ORIGINAL ARTICLE

ANTI-OXIDATIVE PROTECTION OF VITAMIN E AGAINST DOXORUBICIN INDUCED OVARIAN TOXICITY IN ANIMAL MODEL

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Background: Chemotherapy has diverse pathologic consequences on the female reproductive system. Current study aimed to ascertain whether vitamin-E administration shielded female rats against doxorubicin-induced ovarian damage. **Methods:** This laboratory experimental study was conducted at the Department of Anatomy, Khyber Medical University, Peshawar. In this study, 24 female rats weighing between 200 and 250 gm were used and divided into three groups, the control group, research group-I, and research group-II. All animals got weighed at the start of the study and again prior to their sacrifice. On the 28th day, animals were sacrificed, and organs were taken out. To observe any change in the ovarian follicles, 5 um thick sections were cut on a rotary microtome and stained with Hematoxylin and Eosin (H&E) and Massan Trichome. The SPSS-25 was used to analyse the data. The difference between and within groups was measured using the independent sample *t*-test and chi square test. $p \leq 0.05$ was considered statistically significant. **Results:** Rats in both the control and experimental groups-II showed a substantial rise in mean weight ($p<0.05$), however rats in experimental group-I showed a significant drop in mean body weight ($p>0.05$) after the experiments. The number of primordial and secondary ovarian follicles did not differ significantly across groups ($p>0.05$), however there was a significant difference in the number of primary ovarian follicles, atretic follicles, and nuclear fragments ($p<0.05$). **Conclusion:** In female rats, simultaneous administration of vitamin E has demonstrated protective effects against doxorubicin-induced ovarian damage.

Keyword: Doxorubicin, Ovarian toxicity, vitamin E, chemotherapy, antioxidant.

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INTRODUCTION

The female ovary harbours all of the oocytes or germ cells needed for the individual's entire life at birth. Pre-granulosa cells encircle the follicular cells in which these oocytes are present. Roughly 2 million follicles are present inside the ovary of a female, which are reduced to three hundred thousand after adolescence. These cells remain in passive state for the whole reproductive phase of life until they are activated at puberty. One germ cell is triggered and released at each menstrual phase, and during a woman's whole reproductive lifespan (about thirty years), approximately 500 mature egg cells are discharged.[1] When a woman reaches the age of 45, or when fewer than a thousand follicle cells remain in her body, her reproductive life enters the premenopausal period. Around the age of fifty, a healthy woman often experiences the total loss of her ovarian follicles. Apoptosis or damage brought on by cancer chemotherapy may also trigger early menopause. This condition, which typically affects young females, is referred to as 'chemotherapy induced infertility'. This indicates that the ovary of the female cancer patient has follicles similar to those of postmenopausal women.[2] Chemotherapy has complex pathophysiological effects on the female reproductive system that depends upon the potency and number of drug therapies. The age and previous history of the patient might also have an impact on how the ovary operates. The primary issue arising from cancer treatment is the harmful impact of pharmacologically active drugs on the gonads of both sexes.[3] This could occasionally lead to infertility.

Numerous anti-cancer medications, including cyclophosphamide, cisplatin, and doxorubicin (DOX), can directly harm ovarian follicles, causing damage to oocytes, granulosa, and cumulus cells, or they can indirectly harm the ovarian parenchymal cells and vascular system, which inhibits the ovarian follicular cells' ability to receive nutrients.[4] Follicle atresia, which lowers the ovarian reserve, will be the eventual outcome in both situations. This is the reason why gonad preservation procedures are utilized today, such as cryopreservation of reproductive cells for future transplantation in female cancer patients vulnerable to premature ovarian failure (POF).[5]

DOX is an anticancer drug, belonging to the anthracycline class and targets topoisomerase II, that prevents DNA synthesis. The cytotoxic mechanism of DOX is based on the generation of DNA intercalation between base pairs of guanine and cytosine, which disrupts the synthesis of DNA and RNA.[6] Follicle-dependent and independent ovarian toxicity are both caused by DOX, which is classified as an intermediate risk group member. It has been proposed that activation

of ataxia-telangiectasia mutated (ATM) kinase, a crucial downstream modulator of the RH-DNA damage response, is the mechanism behind DOX-induced ovarian toxicity. Studies in mouse models have demonstrated that inactivating ATM can result in the inhibition of apoptosis and an acceleration of tumour formation. The dysregulation of ATM activity can affect cell survival.[7] DOX also harms the integrity of DNA inside the oocytes and granulosa cells.[8] Work on animal models has provided more proofs that besides DNA, it also causes mitochondrial dysfunction resulting in activation of pro-apoptotic mediators such as caspase-3 and 12, hence activating the apoptotic signalling pathways in oocytes and granulosa cells.[9]

Vitamin E, a vital nutrient, was identified in the 1920s. Many of vitamin E's physiological roles, particularly its antioxidant effects, have been investigated for more than a century. The vitamin E family has eight isomers: four tocopherols (α-, β-, γ-, and δ-tocopherol) and four tocotrienols (α-, β-, γ-, and δ-tocotrienol). Vitamin E is an essential nutrient and cannot be produced in the body endogenously, therefore required exogenously through our diet for fulfilment of body requirements. Vitamin E is necessary for various biochemical processes and have anti-allergic, anti-atherogenic, anti-cancer, anti-cardiovascular, anti-diabetic, anti-lipidemic, antihypertensive, anti-inflammatory, anti-obesity and neuroprotective activities.[10] Redox reactions in the body produces reactive oxygen species (ROS) which are linked to a number of illnesses. As vitamin E is a lipid soluble vitamin, sufficient concentration of this essential vitamin is known to control body's redox equilibrium. It is found in cell membranes and lipoproteins throughout the body. The anti-oxidative activity of vitamin E is only shown *in vitro* and not significantly *in vivo*.[11] The present study was carried out to observe the protective effect of oral administration of vitamin E against doxorubicin induced ovarian toxicity in animal model.

MATERIAL AND METHODS

The current experimental study was conducted at the Department of Anatomy, Khyber Medical University, Peshawar from 15 Feb to 15 Jul 2022. Twenty-four female rats, weighing 200–250 grams, were obtained from the animal house of Institute of Pharmaceutical Sciences, Khyber Medical University and split into three groups (Table-1). The number of rats and the groups were calculated using the one-way ANOVA formula. International guidelines for sheltering and feeding research animals were met by the animal house. For this investigation, a separate room with a 12-hour light and dark cycle and good ventilation was assigned. The temperature was kept at 18–26 °C.

Healthy female rats that had previously been pregnant and weighed 200–250 g were chosen. Rats that had any ailment prior to the start of the experiment or developed disease throughout the trial were eliminated.

The initial weight (W_i) of each rat was measured before starting the experiments and shortly before their sacrifice, i.e., final weight (W_f). Doxorubicin injection (10 mg/dL) (AJ Mirza Pharmaceuticals, Lahore, Pakistan) was acquired from Institute of Radiotherapy and Nuclear Medicine, Peshawar. Vitamin E was procured from the local market. A dose of 500 mg/Kg of vitamin E was administered orally to the rats of group-II with the help of a flexible rubber feeding tube. The animals were fed on water and commercial mash diet. The animals were killed on the 28th day, and the organs were removed and placed in 10% neutral buffered formalin to check for micro-pathological changes. For regular microscopy, 5 µm thick slices were cut using a rotary microtome, and stained with Hematoxylin and Eosin (H&E). Massan Trichome was also used to stain sections in order to highlight changes in the connective tissue of the ovarian follicle. Every follicle that had a nucleus visible was counted. Apoptotic granulosa cells, or apoptotic bodies in granulosa cell layers, disorganized granulosa cells, a degenerating oocyte, or fragmentation of the oocyte nucleus were all considered indicators of atretic follicles.

Data were analysed using SPSS-24. Quantitative variables were given as means and standard deviations, whereas categorical variables were calculated as frequencies and percentages. The difference between and within groups was measured using the independent sample *t*-test and the Chi-square test, and $p \leq 0.05$ was considered statistically significant.

Table-1: Groups demographics

Groups	Description
Control	Received Vitamin-E (mixed in water) once a week for 4 weeks.
Group-I	Received intraperitoneal injection (7.5 mg/Kg) of DOX once per week for 4 weeks.
Group-II	Received intraperitoneal injection (7.5 mg/Kg) of DOX once per week for 4 weeks + treatment with Vit E for 4 weeks

RESULTS

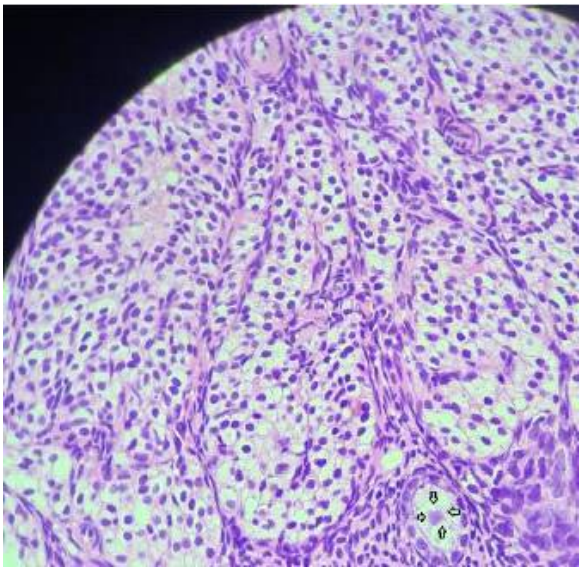
Rats in the control group had an average weight of 105.30±1.78 g prior to the trial, however their mean weight increased significantly ($p<0.05$) following the experiment. Rats in experimental group I had mean weights of 115.25±4.45 g and 95.21±5.95 g before and after the experiment, respectively. A statistically significant decrease in mean body weights of animals was seen in research group-1 at the end of the study (Table-2). There were significant differences in the number of primary ovarian follicles in right ovary, atretic follicles in both ovaries and nuclear fragments in left ovary between the study groups (Table-3). No significant difference was observed in number of primordial and secondary ovarian follicles within the study groups. The results of H&E stains are shown in Figure-1.

Table-2: Changes in mean body weight (n=24) (gm)

Group	Initial weight (W_i)	Final weight (W_f)	Weight change	*p*
Control	105.30±1.78	110.42±3.95	-5.12±2.17	<0.05
Group-I	115.25±4.45	95.21±5.95	20.04±1.50	<0.05
Group-II	114.60±4.85	158.40±22.50	-43.80±20.25	<0.05

Table-3: Statistical Analysis within the groups (n=24, Chi-square test) (Mean±SD)

Follicles	Ovary	Control	Group-I	Group-II	*p*
Primordial	**Right**	1.73±1.62	2.12±2.2	2.35±1.90	0.79
	Left	1.65±1.95	1.66±1.75	1.66±1.75	1.02
Primary	**Right**	1.1±1.06	2.12±1.80	3.23±2.15	**0.07**
	Left	1.12±1.35	1.11±1.24	1.36±1.10	**0.05**
Secondary	**Right**	1.6±1.95	3.11±2.34	3.77±2.45	0.16
	Left	1.74±1.25	1.23±0.48	1.65±0.75	0.59
Atretic	**Right**	0.001	1.75±0.88	4.12±2.95	**0.002**
	Left	0.001	1.24±0.47	1.52±0.95	**0.003**
Nuclear Fragment	**Right**	0.001	0.52±0.76	0.27±0.47	0.184
	Left	0.001	1.52±0.95	0.78±0.89	**0.006**



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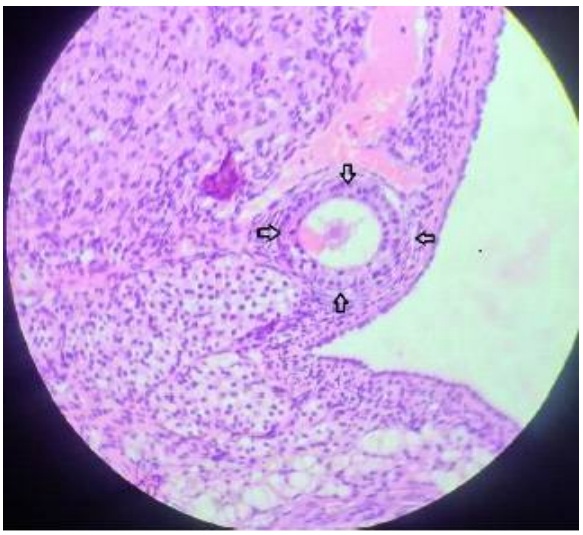

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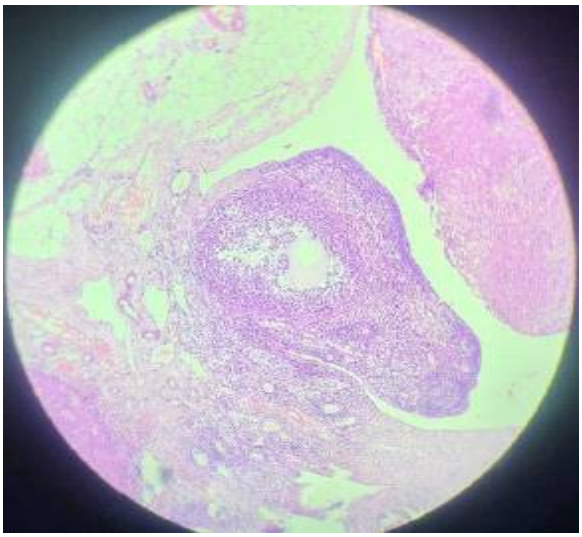

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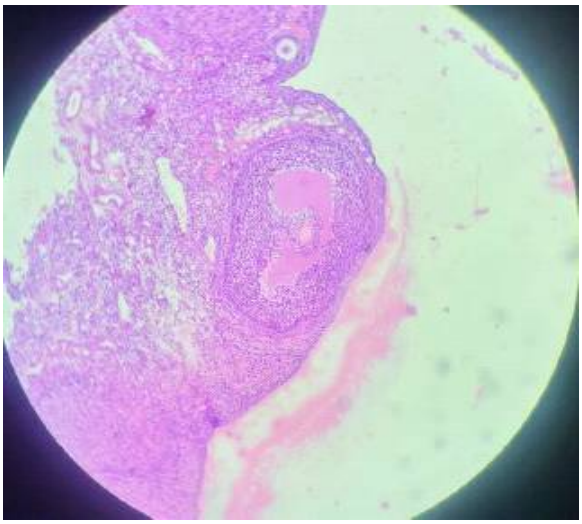

D

Figure-1: Photomicrograph of ovarian follicles in different study groups.

A: Group-1 Primary ovarian follicles, B: Group-1 Secondary ovarian follicles, C: Research Group-I: Doxorubicin-induced follicular atresia, D: Research Group-II: Vitamin E induced protection

DISCUSSION

Despite the fact that chemotherapeutic drugs have proven helpful in the treating different types of cancer, toxic side-effects are a common and undesirable aspect of treatment that can happen at standard dosage. Infertility, which results from premature ovarian insufficiency (POI), is one of the most dangerous long-term effects of cytotoxic chemical treatment. Because of this, patients and their physician's major priorities now include preventing infertility and maintaining ovarian reserve. The current study tested the harmful effects of doxorubicin on the ovulatory phase across a 28-day treatment period. The rats treated with doxorubicin for 28 days showed a decrease in body weight ($p<0.05$),

indicating that increased oxidative stress may have contributed to their weight loss. Swamy *et al*[12] revealed that albino rats treated with doxorubicin had significantly lower body weights than animals in the normal control group. The rats receiving doxorubicin plus oral vitamin-E showed a substantial increase in body weight compared to the control group ($p<0.05$). The rats in this group gained weight because vitamin E, which works as an antioxidant, protected them from the adverse effects of DOX therapy. Azman *et al*[13] showed that vitamin E supplementation to female rats resulted in increase in body weight due to increase in fat masses. The number of primordial follicles is usually a determinant of female fertility in chemotherapy survivors. Primordial follicular oocytes are extremely susceptible to the effects of chemotherapy. P53 accumulates in the cell as a result of damage to the DNA. A transcription factor or tumour suppressor protein involved in cellular growth and stress is called p53. It has been observed that doxorubicin increases the expression of the p53 protein, which causes the damaged cells to undergo apoptosis. Rats treated with doxorubicin showed increased p53 activity in their primordial follicles, which may be attributed to the same mechanism. Our study revealed that the mean number of primordial follicles did not significantly change between the research and control groups ($p>0.05$). Morgan *et al*[14] observed that the group of mice treated with doxorubicin had a marked reduction in the quantity of primordial follicles.

Larger follicles with more primary, secondary, and antral follicles, are susceptible to DNA damage from doxorubicin. Doxorubicin has been shown previously by Bar-Joseph *et al*[15] to be able to cross the follicular basement membrane and deposit in the mitochondria and DNA of the oocyte. This results in significant oxidative stress in the nucleus and mitochondria, damaging DNA, and a decrease in the number of targeted ovarian follicles. Doxorubicin not only targets primordial follicles but also has an impact on later stages of follicle development, which lowers the number of primary and secondary ovarian follicles.[16] In the current study, the mean value of primary ovarian follicles in right ovaries of rats was not significantly different between the control and research groups (1 and 2), however, the mean number of primary ovarian follicles in the left ovaries change significantly between the control and research groups (1 and 2). The mean value of secondary ovarian follicles (right and left) ovaries did not change significantly between the control and research groups (1 and 2). It was initially thought that doxorubicin was only slightly hazardous to reproductive organs, particularly those of women. Nevertheless, recent data[17] refutes this previously proposed theory. Reduced ovarian size and weight are the hallmarks of dose-dependent acute ovarian toxicity caused by doxorubicin therapy, which may also be linked to parenchymal fibrosis and ischemia. The rat ovaries treated with a single dose showed enhanced follicular atresia, which may have been brought on by severe oxidative stress.[18,19] A statistically significant difference was observed in our study in the mean number of atretic ovarian follicles in both right and left ovaries between the control and research groups (1 and 2). Samare-Najaf *et al*[20] found a similar discovery in a rat model, where doxorubicin caused follicular atresia. DOX causes DNA damage in normal tissues, resulting in dysregulation of apoptotic signalling pathways. These included a drop in Bcl-2, the activation of caspase, the increase of p53, and ultimately the death of cells.[21–23] In current study, inside the atretic follicles, nuclear fragments were seen which may be due to the severe ovarian atresia and apoptosis. Besides this, the mean number of nuclear fragments in left ovaries were statistically significant between the study groups as compared to the right ovaries. Reactive oxygen species (ROS) or free radicals are produced by doxorubicin at high, repeated doses, which leads to toxicity.

Numerous adjuvant antioxidant treatments have shown promising results to counter the adverse effects of DOX therapy. Roti *et al*[24] have shown that the iron chelating drug dexrazoxane considerably increased the overall survival rate of ovarian follicles in female mice against the harmful effects of doxorubicin during the course of chemotherapy. Promising supplements with strong antioxidant properties such as phytochemicals counteract the harmful effects of doxorubicin and other chemotherapy drugs. In an earlier research, Samare-Najaf *et al*[20] showed that the antioxidant vitamin E and the plant-derived flavonoid quercetin provide protection against the doxorubicin's ovo-toxic effects in a rat model. Mohajeri *et al*[25] showed that curcumin guards against the harmful effects of doxorubicin.

We used vitamin E as an antioxidant to study its protective effect against the toxic activity of DOX therapy in animal model. It was observed that after 28 days of the study, the mean body weight the rats in research group 2 increased by 43.80±20.25 gm. In contrast, the mean body weight of rats in research group 1 was decline by 20.04±1.50 gm. Rats receiving doxorubicin and vitamin E concurrently showed an increase in the number of ovarian follicles due to the preventive action of vitamin E. Our findings confirmed that vitamin E supplementation protects the ovary against the damaging effects of doxorubicin.

CONCLUSION

Simultaneous administration of vitamin E has demonstrated encouraging results in preventing doxorubicin-induced ovarian damage in animal model. More extensive and prospective research is needed to clarify the safety and efficacy of vitamin E in human individuals.

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SH: Principal Author
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