation protection [32]. The aim of this study was to show the effect of vitamin C in a low dose of 25 mg/kg in radiation induced apoptosis in human peripheral blood lymphocytes in vivo conditions. We revealed that receiving skin dose 385.6 mGy in the chest area in patients undergoing cardiac interventional procedures, and the apoptosis increased significantly by 6.46% in 24 hours after examination.

Various types of compounds have been tested for their free radical scavenging abilities against radiation like amifostine suppressive activity on gamma-induced apoptosis in

mouse bone marrow cells, inhibition of apoptosis induced by melatonin and famotidine, reduced apoptosis in rats treated with hesperidin [30, 33-35]. It has been known that the formation of hydroxyl radicals (OH•),and the most common form of damaging of free radical cause approximately 70% damage to the DNA, hence, OH• scavengers are important and potent antioxidants [36]. It has recently been reported that the cause of the decrease in apoptosis induced by vitamin C was probably due to the radical-OH• scavenging properties, and an intracellular mechanism [30]. Various studies have shown the protective properties of vitamin C in protecting normal cells [19, 37, 38]. The protective effect of vitamin C is shown against DNA damage caused by radiation of gamma in human lymphocytes [39]. Administration of ascorbic acid maintained mice against radiation-induced lethality [40]. The consumption of vitamin C before radiation of gamma prevented chromosomal damage, micronuclei formation in bone marrow cells, peripheral blood lymphocytes and leukocytes [19, 41]. We also observed it in this study that oral administration of 25 mg/kg of vitamin C in patients 3 hours before examination controls the apoptosis in 24 hours after irradiation. In other words, although the mean of entrance skin dose was approximately 2.6 times higher than that of the first group, apoptosis was lower than that of the first group (4.6%), which probably could be the result of vitamin C controlling apoptosis by its radioprotection property.

Bax and Bcl-2 genes have very important roles in the intrinsic apoptosis process [9, 42]. This means that the Bax- Bcl-2 ratio has a major role in detection of apoptosis. Reducing this ratio may result in inhibition of bax translocation from cytoplasm to mitochondria. Reducing mitochondrial membrane permeability can lead to decreasing in the release of cytochrome C into the cytosol and consequently, the caspase cascade is inactivated. These processes reduce radiation-induced apoptosis.

Various studies have attributed the change in apoptosis with the change in the level of Bax/Bcl2 ratio and they have shown that an antioxidant, such as vitamin C, can reduce the Bax/Bcl2 ratio [43-46]. Our study showed that in patients undergoing cardiac interventional procedures with mean skin dose 385.6 mGy in the chest area, the Bax protein expression increased, the Bcl-2 protein expressions decreased that resulted in the Bax/Bcl-2 ratio increase, these differences were significant. These results were consistent with the increase in apoptosis. In addition, our findings demonstrated that the administration of 25 mg/kg of vitamin C 3 hours before the examination with mean skin dose 1001 mGy in the chest area resulted in the decreased expression of Bax protein and increased expression of Bcl-2 protein that resulted in decreased Bax/Bcl-2 ratio in 24 hours after examination in comparison to before it. This can be due to the effect of vitamin C on the expression levels of Bax and Bcl-2 proteins. (as well as, there was no significant difference in the amount of apoptosis measured in 24 hours after the examination than before it).

On the other hand, interventional radiology techniques (fluoroscopically- guided) are on the rise worldwide, and are being used by an increasing number of clinicians who are not adequately trained in radiation safety or radiobiology. Prolonged fluoroscopy time and multiple exposures result in the increased patient dose and subsequent risks [3, 4]. In this study, the mean fluoroscopy time for the CA procedure was 4.28 minutes, and for the PTCA procedure 20.43 minutes, as well as the mean DAP for CA procedure was 10.94 $Gy.cm^2$ and for PTCA procedure 100.53 $Gy.cm^2$.

The DAP value obtained in our study for CA procedure was lower than that of reported by Viktorie Stisova [1] for four cardiac catheterization laboratories of three hospitals, but for the PTCA procedure, the DAP value reported by the GE Adrantx device (10^4 Gy/cm^2) was comparable with our finding. By using the

computer program PCXMC, the amounts of dose in organs, including active bone marrow, lung, heart, liver, and skin were obtained for the CA and PTCA procedures (Figure 3). The highest equivalent dose for the lung (6.88 mSv for CA and 53.61 mSv for PTCA) and the lowest value for liver (0.67 mSv for CA and 10.69 mSv for PTCA) were obtained, which are dependent on the distance between organ and radiation field, as well as tissue type. Above all, as expected, the risk of exposure induced cancer death (REID) for lung than other organs was higher in both CA (0.007%) and PTCA (0.058%) procedures, which can be a concern for patients undergoing PTCA procedure who receive high doses and have hereditary lung cancer or are highly sensitive. For four patients with PTCA, the amount of air kerma was higher than 1 Gy, which can be of a concern for deterministic effects.

In a study conducted by Luca Vigna et al., the highest equivalent dose was obtained for the lung in the CA (32.5 mSv) and PTCA (58.5 mSv) procedures [47], and the equivalent dose obtained in their study for the PTCA procedure was comparable to ours. In addition to in their study, the highest REID for the lung was obtained in both the CA and PTCA procedures. To the best of our knowledge, few studies have reported the effective dose in cardiology procedures. Different mounts were reported by Viktorie Stisova [1] for GE Advantx device (7.9 mSv for CA and 15.3 mSv for PTCA), Betsou et al., [48] (5.6 mSv for CA and 13.0 mSv for PTCA), Neofotistou et al., [49] (4.6±15.8 mSv for CA and 5.4± 41.0 mSv for PTCA), Efstathopofulos et al., [50] (5 mSv for CA and 14 mSv for PTCA) and Luca Vigna et al., [47] (9.1 mSv for CA and 20.3 mSv for PTCA). The effective dose value of the PTCA (17.65 mSv) achieved by us is in line with the articles listed, however, the effective dose of CA (1.92 mSv) obtained by us was less than the mentioned amount in the articles, one reason for this might be the high skill of our cardiologist in performing CA.

Conclusion

According to the results obtained in this study in vivo condition on patients undergoing cardiac interventional procedures, it is concluded that in the physical state, increasing fluoroscopy time, and ultimately skin dose in the chest area in patients cause increases in equivalent dose, effective dose, and consequently the risk of exposure induced cancer death (REID). In biological state, apoptosis increases in human peripheral blood lymphocytes by causing DNA damage through produced free radicals. Moreover, according to the results, it can be suggested that administration of 25 mg/kg of vitamin C, 3 hours of before cardiac interventional procedures can modify the Bax/Bcl-2 proteins ratio and ultimately reduce apoptosis by possibly antioxidant and scavenging of free radicals properties.

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Conflict of Interest

None

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