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Full Length Research Paper

Effects of diazinon on the ovarian micro-anatomical and micrometric parameters of pregnant mice

Khawaja Raees Ahmad[1] , Muhammad Zahir Tahir[1], Shafaat Yar Khan[1]*, Hafiz Muhammad Tahir[1], Kausar Raees[3], Muhammad Arshad[1] and Tahir Abbas[2]

[1]Department of Biological Sciences, University of Sargodha, Pakistan.
[2]University of the Punjab, Lahore, Pakistan.
[3]Government College (for women) Farooq Colony Sargodha, Pakistan.

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Pregnant mice (10 in 4 groups each) were exposed to diazinon (DZN) at 10 and 20 mg/kg on gestation day 6 (GD 6), 9 and 12. A group of 10 pregnant animals was maintained as untreated control. The ovaries were exteriorized on GD 18 by euthanizing the animals. The tissues were processed for histopathological and micrometric analysis. Oocytic nuclear derangements in terms of size increase followed by leakage of nuclear contents with increased duration of exposure were seen. Follicular and luteal cells also showed necrosis. The morphometric data showed significant increase in oocyte and para-follicular size (P_0.0001) along with similar increase in nuclear sizes (P_0.001). These findings indicate that DZN is toxic to bring about histopathological and micrometric derangements in the ovaries of the treated pregnant females.

Key words: Pesticides, diazinon, toxic, gestation, ovaries, micro-anatomical, micrometric, derangements.

INTRODUCTION

Pesticides are group of chemicals synthesized to kill or repel a pest or to stop its reproduction (Gilden et al., 2010). Insecticides are chemicals used to kill insects selectively, although, most of the chemical insecticides are not specific and kill/harm non-target organisms (Shah and Sarivastava, 2000). Organophosphates are the most frequently used insecticides. These are powerful acetyl cholinesterase inhibitors. Diazinon (DZN) is moderately toxic class II organophosphate (WHO, 1998). In mouse sub-cytotoxic levels of DZN have neurotoxic effects on differentiating cells (Sidiropoulou et al., 2009). The developmental exposure to nontoxic doses of DZN in rats alters the acetylcholine synaptic function in adolescence and adulthood (Slotkin et al., 2008).

Diazinon exposures cause organ pathologies in mouse such as necrotic degeneration of spleen and thymus, hyperplasia of thymus, spleen and lymph nodes, and sometimes hemorrhage from all tissues (Handy et al., 2002). Its exposure causes diarrhea, pleural effusion, hemorrhage and ulceration of proventiculus, and swollen and hemorrhagic liver in broiler chicken (Hill et al., 1994). In rabbits, DZN causes decrease in RBCs, haemoglobin and plasma total proteins, and increases cholesterol and microsomal protein levels (Yehia et al., 2007). Diazinon causes hepatotoxicity by causing swelling of mitochondria and breakage of mitochondrial cristae of hepatocytes in Wistar rats. Vitamin E decreases this toxicity, but does not protect completely (Kalender et al., 2005).

The major function of the female gonad is to release mature oocytes for fertilization and propagation of the species. In mammalian ovaries, an individual follicle consists of an innermost oocyte, surrounding para-follicular cells and an outer layer of stromal cells. The fate of each follicle is controlled by endocrine and paracrine factors (Gougeon, 1996; Greenwald and Roy, 1994; Hirshfield, 1991). The follicles develop through primordial, primary and secondary stages. At this stage, most follicles undergo atretic degeneration and few reach the pre-ovulatory stage under the cyclic effect of gonadotropins (Soji and Herbert, 1990). At birth, the mammalian ovary contains a finite number of primordial follicles. The majority of these about 99% undergo degeneration or atresia by cell death or apoptosis

*Corresponding author. E-mail: shafaatyarkhan@hotmail.com.

(Erickson et al., 1985). In mammals, the corpus luteum is formed at the site of ovulation. Its primary function is to synthesize and secrete progesterone, which regulates menstrual or estrous cycle and prepare the uterus for reproduction (Soji et al., 1990). In mice, DZN causes a significant reduction in the body weight, number of primordial, primary and growing follicles, corpus luteum and its cells and the diameter of Graffian follicles and their oocyte and nuclei, theca and granulosa layers and also the diameter of corpus luteum and the blood levels of estrogens and progestins. It also shows a marked increase in the number of Graffian and atretic follicles and diameter of ovaries (Homa et al., 2008).

The aforementioned literature indicates that there are only a few studies which directly put spotlight on the gonado-toxicity especially ovotoxicity of this otherwise a proven systematic toxicant. The present research was aimed to be carried out on pregnant female mice for micro-anatomical and micrometric derangements of this commonly used insecticide on the ovary- an organ bearing vital germplasm and simultaneously executing endocrine functions.

MATERIALS AND METHODS

Animal groups and diazinon treatment

Pregnant albino mice (*Mus musculus*) of the Swiss Webster strain were used in the present study to check the ovarian histopathological responses of the co-gestational exposure of diazinon. The animals were maintained in a wire gauzed 12"×18" steel frame cages under 12-12 h dark light cycles. Food and water were provided *ad libitum*. The ambient temperature and humidity were maintained at 23 ± 2°C and 40 to 45%, respectively. Fifty pregnant animals weighing 30 ± 3 g were randomly divided in to 3 groups namely, the vehicle, 10 and 20 mg/kg groups. The vehicle group (10 animals) was maintained as untreated control. The 10 and 20 mg/kg groups (20 animals each) were further divided in single and triple treatment subgroups. These subgroups received their respective dose of DZN correspondingly on GD 6, 9 and 12. All doses were applied orally by gavage.

Animal recoveries and histological processing

On GD18, the gravid uteri and ovaries exteriorized from the etherized dams. Intact ovaries were carefully separated from the adjoining connective tissue and washed in normal saline.Finally, for the histological and micrometric estimations, ovaries were fixed in alcoholic Bouin's solution for 48 h. After fixation, these tissues were processed for wax embedding and microtomy. The serial sections of 8µ thickness of these ovaries were obtained on a rotary microtome (ERMA TOKYO 422). The histological sections were further processed for hematoxylin and eosin staining.

Digital photography and computer based micrometry

Micro-photographs of the selected sections from control and each subgroup from 10 and 20 mg/kg groups were obtained using Labomed CXR2 trinocular microscope mechanically fitted with Sony Cybershot (Model: DSC-W35) 7.2 megapixel digital camera at 100, 400 and 1000x for digital micrometry and histopathological presentations. Digital micrometry was carried out using copy- paste mode in corelDRAW11. The calibrated wireframe mode was applied on these digital images for this purpose. The wireframe calibrations were carried out with the help of similar images of the stage micrometer on the respective magnifications. For highlighting the histological abnormalities, the digital images of the selected sections from each group (and subgroups) were processed in color balance, bright-contrast and gamma modes in CorelDRAW11. To highlight various abnormalities these digital images were cropped using corelPHOTO-PANT 11 and finally the digital marks (labeled arrows) were added in corelDRAW11.

Morphometric data analysis

Microscopic data of various ovarian parameters like primary follicular size, size of the oocyte, oocyte nuclear size, mean size of the parafollicular cells and mean size of the endocrine cells of corpora lutea were obtained in corelDRAW11. Data obtained on these parameters were analyzed on the bases of single factor ANOVA and descriptive statistics in data analysis mode using MS excel data sheets. The results are shown in the form of histograms.

RESULTS

Follicular size

Average follicular size in control group animals was 3584.16 μ^2. A dose and exposure dependent significant increase ($P \leq 0.01$) in mean follicular size was observed in DZN exposed groups (Figure 1).

Size of the oocyte

In the control group, mean oocyte size remained 1510.06 µM². A similar trend has already been noted in mean follicular size was observed in the oocyte size. Overall data showed a significant divergence ($P \leq 0.01$) (Figure 2).

Oocyte nuclear size

The control nuclear size remained 190.12µ². The nuclei in the DZN treated groups showed an increasing trend in size. The mean value (400.84 µ²) for 20 mg/kg triple exposure subgroup showed above two folds increase in nuclear size to that of control group value (Figure 3).

Parafollicular cell size

Slight increase in all DZN treated subgroups to that of cell size in untreated group (66.29 µM²) were noted. Means ± SEM in each DZN treated and untreated groups and sub groups are shown in Figure 4.

Mean size of the endocrine cells of corpora lutea

Apart from 10 mg/kg single exposure sub groups where

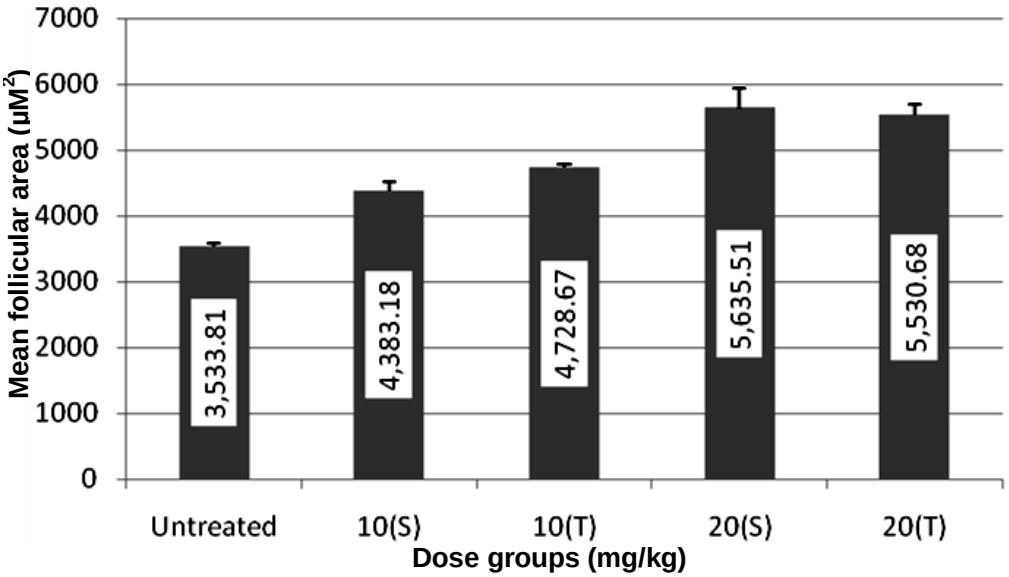

Figure 1. Effect of different doses of diazinon (control, single 10mg/kg, triple 10mg/kg, single 20mg/kg, triple 20mg/kg) on mean follicular area.

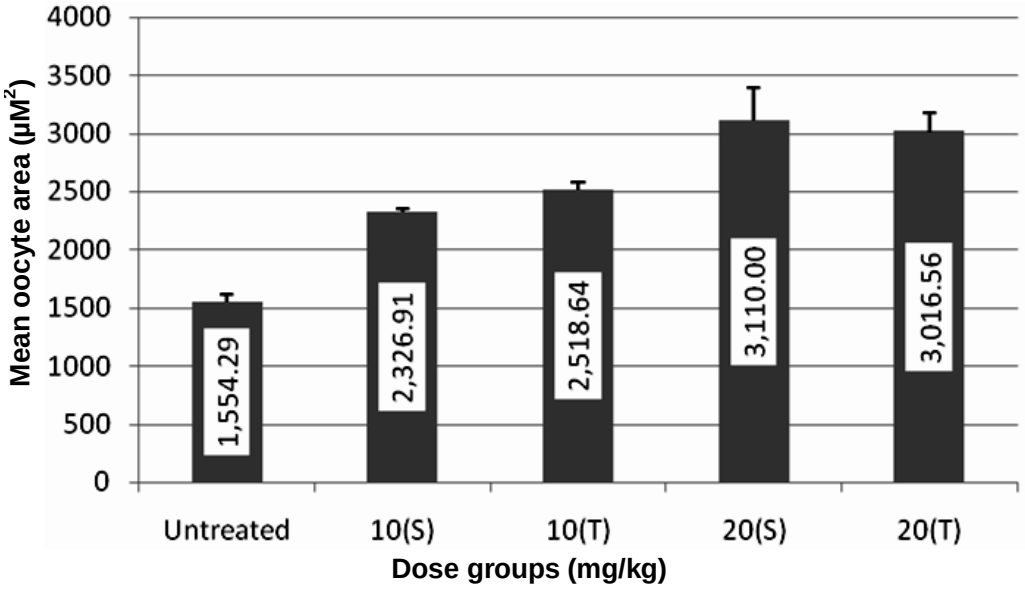

Figure 2. Effect of different doses of diazinon (control, single 10 mg/kg, triple 10mg/kg, single 20mg/kg, triple 20mg/kg) on mean oocyte area.

an increase in mean luteal cell size (149.90 µm²) to that of control group (126.97 µm²) was seen, all other sub groups showed a decline in mean luteal cell size as shown in Figure 5.

Histological results

In the control group, histological slides revealed that oocytes were well placed and embedded in ovarian stroma surrounded by parafollicular cells. The corpus lutea appeared as normal possessing functional endocrine cells. No signs of cellular apoptosis or involutions were observed (Figures 6A and 7A). Oocytic enlargements along with nuclear derangements in terms of increase in size and finally leakage of the nuclear material were seen in both DZN treated groups especially at triple exposure. Moreover, oocytic atresia within the regressing individual primordial follicles was observed in 20 mg/kg triple exposure sub group (Figure 6E).

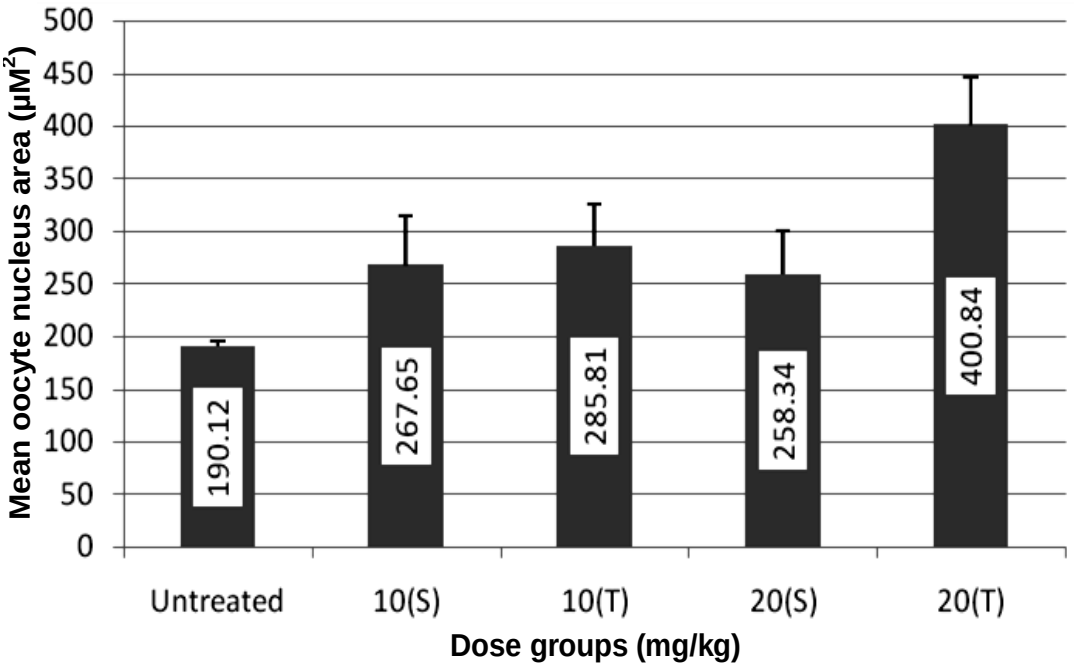

Figure 3. Effect of different doses of diazinon (control, single 10 mg/kg, triple 10 mg/kg, single 20 mg/kg, triple 20 mg/kg) on mean size of oocyte nucleus.

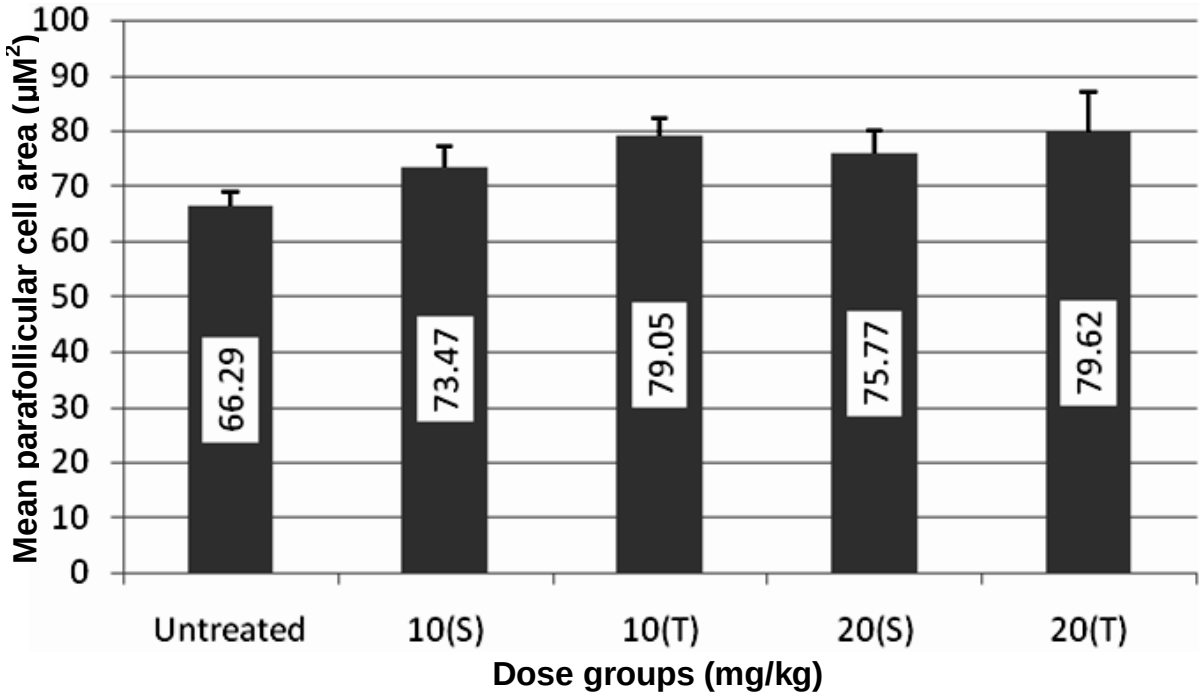

Figure 4. Effect of different doses of diazinon (control, single 10mg/kg, triple 10mg/kg, single 20mg/kg, triple 20mg/kg) on mean area of parafollicular cells.

Primordial follicles that appeared embedded deep within the ovarian stroma in untreated group were seen more superficial in position closer to the outer ovarian margins in DZN treated groups (Figure 6). Follicular cells showed involutions and apoptosis as indicated by the appearance of the fibrosis around the oocyte in both

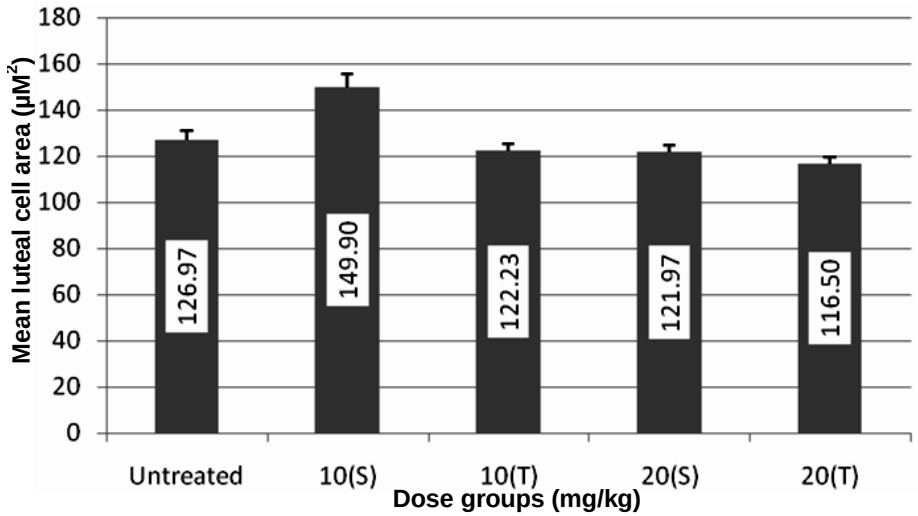

Figure 5. Effect of different doses of diazinon (control, single 10mg/kg, triple 10mg/kg, single 20mg/kg, triple 20mg/kg) on mean luteal cell area.

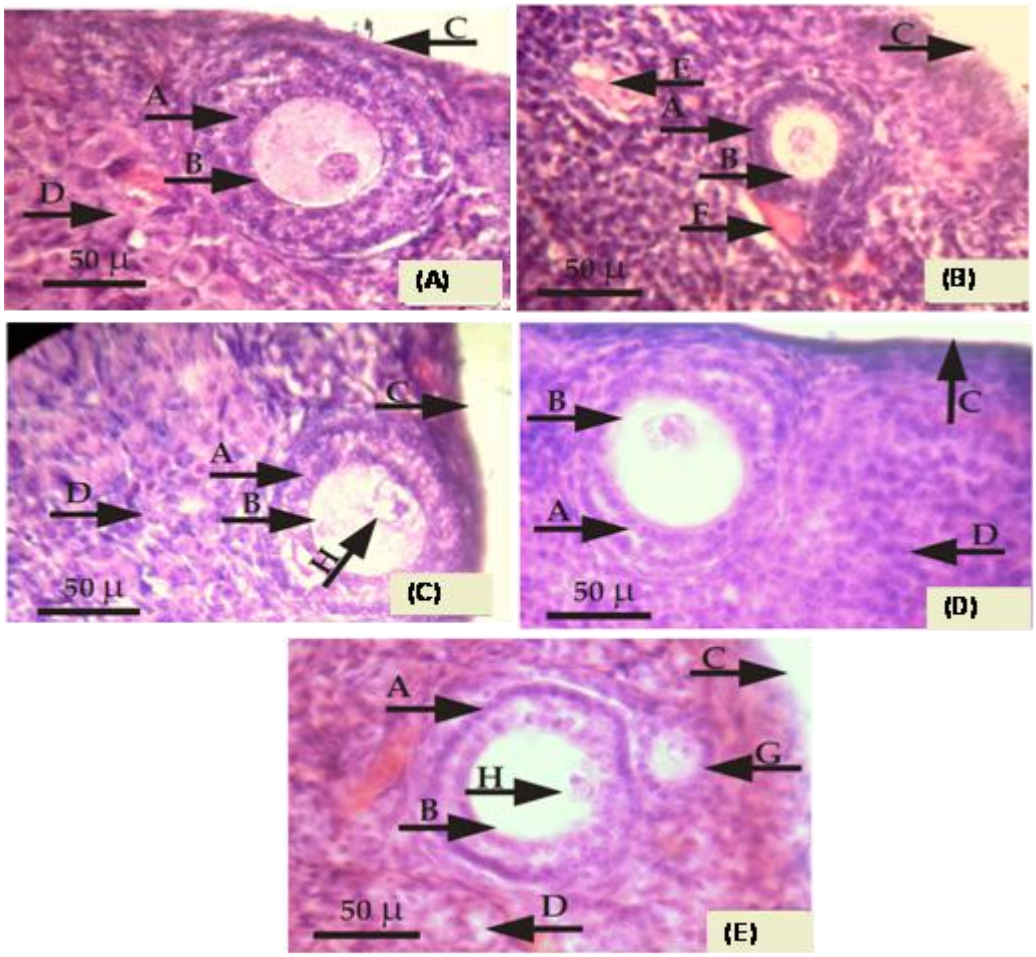

Figure 6. Sections of Ovary treated with different doses of DZN (400X). (A) Untreated group; (B) 10 mg/kg Single dose; (C) 10 mg/kg Triple dose; (D) 20 mg/kg Single dose; (E) 20 mg/kg Triple dose. A: Parafollicular cells; B: oocyte; C: outer margin; D: corpus luteal cells; E: follicular atresia; F: parafollicular stromal involution; G: primary oocytic atresia; H= nuclear material leakage.

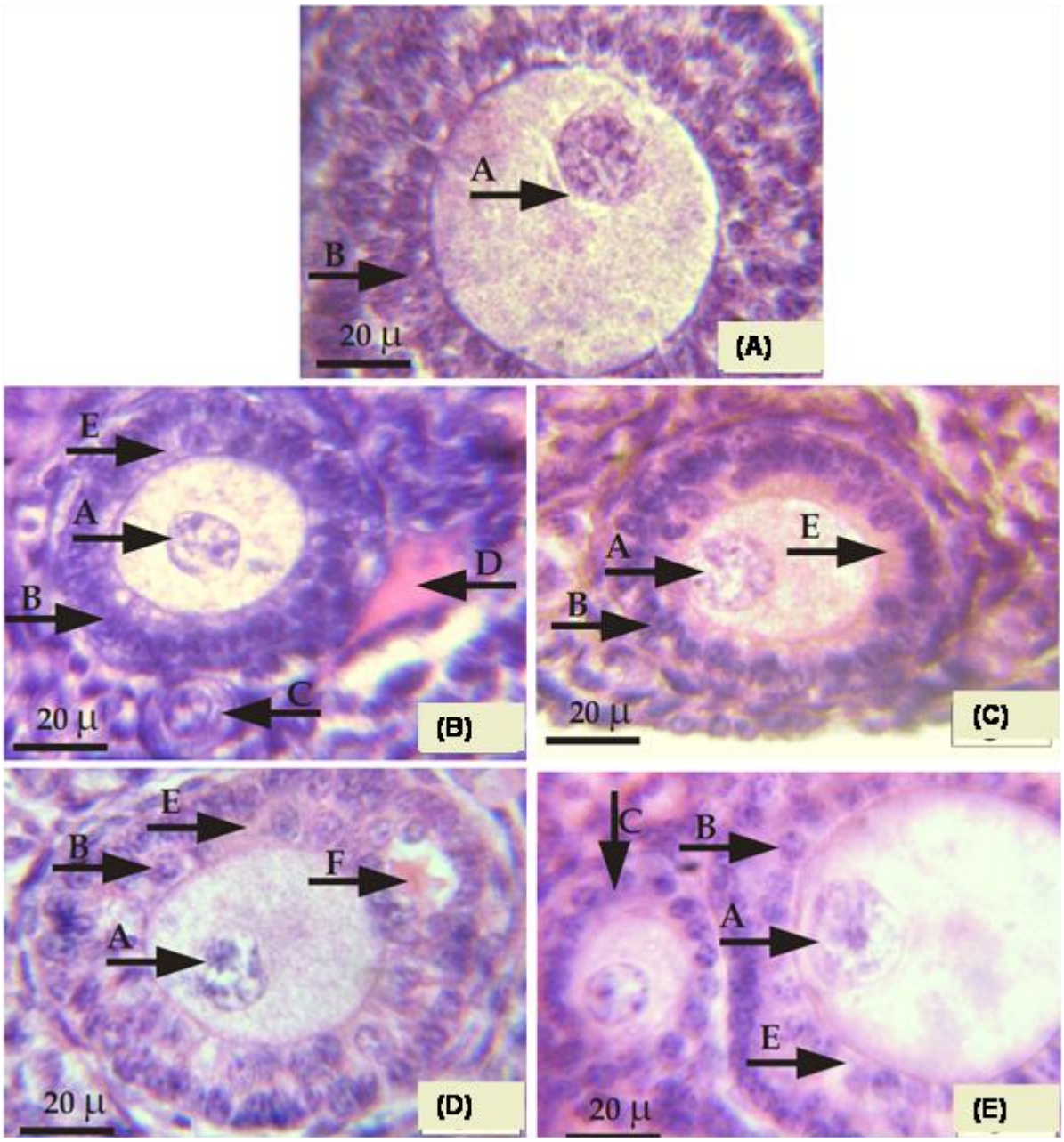

Figure 7. Sections of ovary treated with different doses of DZN (1000X). (A): Untreated group; (B): 10 mg/kg single dose; (C): 10 mg/kg triple dose; (D): 20 mg/kg single dose; (E): 20 mg/kg triple dose. A, *Oocyte nucleus;* B, *Parafollicular cells;* C, *Shrunken Follicles;* D, *parafollicular* stromal involutions;. E, parafollicular cell apoptosis; F, parafollicular cell fibrosis and involution.

subgroups of 10 and 20 mg/kg DZN groups. Similar involutions are also evident in parafollicular stroma (Figure 7).

DISCUSSION

Diazinon (DZN) has been found to exert various toxicological effects on mammalian spermatogenesis. On the other hand, little is known about its effects on oogenesis (Bonilla et al., 2008). In a study on bluegill (*Lepomis macrochirus*) ovaries examined after various intervals of exposure of 60 µg/L DZN have shown primary follicle adhesions and cytoplasmic retractions after 24 h of exposure. The degree of adhesion and retraction enhanced with the increase of exposure time along with cytoplasmic degeneration. Complete destruction of follicles was found at 2 to 3 weeks of exposure along with

the extrusion of karyoplasms (Dutta and Maxwell, 2003). The limit and duration of exposure has further been related with the levels of estradiol, which has indicated a gradual decline in comparison with the control sample along with an increase in the duration of exposure (Maxwell and Dutta, 2005).

Unfortunately, there is far little literature available to compare the results of present study. In a similar study on DZN where 40 mg/kg insecticide was given intraparitoneally for seven consecutive days, results revealed significant decrease in body weight, number of primordial and primary follicles, and corpora lutea along with a decline in the diameter of corpora lutea. On the other hand, the estrogens and progesterone levels were also decreasing, while Graffian and atretic follicles were increasing in number (Homa et al*.,* 2008). In present study, it has been observed that the mean follicular size, mean oocytic size, oocytic nuclear size and mean parafollicular cell size showed an increasing trend in a dose and duration of exposure dependent manner, whereas an inverse trend was seen in average luteal cell size. Taken together with histological data where disruption of the oocyte nuclei, expansion and declining cytoplasmic density along with individual oocytic and follicular atresia in both the treated groups has been found in pregnant female ovaries, it has been seen with concern that follicular cells showed necrosis along with fibrosis in parafollicular ovarian stroma on enhanced dose and duration of exposure.

The results obtained in this study gave a clear preview that DZN is toxic to ovarian tissue in general, and developing oocytes and endocrine parafollicular cells in particular. It also inflicts size reduction on the luteal cells. This seems to bring a decline in oogenesis, ovulations and corpus luteal formation that must lead to a decline in fertility index if such exposure has to be applied in virgin females. To develop a further insight into the ovarian toxicology hormonal bioassay in various female conditions such as adolescent virgins, pregnant and lactating mothers, exposed to DZN in conjecture with histologic and micro morphometric analysis of this study is recommended.

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