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Protective effect of *Spirulina* in the ovary of rats against Doxorubicin toxicity

Efecto protector de la espirulina en el ovario de ratas contra la toxicidad de la Doxorrubicina

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ABSTRACT

Doxorubicin (Dox) is an anti-cancer agent used of ovarian, breast, liver, lung cancers and solid tumors such as leukemia and lymphoma treatment. However, since it is an antineoplastic agent with a high toxic effect, it causes toxicity on many organs and tissues in the organism. *Spirulina* spp. (SP) contains phytopigments such as β-carotene, tocopherols and xanthophylls and is a natural source of vitamin A as well as its anticarcinogenic effect. For these reasons, this study was planned to experimentally reveal the antioxidant and protective effects of SP on Dox-induced reproductive toxicity in female rats. In the study, histopathological evaluation was performed after Hematoxylin-Eosin staining in female rats with Dox toxicity. The distribution of GSK-3β for cell proliferation, HIF-1α for oxidative stress and VEGF for vascularization were evaluated. TUNEL staining was performed to determine apoptosis. It was determined that SP prevented tissue damage on the ovarian tissue of rats with Dox toxicity, and this positive effect was achieved through factors such as oxidative stress (HIF-1α), vascularization (VEGF) and inhibition of cell death (Apoptosis). It has been observed that SP treatment to be applied after chemotherapy is effective in protecting both the developing follicles and the primordial follicle pool.

Key words: *Spirulina* spp.; apoptozis; ovary; infertility; doxorubicin

RESUMEN

La doxorrubicina (Dox) es un agente anticancerígeno que se utiliza en el tratamiento de cánceres de ovario, mama, hígado, pulmón y tumores sólidos como la leucemia y el linfoma. Sin embargo, al ser un agente antineoplásico con un alto efecto tóxico, provoca toxicidad en muchos órganos y tejidos del organismo. *Spirulina* spp. (SP) contiene fitopigmentos como β-caroteno, tocoferoles y xantofilas y es una fuente natural de vitamina A, además de su efecto anticancerígeno. Por estas razones, se planeó este estudio para revelar experimentalmente los efectos antioxidantes y protectores del SP sobre la toxicidad reproductiva inducida por Dox en ratas hembra. En el estudio, la evaluación histopatológica se realizó después de la tinción con hematoxilina-eosina en ratas hembra con toxicidad por Dox. Se evaluó la distribución de GSK-3β para la proliferación celular, HIF-1α para el estrés oxidativo y VEGF para la vascularización. Se realizó tinción TUNEL para determinar la apoptosis. Se determinó que SP previno el daño tisular en el tejido ovárico de ratas con toxicidad por Dox, y este efecto positivo se logró a través de factores como el estrés oxidativo (HIF-1α), la vascularización (VEGF) y la inhibición de la muerte celular (Apoptosis). Se ha observado que el tratamiento con SP aplicado después de la quimioterapia es eficaz para proteger, tanto los folículos en desarrollo como el conjunto de folículos primordiales.

Palabras clave: *Spirulina* spp.; apoptosis; ovario; esterilidad; doxorrubicina

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INTRODUCTION

Doxorubicin is classified as anthracycline group antibiotics. It is commonly known by its trade name adriamycin and is the hydroxylated analogue of Daunorubicin. Anthracyclines have varying effects depending on the cell type. These drugs do not selectively enter between base pairs close to each other and bind to the sugar–phosphate structure of Deoxyribonucleic acid (DNA) and prevent its synthesis [1]. Dox is used in the treatment of sarcomas, carcinomas including breast and lung cancers, acute lymphocytic leukemias and lymphomas [2]. The long-term effects of chemotherapy on ovarian tissue are reduced premature ovarian failure and ovarian reserve, which causes infertility. The number of follicles is an indicator of ovarian reserve in the ovary. The ultrastructural indicators of chemotherapy–induced ovarian damage are diffuse follicle loss and ovarian fibrosis [3, 4]. Alkylating agents are the substances that cause the highest follicle loss. In addition to follicular damage, they also cause widespread granulosa cell damage characterized by decreased steroid synthesis by forming DNA cross–links [3, 5].

Studies on microalgae species at the national and international level continue intensively, and the number of commercial enterprises serving in this field is gradually increasing [6]. *Spirulina* spp. (SP) İs the only type of blue–green algae grown and traded for use as a food supplement among algae [7]. The properties of SP have attracted the attention of researchers due to its ability to break the cell wall barrier and easy access to its components. SP is consumed as a food substance that has proven its safety in many toxicological studies [8]. Recently, algae has emerged as a new food source with the potential for multi–purpose use in Medicine Human. In addition, algae are a rich source of natural bioactive compounds with various biological activities. Apart from these, they carry many critical compounds with their unique properties such as carotenoids, amino acids and micronutrient accumulations, which have very important roles for Human Health. Therefore, there is an increasing interest in investigating the positive effect of algae on Human Health.

SP is a filamentous, spiral–shaped, multicellular and photosynthetic cyanobacteria. This cyanobacterium is cultivated Worldwide and is used as a primary human dietary supplement. It contains a wide range of prophylactic and healing nutrients including vitamins, minerals, proteins, γ–linolenic acid, β–carotene and undiscovered bioactive compounds. The study was planned to consider the role of chemical compounds in SP in preventing ovarian damage induced by the chemotherapeutic agent Dox and improving damage repair mechanisms. It is important to obtain a protective substance or substances against the toxic effects of Dox on reproductive cells.

MATERIALS AND METHODS

In the study, 24 Wistar albino female rats (*Rattus norvegicus*), 2–3 months old, weighing 200–300 g, were preferred. The rats were included in the study after being observed for two weeks to avoid adaptation problems. Subjects included in the study were kept in optimized conditions (12–hour day and night cycle, 22°C temperature, humidity 30–70%). They were fed with standard feed and water.

Toxicity model and application of Spirulina

Dox was obtained as Adrimisin (50 mg/25 ml injectable solution) from Saba Pharmaceuticals (Istanbul, Turkey). SP (Spirulina 99% green powder) was purchased from (of Naturalebio, Italy). In order to compose toxicity, Doxorubicin was administered as 1 dose (2 mg·kg^{-1} / i.p) every 3 days, a total of 12 mg·kg^{-1}. The dose of doxorubicin was selected minimal lethality [9]. After the administiration of Dox, 500 mg·kg^{-1} SP was given intragastrically (by gavage) once a day for 3 weeks. On the 21st day of the experiment, the animals were sacrificed under anesthesia and tissue samples were taken [10].

For experimental study, female rats were divided three groups as follows:

Group–1 (n:6): Control group, no treatment was applied, only 100 µL volume of physiological saline (SF) was given intraperitoneally every day during the experiment (n:6).

Group–2 (n:6): Doxorubicin group (DOX), Dox 2 mg·kg^{-1} 100 µL was administered intraperitoneally every three days (cumulative doses 12 mg·kg^{-1}) [9]. Since severe toxicity and lethal effects occurred with the application of high amounts of Dox at a time, induction was done in lower doses and at certain intervals.

Group–3 (n:6): DOX + SP group, Doxorubicin was administered at a dose of 2 mg·kg^{-1} in a volume of 100 µL intraperitoneally once in three days (6 doses in total), while SP was administered once daily as 500 mg·kg^{-1} intragastric (gavage).

Group–4: SP group, SP was dissolved in distilled water to achieve a final concentration of 200 mg·ml^{-1}. 500 mg·kg^{-1} of SP was administered intragastric (gavage) once a day. All samples were taken on the 21st day of the experiment.

Follicle count

After Dox and SP application, sections were taken from the right and left ovarian tissue, stained with Hematoxylin–Eosin (H–E) staining and examined under a light microscope. Ovarian reserve was determined by counting primordial follicles. Primordial, primary, preantral, secondary and tertiary follicles in the ovarian cortex, which can clearly distinguish both nuclei and nucleoli, were counted separately in each section. Evaluation of follicle quality is based on basement membrane integrity, cell density, and oocyte integrity. Unhealthy follicles were differentiated from healthy follicles by loss of granulosa cells and pycnotic nuclei.

Performing histochemistry, immunohistochemistry, TUNEL assay

At the end of the experiment, ovarian tissue samples were taken from female rats and fixed in 10% formalin solution for 72 hours. Then, histochemical and immunohistochemical stainings were performed. Since the severity of the staining in the tissue is important in this evaluation, it was accepted as weak (+), moderate (++) and severe (+++). The amount of stained cells was calculated by the H–score method and the data were evaluated statistically [11]. Glycogen synthase kinase (Gsk–3β), hypoxia inhibition factor (HIF–1 alpha) and vascular endothelial growth factor (VEGF) staining was performed on serially sectioned tissues using the indirect immunohistochemical method. GSK–3β is a protein that is a member of protein kinases and redox–sensitive multifunctional serine/threonine protein kinase and is expressed in all cell types. Many studies have shown that GSK–3 inhibition plays a protective role against oxidative and apoptotic damage caused by chemotherapy [12]. The transcription factor HIF– 1α is thought to be a critical regulatory factor during the development of physiological systems and a key regulator of body tissue homeostasis involved in the regulation of cell survival/adaptation, anaerobic metabolism, immune response, cytokine secretion, and angiogenesis [13]. New blood vessels are formed in the

ovary to facilitate the distribution of oxygen, nutrients and hormone substrates, as well as the transfer of hormones to target cells. The ovarian follicle and corpus luteum produce a variety of angiogenic factors, but vascular endothelial growth factor (VEGF) is thought to play a crucial role in regulating normal and abnormal angiogenesis in the ovary. Inhibition of VEGF expression prevents the development of mature antral follicles by reducing follicle angiogenesis [14].

Terminal Transferase dUTP Nick End Labeling (TUNEL) staining method was used to determine apoptotic cell death. TUNEL staining method is used to detect DNA fragmentation resulting from apoptosis. Follicles showing apoptotic activation were evaluated using the TUNEL method. One of two serial sections taken from 5 different levels by random sampling method from the right (n=6) and left (n=6) ovarian tissue blocks was stained with the TUNEL method. The number and percentage of follicles showing apoptotic activation were counted and calculated separately. Apoptotic activation of ovarian follicles was scored semiquantitatively between 0 and 2.

Score 0: Presence of at most 3–5 TUNEL positive follicle cells in the granulosa cell layer, no staining in the primary oocyte

Score 1: Less than 50% TUNEL positive staining in the granulosa cell layer, no staining in the primary oocyte

Score 2: Intense TUNEL positive staining covering more than half of the granulosa cell layer and TUNEL positive staining of the primary oocyte

Follicles with a TUNEL 2 score were considered as atretic follicles, and the apoptotic follicle index was calculated using the formula given below for follicles at different developmental stages.

%Apoptotic index = (number of positively stained apoptotic follicles / total number of follicles) × 100 [15].

Statistical analysis

The difference between the obtained findings and the groups was determined by one-way-ANOVA test and Tukey test was used for multiple comparisons. $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Evaluation of body weights

The average weights of the rats were determined before starting the experiment. Before the experiment was terminated after 21 days, each subject was weighed again and their average weight was determined. Each group was evaluated statistically according to its pre-experiment and post-experiment weights. No statistical difference was detected in the subjects of the control group according to their body weight ($P<0.08$). Post-experiment weight gain was noted in the subjects in the SP group, and a statistical difference was detected as $P<0.02$. A statistically significant decrease was determined in the post-experiment weights of the subjects in the Dox group compared to the pre-experiment ($P<0.001$)(FIG. 1).

Evaluation of ovarian follicle counts

The number of healthy follicles is important in ovarian toxicity. Especially the number of secondary and tertiary follicles is very important in drug toxicity. However, in the developing follicles, the number of unilaminar (seen of primary oocyte, zona pellucida and a single layer of cuboidal follicle cells surrounding it) and multilaminar (seen of primary oocyte, zona pellucida and a single layer of cuboidal follicle cells surrounding it) primary follicles is less due to the formation of an atretic structure. is taken into account. In our findings, although there was no statistically significant difference in the number of unilaminar and multilaminar primary follicles, a statistically significant decrease was observed in the number of secondary and graaf follicles. When the groups were compared in terms of primary follicle numbers, a significant difference was found between Control–Dox, Dox–Dox+SP and Dox–SP. When the groups were compared in terms of secondary and graaf follicle numbers, a significant difference was found between Control–Dox, Dox–Dox+SP and Dox–SP (FIG. 2). There was a significant increase in the number of atretic follicles in Dox administered subjects. Statistical data of atretic follicles belonging to the ovaries of the subjects are shown in FIG. 3. Between Control–Dox, Dox–Dox+SP, Dox–SP.

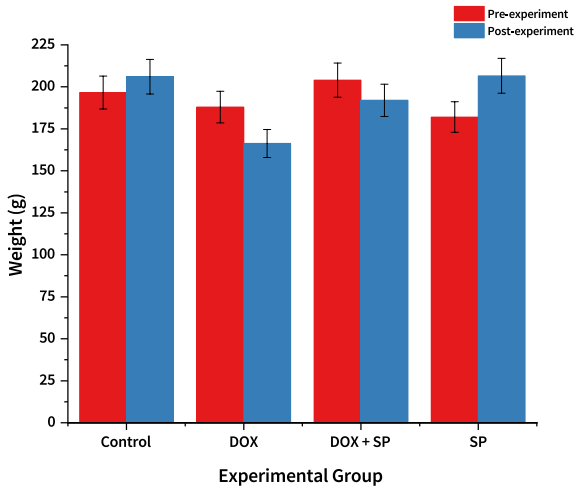

FIGURE 1. Body weights of the subjects before and after the experiment

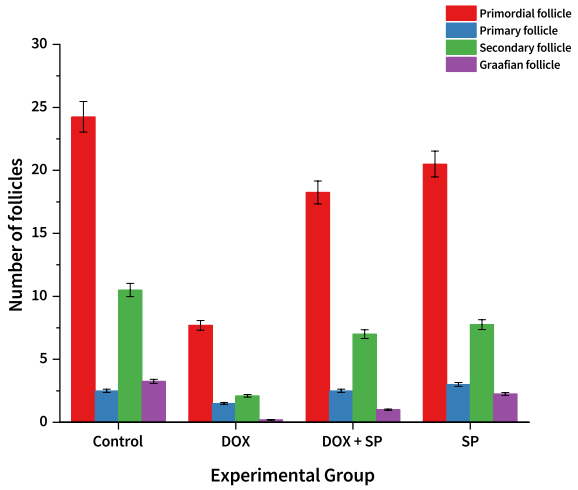

FIGURE 2. Number of ovarian follicles of the experimental groups; primary follicle: Control–Dox ($P<0.001$), Dox–Dox+SP ($P<0.01$) and Dox–SP ($P<0.001$), secondary and Graafian follicle: Control– Dox ($P<0.001$), Dox–Dox+SP ($P<0.02$) and Dox–SP ($P<0.001$)

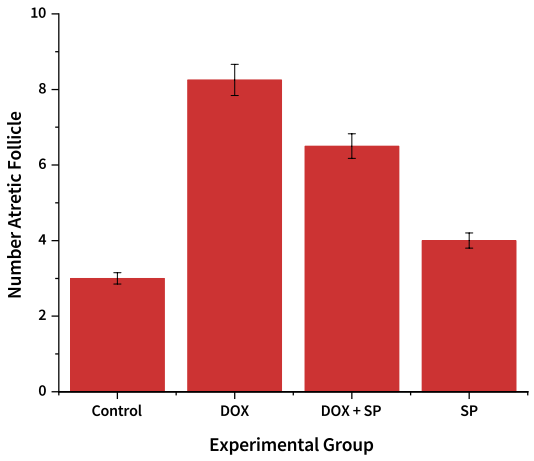

FIGURE 3. The number of atretic follicles in the ovarian tissues of the experimental groups; Control–Dox (*P*<0.001), Dox–Dox+SP (*P*<0.01), Dox–SP (*P*<0.001)

Microscopic findings

H–E stained ovarian sections of all experimental groups were used to determine the general morphological features and follicle numbers of the follicles in the ovarian cortex (FIG. 4A1). It was observed that the normal histological structure was disrupted in the ovarian sections of the DOX–treated experimental groups, and follicle degeneration, vascular congestion, hemorrhage, and polymorphonuclear leukocyte infiltration (PMNL) were quite common (FIG. 4B1). In the groups given Dox + SP together, the histological structure was preserved compared to the experimental groups given Dox, histophatological features were reduced, and there were newly developing follicles (FIG. 4C1). When the ovarian tissues taken from rats belonging to SP group, a generally normal histological appearance was detected. A very rare pathological picture was observed in the ovarian sections, stroma and parenchyma of the cortex. However, vascular congestion areas were observed in some sections (FIG. 4D1).

Immunohistochemical findings

In the ovarian sections stained with GSK–3β primary antibody, a statistically significant decrease was observed in the Dox group (FIG. 4B2) compared to the control group (FIG. 4A2) (*P*<0.001). A statistically significant increase was observed in the SP (FIG. 4D2) compared to the control (*P*<0.05) (FIG. 5). Although the immunoreactivity intensities increased in the groups where Dox and SP were administered together (FIG. 4C2) compared to the Dox group, there was a lesser increase compared to the SP and control groups.

It was observed that HIF–1α immunoreactivity was prominently manifested in the oocytes, granulosa cells and theca cells of secondary and early tertiary follicles in the Dox group (FIG. 4B3). While it was observed that the immunoreactivity was quite low in the ovarian tissue of the control group (FIG. 4A3), it was observed that the immunoreactivity was mild in the ovarian tissue of the SP group only (FIG. 4D3) and in the ovarian tissue of the Dox+SP (FIG. 4C3) group. A significant difference was also observed in the statistical comparison between Dox and other groups in terms of the number of positive cells (FIG. 5).

VEGF expression in ovarian follicles is dependent on follicle size. Inhibition of VEGF expression results in decreased follicle angiogenesis and failure to develop mature antral follicles. While the group with the lowest VEGF expression was Dox (FIG. 4B4), the increase in immunoreactivity was noted in the control (FIG. 4A4) and SP groups (FIG. 4D4). A significant difference was found between the Dox and the control and SP (*P*<0.001) in terms of VEGF immunoreactivity, and between the Dox–Dox+SP (FIG. 4C4) group (*P*<0.01) (FIG. 5).

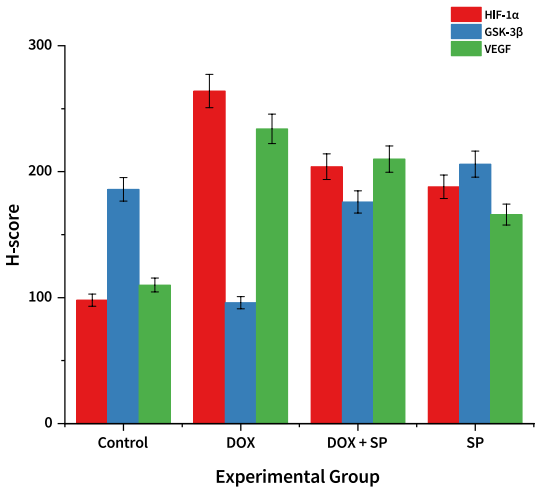

FIGURE 5. Immunoreactivity graph of the experimental groups

Tunel findings

Apoptotic activation and the number of atretic follicles in the ovarian cortex and medulla stroma and in the granulosa cells of the developing follicles were statistically increased in the Dox (FIG. 6A) group compared to the other groups (FIG. 7). After Dox application, it was observed that apoptotic activation decreased in the cortex and medulla of the ovarian tissues of the subjects given SP, and there was a decrease in the number of atretic follicles with an increase in the number of follicles (FIG. 6C). It was observed that the TUNEL activity in the ovarian tissues of the control group (FIG. 6A) and only the SP group (FIG. 6D) was mild and in the follicle granulosa cells.

The protective effects of SP against Doxorubicin–induced ovarian toxicity were evaluated at the histochemical and immunohistochemical levels. It has been shown that when SP is administered at a dose of 500 mg·kg^{-1} *in vivo*, it can provide a protective effect against the gonadotoxic effect of Doxorubicin. This effect was seen as preserving the number of primordial and developing follicles and decreasing the number of atretic follicles, excluding reduction in body weight. This is the first study in the literature to show the protective effect of SP against Dox–induced gonadotoxicity.

Considering the results of studies investigating the gonadotoxic effect of Doxorubicin; Ben–Aharon *et al.* [16] reported that Doxorubicin administration causes an increase in the number of atretic follicles in the ovaries and this effect causes a significant decrease in ovulation rate [16]. Although follicular atresia is a physiological function of the ovaries, damage to the follicles at the level of oocyte and granulosa

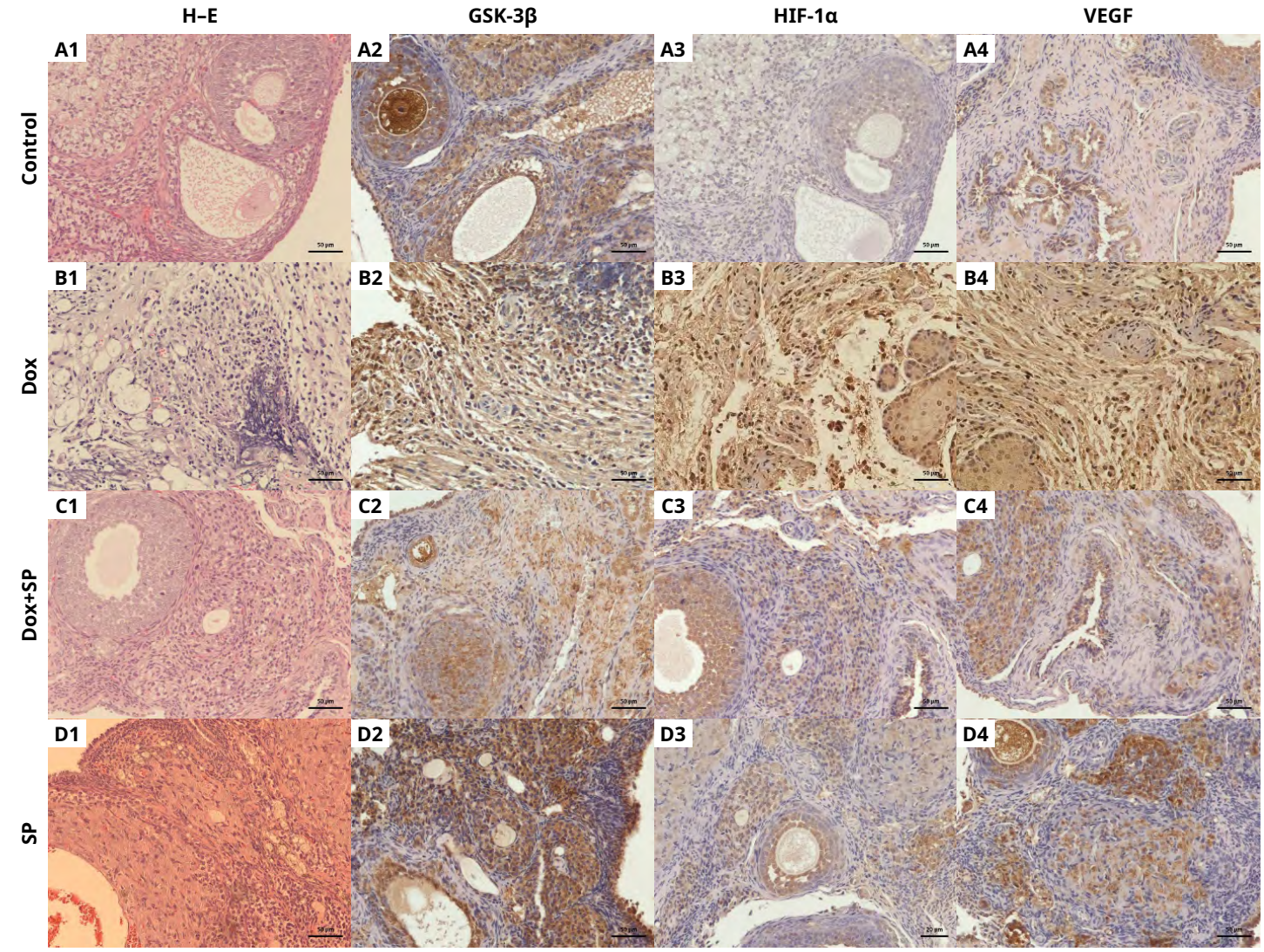

FIGURE 4. Histochemical and immunohistochemical staining of control and experimental groups

cells can lead to excessive follicle atresia, which may induce ovarian dysfunction. As a result of histological examination of ovarian biopsies of women receiving chemotherapy, it was observed that germ cell damage occurred due to lower estradiol production [17, 18]. Therefore, in chemotherapy-induced gonadotoxicity, apart from the interactions between granulosa cells and oocyte, disruptions in the steroidogenesis mechanism due to losses in granulosa and theca cells cause follicle loss. It has been reported that Doxorubicin causes deterioration in endothelial function by inducing free oxygen radicals and causing apoptosis in endothelial cells [19, 20]. It has been

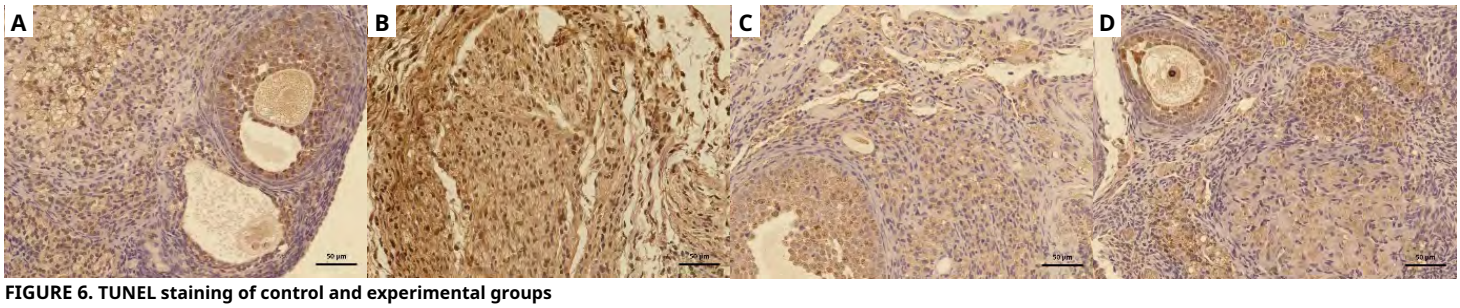

FIGURE 6. TUNEL staining of control and experimental groups

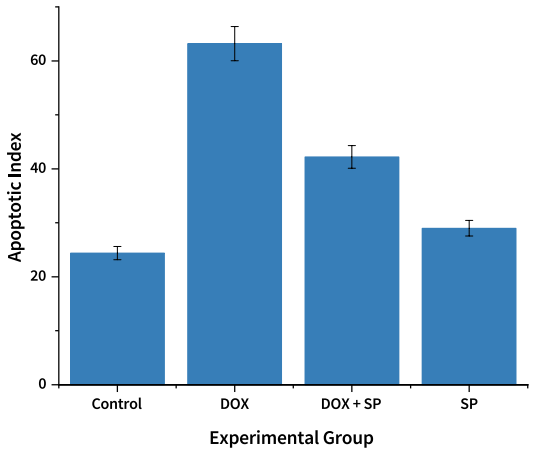

FIGURE 7. Apoptotic index data graph of experimental groups

reported that after Doxorubicin administration, ROS production in granulosa cells increased in a dose-dependent manner, decreased mitochondrial membrane potential, increased Bax, Bcl-2 and p-53 mRNA expression, which led to apoptosis [21]. In this study, it has been shown that Doxorubicin administration causes follicle loss by increasing apoptotic activation in ovarian follicles.

Studies have reported that TUNEL positive cell staining in atretic follicles in the ovary is more common in granulosa and theca cells [22]. When Slot *et al*. compared atretic and healthy follicles, they observed that the release of Fas ligand, Bax, TUNEL and caspase-3 was more intense in atretic follicles [23]. Vaskivuo *et al*. [24] reported TUNEL positive staining in granulosa cells in the human ovary at the level of secondary and graaf follicles. In a different study, it was stated that after TUNEL staining and caspase-3 immunohistochemistry in human ovaries, atretic follicle numbers were rare in the early follicle stage, and apoptosis could not be demonstrated by caspase-3 and TUNEL method in follicles at early maturation stage [25]. In this study, TUNEL positivity was obtained in developing and graaf follicles. It can be interpreted that apoptotic activation in primordial and unilaminar primary follicles results in direct loss of follicle due to rapid death of few follicle cells and degeneration of primary oocyte. This interpretation also supports the differences in the number of primordial follicles between the groups.

In studies evaluating the efficacy of tiopronin or cannabinoids for the treatment of nephrotoxicity caused by cisplatin, a broad-spectrum platinum-derived antineoplastic agent that has a toxic effect on the gastrointestinal system similar to Doxorubicin in the literature, it has been reported that therapeutic agents are similarly ineffective in preventing weight loss. They interpreted this result as the lack of effectiveness of the agents used for treatment on the gastrointestinal system. However, although anorexia and weight loss due to anorexia developed in rats due to the toxic effect of Doxorubicin on the gastrointestinal system in this study, it is possible that there will be reductions in weight loss due to the therapeutic effect of SP on the gastrointestinal system. Kaygusuzoglu *et al*. emphasized in their study that more apoptotic positive cells were detected in the cisplatin group compared to the control group in ovarian toxicity induced by cisplatin [26]. It was demonstrated increased apoptosis in the ovarian tissues of rats treated with cyclophosphamide in ovarian damage with cyclophosphamide, compared to the control group, with increased caspase-3 immunoreactivity [27]. They also reported that this caspase-3 immunoreactivity mainly occurs in the granulosa, stromal cells and corpus luteum.

Said *et al*. examined the uterus and ovaries of female rats exposed to radiation in their study and showed that caspase-3 immunoreactivity was more intense in the granulosa, theca interstitial cells and uterine epithelial cells compared to the control group [28]. They also emphasized in their study that uterine degeneration was prevented by sodium selenite treatment. Similarly, in the present study, was found that DOX induced apoptosis in both follicle cells and stromal cells in the ovary and uterus. It was observed that the number of caspase-3 positive apoptotic cells increased in DOX-induced ovarian damage compared to the control, and this number decreased significantly in the DOX+SP group. As in this study, Maltaris *et al* [29]. showed that Doxorubicin has negative effects on ovarian reserve, with a decrease in the number of ovarian follicles and a significant increase in the number of degenerated follicles in the mouse (*Mus musculus*) group given Doxorubicin, and follicular apoptosis was determined by the TUNEL method, as in this study.

The steroid content in green algae can contribute to balanced hormonal regulation by forming hormones that play an important role in female fertility and regulate the function of the reproductive system in the best way. Therefore, algae can be used as an alternative herbal treatment to overcome the toxic effects of free radicals that cause female infertility as a result of disruption of the hormonal regulation of the reproductive system [30]. However, although the effect of SP, a microalgae species, on reproductive functions is not well known, it has been reported that *S. maxima* extract inhibits body and testicular weights, metabolic parameters, normal seminiferous tubules degeneration, increases Leydig cell number, Testosterone levels and steroidogenic enzymes in studies conducted in rats so far [31]. SP is a powerful antioxidant molecule and is known for its antioxidant and antiapoptotic properties. It consists primarily of various components such as B-complex vitamins, β-carotene, chlorophyll, vitamin E, superoxide dismutase, and numerous minerals. It highlights that the antioxidants in SP, particularly C-phycocyanin, SOD, B-complex vitamins, β-carotene, chlorophyll and vitamin E, may act synergistically to restore the antioxidant status of the ovary [32]. In addition to its nutritional advantages, SP has extra beneficial characters such as antibacterial, antifungal, antiviral, anticancer, anti-inflammatory and antioxidant activities. Also SP is used as a feed supplement in the aquaculture and poultry industry. SP aqueous extract has been reported to act against MSG-induced ovarian dysfunction in mice (*Mus musculus*) by weakening the changed oocyte quality, ovarian histopathology, sex hormone and antioxidant enzymes as a result of mono sodium glutamate (MSG) supplementation [33].

CONCLUSION

Chemotherapy has extremely harmful effects on the female reproductive system, especially on the ovary, which has a fast cellular cycle. Although most women with cancer are treated with chemotherapy or radiotherapy, they are faced with infertility and gonadal insufficiency due to the toxic effects of the agents used after the treatment. It has been observed that SP treatment to be applied after chemotherapy is effective in protecting both the developing follicles and the primordial follicle pool. The apoptotic activation

caused by Doxorubicin in the follicular granulosa cells and stromal cells by causing calcium accumulation in the cell and causing DNA damage together with the ROS accumulation, the inhibiting effect of the calcium in the cell into the cell and the free oxygen radicals minimizing the developing follicles in the normal ovarian tissue by chemotherapeutic agents. It is thought to protect from toxic effects. This study is the first to show that SP has a protective effect on female reproductive system toxicity after doxorubicin chemotherapy and will guide approaches to preserve fertility as an alternative treatment option. Further studies are needed to support this view.

Ethical approval

The research protocol was approved by Çanakkale Onsekiz Mart University Animal Research Ethics Committee (2021-03/02).

Disclosure statement

The authors report there are no competing interests to declare.

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Conflicts of interest

The authors declare that they have no conflicts of interest with respect to the work presented in this report.

BIBLIOGRAPHIC REFERENCES

[1] Zhang W, Azibani F, Okello E, Kayima J, Walusansa V, Orem J, Sliwa K. Rational and design of SATRACD study: detecting subclinical anthracycline therapy related cardiac dysfunction in low income country. Afr. Health Sci. [Internet]. 2021; 21(2):647-654. doi: https://doi.org/grcdt7

[2] Peter S, Alven S, Maseko RB, Aderibigbe BA. Doxorubicin-Based Hybrid Compounds as Potential Anticancer Agents: A Review. Molecules. [Internet]. 2022; 27(14):4478. doi: https://doi.org/k9tt

[3] Sonmezer M, Oktay K. Assisted reproduction and fertility preservation techniques in cancer patients. Curr. Opin. Endocrinol. Diabetes Obes. [Internet]. 2008; 15(6):514-522. doi: https://doi.org/dqx8qk

[4] Kim SE, Kim WJ, Choi D, Lee DY. Comparison of goserelin and leuprorelin for ovarian protection during chemotherapy in young patients with breast cancer. Breast Cancer Res. Treat. [Internet]. 2023; 198(2):231-237. doi: https://doi.org/k9tv

[5] Liu S, Jia Y, Meng S, Luo Y, Yang Q, Pan Z. Mechanisms of and Potential Medications for Oxidative Stress in Ovarian Granulosa Cells: A Review. Intern. J. Mol. Sci. [Internet]. 2023; 24(11):9205. doi: https://doi.org/k9tw

[6] Stefanutti D, Tonin G, Morelli G, Zampieri RM, La Rocca N, Ricci R. Oral Palatability and Owners' Perception of the Effect of Increasing Amounts of *Spirulina* (*Arthrospira platensis*) in the Diet of a Cohort of Healthy Dogs and Cats. Anim. (Basel). [Internet]. 2023; 13(8):1275. doi: https://doi.org/k9tx

[7] Brito AF, Silva AS, de Oliveira CVC, de Souza AA, Ferreira PB, de Souza ILL, da Cunha-Araujo LC, da Silva-Félix G, de Souza-Sampaio R, Tavares RL. *Spirulina platensis* prevents oxidative stress and inflammation promoted by strength training in rats: dose-response relation study. Sci. Rep. [Internet]. 2020; 10(1):6382. doi: https://doi.org/gm575v

[8] Maddiboyina B, Vanamamalai HK, Roy H, Ramaiah, Gandhi S, Kavisri M, Moovendhan M. Food and drug industry applications of microalgae *Spirulina platensis:* A review. J. Basic Microbiol. [Internet]. 2023; 63(6):573-583. doi: https://doi.org/gs7fq6

[9] Samare-Najaf M, Zal F, Safari S. Primary and Secondary Markers of Doxorubicin-Induced Female Infertility and the Alleviative Properties of Quercetin and Vitamin E in a Rat Model. Reprod Toxicol. [Internet]. 2020; 96:316-326. doi: https://doi.org/k9tz

[10] Elmorsi RM, Kabel AM, El Saadany AA, Abou El-Seoud SH. The protective effects of topiramate and spirulina against doxorubicin-induced cardiotoxicity in rats. Hum Exp Toxicol. 2023; 42:9603271231198624. doi: https://doi.org/k9t2

[11] Irkin LC, Öztürk Ş. Ameliorative effects of *Ulva rigida* (C. Agardh, 1823) on cadmiuminduced nephrotoxicity in Wistar albino rats. Pak. Vet. J. [Internet]. 2022 [cited 20 July 2023]; 42(3): 419-423. Available in: https://bit.ly/4aBmtf8.

[12] Wang Z, Bao H, Ge Y, Zhuang S, Peng A, Gong R. Pharmacological targeting of GSK3β confers protection against podocytopathy and proteinuria by desensitizing mitochondrial permeability transition. Br. J.Pharmacol. 2015;172(3):895-909. doi: https://doi.org/k9t3

[13] Palazon A, Goldrath AW, Nizet V, Johnson RS. HIF transcription factors, inflammation, and immunity. Immunity. 2014; 41(4):518-528. doi: https://doi.org/f3n5rh

[14] Kaczmarek MM, Schams D, Ziecik AJ. Role of vascular endothelial growth factor in ovarian physiology - an overview. Reprod. Biol. 2005; 5(2):111-136. Cited in PubMed; PMID: 16100562.

[15] Garrett WM , Guthrie HD. Expression of Androgen Receptors and Steroidogenic Enzymes in Relation to Follicular Growth and Atresia Following Ovulation in Pigs. Biol. Reprod. [Internet].1996; 55(5):949-955. doi: https://doi.org/bpptd2

[16] Ben-Aharon I, Bar-Joseph H, Tzarfaty G, Kuchinsky L, Rizel S, Stemmer SM, Shalgi R. Doxorubicin-induced ovarian toxicity. Reprod. Biol. Endocrinol. [Internet]. 2010; 8:20. doi: https://doi.org/fm26cj

[17] McLaughlin M, Kelsey TW, Wallace WHB, Anderson RA, Telfer EE. Non-growing follicle density is increased following adriamycin, bleomycin, vinblastine and dacarbazine (ABVD) chemoterapy in the adult human ovary. Human Reprod. [Internet]. 2017; 32(1):165-174. doi: https://doi.org/k9t4

[18] Oktay K, Oktem O, Reh A, Vahdat L. Measuring the impact of chemotherapy on fertility in women with breast cance. J. Clin. Oncol. [Internet]. 2006; 24(24):4044-4046. doi: https://doi.org/ctpvgh

[19] Chow AY, Chin C, Dahl G, Rosenthal DN. Anthracyclines cause endothelial injury in pediatric cancer patients: a pilot study. J. Clin. Oncol. [Internet]. 2006; 24(6):925-928. doi: https://doi.org/fvf7w3

[20] Schneider C, González-Jaramillo N, Marcin T, Campbell KL, Suter T, Bano A, Wilhelm M, Eser P. Time-Dependent Effect of Anthracycline-Based Chemotherapy on Central Arterial Stiffness: A Systematic Review and Meta-Analysis. Front. Cardiovasc. Med. [Internet]. 2022; 9:873898. doi: https://doi.org/grcc83

[21] Zhang T, He WN, Feng LL, Huang HG. Effect Of Doxorubicin-Induced Ovarian Toxicity On Mouse Ovarian Granulosa Cells. Regulat. Toxicol. Pharmacol. [Internet]. 2017; 86:1-10. doi: https://doi.org/gbhg8z

[22] Liu M, Zhang D, Zhou X, Duan J, Hu Y, Zhang W, Liu Q, Xu B, Zhang A. Cell-free fat extract improves ovarian function and fertility in mice with premature ovarian insufficiency. Stem Cell Res. Ther. [Internet]. 2022: 13:320. doi: https://doi.org/k9t5

[23] Slot K, Voorendt M, Boer-Brouwer M, Vugt H, Teerds K. Estrous cycle dependent changes in expression and distribution of Fas, Fas ligand, Bcl-2, Bax, and pro- and active caspase-3 in the rat ovary. J. Endocrinol. [Internet]. 2006; 188:179-192. doi: https://doi.org/cfmhbb

[24] Vaskivuo TE, Anttonen M, Herva R, Billig H, Dorland M, Te Velde ER, Stenback H, Heikinheimo M, Tapanainen JS. Survival of human ovarian follicles from fetal to adult life: apoptosis, apoptosis-related proteins, and transcription factor GATA-4. J. Clin. Endocrinol. Metab. [Internet]. 2001; 86(7):3421-3429. doi: https://doi.org/k9t6

[25] Hou J, Lei Z, Cui L, Hou Y, Yang L, An R, Wang Q, Li S, Zhang H, Zhang L. Polystyrene microplastics lead to pyroptosis and apoptosis of ovarian granulosa cells via NLRP3/Caspase-1 signaling pathway in rats. Ecotoxicol. Environ. Saf. [Internet]. 2021; 212:112012. doi: https://doi.org/gk7fh7

[26] Kaygusuzoğlu E, Caglayan C, Kandemir FM, Yıldırım S, Kucukler S, Kılınc MA. Zingerone ameliorates cisplatin-induced ovarian and uterine toxicity via suppression of sex hormone imbalances, oxidative stress, inflammation and apoptosis in female wistar rats. Biomed. Pharmacother. [Internet]. 2018; 102:517-30. doi: https://doi.org/gdngvn

[27] Hamzeh M, Hosseinimehr SC, Mohammadi HR, Beklar SY, Dashti A, Amiri FT. Atorvastatin attenuates the ovarian damage induced by cyclophosphamide in rat: An experimental study. Intern. J. Reprod. Biomed. 2018; 16(5): 323-334. Cited in PubMed; PMID: 30027148.

[28] Said RS, Nada AS, El-Demerdash E. Sodium selenite improves folliculogenesis in radiation-induced ovarian failure: a mechanistic approach. PLoS One. [Internet]. 2012; 7(12):e50928. doi: https://doi.org/f4f56f

[29] Maltaris T, Seufert R, Fischl F. The Effect of Cancer Treatment on Female Fertility and Strategies for Preserving Fertility. Eur. J. Obstet. Gynecol. Reprod. Biol. [Internet]. 2007; 130(2):148-155. doi: https://doi.org/fsq9g3

[30] Arini LA. The study of sex steroid hormone compound in green algae *(Chlorophyta)* for female fertility: A literature review. IOP Conf. Ser: Earth Environ. Sci. [Internet]. 2021; 913:012085. doi: https://doi.org/k9t7

[31] Won HN, Il KK, Hae SA, Mi JK, Hee-Gyoo K, Jin HJ, Myung Effect of *Spirulina maxima* on spermatogenesis and steroidogenesis in streptozotocininduced type I diabetic male rats. Food Chem. [Internet]. 2012; 134(1):173-179. doi: https://doi.org/k9t9

[32] Yener NA, Sinanoglu O, Ilter E, Celik A, Sezgin G, Midi A, Deveci U, Aksungar F. Effects of Spirulina on cyclophosphamide-induced ovarian toxicity in rats: biochemical and histomorphometric evaluation of the ovary. Biochem. Res. Int. [Internet). 2013; 2013:764262. doi: https://doi.org/gb5f3n

[33] Abdel-Aziem SH, Abd El-Kader HAM, Ibrahim FM, Sharaf HA, El Makawy AI. Evaluation of the alleviative role of *Chlorella vulgaris* and *Spirulina platensis* extract against ovarian dysfunctions induced by monosodium glutamate in mice. J. Genet. Eng. Biotechnol. [Internet]. 2018; 16(2):653-660. doi: https://doi.org/k9vb