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Effect of *Astragalus microcephalus* wild extract and Vitamin E–Selenium combination on Cadmium–induced damage in rat ovaries

Efectos del extracto de *Astragalus microcephalus* y la combinación de vitamina E y Selenio sobre el daño tisular inducido por Cadmio en ovarios de rata

***Begum Kurt*[1], *Haki Kara*[2]*, *Mahmut Sahın*[2], *Alper Serhat Kumru*[2], *Mustafa Ozkaraca*[3]**

[1]*Sivas Cumhuriyet University, Faculty of Medicine, Department of Obstetrics and Gynecology. Sivas,Türkiye.*
[2]*Sivas Cumhuriyet University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology. Sivas,Türkiye.*
[3]*Sivas Cumhuriyet University, Faculty of Veterinary Medicine, Department of Pathology. Sivas,Türkiye*
**Corresponding author: hakikara@cumhuriyet.edu.tr*

ABSTRACT

This study was conducted to investigate the protective effect of Astragalus (AST) extract alone and in combination with vitamin E (vit E) + selenium (Se) against the toxicity induced by cadmium ($CdCl_2$) in rat ovaries. Thirty–six female Wistar rats were divided into six groups. AST was administered at a dose of 5 mg·kg^{-1}, Cd at a dose of 2 mg·kg^{-1}, and Vit E (60 mg·kg^{-1}) + Se (1 mg·kg^{-1}) orally for a duration of 15 days. The levels of MDA, GSH–Px, SOD, and CAT were analyzed in the blood and tissue samples to assess oxidative stress. Additionally, the levels of estrogen, FSH, LH, Inhibin B, and Antimullerian hormones were measured in the serum samples. The ovarian tissues were examined histopathologically and immunohistochemically for 8–OhDG, Caspase 3, and LC3B immunoreactivity. In the group exposed to $CdCl_2$, MDA levels significantly increased, while antioxidant parameters showed significant decreases ($P<0.05$). Although significant improvements were observed in the groups treated with AST alone, more significant improvements were seen in the groups treated with both AST and Vit E + Se ($P<0.05$). It was concluded that AST extracts alone and in combination with Vit E + Se exhibited protective effects against ovarian toxicity caused by Cd exposure and may be effective against metal toxicity.

Key words: Cadmium; ovarium damage; Astragalus; oxidative stress

RESUMEN

Este estudio se realizó para investigar el efecto protector del extracto de astrágalo (AST) solo y en combinación con vitamina E (vit E) + selenio (Se) contra la toxicidad inducida por cadmio ($CdCl_2$) en ovarios de rata. Treinta y seis ratas Wistar hembra se dividieron en seis grupos. Se administró AST a una dosis de 5 mg·kg^{-1}, Cd a una dosis de 2 mg·kg^{-1} y Vit E (60 mg·kg^{-1}) + Se (1 mg·kg^{-1}) por vía oral durante 15 días. Se analizaron los niveles de MDA, GSH–Px, SOD y CAT en muestras de sangre y tejido para evaluar el estrés oxidativo. Además, en las muestras de suero se midieron los niveles de estrógeno, FSH, LH, inhibina B y hormonas antimullerianas. Los tejidos ováricos se examinaron histopatológica e inmunohistoquímicamente para determinar la inmunorreactividad de 8–OhDG, caspasa 3 y LC3B. En el grupo expuesto a $CdCl_2$, los niveles de MDA aumentaron significativamente, mientras que los parámetros antioxidantes mostraron disminuciones significativas ($P<0,05$). Aunque se observaron mejoras significativas en los grupos tratados con AST sola, se observaron mejoras más significativas en los grupos tratados tanto con AST como con Vit E + Se ($P<0,05$). Se concluyó que los extractos de AST solos y en combinación con Vit E + Se exhibieron efectos protectores contra la toxicidad ovárica causada por la exposición al Cd y pueden ser eficaces contra la toxicidad de los metales.

Palabras clave: Cadmio, daño ovárico, astrágalo, estrés oxidativo

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INTRODUCTION

For the survival and growth of species, reproduction is seen as essential [1]. The reproductive system influences the organism's behavior and controls the morphological evolution and physiological distinctions between males and females. Exposure to hazardous compounds can cause teratogenic, carcinogenic, and mutagenic consequences, infertility, tissue damage, oogenesis deficits, and other reproductive diseases [1, 2].

Due to increased industrial and agricultural activities, metal-induced environmental pollution and contamination of heavy metals in the food chain are rising. Toxic mechanisms can occur as ion-like effects, disruption of cellular signaling pathways, oxidative stress, disruption of gene structure, apoptosis, inflammation, and impaired endocrine metabolism [3]. Cadmium (Cd), which is found among heavy metals, is a highly toxic element. The increase in its quantity in soil and water due to intensive industrial activities, consumption of foods grown in contaminated areas, consumption of contaminated animal products, and consumption of tobacco products with high Cd content can pose significant risks to human health [4, 5]. Due to the effects of Cd toxicity on the body's major organs, it is considered one of the most toxic compounds to human health [5].

Cadmium entering the body is associated with Metallothionein (MT) protein. MT is known to be responsible for both the transport and detoxification of cadmium in organ tissues. Due to its low elimination rate, cadmium accumulation in the liver and kidneys increases cadmium toxicity in long-term exposures. Cadmium disrupts apoptotic mechanisms by causing the formation of reactive oxygen species (ROS) in the kidney, liver, lungs, brain, bone tissue, blood components, testes, and ovaries, leading to cellular and DNA damage. As a result, it can lead to cancer [6]. In cadmium-induced ovarian dysfunction, the increase in ROS, changes in gene expression, DNA damage, apoptosis, and increased membrane lipid peroxidation occur due to oxidative stress [7, 8].

Cadmium chloride ($CdCl_2$) is a potential endocrine disruptor [9]. It has been reported that exposure to Cd can cause reproductive and developmental disorders, especially in embryonic and young animals and humans [10]. $CdCl_2$ can cause damage to both male and female reproductive organs. It induces histopathological disorders, disturbances in spermatogenesis, decreases testosterone levels and has carcinogenic effects on the testes. At the same time, in female reproductive organs, it causes histopathological disorders, delayed pubertal disorders, prolonged estrus periods, and oxidative stress [2]. It also increases enzyme activity in granulosa cells of the ovaries, decreases gonadotropin binding, and decreases serum progesterone and estradiol levels [8, 10, 11].

The formation of ROS and disruption of fundamental molecular mechanisms through oxidative stress, which is associated with mitochondrial damage, has been reported to play a significant role in Cd toxicity, involving both caspase-dependent and caspase-independent apoptotic pathways [12, 13].

In addition to classical chelating agents, certain antioxidants such as vitamin E, Selenium, and melatonin have been successful against Cd toxicity [14]. Recently, extracts obtained from medicinal and aromatic plants have started to be used in health. The beneficial effects of flavonoids found in plant extracts, in particular, have attracted the attention of an increasing number of researchers. Some previous studies have indicated the protective effects of herbal and natural substances such as quercetin, tualang honey, and Hibiscus sabdariffa extract against ovarian toxicity [15, 16, 17].

This study examines the effectiveness of *Astragalus microcephalus* wild extract, known for its potent antioxidant properties, against the toxic effects of cadmium in the ovaries. Astragalus (AST) species are plants that can be found in countries with temperate climates around the world and have approximately 2,000 different species [18]. The root parts are more commonly used [19]. It is stated that it has been used in China for about 2,000 years [20]. Astragalus tea and capsules are sold as over-the-counter dietary supplements in the US health food market [21]. AST species have applications as food additives and nutritional supplements in many countries worldwide.

The active ingredients found in Astragalus species include saponins, flavonoids, polysaccharides, and trace elements such as selenium, copper, zinc, iron, and volatile fatty acids. It has also been reported to have a high selenium retention capacity [21, 22].

The main pharmacological effects of Astragalus polysaccharides include anticancer, antiaging, antiviral, antibacterial, immune system regulatory, blood sugar level-regulating, lipid-lowering, radiation-protective effects, and antioxidant properties with very low toxicity [17, 22, 23, 24, 25]. It is stated that AST exhibits a strong antioxidant effect, reduces lipid peroxidation, increases superoxide dismutase activity, decreases malondialdehyde (MDA) production, and exhibits protective and anti-aging properties [26].

AST species have been reported to increase the proliferation of T and B lymphocytes, increase cytokine production, activate macrophages and B cells, increase the expression of IL2, IL3, IL4, IFNy, IgM, and IgG, and decrease IgE levels [23, 24, 25, 27].

This study aims to determine the protective effect of AST extract, which has a substantial antioxidant property, against the potential damage caused by cadmium in the ovaries. Additionally, the study aims to determine the feasibility of using AST alone or combined with Selenium and vitamin E in treatment by performing applications with AST, Selenium, and vitamin E combinations.

MATERIALS AND METHODS

The chemicals used in the study were of analytical purity, cadmium chloride ($CdCl_2$),vitamin E (α-tocopherol), and selenium (sodium selenite) were purchased. (Sigma, St.Louis, MO, USA).

Experimental animals

This study used 36 Wistar female rats (*Rattus norvegicus*)(average live weight 220-250 g). Animals were obtained from Sivas Cumhuriyet University Experimental Animals Unit, and the study was carried out in the same place. The rats were housed in 12 hours of light and 12 hours of darkness during the trial, and all guidelines for animal care were observed. The rats received unlimited amounts of food and water. Sivas Cumhuriyet University Animal Experiments Local Ethics Committee granted clearance for this investigation with its letter dated April 20, 2021, and number 538.

While forming the groups, a total of 36 female rats were used in 6 groups, with six animals in each group. The application period was determined as 15 days in all groups.

Application groups were created as follows:

1. Control group (saline i.p.)

2. Astragalus (AST) (5 mg·kg^{-1} oral gavage)
3. Cadmium chloride ($CdCl_2$) (2 mg·kg^{-1}·day^{-1} i.p.)
4. $CdCl_2$ + AST (2 mg·kg^{-1}·day^{-1} i.p. + 5 mg·kg^{-1} oral gavage)
5. $CdCl_2$ + Vitamin E + Selenium (2 mg·kg^{-1}·day^{-1} i.p. + Vit E 60 mg·kg^{-1}·day^{-1} + Se 1 mg·kg^{-1}·day^{-1} oral gavage)
6. $CdCl_2$+Vitamin E+Selenium+AST (2 mg·kg^{-1}·day^{-1} i.p.+ Vit E 60 mg·kg^{-1}·day^{-1} + Se 1 mg·kg^{-1}·day^{-1} oral gavage + 5 mg·kg^{-1} oral gavage)

At the end of the application, the rats were euthaniased under ketamine/xylazine (90/10 mg·kg^{-1}, ip.) anesthesia. The blood taken from the animals was centrifuged (Nuve, NF800, Turkey) and separated for hormone analysis and other biochemical analyzes and kept at -18 C° (Arçelik, 2350E, Turkey). The ovaries of the rats were removed, and one was separated for biochemical analysis, while the other was preserved in a 10% formaldehyde solution until histopathological examination.

Plant extraction

The wild plant *Astragalus microcephalus* used in this study was obtained from the rural area of Sivas/Türkiye. The identification of the collected plants was made by Prof. Dr. H. Aşkın AKPULAT, a faculty member of the Biology Department of the Faculty of Science, Sivas Cumhuriyet University. The root parts of the collected events were dried and ground. Then, 1 g of plant was extracted with 10 mL of ethanol (10:1 mL extraction solvent·g^{-1} herb) for 24 hours. After the extraction, it was centrifuged (Nuve, NF800, Turkey) at 3075 G for 10 min, passed through the evaporator (IKA, CMF300, People's Republic of China).

Biochemical assay

Rats were euthanasied, blood and the ovarian tissues collected. Ovarium tissues were immediately removed under ice–cold conditions, blotted free of blood and tissue fluids, weighed, and kept at -80 C°. (Antech, Eco Toch, Turkey).

Determination of MDA level

Lipid peroxidation was measured in terms of malondialdehyde (MDA) as specified by Ohkawa *et al*. [28]. Thiobarbituric acid reactive substances (TBARS), which are produced when MDA and thiobarbituric acid react, were used to measure the MDA level in ovarian tissue homogenate. Thiobarbituric acid and MDA reacted aerobically to produce the MDA–(TBA) 2 complex, which was detected at 532 nm using a spectrophotometer (Perkin Elmer, Lambda365, USA). For serum, MDA concentration (TBARS) was estimated as "nmol·ml^{-1}," and for ovarium tissues, "nmol·mg^{-1} protein." Standard solutions of 1, 1, 3, and 3–tetra ethoxy–propane were used to compare absorbance readings (TEP).

Measurement of SOD activity

A commercially available standard enzymatic kit (Cayman Chemical Company, Ann Arbor, MI) that uses a tetrazolium salt for the detection of superoxide radicals produced by xanthine oxidase and hypoxanthine was used to measure superoxide dismutase (SOD). All three forms of SOD are measured by the SOD assay. The quantity of enzyme required to demonstrate a 50% dismutation of the superoxide radical is referred to as one unit of SOD activity. Using a plate reader, the absorbance was read between 440 and 460 nm (Thermo, Multiskan GOMicroplate, Massachusetts, USA). "U·mL^{-1}" was used to express the SOD activity.

Measurement of GSH–Px activity

Glutathione peroxidase enzyme levels in blood and ovarian tissues were measured with a commercial assay kit (Cayman Chemical Company, Ann Arbor, Michigan,USA). Glutathione peroxidase is an important antioxidant enzyme that helps protect cells from damage caused by reactive oxygen species (ROS).

Measurement of CAT activity

Catalase enzyme activities (CAT) in blood and ovarian tissues were determined with Catalase Colorimetrik Acitivity Kit (ThermoFischer Scientific, cat no EIACATC, USA).

Histopathological Examination

Rats were necropsied, and the ovarian tissues were preserved in a buffered formalin solution at a 10% concentration. The samples were subsequently fixed in paraffin blocks and subjected to standard follow–up procedures. Hematoxylin–Eosin was used to analyze the 5 µm slices from the blocks to the slides under a light microscope for histopathological results. Semiquantitative ratings of the histopathological findings included absent (0), mild (1), moderate (2), and severe (3).

Immunohistochemical Examination

After washing with phosphate buffer solution (PBS) and xylol and alcohol series on 5 µm slices on polyzed slides, endogenous peroxidase inactivation is made sure of by soaking them in 3 percent H_2O_2 for 10 min. Tissues were exposed to antigen retrieval solution for 2×5 min at 500 watts in order to reveal the antigen within them. After protein blocking, tissues were rinsed with PBS before being treated with 8–OhDG, Caspase 3 (Biorbyte, Cat. No. Orb382909, 1/100 dilution ratio), and LC3B (Santa Cruz, Cat. No. sc–271625, 1/300 dilution ratio) before being incubated with primary antibodies overnight at +4 C°. In addition, the manufacturer's advice was followed when using the Large Volume Detection System: anti–Polyvalent, HRP (Thermo Fisher, Catalog no: TP–125–HL). As the chromogen, DAB (3,3–Diaminobenzidine) was employed. It was coated with Stellan, counterstained with Mayer's Hematoxylin, and then viewed under a light microscope (Olympus, IX70, Tokyo, Japan). The analysis was semiquantitative and classified the immunopositivity in the ovarian tissues as absence (0), mild (1), moderate (2), severe (3), and very severe (4).

Hormone analysis

Anti–Mullerian Hormone (AMH), Estradiol 2 (E2), Folicul Stymulan Hormone (FSH), Inhibin B (INH B), and Luteinizing (LH) hormone levels were determined using the ELISA method following the manufacturer's manual (BT Lab, China). The manufacturer's catalog numbers are given. Rat Anti–Mullerian Hormone (AMH Elısa kit Cat. No: EA0083Ra), Rat Estradiol (E2 ELISA Kit Cat.No: EA0011Ra), Rat Follicle–stimulating Hormone (FSH ELISA Kit Cat.No: EA0015Ra), Rat Inhibin B (INHB ELISA Kit Cat.No: EA0059Ra).

Statistical analysis

The SPSS version 20.00 application was used to analyze the data that was obtained. The Kruskal Wallis test, one of the non–parametric tests, and the Mann–Whitney U test for the group that caused the difference were used to determine the difference between the groups. ($P<0.05$). *P*–value less than 0.05 was considered statistically significant.

RESULT AND DISCUSSION

Hematoxylin–Eosin staining findings

Between the groups, a statistically significant difference was found (TABLE I, $P<0,05$). Rats in the control and AST groups' ovarian tissues displayed a typical histological appearance. Degeneration and edema in the stromal tissue of the interstitial zones were the identified histological findings. While these histological results were moderate in the Cd+AST and Cd+Vit E–Se groups and mild in the Cd+Vit E–Se+AST group, they were severe in the Cd group (FIG. 1).

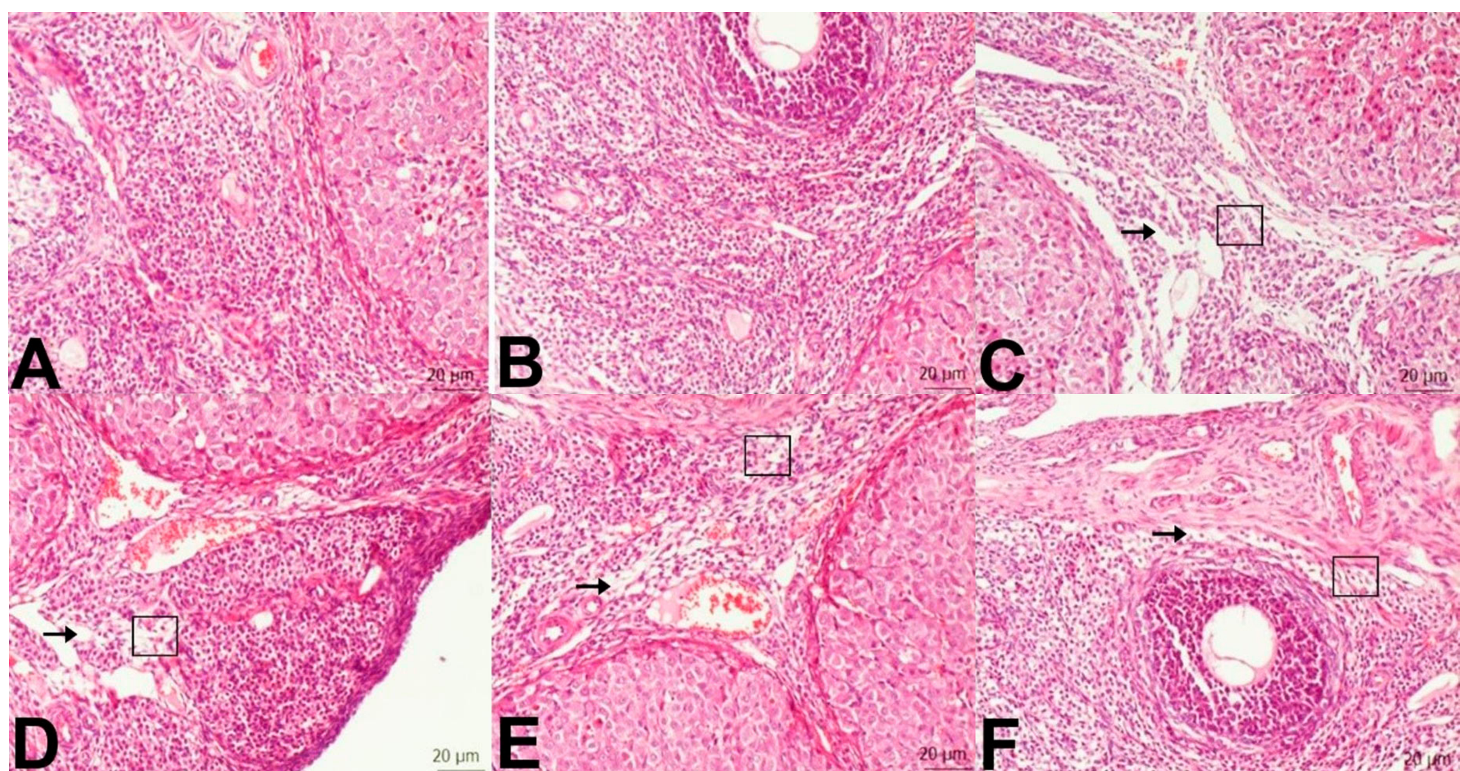

FIGURE 1. Ovarium stromal degenerasyon and edeme levels. A– Control group and B– Astragalus group. Normal histological appearance, C– Cd group. Severe level, D– Cd +AST group. Intermediate level, E– Cd +Vit E–Se group. At intermediate level, F– Cd +Vit E–Se group+AST group. Mild stromal degeneration (□) and edema (arrow), Hematoxylin–Eosin staining

Immunohistochemical Staining Findings

In immunohistochemical staining, statistically significant differences were found between the groups in terms of 8–OhDG, Caspase 3, and LC3B immunoreactivity (TABLE I, $P<0.05$).

Staining with 8–OhDG, Caspase 3, and LC3B showed mild immunopositivity in the control and AST group. Among the other groups, very severe positivity was found in Cd, severe positivity in Cd+AST and Cd+Vit E–Se, and moderate positivity in the Cd+Vit E–Se+AST group. Immunopositivity was in stromal cells and granulosa cells in the follicular (FIGS. 2, 3 and 4).

TABLE I
Immunohistochemical staining and histopatological (HE) findings

Groups	8–OhDG	Caspase 3	LC3B	Stromal degeneration (HE)	Edema (HE)
Control	1.00 ± 0.40^{a}	1.00 ± 0.00^{a}	1.16 ± 0.40^{a}	0.16 ± 0.40^{a}	0.33 ± 0.51^{a}
AST	1.00 ± 0.00^{a}	1.16 ± 0.40^{a}	1.16 ± 0.40^{a}	0.16 ± 0.40^{a}	0.16 ± 0.40^{a}
Cd	3.66 ± 0.51^{b}	3.83 ± 0.40^{b}	3.66 ± 0.51^{b}	2.83 ± 0.40^{b}	2.83 ± 0.40^{b}
Cd +AST	2.66 ± 0.51^{c}	2.83 ± 0.40^{c}	2.66 ± 0.51^{c}	2.00 ± 0.00^{c}	2.16 ± 0.40^{c}
Cd +Vit E–Se	2.83 ± 0.40^{c}	2.66 ± 0.51^{c}	2.83 ± 0.40^{c}	2.16 ± 0.40^{c}	1.83 ± 0.40^{c}
Cd +Vit E–Se+AST	1.83 ± 0.40^{d}	1.83 ± 0.40^{d}	2.00 ± 0.00^{d}	1.16 ± 0.40^{d}	1.00 ± 0.00^{d}

[a,b,c,d]: Different letters in the same column represent differences between groups ($P<0.05$)

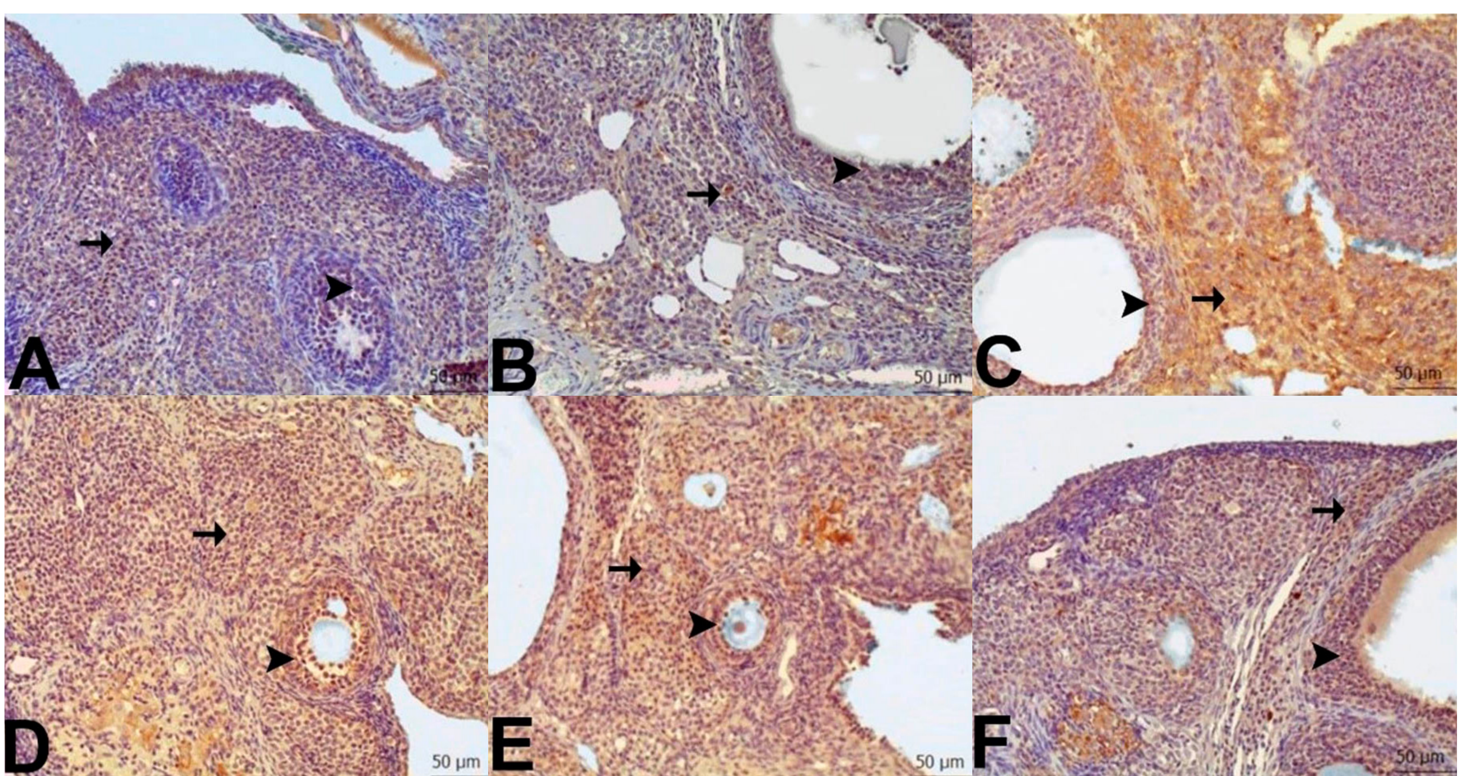

FIGURE 2. 8–OhDG Immunohistochemistry levels. A– Control group and B– Ast group. Mildly, C– Cd group. At very severe level, D– Cd+AST group and E– Cd+Vit E–Se group. At severe level, F– Cd+Vit E–Se group+AST group. Medium–level. 8–OhDG immunopositivity is shown in stromal cells (arrow) and granulosa cells of follicles (arrowhead)

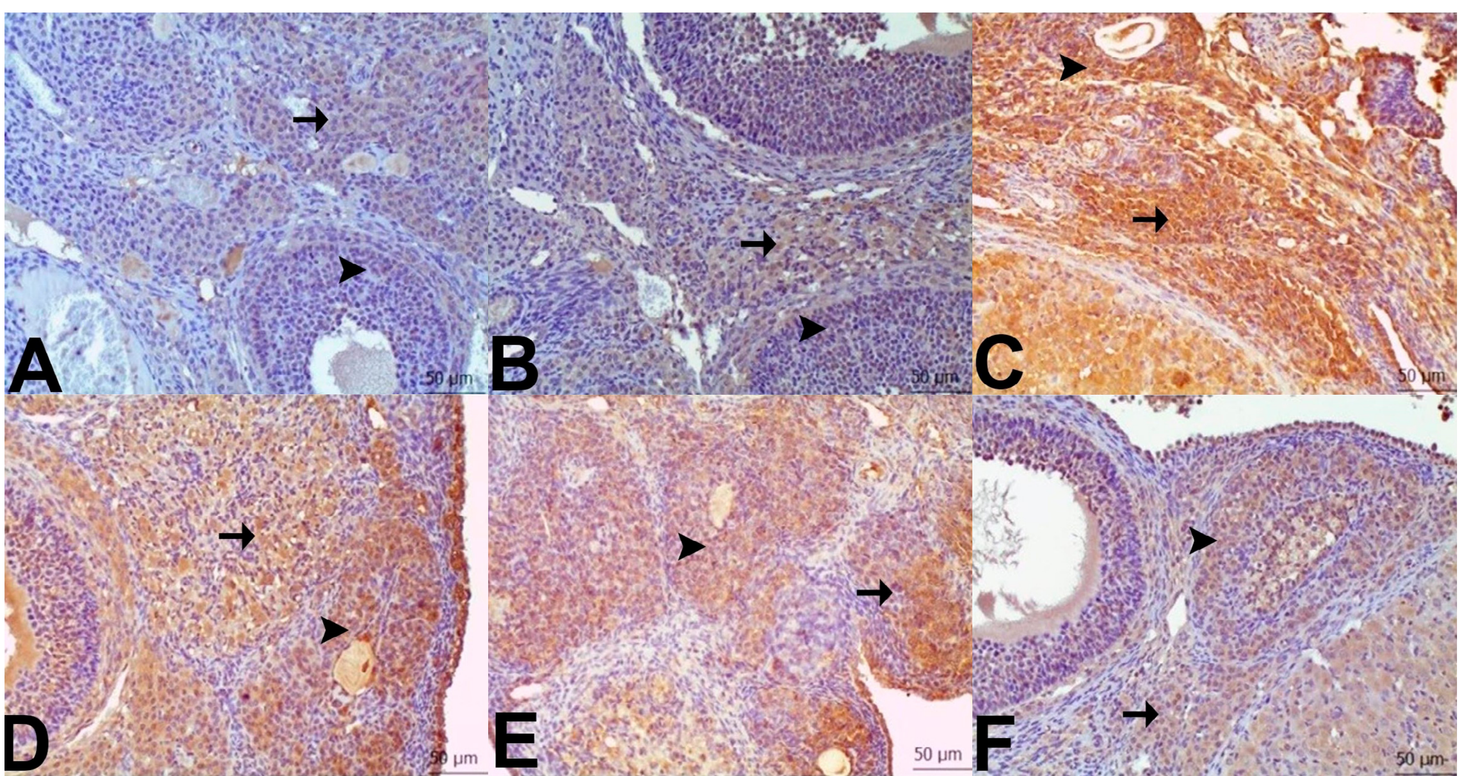

FIGURE 3. Caspase–3 Immunohistochemistry levels. A– Control group and B– Ast group. Mildly, C– Cd group. At very severe level, D– Cd+AST group and E– Cd+Vit E–Se group. At severe level, F– Cd+Vit E–Se group+AST group. Medium–level. Caspase–3 immunopositivity is shown in stromal cells (arrow) and granulosa cells of follicles (arrowhead)

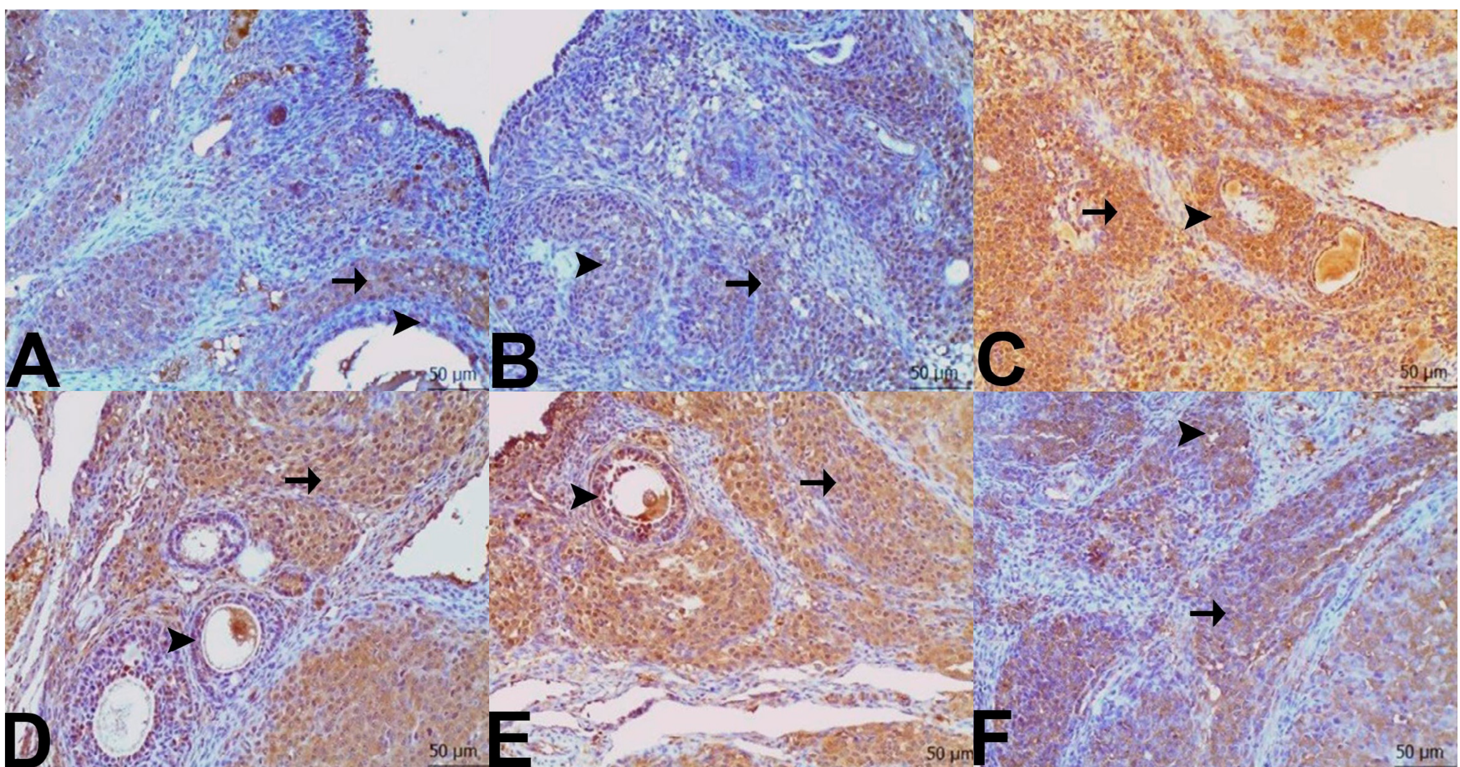

FIGURE 4. LC3B Immunohistochemistry levels. A– Control group and B– Ast group. Mildly, C– Cd group. At very severe level, D– Cd+AST group and E– Cd+Vit E–Se group. At severe level, F– Cd+Vit E–Se group+AST group. Medium–level. LC3B immunopositivity is shown in stromal cells (arrow) and granulosa cells of follicles (arrowhead)

Antioxidant parameter findings

Ovarian tissue and blood MDA, SOD, CAT, and GSH–Px values obtained in the study are given in TABLE II.

Hormon Levels Findings

Estradiol, Luteinizing hormone, Follicle stimulating hormone, Antimullerian hormone, and inhibin B levels were measured in the sera obtained from the rats used in the study. While there were significant decreases in hormone levels in all groups given cadmium ($P<0.05$), hormone levels in the treatment groups increased significantly ($P<0.05$) and approached control levels (FIG. 5).

With the advancement of technology and industrialization, exposure to heavy metals, particularly cadmium, is becoming increasingly common. Heavy metal toxicity can lead to damage in various organs and tissues. It is well–known that exposure to cadmium can cause significant damage to the reproductive systems and hormonal changes in both males and females. Accumulation in the female reproductive organs can lead to cytotoxicity, decreased FSH, LH, and Estradiol hormone levels, and menstrual cycle delays [29]. This study is the first to look into the adverse effects of AST on cadmium toxicity–related ovarian damage.

Ovaries play a crucial role in the production of female steroid hormones. Teca and granulosa cells regulate the estrous cycle in

TABLE II
Blood and ovarium antioxidant parameters of rats given cadmium chloride

Groups	MDA (nmol/protein)		SOD U·mg^{-1} protein		CAT U·mg^{-1} protein		GSH–Px nmol·mg^{-1} protein	
	Blood	Ovarium	Blood	Ovarium	Blood	Ovarium	Blood	Ovarium
Control	5.2 ± 1.0^{a}	2.8 ± 0.3^{a}	3.5 ± 0.7^{a}	1.5 ± 0.3^{a}	6.5 ± 0.8 a	1.3 ± 0.2^{a}	85 ± 7.4^{a}	55 ± 4.5^{a}
Cd	11.6 ± 2.1^{a}	5.6 ± 1.2^{a}	1.6 ± 0.2^{a}	0.6 ± 0.1 a	2.4 ± 0.2^{a}	0.5 ± 0^{a}	41 ± 3.6^{a}	28 ± 3.5^{a}
AST	6.1 ± 1.8^{b}	3.2 ± 0.5^{b}	3.2 ± 0.6^{b}	0.9 ± 0.2^{b}	5.2 ± 0.6^{b}	1.2 ± 0.3^{b}	88 ± 6.4^{a}	45 ± 3.6^{b}
Cd+AST	7.1 ± 1.5^{c}	3.8 ± 0.6^{c}	2.5 ± 0.3^{c}	1.0 ± 0.2^{b}	4.3 ± 0.4^{c}	1.0 ± 0.2^{c}	62 ± 6.5^{b}	41 ± 3.8^{c}
Cd+Vit E–Se	6.5 ± 0.9^{b}	3.2 ± 0.4^{c}	2.9 ± 0.5^{c}	1.1 ± 0.2^{c}	5.2 ± 0.6^{b}	1.3 ± 0.3^{d}	72 ± 5.8^{c}	52 ± 4.0^{d}
Cd+AST+ Vit E–Se	5.8 ± 0.8^{d}	3.0 ± 0.2^{d}	3.2 ± 0.5^{d}	1.2 ± 0.4^{d}	5.9 ± 0.8^{d}	1.4 ± 0.3^{d}	79 ± 6.7^{d}	50 ± 4.3^{d}

^{a,b,c,d}: Different letters in the same column represent differences between groups ($P<0.05$).

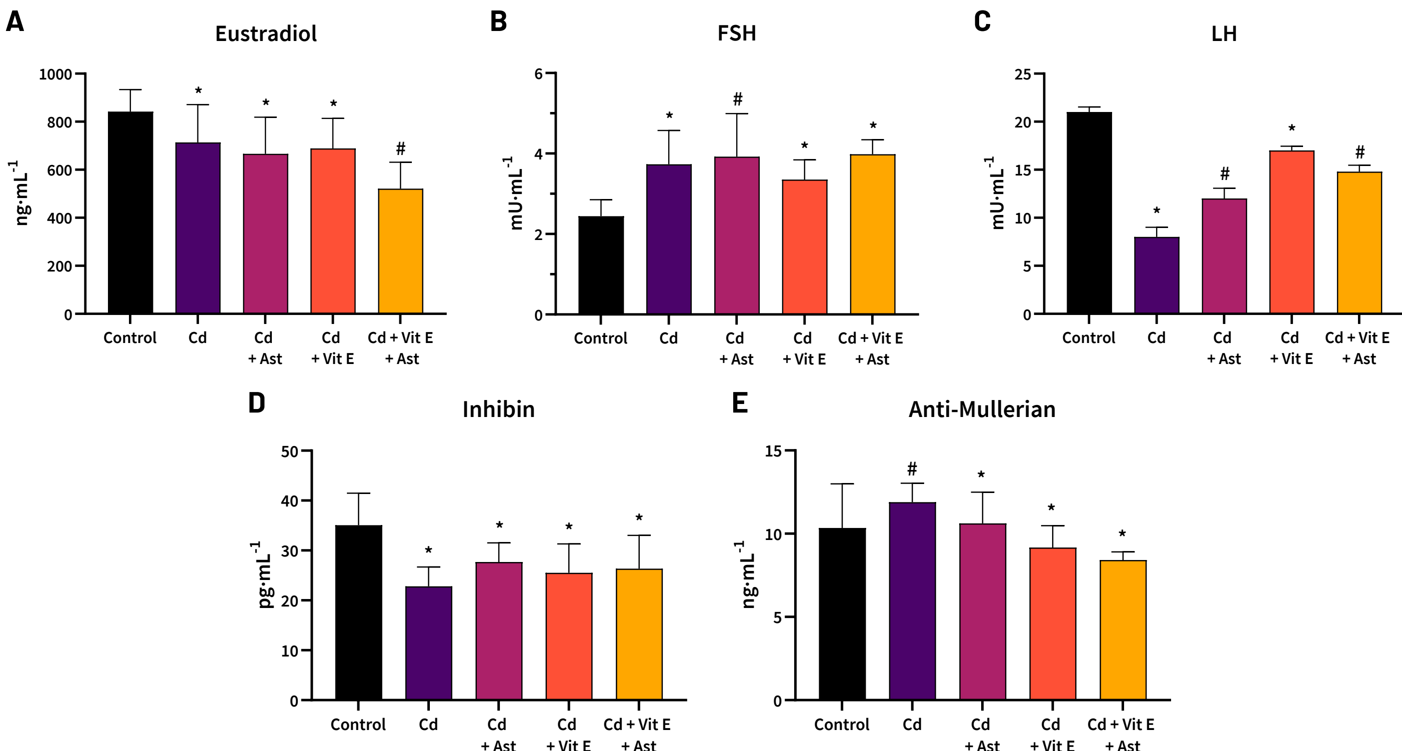

FIGURE 5. A: Estradiol, B: Follicle stimulating hormone, C: Luteinizing hormone, D: Inhibin B and E: Antimullerian hormone levels were measured in the serum (*,# *P*<0.05)

response to these steroids [29]. It is known that cadmium reduces the number of follicles and leads to folliculogenesis dysfunction [8, 13, 30]. Follicular dysfunction due to ovarian toxicity affects fertility and accelerates the menopausal process, increasing the risk of osteoporosis and cardiovascular diseases. Gonadotropic hormones (LH, FSH) need to bind to surface–specific receptors on granulosa cells to stimulate the production of gonadal steroid hormones. It has been shown that cadmium toxicity inhibits the binding of LH and FSH hormones to these receptors [30]. The effects of cadmium on ovarian follicles were shown to be connected with changes in gonadotropin hormones and decreases in follicle–stimulating hormone (FSH) and luteinizing hormone (LH) in a study by Ruslee et al. that was comparable to this study [15]. In our study, observed a decrease in all hormone levels in rats exposed to cadmium. When AST was added to the treatment groups, the adverse effects were observed to decrease. The best results were observed in the group where AST was combined with vitamin E–Se, known for its antioxidant activity.

Histopathological examination of ovaries exposed to cadmium revealed severe degeneration and edema in the stromal tissue. Although the use of AST in the treatment groups reduced the level of histopathological damage, the most effective protection was observed in the groups where vitamin E–Se was used in combination with AST.

There is growing proof that one of the primary molecular processes behind cadmium toxicity is oxidative stress, which results in the production of ROS and mitochondrial damage [31]. For many cell lines exposed to cadmium, apoptotic processes involving both caspase–dependent and caspase–independent pathways have been discovered [32]. In this study, immunohistochemical parameters (8–OhDG, Caspase 3, and LC3B) were examined, and it was found that intense staining occurred only in the ovarian tissues exposed to cadmium toxicity. In contrast, the staining decreased when AST was administered. Furthermore, the combination of vitamin E–Se and AST resulted in the least staining.

It is commonly acknowledged that one of the main mechanisms of cellular malfunction and death brought on by cadmium exposure is oxidative stress (OS) [31]. According to numerous studies, cadmium can cause granulosa cells to undergo apoptosis by producing ROS that come from the mitochondria. Cellular homeostasis depends on maintaining a balance between the generation of reactive oxygen species and the ability of the antioxidant system. The equilibrium of oxidation–antioxidant systems can be upset by excessive ROS generation, which can result in illness and malfunction in cells [12].

Cd has a high affinity for the sulfhydryl groups of proteins, which can affect the activities of specific enzymes, particularly glutathione, the major sulfhydryl reserve in granulosa cells [29]. Furthermore, investigations have shown that in the group exposed to Cd, SOD activity is significantly inhibited, while CAT activity shows a significant increase. Another study has also found that cadmium reduces antioxidants in rats' ovaries and increases MDA and hydrogen peroxide (H_2O_2) [33]. TABLE II showed the tissue and blood antioxidant values. The Cd–exposed group had significantly higher MDA levels than the control group, whereas SOD, CAT, and GSH–Px levels were significantly lower. These indicators are seen to vary significantly across all treatment groups and get closer to the levels of the control group ($P<0.05$).

In previous studies, it has been reported that 8–OHdG, a commonly used biomarker for DNA damage, is formed in damaged tissues by removing a hydrogen atom from nucleic acids by toxic oxygen radicals such as hydroxyl radicals [34]. There is a relationship between the

formation of 8–OHdG and the production of ROS, indicating that ROS can contribute to the formation of 8–OHdG. Studies demonstrate significantly increased levels of 8–OHdG in damaged ovarian tissue caused by oxidative stress compared to healthy tissues [34]. Immunohistochemical staining using 8–OHdG as a DNA damage marker revealed intense immunopositivity only in ovarian tissues exposed to Cd toxicity. However, when AST was administered, the immunopositivity started to decrease, and when AST was combined with vit E–Se, the immunopositivity was found to be minimal. Similar findings were also observed in the staining performed with Caspase–3 and LC3B. In the group treated with Cd alone, advanced levels of positivity were detected, which started to decrease with the use of AST and Vit E–Se. The combination group, Cd + Vit E–Se + AST, showed the lowest levels of positivity, indicating that this combination reduced cell death. Caspase–3, one of the markers used in the study, is an indicator of apoptosis, which is defined as a typical cellular death process that maintains tissue homeostasis and is triggered by various pathological–physiological stimuli such as oxidative damage [35, 36].

LC3B, another marker used in the study, is a marker that indicates autophagic cell death. It has been noted that oxidative stress can trigger autophagy, causing tissue and cellular damage. LC3B is a structural protein that can bind to the membrane of autophagosomes and plays a key role in autophagosome formation [37]. Therefore, in this study, the minimal immunopositivity of both Caspase–3 and LC3B in the Cd + Vit E–Se + AST group indicates that the combination treatment reduced apoptotic and autophagic cell death induced by Cd.

CONCLUSION

In conclusion, when histopathological, immunohistochemical, hormonal, and antioxidant parameters were examined, Cd–induced ovarian toxicity was observed consistently. It has been concluded that the phenolic and flavonoid compounds found in AST extract may play a role in these effects. While AST alone was relatively effective in protecting against this toxicity, the combination of Vit E–Se and AST showed the highest efficacy in mitigating the toxic effects.

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Ethical Approval

All experimental procedures applied in this study were examined by Sivas Cumhuriyet University Experimental Animal Research Ethics Committee and approved on 20.04.2021 with the number 538.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Begum Kurt, Haki Kara, Mahmut Sahin, Alper Serhat Kumru and Mustafa Ozkaraca. The first draft of the manuscript was written by Begum Kurt and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability statement

The data are available by the corresponding author upon.

Conflicts of interest

The authors declare no conflict of interest.

Sample availability

Samples of the compounds are available from the authors.

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