ORIGINAL ARTICLE

PROTECTIVE ROLE OF OLIVE OIL IN THE DOXORUBICIN INDUCED OVARIAN TOXICITY IN FEMALE RATS

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Background: The pathophysiological effect of chemotherapy on female reproductive system is complex. The current study aimed to determine the protective effect of olive oil extract against doxorubicin induced ovarian toxicity in female rats. **Methods:** This was an analytical experimental randomized control study carried out in the Department of Anatomy, Peshawar Medical College. Twenty-four female rats weighing 200–250 g were included and were divided into 3 groups, i.e., control group, experimental group-I and experimental group-II. Animals were weighed at the beginning of the experiment and before their sacrifice. The ovaries were obtained after their sacrifice. Five (5) µm thick sections were cut and stained with H & E stain, and Massan Trichome to see the changes the ovarian follicles. Data was entered and analysed on SPSS-20. Independent sample *t*-test and Chi-square test were used to measure the difference between and within groups, and $p \leq 0.05$ was considered statistically significant. **Results:** A significant increase in the mean weight of rats in control group and experimental group-II was observed ($p<0.05$) while a post-experiment significant decrease in the mean body weight of rats in experimental group-I was seen ($p>0.05$). There was no significant difference in number of primordial and secondary ovarian follicles within the groups ($p>0.05$) while a statistical significant difference in number on primary ovarian follicles, atretic follicles and nuclear fragments within the groups ($p<0.05$). **Conclusion:** The co-administration of olive oil has shown a protective effects against the doxorubicin induced ovarian toxicity in female rats.

Keyword: Doxorubicin, Ovarian toxicity, Female rats, Chemotherapy, Olive oil

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INTRODUCTION

Female ovary contains all the germ cells (oocytes) which are required for the whole life span by the time of their birth. These oocytes are kept in follicular cells which are surrounded by pre-granulosa cells. There are approximately 2 million follicles inside the ovary which are reduced to 300,000 at the time of puberty.[1] The premenopausal phase in women's reproductive life starts when less than a thousand follicle cells are left, normally around 45 years of age. The complete loss of ovarian follicles in healthy woman usually occurs around 50 years of age when all the ovarian follicles are vanished. However, early menopause can also result due to apoptosis or destruction caused by cancer chemotherapy. This phenomenon is known as 'chemotherapy-induced infertility', usually in young women. The cancer survivor women's ovary contain follicles same as those of post-menopausal women.[2] The pathophysiological effects of chemotherapy on female reproductive system is complex and depends upon its potency and frequency of administration, the age of the patient and her medical history also play their role in manipulating the functions of ovary.[3] Many anti-cancer drugs like cyclophosphamide, cisplatin, and doxorubicin (DOX) cause ovarian toxicity by direct damage to ovarian follicles, oocytes, granulosa, and cumulus cells, or may indirectly renders damage to ovarian parenchymal cells and vascular system which leads to inhibition of nutrients supply to the ovarian follicular cells.[4] In both cases, the ultimate result will be follicular atresia reducing the ovarian reserve. For this purpose, gonads preservative strategies are followed nowadays, i.e., cryopreservation of reproductive cells which can be implanted later especially in female cancer patients who are prone to chemotherapy-induced premature ovarian failure (POF).[5]

DOX is a cytotoxic agent from the anthracycline class of anti-tumour antibiotics which inhibits DNA synthesis by targeting topoisomerase II. The DOX cytotoxicity mechanism involves the induction of DNA intercalation between guanine and cytosine base pairs which interferes with DNA and RNA synthesis.[6] DOX is identified as an intermediate risk group member for causing ovarian toxicity, and causes both follicle-dependent and independent ovarian toxicity. A possible mechanism for DOX-induced ovarian toxicity could be due to activation of ataxia-telangiectasia mutated (ATM) kinase, which is an important downstream mediator of the RH-DNA damage response. The deregulation of ATM activity can influence cell survival. Studies in mouse models have shown that inactivation of ATM can lead to a suppression of apoptosis and an acceleration of tumour

development.[7] DOX could damage oocyte's DNA integrity in the ovarian follicle, but it has also been reported to induce damage to granulosa cells, the capillary system, and also induce oxidative stress.[8] Increasing evidence from studies in animal model has shown that DOX targets DNA and also mitochondria where it reduces mitochondrial functions and possibly triggers apoptotic signalling pathways by increasing the expression of pro-apoptotic mediators such as caspase-3 and caspase-12 in egg and granulosa cells.[9]

The leaves of the olive tree (*Olea europaea L.*) have been used as traditional therapies for many years in the Mediterranean countries. Several experimental studies have shown that olive leaves are useful in lowering blood pressure. Besides this, they have anti-atherogenic, anti-inflammatory, sugar and cholesterol lowering activities. These effects are attributed to the antioxidant components of the olive leaves. Oleuropein and its derivatives such as hydroxytyrosol and tyrosol are the most important phenolic compounds of olive leaves that are suspected to be responsible for their pharmacological properties. In addition, olive leaves contain caffeic acid, p-coumaric acid, vanillic acid, vanillin, Luteolin, Diosmetin, Rutin, Verbascosid, Luteolin-7-glucoside, Apigenin-7-glucoside and Diosmetin-7-glucoside. OLE or its constituents, especially oleuropein, protect tissues as an antioxidant when administered for therapeutic purposes.[10] Several researchers have examined the protective potential of OLE or its components, in various pathologies induced by oxidative stress such as arteriosclerosis, diabetes, the brain ischemia and lead-induced neuropathy.

The current study aimed to determine the protective effect of oral olive oil extract against doxorubicin-induced ovarian toxicity in female rats.

MATERIAL AND METHODS

This analytical experimental randomized control trial was carried out in the Department of Anatomy, Peshawar Medical College, Peshawar. The duration of study was six months from 1st January to 30th June 2021. The sample size and number of groups were calculated through the one-way ANOVA formula for animal studies. Twenty-four female rats weighing 200–250 g, were procured from the animal house of Peshawar Medical College, Peshawar and divided into three groups. The animal house facility of Pakistan Council of Scientific and Industrial Research (PCSIR), Peshawar, was used. An independent room was allocated for this research which was well ventilated and 12 hours light and dark cycle was maintained. The temperature was maintained as 18 to 26 °C. Healthy female rats were selected which had been previously pregnant. Rats with any disease prior to the onset of experiment, or the ones that developed disease during the study were excluded from the study. All animals were weighed at the beginning of the experiment. This weight was abbreviated as W_i (initial weight). They were then weighed just before their sacrifice and this weight was abbreviated as W_f (final weight). Doxorubicin injection (10 mg/dL) (ONCODOX-10 by AJ Mirza Pharmaceuticals, Lahore Pakistan) were procured. Olive oil extract was purchased from the local market. The animals were kept in solid bottom polypropylene cages and fed on commercial standard mash feed and water for rat. Experiments were conducted as per the protocol approved by the Institutional Animal Ethics Committee. The animals were divided into 3 groups comprising of eight (8) female rats each, i.e., control group, experimental group-I and experimental group-II (Table-1). On 28th day, animals were euthanized, and organs were collected in 10% neutral buffered formalin for micro-pathological changes and were processed for paraffin embedding, 5 µm thick sections were cut on rotary microtome and stained with H and E, for routine microscopy. Sections were also stained with Massan Trichome to see the changes in the connective tissue elements of the ovarian follicles. The follicles having visible nuclei were counted. Follicles containing 20 or more apoptotic granulosa cells (appearance of apoptotic bodies in granulosa cell layers), disorganized granulosa cells, a degenerating oocyte, or fragmentation of oocyte nucleus were categorized as atretic follicles.

Data was entered and analysed on SPSS-20. Quantitative variables were presented as Mean±SD. Categorical variables were computed as frequencies and percentages. Independent sample *t*-test and Chi-square test was used to measure the difference between and within groups, and $p \leq 0.05$ was considered statistically significant.

Table-1: Groups Demographics

Groups	Description
Control Group	Received 12 mL/L olive oil mixed in water once a week for 4 weeks.
Experimental Group-I	Doxorubicin injected intraperitoneally at 7.5 mg/Kg body weight once weekly for 4 weeks.
Experimental Group-II	Doxorubicin injected intraperitoneally at 7.5 mg/Kg once weekly for 4 weeks and then treated with olive oil, i.e., 12 mL/L of water for 4 weeks

RESULTS

The mean weight of rats in control group was 107.25±1.83 g before the experiment, while there was a significant increase in the mean weight of rats in control group after the experiment ($p<0.05$). The mean weight of rats in experimental group 1 before and after the experiment was 117.13±3.56 g and 93.0±6.41 g respectively and a significant decrease in the mean body weight of rats in experimental group 1 was found ($p<0.05$). The mean weight of rats in experimental group 2 before the experiment was 112.75±4.80 g. A significant increase in mean body weight of rats in

experimental group 2 was found after the experiment (p<0.05). The results are summarized in Table-2.

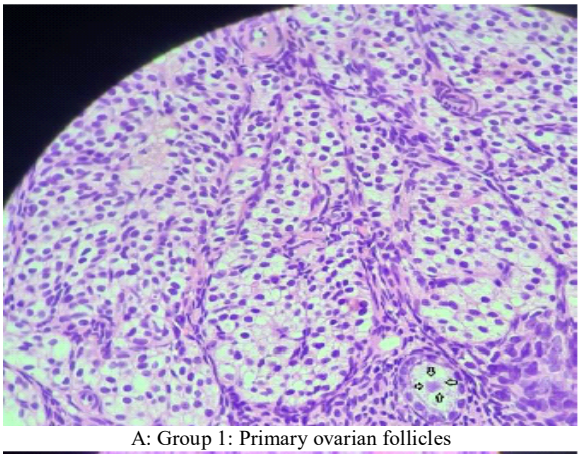
The H and E stain results are shown in Figure-1. There was no statistical significant difference between primordial and secondary ovarian follicles within the groups (p>0.05). However, a statistical significant difference was observed in number on primary ovarian follicles (right ovary), atretic follicles (in both ovaries) and nuclear fragments (left ovary) within the groups (Table-3).

Table-2: Mean body weight of animals (g)

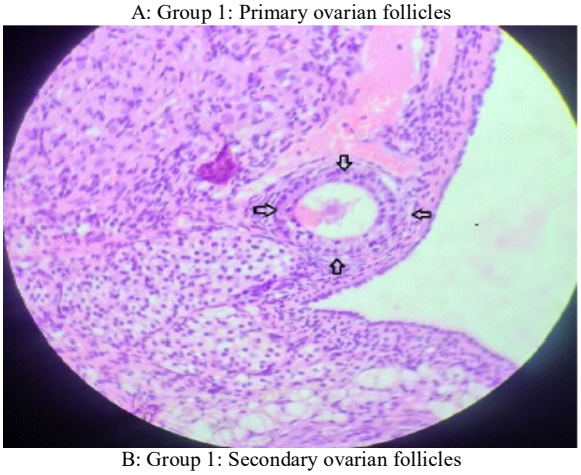
Group	Before experiment	After experiment	Weight change	*p*
Control group	107.25±1.83	111.50±4.63	-4.25±3.28	<0.05
Experimental Group-I	117.13±3.56	93.0±6.41	24.13±5.19	<0.05
Experimental Group-II	112.75±4.8	155.25±25.5	42.50±21.86	<0.05

Table-3: Follicle analysis within groups (Mean±SD)

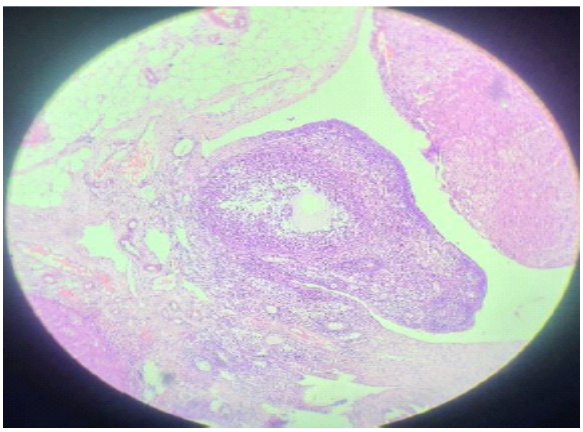
Follicles	Ovary	Control Group	Experimental Group-1	Experimental Group-2	*p*
Primordial	Right	1.75±1.65	2.13±2.1	2.37±1.92	0.78
	Left	1.63±1.99	1.63±1.77	1.63±1.77	1.00
Primary	Right	1.0±1.07	2.13±1.82	3.25±2.12	**0.05**
	Left	1.13±1.36	1.13±1.25	1.38±1.06	0.89
Secondary	Right	1.5±1.93	3.13±2.36	3.75±2.43	0.14
	Left	1.75±1.28	1.25±0.46	1.63±0.74	0.52
Atretic	Right	0.000	1.75±0.88	4.00 ±2.98	**0.001**
	Left	0.000	1.25±0.46	1.50±0.93	**0.002**
Nuclear Fragment	Right	0.000	0.50±0.75	0.25±0.46	0.173
	Left	0.000	1.50±0.93	0.75±0.89	**0.002**



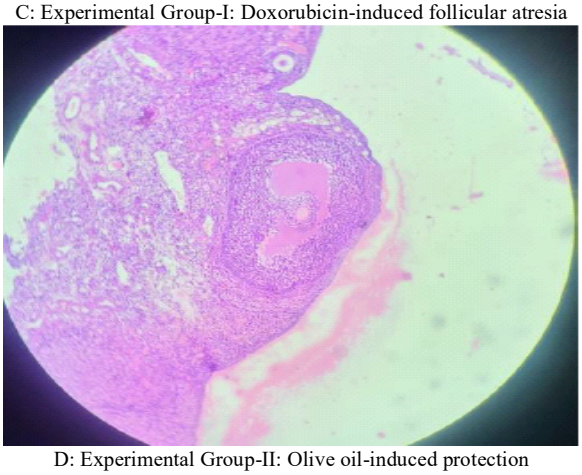
A: Group 1: Primary ovarian follicles



B: Group 1: Secondary ovarian follicles



C: Experimental Group-I: Doxorubicin-induced follicular atresia



D: Experimental Group-II: Olive oil-induced protection

Figure-1: Ovarian follicles in different experimental groups

DISCUSSION

Although chemotherapeutic medicines have been effective in the treatment of cancer, toxic effect from chemotherapy is indeed a common and unfortunate side-effect of treatment that can occur even at standard doses. One of the most serious long-term consequences of cytotoxic drug treatment is infertility, which is caused by premature ovarian failure (POF) or insufficiency (POI). As a result, the preservation of ovarian reserve and the prevention of infertility has risen to the top of the priority list for patients and their doctors. The specific causes of ovarian toxicity are unknown, and they vary depending on the medication and the cell type.

In the current study, the 28-day treatment period was chosen to test the effects of the drug on the ovulatory phase. It was observed that the 28 days doxorubicin-treated rats had reduced body weight (p<0.05), which suggests the loss of weight associated to enhanced oxidative stress. Previously, Swamy *et al*, also found that there was a significant reduction in the body weight of doxorubicin-treated albino rats as compared to animals in the normal control group.[11] However, a significant increase in body weight was observed in the rats included in control group and the

group which was receiving doxorubicin along with oral olive oil (p<0.05). The body weight of the rats in this group was increased since olive oil contain antioxidant compounds, it had a protective role against the toxic effects of doxorubicin treatment.

The density of primordial follicles determines female fertility. Chemotherapy survivors' ovarian reproductive function is investigated by monitoring menstrual behaviour or certain serum indicators as well as the number of primordial follicles to accurately predict fertility. Primordial follicular oocytes are very vulnerable to chemotherapeutic substances. Damage to the DNA causes the deposition of p53 in the cell. p53 is a transcription factor or tumour suppressor protein that plays a role in cellular stress and development. Doxorubicin has been reported to increase the expression of the p53 protein, which triggers apoptosis in the injured cells. The same mechanism may be assumed to be responsible for the enhanced p53 activity in primordial follicles of doxorubicin-treated rats. As per our study, there was no significant difference in the mean number of primordial follicles between the control group and the experimental groups (p>0.05). Morgan *et al*[12] have observed a significant decrease in number of primordial follicles in doxorubicin-treated mice group.

Doxorubicin can cause DNA damage in larger follicles with more granulosa cells, such as primary, secondary, and antral follicles. Bar-Joseph *et al*, observed that doxorubicin can pass through the follicular basement membrane and deposits in the oocyte's DNA and mitochondria.[13] This causes serious damage to DNA and oxidative stress in nuclei and mitochondria leading to decrease count of targeted ovarian follicles. Besides targeting primordial follicles, doxorubicin also affects later phases of follicle development, resulting in a decrease in primary and secondary ovarian follicles.[14] In our study, it was found that there was a significant difference between the mean number of primary ovarian follicles in right ovaries of rats in control group and the experimental groups (1 and 2) (p=0.05). However, no significant difference was found between the mean number of primary ovarian follicles in left ovaries in control group and the experimental groups (1 and 2) (p>0.05). Similarly, no significant difference was observed between the mean number of secondary ovarian follicles in right and left ovaries of rats in control group and the experimental groups (1 and 2) (p>0.05). Doxorubicin was formerly thought to be only mildly toxic to reproductive organs especially the female reproductive organs. However new evidence contradicts this previous assumption.[15]

Doxorubicin therapy results in dose-dependent acute ovarian toxicity characterized by decreased ovarian size and weight and may be associated to ischemia and parenchymal fibrosis. Single dose treatment resulted in increased follicular atresia in the rat ovaries, which could be caused by significant oxidative stress.[16,17] In our study, a significant difference was found in the mean number of atretic ovarian follicles in both right and left ovaries of control group as compared to the experimental groups (1 and 2) (p<0.05). The similar observation was seen by Samare-Najaf *et al*, in rat model, i.e., doxorubicin produced a dose-dependent follicular atresia which was prevented by concomitant administration of antioxidant therapy.[18] Several studies had been published indicating doxorubicin-induced DNA damage in several normal tissues, resulting in dysregulation of apoptotic signalling pathways such as decreased Bcl-2, caspase activation, p53 accumulation, and eventually cell apoptosis.[19–21] In our study, due to severe ovarian atresia and apoptosis, nuclear fragments were seen in the atretic follicles, and a significant difference was found in mean number of nuclear fragments in both left and right ovarian atretic follicles in experimental groups (1 and 2) as compared to the control group (p<0.05).

Doxorubicin induced toxicity arises because of its large, repeated doses and production of reactive oxygen species (ROS) or free radicles. Several adjuvant antioxidant therapies have been studies with significant advantages. Roti *et al*, found that Dexrazoxane —an iron chelating agent, significantly protected the overall survival rate of ovarian follicles against the toxic effects of doxorubicin in female mice till the course of chemotherapy.[22] Phytochemicals are promising supplements having significant antioxidant activities against the toxic effects of various chemotherapeutic agents including doxorubicin. Samare-Najaf *et al*[18], proved that quercetin (plant derived flavonoid) and vitamin E (antioxidant) have protective effect against the ovo-toxic effects of doxorubicin in rat model. Mohajeri *et al*[23], also demonstrated the protective effects of curcumin against the toxic effects of doxorubicin.

In the current study, olive oil was used as an antioxidant to study its potential to protect the ovarian follicular reserves against the toxic effects of doxorubicin in female rat model. After 28 days of the study, the mean body weight of experimental group 2 was increased by 42.50±21.86 gm as compared to experimental group 1 in which the mean body weight was decreased by 24.13±5.19 gm. Because of protective effect of olive oil, an increase in number of ovarian follicles was observed in those rats which were receiving doxorubicin and olive oil simultaneously. On the other hand, a decrease in number of ovarian follicles was seen in rats to which only doxorubicin was administered.

Our results have confirmed the protective role of olive oil against the toxic effects of doxorubicin in ovary in animal models. The results of our study coincide with the results of a study by Nishi *et al*[24], which also demonstrated the protective effect of olive

oil against the doxorubicin induced ovarian toxicity in Wistar rats.

CONCLUSION

The co-administration of olive oil can be a new adjuvant therapy which has shown promising effects against the doxorubicin induced ovarian toxicity in animal models.

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SF: Principle Researcher and manuscript writing
MK: Animal handling and laboratory work
SH: Animal handling and laboratory work
SB: Animal handling and data analysis
MF: Results compilation and statistical analysis
JB: Manuscript writing

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