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Fertility protective effect of taxifolin in cisplatin-induced ovarian damage

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Abstract. – OBJECTIVE: The aim of our study was to investigate the protective effect of taxifolin on ovarian damage and reproductive dysfunction created by cisplatin administration.

MATERIALS AND METHODS: A total of 36 albino Wistar female adult rats were equally divided into 3 groups as cisplatin administered only (CIS), taxifolin+cisplatin (T+C) and healthy control group (HG). Taxifolin 50 mg/kg was administered orally by gavage in the T+C (n=12) group. In the HG (n=12) and CIS (n=12) groups, the same volume of distilled water as a solvent was orally administered. One hour after administration of taxifolin or distilled water, animals in the T+C and CIS groups were injected with cisplatin at a dose of 2.5 mg/kg intraperitoneally. This procedure was repeated once a day for 14 days. Six animals from each group were sacrificed on day 15, and their ovaries were removed for histopathological and biochemical analysis. Ovarian tissue malondialdehyde (MDA), total Glutathione (tGSH), Nuclear Factor-Kappa B (NF-kB), Tumor Necrosis Factor-α (TNF-α), Interleukin 1 beta (IL-1β), and Interleukin-6 (IL-6) levels were measured. The remaining animals (n=6 in each group) were kept in the laboratory with mature male rats for two months to breed.

RESULTS: CIS administration led to an increase in inflammatory molecules and membrane lipid peroxidation products, and decreased the synthesis of antioxidant molecules. Compared to the CIS group, the ovarian tissue MDA, NF-kB, TNF-α, IL-1β and IL-6 levels were found to be significantly decreased in the T+C group ($p<0.001$ for all comparisons). On the other hand, the tGSH levels of the T+C group were significantly higher than the CIS group ($p<0.001$).

Milder ovarian necrosis, fibrosis and follicle damage were detected in animals which were given taxifolin. Four out of the six rats (67%) treated with taxifolin gave birth within 27 days.

CONCLUSIONS: We demonstrated, for the first time, that taxifolin ameliorates cisplatin-induced ovarian injury by decreasing MDA and proinflammatory cytokines and increasing the antioxidant enzyme. The fact that more than half of the animals receiving taxifolin became pregnant suggests that the cytoprotective effect of taxifolin is strong enough to preserve fertility.

Key Words:
Taxifolin, Ovarian injury, Cisplatin, Antioxidant, Anti-inflammatory.

Introduction

Fertility preservation by freezing eggs or ovarian tissue is widely used in cancer patients undergoing chemo or radiotherapy. Despite the improvement in fertility preservation methods in patients receiving chemotherapy, cisplatin-induced ovarian injury continues to be an important problem in women of reproductive age. Cisplatin [cis-diamminedichloroplatinum (II)] was approved for use in cancer chemotherapy by Food and Drug Administration in 1978[1]. Cisplatin is one of the most potential and widely used drugs in the treatment of various solid cancers such as testicular, ovarian, head and neck, bladder, lung, cervical, and melanoma[2]. However, dose-related toxicity is known to be one of the natural factors limiting the administration of cisplatin. It has been document-

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ed that cisplatin has about 40 different toxicities[3]. Experimental studies have proven that cisplatin has nephrotoxic, ototoxic, neurotoxic, cardiotoxic and hepatotoxic side effects[4-8]. It has also been reported that cisplatin is toxic on the ovary[9].

The most widely accepted mechanism of cisplatin toxicity includes the generation of large amounts of reactive oxygen species (ROS) and induction of oxidative stress[10]. Kulhan et al[11] reported that cisplatin administration leads to oxidative damage in ovary of experimental animals. Infertility develops in 70% of animals which are given cisplatin[12]. In chemotherapy-induced ovarian damage, malondialdehyde (MDA), myeloperoxidase (MPO), nitric oxide (NO) levels increase, while total glutathione (tGSH), glutathione peroxidase (GPx) and superoxide dismutase (SOD) levels decrease. Cisplatin has been also shown to increase the level of proinflammatory cytokines, such as nuclear factor kappa-beta (NF-kB), tumor necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β) and interleukin-6 (IL-6) in ovarian tissue[13]. Therefore, it is thought that antioxidant and anti-inflammatory drugs can be used in the treatment of oxidative and inflammatory ovarian damage due to cisplatin-induced infertility.

Taxifolin (dihydroquercetin) is an important flavonoid. This compound has been isolated from various plants such as thistle, onion, and tamarind[14]. Bioactive form of taxifolin has been found to be effective against inflammation infection, oxidative stress, cardiovascular and liver diseases[15]. In a recent study, antioxidant role of taxifolin has been proven[16]. It has also been stated that the cytoprotective effect of taxifolin is due to its inhibition of excessive ROS production[17]. Apart from its antioxidant effects, taxifolin inhibits the excessive production of proinflammatory cytokines including NF-kB, TNF-α, IL-1β, IL-6[18]. The primary aim of this study was to investigate the possible protective effect of taxifolin on ovarian damage and reproductive dysfunction created by cisplatin administration. The possible protective effect of taxifolin in ovarian tissue damage due to cisplatin was evaluated biochemically and histopathologically.

Materials and Methods

Animals

A total of 36 albino Wistar female rats weighing between 260 and 275 grams were included in the experiment. All animals were obtained from Erzincan Binali Yildirim University Medical Experimental Application and Research Center. Before the experiment, the animals were kept in the laboratory room (22°C) with 12 hours of light and 12 hours of darkness. During this period, the rats were fed with animal feed and tap water. The protocols and procedures were approved by the local Animal Experimentation Ethics Committee of Erzincan Binali Yildirim University (Date: 07.02.2022, No: 01).

Chemicals

Thiopental sodium was obtained from I.E Ulagay (Istanbul, Turkey). Cisplatin was obtained from Ebewe Liba (Istanbul, Turkey) and Taxifolin from Evalar (Moscow, Russia).

Experimental Groups

The animals were divided into 3 groups as healthy control group (HG), cisplatin administered only (CIS), and taxifolin+ cisplatin (T+C) groups.

Experimental Procedure

For the implementation of the experiment, taxifolin 50 mg/kg was administered orally by gavage in the T+C (n=12) group. In the HG (n=12) and CIS only (n=12) groups, the same volume of distilled water as a solvent was orally administered. One hour after the administration of taxifolin or distilled water, animals in the T+C and CIS groups were injected with cisplatin at a dose of 2.5 mg/kg intraperitoneally (ip). This procedure was repeated once a day for 14 days. At the end of this period, six (n=6) rats from each group were killed with high-dose anesthesia (50 mg/kg thiopental sodium). Ovarian tissues underwent biochemical and histopathological examinations. The remaining animals (n=6 in each group) were kept in the same environment with mature male rats for two months to breed. The pregnant rats were moved into separate cages. Rats that did not give birth within two months were considered as irreversible ovarian damage.

Biochemical Analysis

Determination of MDA and tGSH in ovarian tissue

The determination of MDA in ovarian tissue is based on the method used by Ohkawa et al[19] which includes the spectrophotometric measurement of the absorbance of the pink complex formed by thiobarbituric acid (TBA) and MDA. tGSH measurement was made according to the method described by Sedlak and Lindsay[20].

Determination of NF-kB, TNF-α, IL-1β and IL-6 in ovarian tissue

We measured weight of samples, and after that we cut all the tissues, rapidly frozen with liquid nitrogen and homogenized by pestle and mortar; samples were then maintained at 2-8°C after melting. We added PBS (pH 7.4), 1/10 (w/v), in a vortex for 10 seconds, then we centrifuged for 20 min at 10,000 xg and collected the supernatants carefully. The levels of Tumor Necrosis Factor α (TNF-α; ng/L), Interleukin 1β (IL-1β pg/L) and Interleukin-6 (IL-6; ng/L) were measured using a commercial ELISA kit (supplied by Eastbiopharm Co. Ltd., China). Tissue-homogenat Nuclear Factor-Kappa B (NF-kB; pg/ml) concentrations were measured using rat-specific sandwich enzyme-linked immunosorbent assays (Rat Nuclear Factor Kappa B ELISA immunoassay kits, Sun-Red, Shangai, China).

Histopathological Examination

For histopathological evaluation, the ovary was fixed in 10% formalin. Following routine tissue follow-up, 5 μm sections were stained with Hematoxylin-Eosin (H&E) and evaluated with a light microscope (Olympus BX 51, Japan) by the pathologist who was unaware of the treatment protocol and photographed with a digital camera (Olympus, DP 71). The severity of histopathological damage in ovarian sections were scored between grades 0 to 3 (0 = depicts normal ovarian histology, 1 = depicts mild injury, 2 = depicts moderate injury, and 3 = depicts severe injury).

Statistical Analysis

Results are expressed as Median (Min-Max) and Mean±SEM. The significance of the difference between groups was determined using the one-way ANOVA test. Then Fisher's post-hoc LSD test (least significant differences) was made. All statistical evaluations were performed in SPSS for Windows, 18.0 statistical program (Chicago, IL, USA) and $p<0.05$ was accepted to be statistically significant.

Results

Biochemical Findings

A complete comparison of biochemical parameters obtained from ovarian samples was presented in Table I. Compared to the healthy group, ovarian tissue MDA, NF-kB, TNF-α, IL-1β and IL-6 levels were found to be significantly increased in the CIS group ($p<0.001$ for all comparisons). However, tGSH levels of the CIS group were significantly lower than the healthy group ($p<0.001$) (Figure 1). CIS application led to an increase in proinflammatory molecules and membrane lipid peroxidation products. On the contrary, concentrations of antioxidant molecules were decreased. Compared to the CIS group, the MDA, NF-kB, TNF-α, IL-1β and IL-6 levels were found to be significantly decreased in the T+C group ($p<0.001$ for all comparisons) (Figures 2 and 3). The tGSH levels of the T+C group were significantly higher than the CIS group ($p<0.001$). Adding taxifolin to CIS activated antioxidant pathways while inhibited the synthesis of anti-inflammatory and lipid peroxidation products. Ovarian tissue inflammatory and antioxidant levels of the T+C group and HG group were found to be similar. Adding taxifolin to the CIS ensured that the inflammatory and antioxidant pathways remained functionally active, similar to healthy controls.

Histopathological Findings

Figure 4 shows the normal structure of healthy ovary. Severe follicular damage (grade 3), increase in cortical connective tissue (grade 3), degeneration in follicular cells (grade 3) and moderate necrosis (grade 2) were observed in the animals treated with cisplatin alone (Figure 5). In the T+C group, a mild connective tissue increase (grade 1), a slight decrease in the number of primordial follicles (grade 1), slight increase in the number of atretic follicles (grade 1) and moderate degeneration of follicular cells (grade 2) were observed (Figure 6). Table II shows histopathological findings of each experimental group.

Fertility Outcome Findings

As shown in Table III, all the rats belonging to the healthy group gave birth within 24 days. However, female rats treated with cisplatin alone did not give birth within two months. Four out of the six rats treated with taxifolin gave birth within 27 days, but two of them did not.

Discussion

Epithelial ovarian cancer is a common malignancy with a high mortality rate, resulting in death within five years in half of the cases. Debulking surgery and platinum-based chemo-

Table I. The statistical values of the biochemical findings of the ovarian tissue.

Groups	MDA (umol/g protein)	tGSH (nmol/g protein)	NF-kB (pg/ml)	TNF-α (ng/l)	IL-1β (pg/ml)	IL-6 (ng/l)
HG	5.05 (4.45-5.23) 4.93±0.13	7.42 (7.10-7.65) 7.39±0.09	2.60 (2.33-2.88) 2.58±0.09	1.83 (1.52-1.96) 1.78±0.06	3.25 (3.12-3.44) 3.26±0.05	2.16 (2.00-2.29) 2.16±0.04
***p*-value**	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
CIS	8.22 (8.16-8.37) 8.25±0.03	3.32 (3.15-3.62) 3.34±0.06	4.15 (4.10-4.89) 4.26±0.12	4.18 (4.00-4.28) 4.16±0.04	5.90 (5.68-6.48) 5.95±0.11	4.56 (4.36-4.69) 4.54±0.04
***p*-value**	-	-	-	-	-	-
T+C	5.49 (5.10-6.12) 5.52±0.15	6.62 (6.42-6.80) 6.60±0.05	2.73 (2.49-2.85) 2.71±0.05	2.16 (2.00-2.21) 2.14±0.03	3.50 (3.27-3.75) 3.49±0.06	2.45 (2.34-2.61) 2.46±0.04
***p*-value**	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

HG: Healthy group; CIS: Cisplatin group; T+C: Taxifolin+Cisplatin group.
Results are expressed as Median (Min-Max) and Mean±SEM, the *p*-values of post-hoc comparisons for variables compared with CIS group. OneWay ANOVA test was used.

therapy are the gold standard in the treatment of ovarian cancers. Despite surgery and chemotherapy, women suffering ovarian cancer succumb to chemoresistance. Neurotoxicity or nephrotoxicity is the most common reason for discontinuation. Additional treatments have been proposed to prevent cisplatin-induced nephrototoxicity. To minimize the side effects of cisplatin, immunomodulatory, anti-inflammatory and antioxidant applications have been used[21]. Unlike other types of cancer, additional treatments have not been very effective in ovarian cancer. Results of early clinical studies[22-25] reported that antioxidant and anti-inflammatory agents reduce cisplatin-related side effects. In the last decade, anti-inflammatory and antioxidant agents have started to be tested in combination with cisplatin in order to increase the clinical efficacy of cisplatin and reduce its toxic effects[8,26,27]. We evaluated the impact of taxifolin on cisplatin-induced ovarian damage in rat model of ovarian injury.

Although not as vital as renal toxicity, ovarian toxicity due to cisplatin is a serious concern in women of reproductive age[9,28]. This anxiety is one of the factors that prevents the use of the drug. Taxifolin is a bioactive flavonoid with antioxidant and anti-inflammatory effect in living cells[15,16]. By cross-linking DNA of tumor cells cisplatin inhibits tumor cell mitosis[29]. However, the cytotoxic side effect of cisplatin on healthy cells develops due to excessive ROS production[12,13]. Increased synthesis of proinflammatory

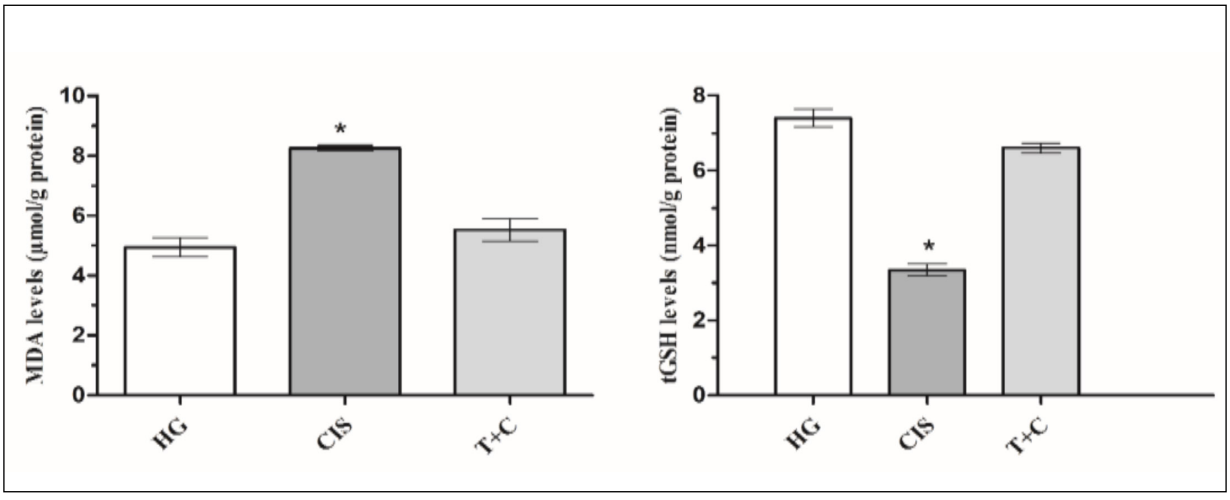

Figure 1. MDA and tGSH levels in the over tissue (MDA: Malondialdehyde, tGSH: Total glutathione, HG: Healthy Control, CIS: Cisplatin administered only, T+C: Taxifolin+Cisplatin groups). CIS group compared to HG and T+C, *means $p<0.05$.

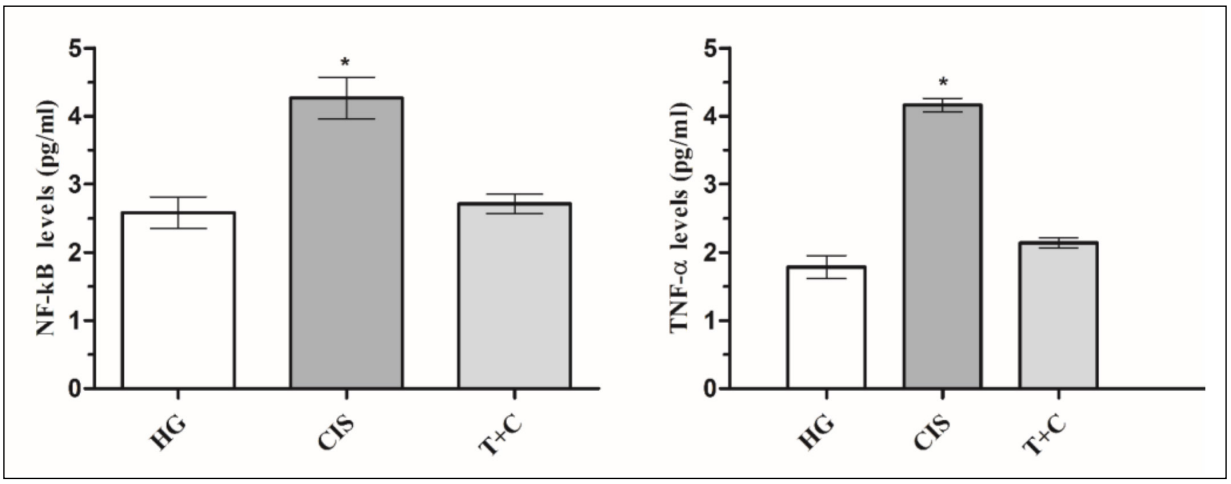

Figure 2. NF-kB and TNF-α levels in the over tissue (NF-kB: Nuclearfactorkappa-beta, TNF-α: Tumor necrosis factor-alpha, HG: Healthy Control, CIS: Cisplatin administered only, T+C: Taxifolin+Cisplatin groups). CIS group compared to HG and T+C, *means $p<0.05$.

cytokines such as TNF-α, IL-1β, and IL-6[12,13] also contribute to the damage of non-tumoral cells. The increase in proinflammatory cytokines activates the NF-kB pathway. The inhibited NF-kB-Ikb complex in the cytoplasm becomes active with proinflammatory cytokines and goes to the nucleus, stimulating target genes and initiating inflammatory events[30]. Inflammatory damage causes lipid peroxidation and stimulates MDA secretion. It also increases the synthesis and release of antioxidant enzymes such as GSH. Taxifolin not only blocks the inflammatory pathways activated by cisplatin, but also stimulates antioxidant pathways[17,18].

In the current study, we investigated the ameliorative effect of taxifolin against cisplatin-induced ovarian toxicity. 2.5 mg/kg/day cisplatin administration induced ovarian damage in animals. 50 mg/kg/day oral taxifolin significantly reduced the lipid peroxidation products including MDA. Decline in MDA indicates that membrane integrity is maintained by taxifolin. In line with this, previous studies[8,26] have shown that taxifolin reduces MDA by preventing membrane destruction. Taxifolin may exert its membrane protective effect by reducing proinflammatory cytokine synthesis. The decrease in inflammatory cytokine levels in taxifolin group supports

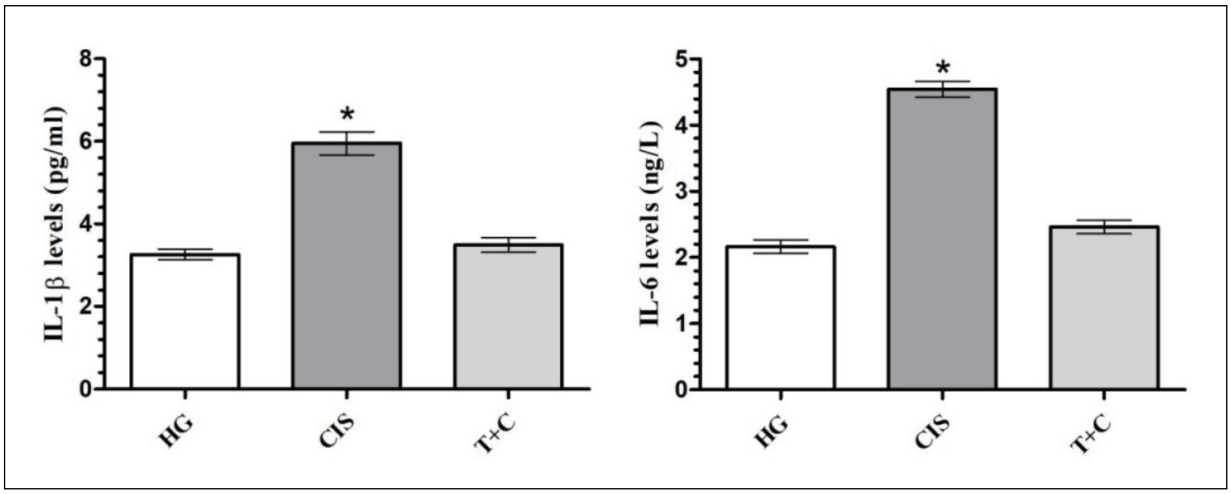

Figure 3. IL-1β and IL-6 levels in the over tissue. (IL-1β: Interleukin 1-beta, IL-6: Interleukin-6, HG: Healthy Control, CIS: Cisplatin administered only, T+C: Taxifolin+Cisplatin groups). CIS group compared to HG and T+C, *means $p<.05$.

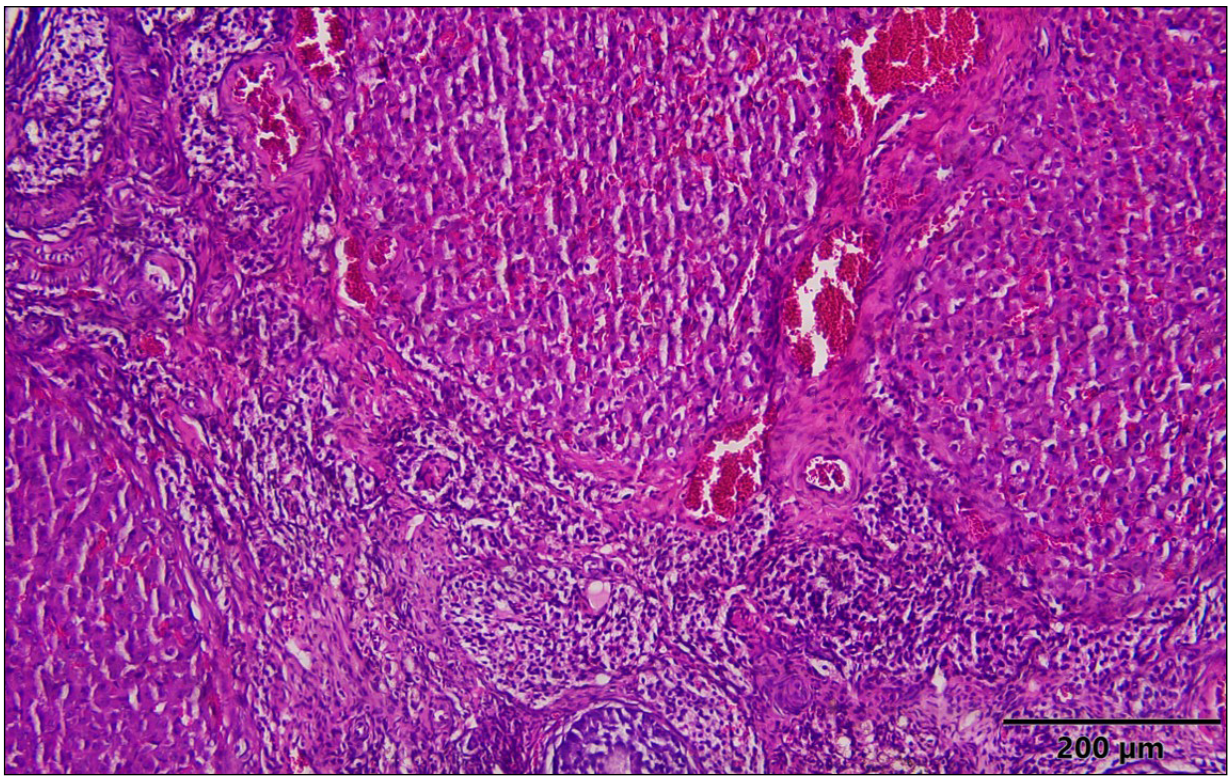

Figure 4. The normal structure of healthy ovarian tissue was observed.

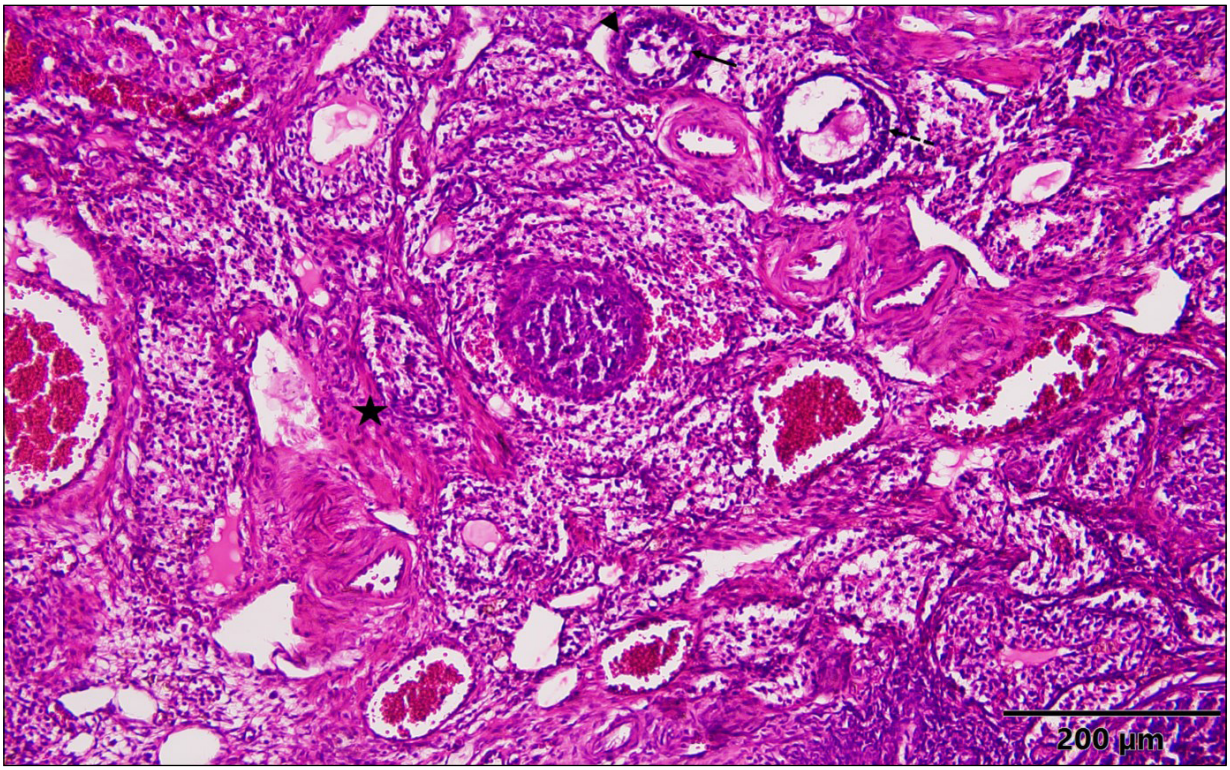

Figure 5. Severe deterioration in follicular cell integrity in the CIS group ovarian tissue (arrow), increase in connective tissue in the cortex (fibrosis) (star) and severe degeneration of follicular cells (dashed arrow) and moderate necrosis (arrow head) were observed.

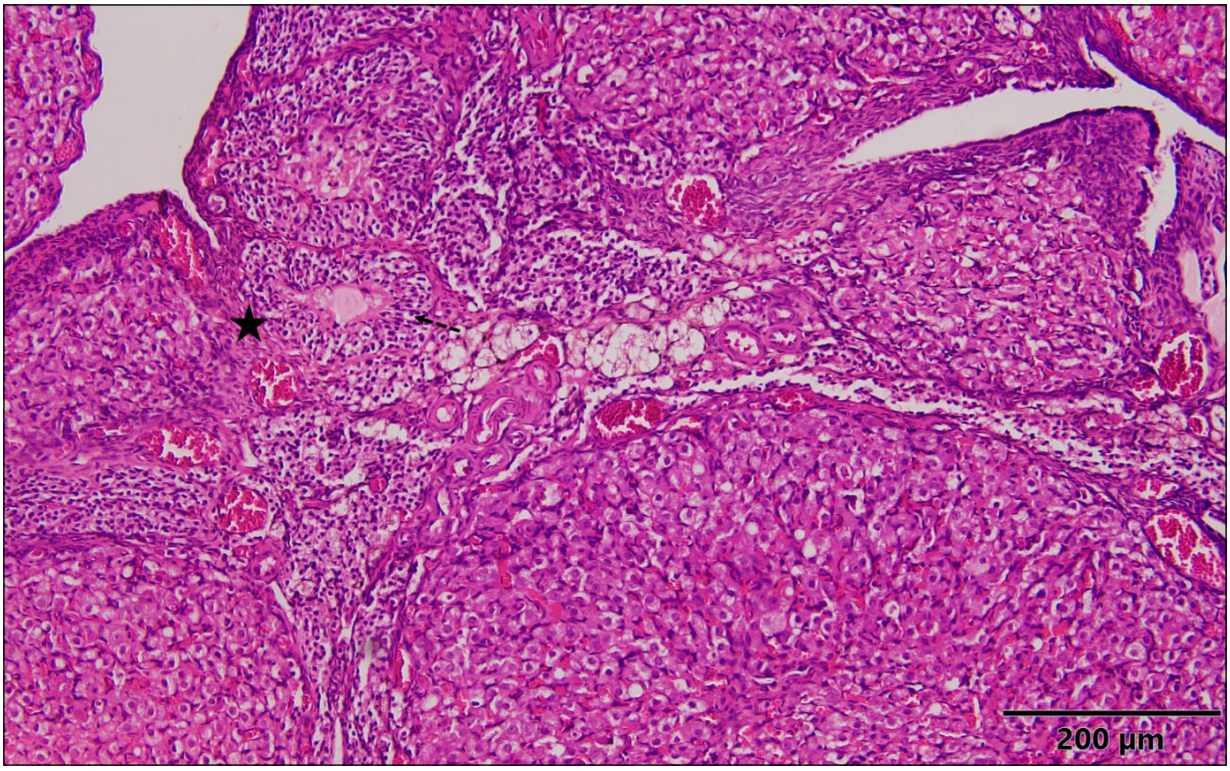

Figure 6. In the ovarian tissue of the T+C group, an increase in mild connective tissue in the cortex (star), a decrease in primordial follicles and an increase in atretic follicular cells, and moderate degeneration of follicular cells (dashed arrow) were observed.

this idea. The significant decrease in NF-kB, TNF-α, IL-1β and IL-6 levels in taxifolin group indicates that taxifolin protects cell membrane via its anti-inflammatory properties[18,22,23]. Another membrane protective effect of taxifolin may be realized with its antioxidant stimulant effect. Consistent with this, tGSH levels increased significantly in animals treated with cisplatin plus taxifolin compared to the group given only cisplatin. Increasing tGSH removes ROS from the environment and weakens cell wall destruction. Previous studies[8,26] have shown that taxifolin caused an increase in tGHS levels. Decreased necrosis, fibrosis and follicle damage in animals given taxifolin suggests that this flavonoid has cytoprotective effects. Consistent with this, the fact that 4 out of 6 rats (67%) in the taxifolin group became pregnant within 27 days is important evidence of the efficacy of taxifolin in preventing follicle damage.

Table II. The statistical values of the histopathological scoring findings of the over tissue.

Groups	Deterioration of follicle cell integrity	Connective tissue increase in the ovarian cortex	Follicular degeneration	Follicular necrosis
HG	0 (0-0)^{a}	0 (0-0)^{a}	0 (0-0)^{a}	0 (0-0)^{a}
	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
CIS	3 (3-3)^{b}	3 (2-3)^{b}	3 (3-3)^{b}	2 (1-3)^{b}
	3.00±0.00	2.83±0.16	3.00±0.00	3 (3-3)^{b}
T+C	1 (1-1)^{ab}	1 (1-1)^{ab}	2 (1-3)^{b}	0 (0-0)^{a}
	1.00±0.00	1.00±0.00	2.00±0.36	0.00±0.00

^{a,b}Groups marked with the same letter are statistically similar, but there is a statistically significant difference at the level of $p<0.05$ between groups with different letters. Results are expressed as Median (Min-Max) and Mean±SEM.
HG: Healthy group; CIS: Cisplatin group; T+C: Taxifolin+Cisplatin group.

Table III. Fertility outcome results of each group.

Groups	Giving birth rats (n)	(%)	Non-breeding rats (n)	(%)	Conception time (day)
HG (n=6)	6	100	-	-	24
CIS (n=6)	-	-	6	100	-
T+C (n=6)	4	67	2	33	27

HG: Healthy group; CIS: Cisplatin group; T+C: Taxifolin+cisplatin group; n: number of animals.

Conclusions

This study demonstrates, for the first time, that taxifolin ameliorates cisplatin-induced ovarian injury *via* decreasing MDA and proinflammatory cytokines and increasing the antioxidant enzymes. The fact that more than half of the rats receiving taxifolin became pregnant suggests that the cytoprotective effect of taxifolin is strong enough to preserve fertility.

Conflict of Interest
The authors declare that they have no conflict of interests.

Ethics Approval
The protocols and procedures were approved by the local Animal Experimentation Ethics Committee of Erzincan Binali Yildirim University (Date: 07.02.2022, No: 01).

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Contributions
Experimental procedures were performed by RO, ZS, FA, SB, CS, KD and HS. Scientific data for writing and discussion were collected by NC, FC, ZK, NG. All authors contributed to statistical analysis. The first draft was written by RO. All authors approved the final version of the manuscript.

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