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Original Article

The effects of pycnogenol on liver damage caused by cisplatin in rats

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Abstract: Objective: In this study, we investigated the effect of Pycnogenol on the liver damage caused by cisplatin. Method: Twenty-four rats were divided into three groups of eight: Group A was the sham group, Group B was the control group, and Group C was the experimental group. At the start of the experiment, Groups A and C were given Pycnogenol via oral gavage at a dose of 20 mg/kg/day. On the third day, a single intraperitoneal dose of cisplatin was given to Groups B and C at a dose of 7.5 mg/kg. The rats were sacrificed after seven days, and tissue samples were taken. Results: The histopathological findings of the binary group analyses were as follows: for vascular congestion $P<0.05$ for Groups A-B and $P<0.05$ for Group B-C; for lipidosis, $P<.001$ for Groups A-B and $P<0.05$ for Groups B-C; for sinusoidal congestion, $P<0.05$ for Groups A-B $P<0.05$ for Groups B-C; for sinusoidal dilation, $P<0.001$ for Groups A-B and $P<0.05$ for Groups B-C; and for mononuclear filtration, $P<0.001$ for Groups A-B and $P<0.05$ for Groups B-C. For the binary group analyses of immunohistochemical free oxygen radicals, there was a significant difference between Groups A and B ($P<0.001$) and Groups A and C ($P<0.001$) and no significant difference between Groups B and C ($P>0.05$). Conclusion: The results of this study showed a significant decrease in liver damage in the group using Pycnogenol. In the immunohistochemical examination, it was observed that the free oxygen radicals decreased but the difference was not significant.

Keywords: Pycnogenol, hepatotoxicity, free oxygen radicals

Introduction

Pycnogenol® is an effective bioflavonoid extracted from the bark of French maritime pine (*Pinus pinaster aiton*) [1]. Its positive effects on cancer, inflammatory diseases, cardiovascular diseases, and immune system diseases have been shown in many studies [2]. Studies have suggested that the mechanism behind these positive effects involves reactive oxygen and nitrogen scavenging features, especially on a molecular basis [3]. It has also been shown through in vivo and in vitro experiments that Pycnogenol has a strong free-radical-scavenging effect and a strong antioxidant effect due to the phenolic acids, polyphenols, and flavonoids in its structure [4, 5].

Cisplatin (cis-Diamineplatinum dichloride) is an antitumoral agent that contains platinum, which is a heavy metal that is frequently used in clinic practice. It exerts its effect mainly by specifically inhibiting cell proliferation [6]. Clinically, cisplatin is one of the preferred agents in the primary treatment of squamous cell tumors, e.g. anal cancers. Although it has positive effects in both the treatment and control of cancer, it also has several side effects. The most well-known side effect is nephrotoxicity, which can cause severe liver damage and result in kidney failure [7]. Although the mechanism of this hepatotoxicity, which is caused even by low doses of cisplatin, remains largely unknown, it is thought to involve oxidative stress caused by reactive oxygen species (ROS) [8, 9]. Therefore, it is important to minimize the potential side effects of cisplatin by using it alongside an effective antioxidant agent to prevent free-radical formation.

Figure 1. Intraperitoneal cisplatin administration.

Some antioxidants protect against cisplatin oxidative stress (OS) in the liver [10, 11]. Antioxidants are endogenous or exogenous structures that prevent the cell damage caused by OS by eliminating ROS or preventing their formation. Endogenous antioxidants include enzymatic antioxidants, such as SOD, CAT, and GSH-PX, and non-enzymatic compounds, such as glutathione, ferritin, albumin, and transferrin proteins. Exogeneous antioxidants include substances such as vitamins C, E, and A; lycopene; and the carotenoids, flavonoids anthocyanin, curcuminoid, and Pycnogenol found in fruits, vegetables, and spices [12].

Although positive results have been reported regarding the use of Pycnogenol to combat the autotoxic and nephrotoxic effects of cisplatin, studies on its use against hepatotoxic effects are limited [1, 13, 14]. Therefore, in this study, we investigated the effect of Pycnogenol, which is known to be an effective antioxidant, on cisplatin-induced liver injury.

Methods

Experimental protocol

This study was performed in the laboratory of Hamidiye Turkey, with the approval of the University's Laboratory Animals Ethics Committee (IRB number: 2019-05/07). The rats were provided by the SBU Centre for experimental animals and kept in special cages under appropriate feeding conditions during the study. The rats were provided with free food and water and kept under controlled temperature (between 19-22°C) and lighting (hours 08:00 to 20:00 were light, hours 20:00 to 08:00 were dark) conditions.

Before the experimental procedure, all the rats were weighed with an analytical scale and their body weights (BWs) were recorded. Twenty-four Sprague Dawley rats weighing 250 to 300 g were divided into three groups of eight: Group A was the sham group, Group B was the control group, and Group C was the experimental group. Pycnogenol was given to Groups A and C via oral gavage at the start of the experiment. On the third day, a single intraperitoneal dose of cisplatin was given to Groups B and C at a dose of 7.5 mg/kg (see **Figure 1**). The experiment was completed after seven days.

All the rats were anesthetized with intramuscular (IM) ketamine (Ketalar 500 mg, 35 mg/kg BW; Pfizer) and Xylazine (Kepro Ksilazin 20, 15 mg/kg BW; Biopharm Veterinary Drugs Company). All rats underwent a laparotomy under ketamine-xylazine anesthesia on the seventh day of the experiment. Tissue samples were taken from the liver for histopathological evaluation and the analysis of immunohistochemical free oxygen radicals before the rats were sacrificed.

Histological examination

All materials were fixed with 10% buffered formaldehyde. Slices that were 4-5 microns thick were taken from each sample (which were embedded in paraffin blocks) and stained with hematoxylin and eosin (H&E). In addition, sections that were 3 microns thick were taken from the paraffin blocks, and immuno-

Table 1. Histopathological scoring table

Score	Vascular congestion	Lipidosis	Sinusoidal congestion	Sinusoidal dilation	Mononuclear filtration	GSTP1, Glut red, SOD1, CAT (Immunohistochemistry)
0	None	None	None	None	None	No stain
1	Mild	Mild	Mild	Mild	Mild	Focal weak positivity
2	Significant	Significant	Significant	Significant	Significant	Moderate positivity
3	Intense	Intense	Intense	Intense	Intense	Intense strong positivity

histochemical staining with GSTP1, Glut-red, SOD1, and CAT was performed with a Leica automated staining device. The histopathological effects were evaluated according to the following parameters: - Vascular congestion, - Lipidosis, - Sinusoidal congestion, - Sinusoidal dilation, - Inflammatory cell infiltration. Effects on all parameters are seen as: No effect: 0, Mild effect: 1. Significant effect: 2. Intense effect: 3. Immunohistochemical staining rates for GSTP1, SOD1, CAT, and Glut red were evaluated as: 0: No stain (0%), 1: Focal weak positive expression (1-25%), 2: Moderate positive expression (26-50%), 3: Intensive positive expression (51-100%), These data are shown in (**Table 1** and **Figure 3**).

Statistical analysis

The data were analyzed using SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., USA). The distributions of the variables were examined using the Kolmogorov Smirnov test. As some of the variables did not have a normal distribution, the Kruskal-Wallis test was used for comparisons between the groups. Descriptive statistics are given as the arithmetic mean ± the standard deviation and median (quarters). A *p*-value lower than 0.05 was considered statistically significant. One-way ANOVA tests were used to compare more than two variables, and differences between groups and total score variables were analyzed using two-way variance analysis. The Bonferroni correction was used for the differences between the groups.

Results

All results are presented according to the order specified in the method section.

Histopathological evaluation

Vascular congestion, lipidosis, sinusoidal congestion, sinusoidal dilation, and mononuclear filtration (**Figure 2**) were evaluated using the Mann-Whitney U test in the paired-group analysis. The histopathological change between Groups A, B, and C for each parameter was significant (P<0.05), and there was a statistical difference between all groups (**Table 2**).

The results of the group comparisons were: P=0.004 (Groups A-B), P=0.264 (Groups A-C), and P=0.020 (Groups B-C) for vascular congestion; P<0.001 (Groups A-B), P=0.025 (Groups A-C), and P=0.006 (Groups B-C) for lipidosis; P=0.001 (Groups A-B), P=0.102 (Groups A-C), and P=0.021 (Groups B-C) for sinusoidal congestion; P<0.001 (Groups A-B), P=0.018 (Groups A-C), and P=0.005 (Groups B-C) for sinusoidal dilation; and P<0.001 (Groups A-B), P=0.025 (Groups A-C), and P=0.003 (Groups B-C) for mononuclear filtration (see **Table 3**).

Immunohistochemical analysis

In terms of the total immunohistochemical score, the mean values were 0.87 in Group A, 7.25 in Group B, and 6.37 in Group C. The *p*-value was <0.001, indicating a significant difference (see **Table 4**). In the binary group analyses for the immunohistochemical score, although there was a significant difference between Groups A and B (P<0.001) and Groups A and C (P<0.001), there was no significant difference between Groups B and C (see **Table 5**). The mean values of the oxygen free radical scores with immunohistochemical for groups are shown (see **Table 6**).

Discussion

The use of chemotherapy drugs in cancer often leads to the interruption of treatment due to undesirable side effects. Therefore, new treatment strategies need to be developed that will minimize the side effects of chemotherapeutic agents. Cisplatin is an important antineoplastic agent used for many types of cancer

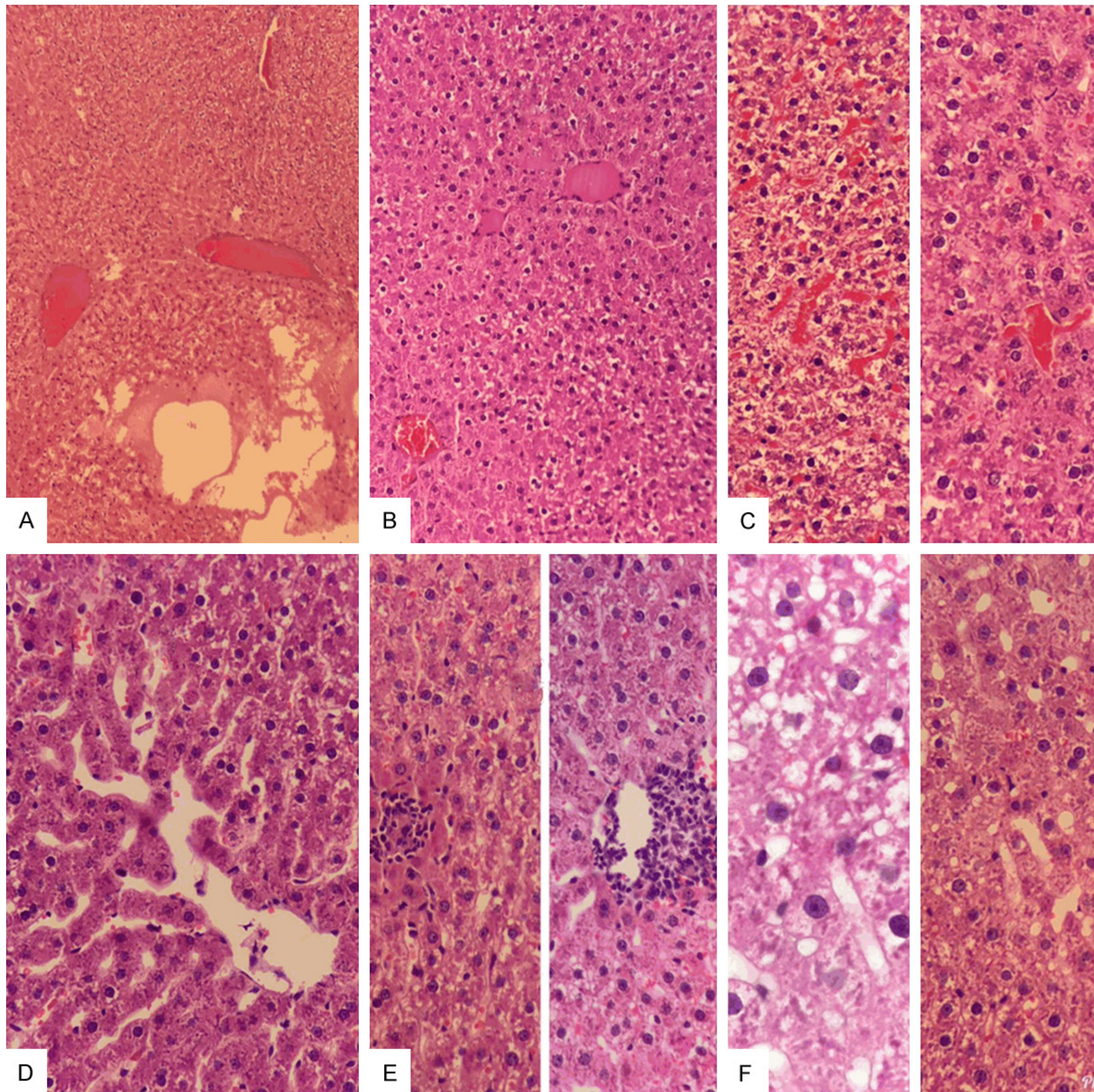

Figure 2. Histopathological effects of Pycnogenol. (A&B) Vascular congestive areas in the liver parenchyma (A, H&E × 200, B, H&E × 400) (Group B). (C) Intense sinusoidal congestion (H&E × 400) (Group B). (D) Intense sinusoidal dilation (H&E × 400) (Group B). (E) Mononuclear cell infiltration in the liver parenchyma (H&E × 400) (Group B). (F) Stellate cell lipidosis in perisinusoidal areas (H&E × 400) (Group B).

worldwide. In addition to being widely used, it also has serious toxic side-effects, such as hepatotoxicity, nephrotoxicity, and ototoxicity [15]. Although the mechanism behind the toxic effect of cisplatin is not fully known, it is thought to involve oxidative stress caused by the increase of free radicals or a decrease of antioxidant structures in the cells [16]. There are a limited number of studies that can explain the histopathology, change in cells, and ultrastructural position of cells caused by cisplatin. Cisplatin has been reported as having cytotoxic effects, especially at the cellular level through sinusoidal obstructions, hepatocellular edema, degeneration, necrosis, apoptosis, and inflammatory cell condensation [7].

Pycnogenol plays a protective role in many diseases associated with oxidative stress. Studies have shown that it protects biomolecules, primarily lipids, proteins, and DNAs (which are the main targets of oxidative damage) against oxidative damage by stabilizing intracellular antioxidant protection systems

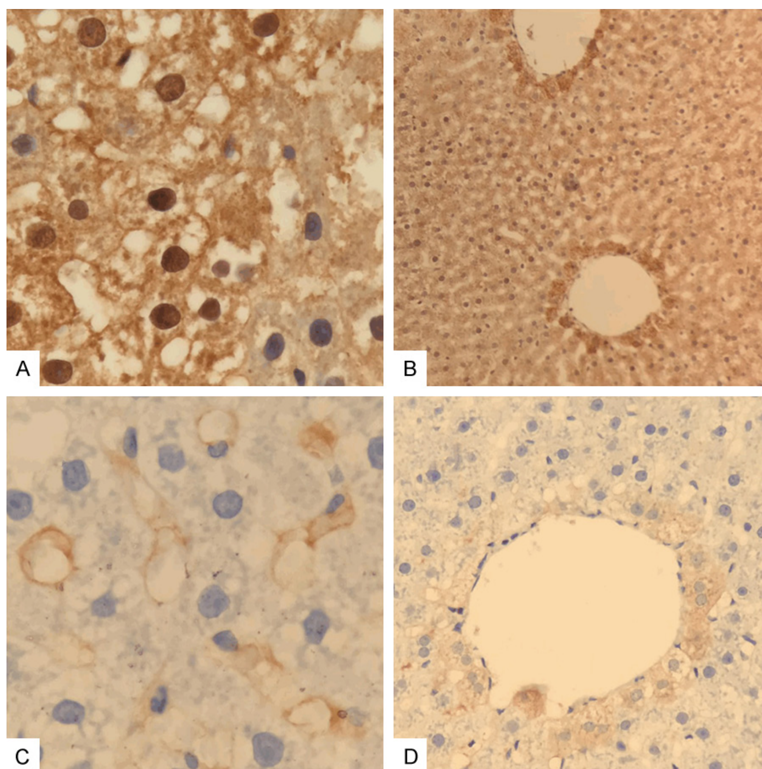

Figure 3. Immunohistochemical staining for GSTP1, SOD1, CAT, and Glut red. A. With SOD 1, intense positivity (Score 3) in liver parenchyma (IHK × 40) (Group C). B. With CAT, especially pericentral positivity with moderate severity (Score 2) (IHK × 400) (Group B). C. With GSTP 1, mild (Score 1) positive staining in the areas of perisinusoidal lipidosis (IHK × 400) (Group C). D. With Glut red, mild (Score 1) positive staining especially in pericentral areas (IHK × 400) (Group C).

[17]. For example, in one study, 25 healthy participants were given 150 mg/day of Pycnogenol for six weeks and their plasma polyphenol levels were found to have increased significantly after just three weeks of supplementation. In this study, the antioxidant potential of plasma was measured by the oxygen radical absorption capacity (ORAC) test, which showed an increase of 40% above the baseline ($P<0.05$). It was concluded that Pycnogenol significantly increased the antioxidant capacity of plasma [18].

The biological effects of Pycnogenol have been evaluated in several studies. For example, Norris et al. caused a traumatic brain injury to rats and then administered a 10 mg/kg Pycnogenol IV for 15 minutes after the brain injury. The results showed that synaptic function was protected in the hippocampus on the seventh and fourteenth day after the injury [19]. In another study, Eryilmaz et al. experimentally evaluated the effect of Pycnogenol on cisplatin-induced ototoxicity and found that it has a protective role against cisplatin ototoxicity and that it was especially protective against cisplatin-induced cochlear apoptosis [14].

In contrast, the effect of Pycnogenol on experimentally cisplatin-induced cardiotoxicity was studied and it was found that Pycnogenol had no effect on CAT activity in healthy tissue and that cisplatin did not cause CAT levels to decrease. When Pycnogenol and cisplatin were used together, however, they were found to trigger the antioxidant CAT level. S100A1 value which was found to be positively correlated with CAT level in the heart tissue was also increased after the Pycnogenol treatment. As expected, serum troponin I level was found to be high in the cisplatin group, and when Pycnogenol was added to the treatment plan, this value significantly decreased [20].

In a study evaluating renal toxicity conducted by Lee et al., which involved a repeated oral dose of Pycnogenol for 10 days before and after cisplatin application, it was found that even at much lower doses (10 and 20 mg/kg/day), Pycnogenol effectively inhibited renal-toxicity-induced serum biochemical changes. It was also found to inhibit cisplatin-induced histopathological changes and provided a protective effect against cisplatin-induced acute kidney damage [13]. Furthermore, Yang et al. used carbon tetrachloride to induce OS and hepatotoxicity and found that carbon tetrachloride significantly increased serum aspartate aminotransferase AST and ALT and caused a large hepatocellular degeneration/necrosis, inflammatory cell infiltration, and sinusoidal dilation. They also found that oral Pycnogenol reduced the hepatotoxic effect and oxidative damage in the subjects [21]. In addition to its antioxidant effects, we also showed the protective effects of Pycnogenol, which has many positive effects on liver damage due to cisplatin,

Table 2. Kruskal-Wallis results

	Group A Mean Rank	Group B Mean Rank	Group C Mean Rank	x^2	P
Vascular congestion	8.31	18.25	10.94	10.562	0.005*
Lipidosis	6.50	19.50	11.50	16.432	<0.001*
Sinusoidal congestion	7.13	18.75	11.63	12.396	0.002*
Sinusoidal dilation	6.06	19.63	11.81	16.516	<0.001*
Mononuclear filtration	6.50	19.75	11.25	17.114	<0.001*

**P*<0.05, the Kruskal Wallis analyses.

Table 3. Histopathological evaluation by group

Groups**	Vascular congestion	Lipidosis	Sinusoidal congestion	Sinusoidal dilation	Mononuclear filtration
Groups A-B	0.004*	0.000*	0.001*	0.000*	<0.001*
Groups A-C	0.264	0.025*	0.102	0.018*	0.025*
Groups B-C	0.020*	0.006*	0.021*	0.005*	0.003*

*P<0.05, post hoc pair-group analysis was performed using a Bonferroni correction. **Group A: sham group, Group B: control group, Group C: experimental group.

Table 4. Results of comparison with immunohistochemical scores

	Groups			F	P
	Group A	Group B	Group C		
Immunohistochemical score	0.87±0.35	7.25±1.98	6.37±2.19	32.231	<0.001*
	0-1	5-10	4-10		

*P<0.05, one-way ANOVA. Each immunohistochemical marker was individually scored, but the statistical evaluation was performed for each rat on the basis of the total score (SOD-1, CAT, GSTP-1, Glut Red). The statistical evaluation between the three groups was done using a one-way ANOVA.

both histopathologically and immunohistochemically.

Similarly, Taner et al. demonstrated the protective effects of Pycnogenol on sepsis-induced oxidative DNA damage in a sepsis model using rats. The rats that were given Pycnogenol showed significantly reduced MDA levels, while their GSH levels and SOD and GPx enzyme activities significantly increased. Pycnogenol treatment also significantly reduced their TNF-α levels. Furthermore, the septic-induced lymphocytes and DNA damage parameters in the kidney and liver tissue of the rats treated with Pycnogenol were found to be reduced compared to the rats not receiving Pycnogenol [22]. The changes in GSTP1, Glut red, SOD1, and CAT levels found in our immunohistochemical results are in line with the results of this study. In the study of the peritoneal adhesion model, its effect on tissue free oxygen radicals was also shown in our other study. These results are thought to be due to the reduction of free oxygen radicals, which occur after OS [22].

Similar to previous studies, we also performed GSTP-1, Glut red, SOD-1, and CAT staining in our study to examine OS parameters in tissue immunohistochemically [22-24]. As expected, cisplatin increased the level of free oxygen radicals in the tissue in our study, but Pycnogenol reduced the free oxygen radical level at a non-significant level in our study. As a result, in terms of the parameters and total scores, it was observed that the liver damage significantly decreased in the group using Pycnogenol. It was also observed that it decreased the free oxygen radicals. More studies are needed to examine this issue in the future.

Disclosure of conflict of interest

None.

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Table 5. Comparison between the groups with respect to the immunohistochemical scores

Groups***	Immunohistochemical score****
Group A-B**	<0.001*
Group A-C**	<0.001*
Group B-C**	0.963

*The mean difference is significant at the level of 0.15. **Post hoc pair-group analysis was performed using a Bonferroni correction. ***The statistical evaluation between the three groups was assessed with a one-way ANOVA. ****Each immunohistochemical marker was individually scored, but the statistical evaluation was performed for each rat on the basis of the total score (SOD-1, CAT, GSTP-1, Glut red).

Table 6. Mean values of free oxygen radicals with immunohistochemical

Groups	Free oxygen radicals with immunohistochemical****
Group A	Mean: 0.8750
	St. deviation: 0.35355
	Median: 1.000
Group B	Mean: 7.2500
	St. deviation: 1.98206
	Median: 7.000
Group C	Mean: 6.3750
	St. deviation: 2.19984
	Median: 6.000

****Each immunohistochemical marker was individually scored, but statistical evaluation was performed for each rat on the basis of the total score (SOD-1, CAT, GSTP-1, Glut red).

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