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ORIGINAL ARTICLE – OPEN ACCESS

Protective Effects of Different Doses of Pycnogenol Extract against Gamma Radiation-induced Liver Damage in Rat

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ABSTRACT

Objective: The study aims to investigate the protective role of pycnogenol extract against liver damage in whole-body gamma-irradiated rats by assessing DNA damage, histopathological changes and biochemical parameters.

Materials and Methods: Rats were irradiated to a single fraction 900 cGy dose of gamma radiation. The pycnogenol extract was dissolved 5% DMSO and daily administrated before starting irradiation. The pycnogenol was administered orally via a gastric tube at a dose of 37.5 mg/kg, 75 mg/kg, 150 mg/kg and 300 mg/kg in 24, 48 and 72 h before irradiation. Irradiation was applied single fraction using Cobalt-60 teletherapy device. The animals were divided into ten groups that included control, pycnogenol extract groups only irradiation group (γ-ray) and pycnogenol extract + γ-ray groups. DNA damage, histopathological changes, catalase (CAT) and superoxide dismutase (SOD) activities, and malondialdehyde (MDA) levels in the liver tissue of rats were evaluated three days after irradiation

Results: Our results obtained that pycnogenol extract was induced to liver damage depend on pycnogenol extract dose, but the pycnogenol extract + γ-ray groups were significantly ameliorated the changes induced in liver antioxidant system; CAT, SOD and MDA. Also, the P300+γ-ray was significantly reduced irradiation-induced liver injury and it was possible to observed significantly preservation in the histopathological evaluation. The pycnogenol extract groups were significantly increased to comet parameter depend on pycnogenol doses compared to control. The pycnogenol extract + γ-ray were significantly decreased the comet parameter compared to the γ-ray group.

Conclusion: The administration of pycnogenol extract might provide substantial protection against radiation-induced oxidative, DNA damage and histopathological changes in the liver.

Keywords: Liver, pycnogenol, gamma ray, CAT, SOD

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INTRODUCTION

The liver is a vital organ with many functions for living organisms, such as protein synthesis, glycogen storage, bile production, digestion of nutrients, and elimination of the waste products. In the whole abdomen or the whole-body radiation therapy, the liver is usually irradiated during radiation therapy of tumors in the distal esophagus, upper abdomen and right lower lung (1). Ionizing radiation has many harmful effects, as well as possible beneficial effects, on humans (2). Exposure to ionizing radiation may induce functional and structural changes in the liver by causing excessive free radicals and oxidative stress (3). Oxidative stress is an important factor in liver damage (4–7), and proteins, nucleic acids and lipids of hepatocytes are primarily among cellular structures that will be affected by reactive oxygen species (ROS). The effects of ROS are eliminated by antioxidants, including the most important antioxidant molecule, glutathione (GSH), catalase (CAT), glutathione peroxidase and superoxide dismutase (SOD). Therefore, supplementation of antioxidants is a recommended strategy to increase the efficacy of radiotherapy because antioxidants may remove free radicals and protect people from ionizing radiation-induced damage (8). Studies on phytochemicals and plant extracts are a new field of research to regulate the effects of radiation (9).

In recent years, scientists have been trying to modulate the response of radiotherapy to the tumor and normal tissue. So far, various radioprotectors and radiosensitizers have been investigated in experimental studies (10).

Plants (such as fruits, vegetables and herbs), phenolic compounds (including tannins, quinones, coumarins, phenolic acids, stilbenes, flavonoids, ligands), terpenoids (including carotenoids), nitrogen compounds (betalains, alkaloids, amines), vitamins and antioxidants may contain a wide variety of free radical scavenging molecules, such as some other endogenous metabolites rich inactivity (11).

Pycnogenol (French Maritime Pine Bark Extract) is a complex of polyphenol/bioflavonoid structure obtained from the bark of marine pine tree. It contains natural compounds called oligomeric proanthocyanidin complexes (OPC) (12). The main components of pycnogenol are polyphenol. In particular, catechin, epicatechin, taxifolin, monomeric and oligomeric are units of this. 65–75% pycnogenol extract consists of subunits of catechin and

epicatechin at varying chain lengths of procyanidins (13). Comparative studies have shown that pycnogenol is more potent than grape seed extract, vitamins C, E, CoQ10 and alpha-lipoic acid in the prevention of lipid peroxidation (14). In addition to laboratory tests, pycnogenol is a potent antioxidant in clinical studies (14, 15).

This study aims to investigate the effects of radiation protection and antioxidant of pycnogenol against 900 cGy whole-body gamma irradiation-induced liver damage. We investigated by evaluating catalase (CAT) and superoxide dismutase (SOD) activities, malondialdehyde (MDA) levels, DNA damage and histopathological changes in liver tissue.

MATERIALS and METHODS

Chemicals

All chemicals were obtained from Merck (Darmstadt, Germany). Pycnogenol was donated by Horphag Research Ltd UK. For chemical and biochemical examinations, ultrapure water received from the two-way water purification system (Purelab ELGA, High Wycombe, UK) was used. All reagents and chemicals were of analytical grade or higher purity.

Animals

A hundred adult male Wistar Albino rats were obtained from the Erciyes University Experimental Research and Application Center. Water and food were available from this center. Animal experimentation was applied according to The Erciyes University Animal Experiments Local Ethics Committee decision (decision 11/127).

Irradiation

Whole-body gamma-irradiation was performed at the Erciyes University Faculty of Medicine, Radiation Oncology using the Co60 teletherapy machine. Animal fixing boxes contained five rats for each irradiation. Physical calculation of radiation dose was calculated as 900 cGy at 2.5 cm depth and 28x24 areas in two anterior and posterior.

Experimental Design

Animals were randomly divided into ten groups, each containing 10 rats. As shown in Table 1, the control group, the pycnogenol extract groups ($P_{37.5}$, P_{75}, P_{150}, P_{300}), the only irradiation group (γ-ray), and the pycnogenol extract + γ-irradiation groups ($P_{37.5}$+ γ-ray, P_{75}+ γ-ray, P_{150}+ γ-ray, P_{300} + γ-ray). Pycnogenol extract was dissolved in 5% dimethyl sulfoxide (DMSO). The control and γ-ray groups received 5% DMSO. The control animals were not irradiated. The γ-ray groups were treated with 900 cGy of gamma-irradiation to the whole-body in one fraction. The $P_{37.5}$, P_{75}, P_{150}, P_{300} groups received 37.5 mg/kg, 75 mg/kg, 150 mg/kg and 300 mg/kg of pycnogenol respectively in 24, 48 and 72 hours. The $P_{37.5}$+ γ-ray, P_{75} + γ-ray, P_{150} + γ-ray, P_{300} + γ-ray groups received 37.5 mg /kg, 75 mg/ kg, 150 mg/kg and 300 mg/ kg of pycnogenol and treated with 900 cGy of gamma-irradiation to the whole-body in one fraction. All irradiations were carried out between 9:30 a.m. and 11:30 a.m. The animals were firstly weighed and then killed anesthetized by intramuscular injection xylazine (0.05 mg/kg) and ketamine (0.1 mg/kg), and after 24 hours post antioxidant and post-irradiation. The livers were quickly removed. The liver tissue was divided into three parts. The first liver was fixed in 10% buffered formal, and the second liver was immediately used for comet assay and another liver was stored at –70°C until antioxidant enzymes assay. The tissue was homogenized in four volumes of 5 mM phosphate buffer (pH 7.4) and then centrifuged at 10,000 x g for 15 min to obtain supernatant which was used for the assay of the antioxidant profile and protein determination of the animals (16).

Table 1. Experiment groups of rats treated with pycnogenol extract and radiation

Groups	Pycnogenol (mm/kg)	Radiation (cGy)
Control	—	—
$P_{37.5}$	37.5	—
P_{75}	75	—
P_{150}	150	—
P_{300}	300	—
γ-ray	—	900
$P_{37.5}$ + γ-ray	37.5	900
P_{75} + γ-ray	75	900
P_{150} + γ-ray	150	900
P_{300} + γ-ray	300	900

Analytical Procedures

Antioxidant Enzymes Assay

The activity of SOD was measured using a method determined by Sun et al. (17). The activity of CAT was measured using a method determined by Aebi (18).

Malondialdehyde (MDA) Determination

The concentration of MDA in the homogenate of the liver was measured using a method determined by Ohkawa et al. (19) as thiobarbituric acid reactive substances (TBARS).

Total Protein Determination

The total protein concentration was evaluated using a method determined by the Bradford (20) as bovine serum albumin (BSA) standard.

Assessment of Tissue DNA Damage

The liver tissue DNA damage was investigated using the comet assay. The comet assay was applied under neutral conditions (21). The images of 100 chosen nuclei were made at a magnification of 200x using a fluorescent microscope (Olympus, BX51, Tokyo, Japan) and were analyzed using the Comet Assay Software Project (CASP-1.2.2, Windows 2010). We used two parameters (tail DNA and tail moment TM) to calculate the quantity of DNA damage. The DNA damage was detected by fragmented DNA that migrated from the nuclei of the liver cells, causing a comet figure. However, nuclei without a comet was not evaluated damaged (22).

Histo-pathology Assessment

After euthanasia of the animals at the end of the experimental period, the liver samples from the animals were excised, and stored in a 10% formalin solution and then dehydrated and paraffin-embedded. The liver tissues were firstly fixed in 10% buffered formal and then dehydrated and paraffin-embedded. Sections of 5 µm thickness were prepared for microtomy and stained with hematoxylin-eosin (H&E) staining order to evaluate the morphology of tissue

Table 2. The liver malondialdehyde (MDA) level, catalase (CAT) and superoxide dismutase (SOD) activities in the experiment groups

Groups	SOD U/mg protein Median (25%–75%)	CAT U/mg protein Median (25%–75%)	MDA nmol/mg protein Median (25%–75%)
Control	0.21 (0.16–0.25)	84.35 (76–104)	0.19 (0.17–0.21)
$P_{37.5}$	0.26 (0.20–0.31)	105 (103–118)	0.55 (0.44–0.61)
P_{75}	0.20 (0.17–0.28)	81 (67–110)	0.18 (0.10–0.22)
P_{150}	0.26 (0.18–0.30)	57 (41–79)	0.29 (0.14–0.4)
P_{300}	0.24 (0.20–0.30)	92 (67–103)	0.14 (0.05–0.24)
γ–ray	0.19 (0.16–0.27)	86 (64–102)	2.18 (1.4–3.6)
$P_{37.5}$ + γ–ray	0.17 (0.13–0.20)	67 (58–79)	1.22 (0.7–1.3)
P_{75} + γ–ray	0.21 (0.19–0.24)	80 (72–135)	1.66 (1.25–2.5)
P_{150} + γ–ray	0.22 (0.19–0.29)	77 (68–101)	0.90 (0.84–1.24)
P_{300} + γ–ray	0.25 (0.17–0.27)	94 (70–115)	1.3 (1.2–1.89)
p	0.213	0.041	<0.001

damage. All the sections were examined with a light microscope (Olympus BX51), and the pieces were photographed.

Statistical Analysis

Analysis of comet, SOD, CAT, and MDA data were evaluated using IBM SPSS Statistics 20.0 (IBM Inc., ILL, USA) software. The suitability of the data to normal distribution was evaluated using the Shapiro-Wilk test and variance homogeneity was evaluated by the Levene test. Comparisons between groups were evaluated with one-way analysis of variance and Kruskal-Wallis H tests. Results of ten different rats were expressed as median (25%–75%). The statistical significance was based on $p<0.05$.

The Student-Newman-Keuls method was used as a multiple comparison test. Data were expressed as mean and standard deviation (±SD). $P<0.05$ was considered statistically significant.

RESULTS

The tissue enzyme values between groups of liver tissue are given in Table 2. SOD and CAT activities were measured as an indicator of the oxidant/antioxidant status of the liver. In the evaluated the SOD activity, the differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference ($p=0.213$). The SOD activity of the γ-ray group was lower than the pycnogenol extract groups and the pycnogenol extract + γ-ray groups. There were no statistically significant between pycnogenol extract groups, γ-ray group and pycnogenol extract + γ-ray groups. The SOD activity of the P_{300}+ γ-ray groups was higher than the other group.

In the evaluated the CAT activity, the differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($p=0.041$). There were no statistically significant differences between the pycnogenol extract groups, the γ-ray group and the pycnogenol extract + γ-ray groups. The CAT activity of the P_{300}+ γ-ray groups was higher than in other groups.

To assess the degree of oxidative stress that arose from ionizing radiation, the level of lipid peroxidation was evaluated in the livers of all rats. The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($p<0.001$). The MDA level of the γ-ray group was statistically significantly higher than in other groups and control groups. The MDA levels of the pycnogenol extract + γ-ray groups were statistically significantly higher than the pycnogenol extract groups. The MDA level of the P_{300}+ γ-ray group was statistically significantly lower than the γ-ray group.

The exposure to whole-body gamma radiation of the rats resulted in cellular DNA damage in the liver tissue. The cellular DNA damage values between groups of liver tissue are given in Table 3, Figure 1. The comet parameters (except Head DNA) of the γ-ray group increased according to the parameters of the other

Table 3. Cellular liver DNA damage in the experiment groups

Groups	L Head AM±SD	L Tail AM±SD	L Comet AM±SD	Head DNA AM±SD	Tail DNA AM±SD	TM AM±SD	OTM AM±SD
Control	160±40	18±13	178±48	98±1	2±1	0.3±1	0.9±1
$P_{37.5}$	165±32	51±16	216±43	92±2	18±2	4±2	6±2
P_{75}	156±32	71±24	228±47	88±4	12±4	9±5	10±5
P_{150}	192±33	100±26	292±51	85±4	15±3	15±7	14.9±5
P_{300}	168±41	109±35	277±65	80±6	20±6	22±11	18±8
γ-ray	172±25	193±56	365±57	67±10	33±10	68±38	42±17
$P_{37.5}$+γ-ray	146±18	202±47	348±56	72±6	28±7	59±23	38±11
P_{75} +γ-ray	181±24	148±43	329±52	77±6	23±5	36±18	27±10
P_{150}+γ-ray	163±24	106±31	269±46	82±4	18±4	20±9	17±5
P_{300}+γ-ray	161±28	82±26	243±50	84±4	16±4	14±7	12±5
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

L Head: Length head; L Tail: Length tail; L Comet: Length comet; TM: Tail moment; OTM: Olive tail moment; AM: Arithmetic mean; SD: Standard derivation

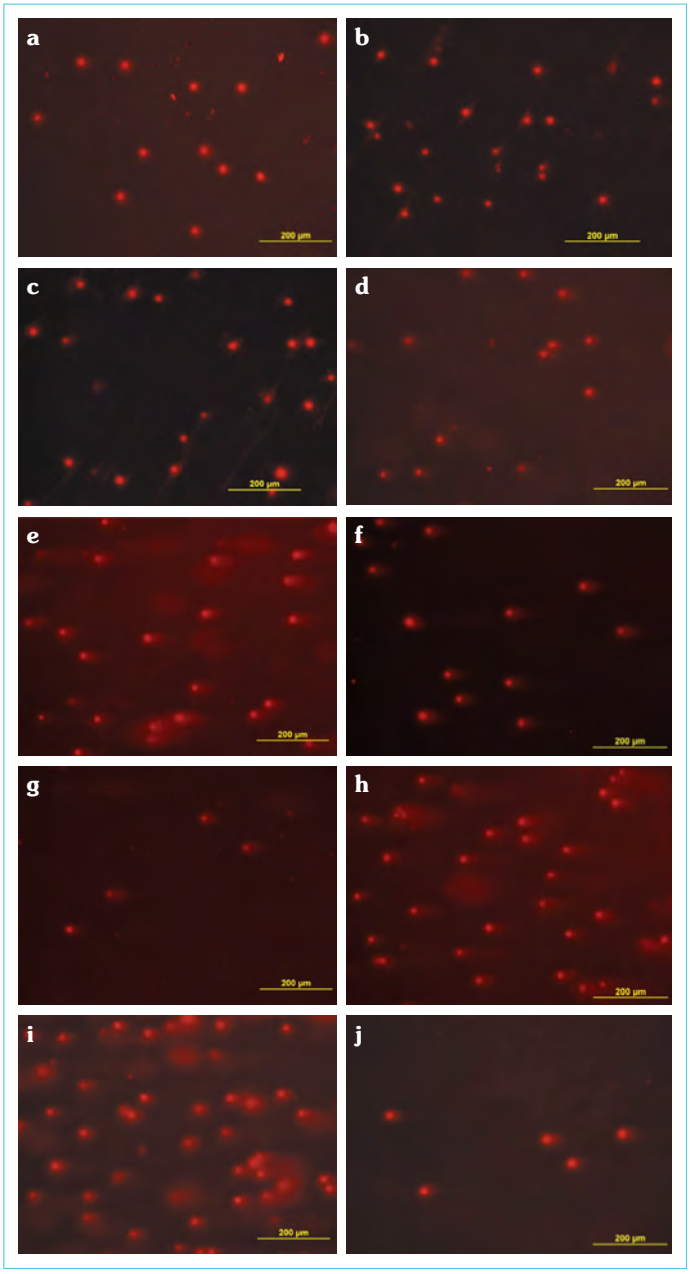

Figure 1. The cell of liver tissue (a) Tail DNA of the control group was 1.7%, (b) Tail DNA of the $P_{37.5}$ group was 7.8%, (c) Tail DNA of the P75 group was 11.6%, (d) Tail DNA of the P_{150} group was 14.7%, (e) Tail DNA of the P_{300} group was 19.6%, (f) Tail DNA of the γ- group was 32.8%, (g) Tail DNA of the $P_{37.5}$+ γ-ray was 28.3%, (h) Tail DNA of the P_{75}+ γ-ray group was 22.9%, (i) Tail DNA of the P_{150} + γ-ray group was 18%, (j) Tail DNA of P_{300} + γ-ray group was 16.4%

Ethidium bromide staining x200, Olympus, Tokyo, Japan

groups (Fig. 1). P<0.05 was statistically significant. The comet parameters of the pycnogenol extract groups ($P_{37.5}$, P_{75}, P_{150}, P_{300}) significantly increased comet parameters depend on pycnogenol doses compared to the control group. In the pycnogenol extract + γ-ray groups decreased the comet parameter compared to the γ-ray group. In the P_{300}+ γ-ray groups, it was possible to observe significant preservation.

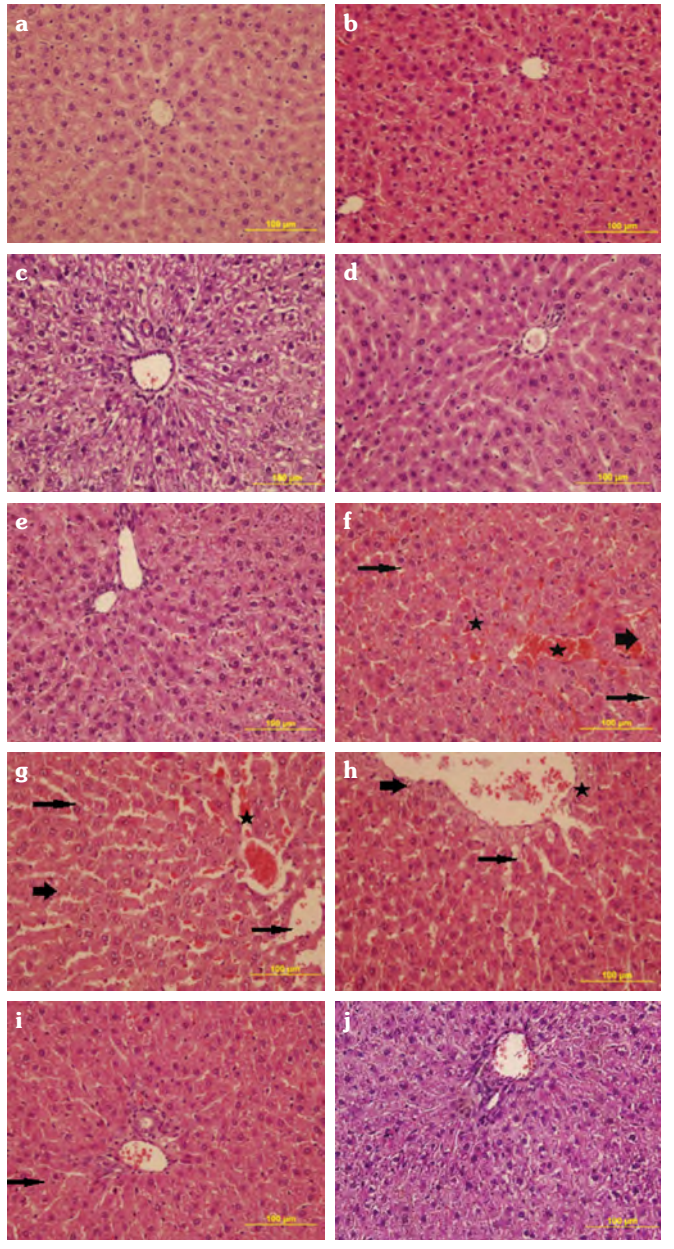

Figure 2. Liver tissue section (a) The control group had normal parenchymal structure, (b–e) The liver tissue of pycnogenol extract groups was shown similar to histological features of the control group, (f) The γ-ray group had intense vascular sinusoidal hemorrhage, necrotic hepatocytes, and swollen hepatocytes, (g–j) There was no protective effect of low dose pycnogenol extract (37.5 mg/kg, 75 mg/kg) against the adverse effects of radiation on liver tissue in the pycnogenol extract + γ-ray groups. But, P300+γ-ray group had close to normal liver tissue appearance and normal liver parenchyma. ➡ cellular damages, * sinusoidal hemorrhage and → sinusoidal dilatation

H&E staining x400, Olympus, BX51, Tokyo, Japan

In this study, Hematoxylin-eosin staining methods were applied to rat liver tissues. As a result of the histopathological evaluations (Fig. 2), the control groups showed their own normal histological tis-

sue characteristics. The control group had a normal parenchymal structure. Also, the control group had normal parenchymal structure characteristics. There were liver cells (hepatocytes) arranged radially around the vena centralis in the lobules. Liver sinusoids were observed around the hepatocytes. There was a triad of the hepatic artery, vein and bile ducts in the connective tissue in the portal areas.

Various cellular damages were observed in the γ-ray group tissues, especially in the areas of vascular and interstitial hemorrhage. When liver sections of the radiation group were examined, unlike the control group, compressing erythrocytes had filled the sinusoids with intense vascular sinusoidal hemorrhage. In all of the irradiated groups, areas of necrotic hepatocytes and swollen hepatocytes were observed. Also, there was a significant expansion of the sinuses extending radially from the vena centralis.

The liver tissue of the pycnogenol extract groups was shown to be similar to the histological features of the control group. Pycnogenol extract had no adverse effects on the liver parenchyma.

There was no protective effect of low dose pycnogenol extract (37.5 mg/kg, 75 mg/kg) against the adverse effects of radiation on liver tissue in the pycnogenol extract + γ-ray groups. In the liver tissue of the experimental group pycnogenol administered 150 mg/kg before irradiation, pycnogenol was found to have a positive effect against the harmful effects of radiation. Treatment with pycnogenol extract before irradiation (P_{300}+γ-ray) ameliorated the effects of radiation exposure. The best protective effect was observed in the P_{300} + γ-ray groups.

DISCUSSION

SOD and CAT activities increased in the γ-ray group compared to the control group in this study, which suggests that the antioxidant system is sufficient against excessive SOR production. CAT and SOD activities were increased in the P_{300} + γ-ray groups compared to the γ-ray groups. The pycnogenol is sufficient against the production of reactive oxygen species (ROS). CAT and SOD activities were increased in the pycnogenol extract + γ-ray groups, which suggest that both antioxidant enzymes potentiate the effects of each other. Jagetia (23) and Mansour et al. (24, 25) evaluated a significant reduction in the antioxidant system, along with increased lipid peroxides after whole-body γ-ray.

Malondialdehyde is a lipid peroxidation product that is an indicator of radiation-induced oxidative damage. Tissue MDA levels were also measured to demonstrate whole-body gamma radiation-induced liver damage in this study. MDA levels significantly increased in the γ-ray groups compared to other experiment groups ($p<0.001$). The increased MDA levels indicated that radiation causes oxidative damage to the liver. Pycnogenol protected membrane lipid against oxidative damage of radiation in the P_{300} + γ-ray groups.

Shedid et al. (26) evaluated 9 Gy γ-ray applied in rats liver. Their results showed significantly increased MDA levels and decreased CAT activity in the irradiation groups.

In our data, as shown in previous studies with oxidant agents, ionizing radiation-induced liver damage may arise from the indirect effect of radiation due to SOR, as well as by the direct effect of ionizing radiation because SOD activity was decreased in the radiation group, whereas CAT activity and MDA levels were increased in the radiation group. Also, although there was a numerical difference between the groups, it was not statistically significant.

As a result, the liver tissue of the γ-ray group showed increased CAT activities, decreased SOD activities and increased MDA levels. However, in the pycnogenol extract +γ-ray groups increased CAT and SOD activities and decreased MDA level. This may suggest that the protective effects of pycnogenol are related to antioxidant activity.

In this study, the neutral comet technique was used to determine radiation-induced single and double-strand breaks in liver tissue DNA. Whole-body gamma-ray irradiated rats resulted in cellular DNA damage in liver tissues and increased comet parameters. Based on the comet results of our study, we can say that 300 mg/kg pycnogenol protects the cell against ionizing radiation-induced damage, but increases the cell damage with increasing doses in healthy groups on which pycnogenol has a toxic effect. Our data were supported by Rohdewald, Trevithick et al. and Masquelier (13, 27, 28). In a similar study, they found decreased SOD, CAT and GSH activity according to the control group in rat liver irradiated with 6 Gy gamma-ray. In the bee venom + irradiated group, biochemical parameters, there were significant changes compared to the irradiated group (29).

As histopathological results, the negative histopathological effects of radiation on liver tissue decreased in the pycnogenol extract + γ-ray groups and liver parenchyma was observed to be close to normal liver tissue appearance. The 300 mg/kg pycnogenol extract may protect enzyme, cell and tissue levels best against ionizing radiation-induced liver tissue damage.

CONCLUSION

To conclude, pycnogenol extract has been found to possess antioxidant effects and their action induced through decreasing DNA damage, ROS, histopathological changes. The use of pycnogenol as a protective agent against ionizing radiation-induced liver damage may be suggested from the data of our study. However, preclinical and clinical studies are also needed to understand better the mechanism of action and molecular basis and to clarify dose, toxicity and tolerability issues.

Ethics Committee Approval: Animal experimentation was applied according to The Erciyes University Animal Experiments Local Ethics Committee decision (date: 23.11.2011, number: 11/127).

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Conflict of Interest: The authors have no conflict of interest to declare.

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