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Electron microscopic examination of the effects of methyl parathion exposure on the ovaries

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Abstract. – OBJECTIVE: In our study, we aimed to investigate histopathological effects of chronic methyl parathion exposure on ovaries at electron microscopic level.

MATERIALS AND METHODS: In this study, Wistar albino type, adult, female rats with an average weight of 190-250 g were used. 30 female rats, included in this study, were divided into 3 groups. Group I received only saline and was evaluated as the control group, whereas Group II received 1/50 percent of LD_{50} dose of methyl parathion and Group III received 1/20 percent of LD_{50} dose of methyl parathion every day at 1^{30} pm orally by gavages during two estrus cycles (8 days). The rats at proestrus stage on the morning of 9^{th} day of the study underwent bilateral ovariectomy. Ovarian tissues of the control and drug groups were examined under the electron microscope; primordial and growing follicles were included in the evaluation, however, corpora lutea were excluded taking into account the presence of remaining regressive corpora lutea from the previous cycles.

RESULTS: Following examination of ovarian tissues of rats exposed to 1/50 and 1/20 percent of LD_{50} dose of Methyl parathion at electron microscopic level, it was detected that significant structural changes had occurred in developing follicles and ovarian stroma in both drug groups, and that primordial follicles had not been affected significantly from methyl parathion but necrosis had been developed in oocyte and granulosa cells of developing follicles, and that in 1/20 group in addition to these changes, apoptotic changes had been found in granulosa cells of developing follicles.

CONCLUSIONS: As a result of chronic exposure to methyl parathion, rat ovaries are significantly affected and follicular development is impaired. This state may explain the cause of infertility due to chronic pesticide exposure.

Keywords:
Methyl parathion, Infertility, Reproductive system, Ovaries.

Introduction

Methyl parathion exposure occurs by skin and respiratory tract in agricultural areas where frequent pesticide is used or by digestive tract as a result of consumption of pesticide-treated fruits and vegetables without abiding 28-35 days consumption standby time[1,2]. It is known that mortality and morbidity causing methyl parathion affects the central nervous system, impairs nerve impulses at peripheral nervous system and has mutagenic and carcinogenic effects[2,3]. In reproductive studies, it had been reported that the number of abnormal sperm increases in a dose-dependent manner in rats exposed to different doses of methyl parathion, pregnancy is terminated in pregnant rats exposed to high-dose methyl parathion, estrous cycle is affected, and that methyl parathion causes decrease in rate of live births and total number of healthy follicles, causes embryo toxicity and a decrease in the number of oocytes in ovaries[4-8]. However, histopathologic effects of methyl parathion on reproductive system and especially on ovaries are not yet clearly understood.

In our study, it was aimed to investigate histopathological effects of chronic methyl parathion exposure on ovaries at electron microscopic level.

Materials and Methods

The study was started after obtaining the permission from the Animal Experiments Ethics Committee. In this study, Wistar albino type, adult, female rats with an average weight of 190-250 g were used. Rats were isolated from male rats for a period of 21 days, which is the period of gestation, in order to eliminate the possibilities of

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presence of pregnant rats. Ambient temperature was kept constant at 22 ± 2°C. Ventilation was provided with a window type exhauster. The windows of the room were painted black, and cycle of brightness and darkness was adjusted to be 12 hours light and 12 hours dark with an automatic controller device (06^{00}-18^{00} bright, 18^{00}-06^{00} dark). The rats were kept in stainless steel wire cages. Sawdust was used in the cage for bedding and was changed once a week. Animals were fed with ready-pellet-feed and tap water was used for water requirements. All the water and nutrient intakes of animals were provided as free.

A vaginal smear was done from all rats every day between the hours of 8^{30}-9^{30} for a month and phases of the cycle were detected. In vaginal smear procedure, 40-50 µl of 0.9% NaCl was transferred into the vagina with the help of a micropipette, and then the liquid was withdrawn and transferred on a slide and stained with methylene blue, and slides sealed with a cover slip were examined under light microscope. Phases of estrus cycle of all animals were recorded according to the examination. The rats with cycles close to each other were included in the study.

30 female rats, included in the study, were divided into 3 groups. Group I received only 0.9% NaCl and was evaluated as the control group, whereas Group II received 1/50 percent of LD_{50} dose of methyl parathion (Folidol M EC 360, Bayer, Leverkusen, Germany) and Group III received 1/20 percent of LD_{50} dose of methyl parathion. Group II and III rats received 1/50 (0.43 mg/kg/day) and 1/20 (1.09 mg/kg/day) of LD_{50} dose (21.8 mg /kg), following dilution with 0.9% NaCl, orally as 1 ml every day at 1^{30} pm by gavages during two estrus cycles. As the age of rats is less than 1 year, the duration of one cycle was accepted as 4 days (24) and methyl parathion was applied for 8 days, as two cycle times. 1 ml of 0.9% NaCl was given by gavage to the rats in Group I at the same time for 8 days. The rats at proestrus stage on the morning of 9^{th} day of the study underwent bilateral ovariectomy.

The animals were positioned in supine position following anesthesia with intraperitoneally (i.p.) administered xylazine hydrochloride (10 mg/kg, Rompun, Bayer, Leverkusen, Germany) and ketamine hydrocloride (80 mg/kg, Eczacibasi, Istanbul, Turkey). Then, thoracic region of rats were cleaned with Povidone-iodine and thoracotomy was performed for perfusion. A pink-tipped IV cannula (16 F gauge catheter) was placed into the left ventricle to access aorta for perfusion and right atrium was cut. During perfusion, to free up the circulating blood, first system was washed with 0.9% NaCl solution for 5 minutes. When clear 0.9% NaCl was seen in the right atrium, perfusion was started with Karnovsky Fixative, used as the perfusion solution, for 15 minutes. After perfusion process had been completed, abdominal cavity was opened with a deep incision right after cleaning the animal's abdominal surface. The skin and peritonea were held with clamps, so a more suitable environment for working in abdominal cavity was provided. The intestines were pushed upwards with the help of a forceps. The exposed ovaries were held with a fine forceps and excised. Ovaries were fixed in 5% glutaraldehyde in Millonig's phosphate buffer at pH 7.4 for 4 hours and postfixed in 1% osmium tetraoxide (OsO4) in phosphate buffer at pH 7.4 for 2 h at 4°C. Tissues were dehydrated in graded ethenol and embedded in araldite. Sections were cut using Leica Reichert OMU3 ultramicrotome (Germany) stained with both uranyl acetate and lead citrate and then examined with Zeiss EM 10 B electronmicroscope (Jena, Germany).

Results

Group I

It was observed that the oocyte and surrounding single layer of flat-shaped follicular cells in primordial follicle were separated from ovarian stroma by a thin basal lamina. Centrally-located oocyte nucleus was observed to be spherical shaped and have euchromatic structure. Organelles were scattered throughout ooplasm, and mitochondria with lamellar type crest, short-RER cisternae and Golgi complexes were observed. In addition, cortical granules were scattered throughout ooplasm (Figure 1).

In developing follicles, oocyte was surrounded by multi-layered cubic or prismatic form granulosa cells. In spherical shaped granulosa cell nuclei, chromatin granules were homogeneously dispersed throughout the nuclei, while the heterochromatin granules constituted small clusters in relation to nuclei membrane. Elongated or spherical-shaped mitochondria, RER cisternae and free ribosomes were widely seen in granulosa cell cytoplasm, and a prominent Golgi complex was also included. Zona pellucida, located between the oocyte and granulosa cells, had a uniform thickness and a homogeneous density. In addition, microvilli of the oocyte and cytoplas-

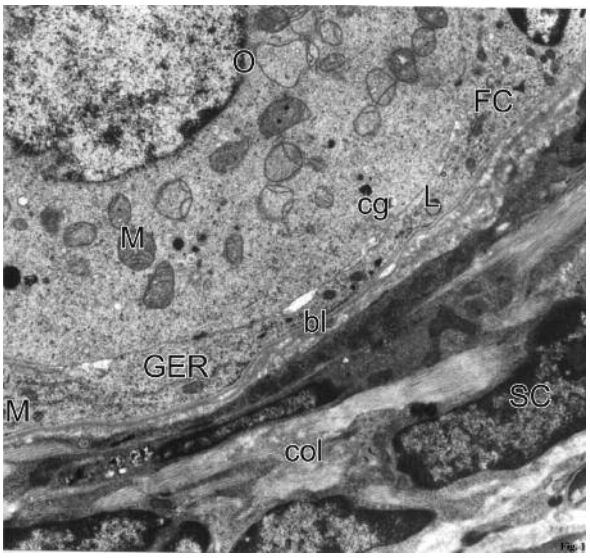

Figure 1. Mitochondria with lamellar type crest (M) and cortical granules (cg) in ooplasm of oocyte (O) in primordial follicle; mitochondria (M), rough endoplasmic reticulum (RER) cisternae and rare lipid droplets (L) in cytoplasm of follicular cells (FC) in close relationship with the oocyte; basal lamina (bl) separating follicle from ovarian stroma are seen. Fibroblast-like stromal cells (SC) and collagen fibers are present in ovarian stroma. × 12400.

mic processes of granulosa cells were within Zona pellucida. Granulosa cells were observed to be separated from theca layer by means of a thin and uniform basal lamina (Figure 2).

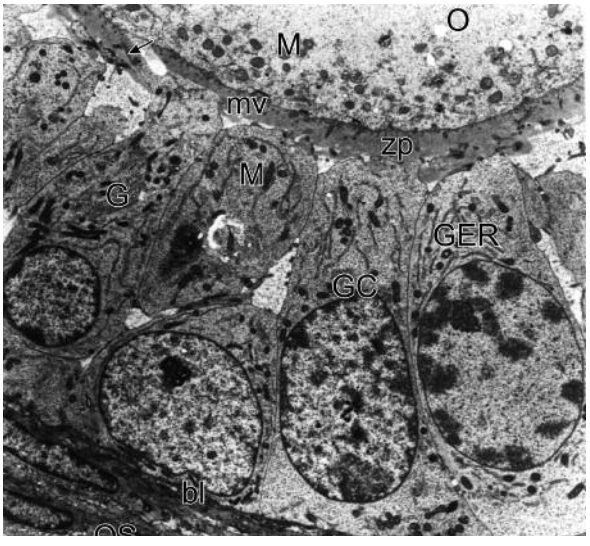

Figure 2. Oocyte (O) of the developing follicle and prismatic-shaped granulosa cells (GC) around the oocyte are seen. Mitochondria (M), rough endoplasmic reticulum (RER) cisternae, Golgi complex (G), zona pellucida (ZP), Basal lamina (bl), microvilli (mv), cytoplasmic processes of granulosa cells (*arrow*), ovarian stroma (OS). × 6300.

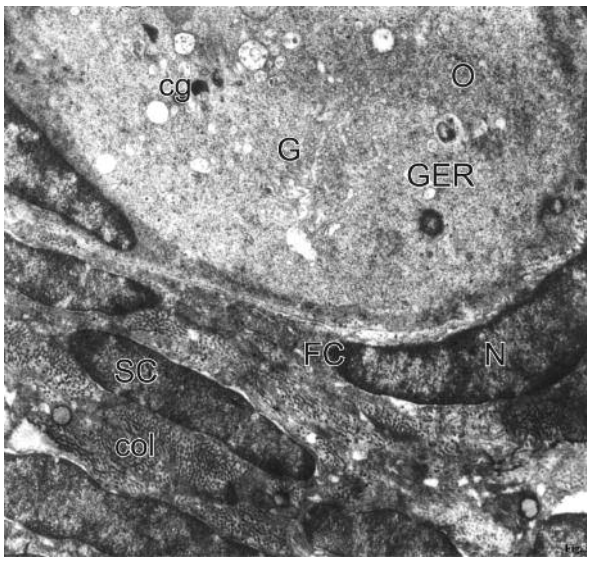

Figure 3. Well-developed Golgi complex (G), cortical granules (CG), short rough endoplasmic reticulum (RER) cisternae, nucleus (N) of flat follicular cells surrounding the oocyte (O) in ooplasm of primordial follicle; fibroblast-like stromal cells (SC) and collagen fibers (col) in ovarian stroma are seen. × 16100.

Group II

Primordial follicles had similar characteristics with control group at the level of fine structure, and well-developed Golgi complex, cortical granules and short-RER cisternae had been present within oocyte cytoplasm (Figure 3).

Heterochromatin granules were increased and perinuclear cisternae were dilated in nuclei of granulosa cells showing degenerative changes of different levels in developing follicles. In addition, besides an increase in cytoplasmic density, widespread development of vacuoles and lipid droplets, enlarged ER cisternae and an increase in densities of mitochondria matrices were present in the cytoplasm of these cells. It was observed that gaps developed between Zona pellucida, located between the oocyte and granulosa cells, and besides the increase in density of microvilli, cytoplasmic extensions and microvilli were found to be absent in some areas of Zona pellucida (Figures 4, 5).

Group III

Oocyte nucleus, settled in the cortex of ovary, continued to preserve its normal structure in primordial follicles. Due to dilatation of Golgi complex cisternae, the cytoplasm appeared as membranous whorl. In addition, reduction in mitochondria cristae and electron lucent areas in matrices were present. The existence of a close rela-

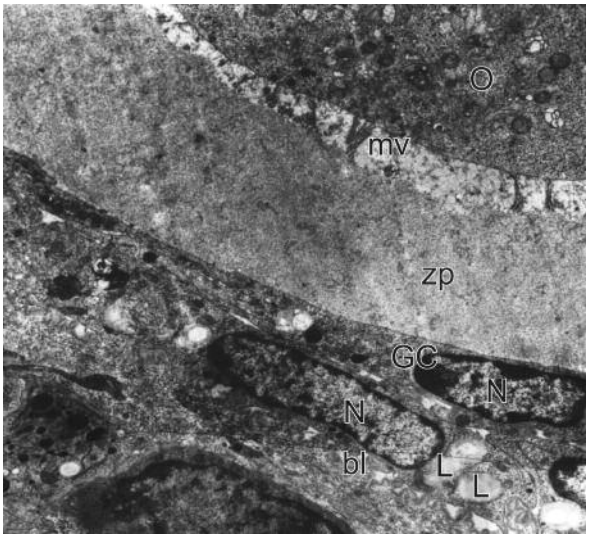

Figure 4. Short and dense microvilli (mv) on the surface of oocyte (O) of follicle at advanced stage of development, zona pellucida (ZP) separated from oocyte; nucleus (N), lipid droplets (L) and twisted basal lamina (bl) separating follicle from theca layer in flat-shaped granulosa cells (GC) which lost their cytoplasmic processes are seen. × 10100.

tionship between the follicular cells and oocytes had been continuing. Basal lamina, that separates follicular cells from stroma, consisted its normal structure (Figure 6).

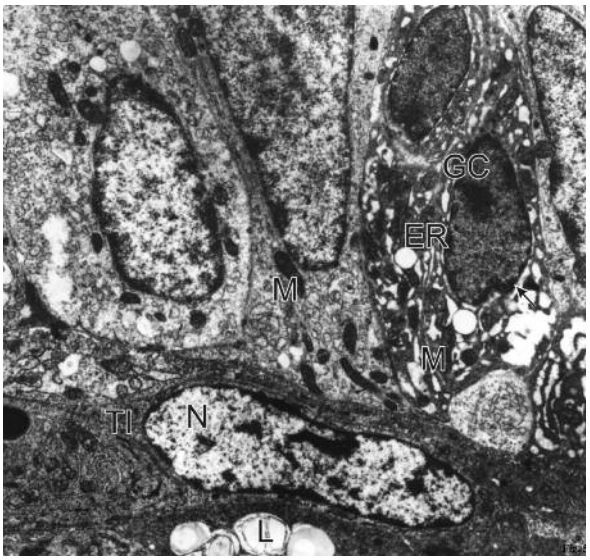

Figure 5. Enlarged endoplasmic reticulum (ER) cisternae and perinuclear cisternae (*arrow*), mitochondria (M) with increased matrix density in granulosa cells (GC) showing degenerative changes of different levels in developing follicles; density loss in nuclei (N) and increased lipid droplets in cytoplasms of enlarged cells (TI) at theca interna layer. × 10100.

Figure 6. Normal spherical shaped oocyte (O) nucleus (N) in primordial follicle; enlarged Golgi complex (G) in membranous whorl shape, mitochondria (M) without cristae, vacuoles (v), cortical granules (cg) in cytoplasm; nuclei (N) of normal-appearing follicular cells (FC) surrounding the oocyte and Golgi complex (G), mitochondria (M) and rough endoplasmic reticulum cisternae in their cytoplasm are seen together. A thin basal lamina (bl) separating follicle from ovarian stroma (OS) is seen. × 12400.

Structural changes present in the cortex of the oocyte of developing follicles were significantly observed in this group too. Oocyte nucleus had lost its spherical shape in developing follicle. Besides multivesicular bodies of different size and volume and membranous structures organized in parallel bundles next to each other in the cytoplasm of oocytes, matrix densities and cristae of mitochondria were observed to be lost. It was observed that microvilli on the surface of the oocyte had been rarefied, a gap between oocyte surface and Zona pellucida had developed and Zona pellucida had lost its uniform thickness (Figure 7).

In some micrographs of the developing follicles, it was seen that there were closely related three oocytes in a follicle containing abnormal nuclei. Chromatin granules of the nucleus were observed to lose their normal ultrastructural features. In addition, mitochondria, lysosomal structures and electron-dense granules formed large clusters gathering together in the area close to the nucleus and peripheral ooplasm. Mitochondria in ooplasm were observed to lose their cristae partly and a relatively homogeneous structure surrounded by an electron opaque membrane was seemed to be composed around

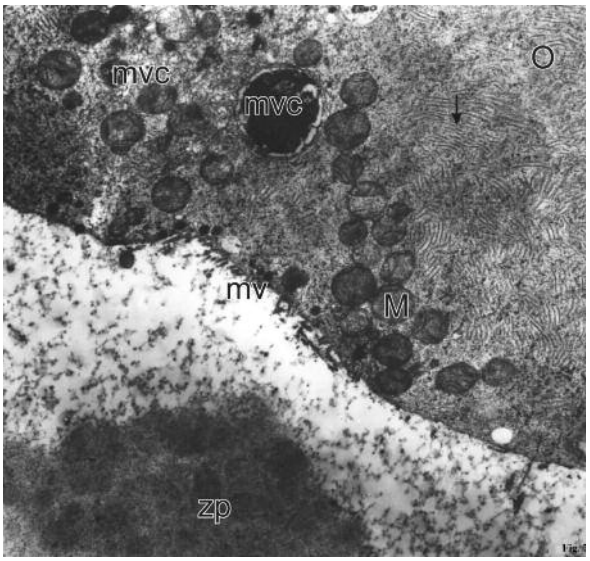

Figure 7. Multivesicular bodies (mvc) of different size and volume, membranous structures (*arrow*) organized in parallel bundles next to each other, mitochondria in the cytoplasm of oocytes (O) which had lost its smooth spherical shape in the developing follicle; rarefied shortened microvilli (mv) on the surface of the oocyte and zona pellucida (zp) separated from oocyte are seen. × 12400.

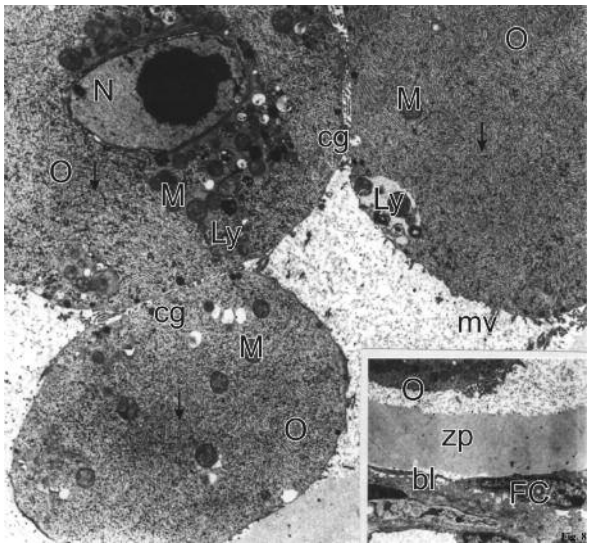

Figure 8. Three oocytes (O) with abnormal nuclei (N) and located close to each other in the follicle in developing follicle; membranous structures (*arrow*) organized in parallel bundles next to each other, mitochondria (M), lysosomal structures (Ly) and cortical granules (CG) in cytoplasm of oocytes; rare microvilli on the surface of oocyte are seen. X6300. At small micrograph, a portion of the oocyte (O) of same follicle and flat-shaped follicular cells (FC) despite well-developed zona pellucida (zp) and indented basal lamina (bl) which had lost its normal structure are present. × 3150.

them. In addition, membranous structures forming bundles arranged parallel to each other were increased and these structures had advanced to oolemma (Figure 8). Although there was a large, well-developed Zona pellucida present in this follicle, follicular cells were flat-shaped and keeping their normal histological features relatively. In addition, basal lamina, separating follicle from stroma, was observed to lose its proper structure and to get into an indented or corrugated shape (Figure 8). Between granulosa cells surrounding the oocyte, apoptotic bodies, characterized by a crescent shaped heterochromatin condensation, were observed. In addition, formation of lipid droplets and vacuoles in the cytoplasm of granulosa cells and presence of structural changes characterized by presence of lysosomal structures were remarkable (Figure 8).

Discussion

Ovaries are located in the pelvic region and have two main functions such as producing female sex cells (Oogenesis) and secreting steroid hormones (Steroidogenesis). Oogenesis is the process of formation of secondary oocyte which will be discarded from primordial germ cells by ovulation. Process of follicular development, found in cortex of ovaries and surrounded by stromal tissue, takes place in four stages as primordial follicle, primary follicle, secondary (antral) follicle and Graaf (mature) follicles. Perfect development of ovarian follicles up to stage of ovulation, growth and differentiation of primary oocyte, proliferation of follicular cells and resulting in ovulation should happen for fertility[9-11]. Degeneration of ovarian follicles before completion of development of stages up to ovulation is called atresia and follicle atresia occurs via apoptosis[12].

In our study, following examination of ovarian tissues of rats exposed to 1/50 and 1/20 percent of LD_{50} dose of methyl parathion for a period of 8 days (for two estrus cycles) at electron microscopic level, it was detected that significant structural changes had occurred in surface epithelium, developing follicles and ovarian stroma in both drug groups, and that primordial follicles had not been affected significantly from methyl parathion but necrosis had been developed in oocyte and granulosa cells of developing follicles, and that in 1/20 group in addition to these

changes, apoptotic changes had been found in granulosa cells of developing follicles. These effects of Methyl parathion on ovaries may be due to directly by causing structural disorders or indirectly by affecting other metabolic pathways. Structural changes as a result of this exposure may be the cause of infertility.

There are very few studies on the effects of methyl parathion on reproductive system and ovaries. In studies, it had been reported that the number of abnormal sperm increases in a dose-dependent manner in rats exposed to different doses of methyl parathion, pregnancy is terminated in pregnant rats exposed to high-dose methyl parathion, estrous cycle is affected, and that methyl parathion causes decrease in rate of live births and total number of healthy follicles, causes embryo toxicity and a decrease in the number of oocytes in ovaries[4-8]. In a study where biochemical effects of 1/8-1/5 percent of LD_{50} dose of organic phosphorus compounds, methyl parathion, monocrotophos and dimethoate on rat ovaries were investigated, each of the three compounds were shown to cause decrease in cytoplasmic concentration, membrane-bound proteins, total lipids, phospholipids and cholesterol[13]. After exposure to 1/5 percent of LD_{50} dose of methyl parathion for a period of 30 days, a significant decrease in the rate of RNA/DNA, a decrease in secondary/primary oocyte rate and necrosis was detected in *Labeo rohita* type fish[14]. In fry carp fish called *Rasbora daniconius*, with increase in exposure time, increase in the percentage of immature oocytes, decrease in the percentage of mature oocytes, deformity in mature oocytes shapes, yolk sac injury, increase in the interfollicular area, tearing in the walls of oocytes were observed following exposure to 1/5 percent of LD_{50} dose of methyl parathion, endosulfan, and carbofuran[15]. In a study investigating the effects of methyl parathion on the growth of Japanese quail, a considerable reduction in egg wall thickness and production of live chicks were found[16].

In studies, organic phosphorus insecticides were found to cause cellular damage on various organs such as nervous system, skin, liver, kidney[17-20]. Organic phosphates are thought to be direct cytotoxic and to target mitochondria that serves as a source of energy and produces ATP in the cell[21-26]. Degeneration in mitochondria of oocytes was also observed in our study after exposure to methyl parathion. In other studies; it was found that tricarboxylic acid cycle and oxidative phosphorylation had been inhibited in fish exposed to methyl parathion, energy production had been reduced due to methyl parathion causing mitochondrial degeneration in cells and mitochondria were the most prominent organelle to be affected by toxicity[25,26]. The disturbance of energy production in these cells after exposure to methyl parathion affects metabolic activity of cells and these cells are likely to suffer irreversible degeneration.

Conclusions

It was found that necrosis had developed in oocytes and granulosa cells of developing rat follicles after chronic exposure to methyl parathion and apoptotic changes had occurred in granulosa cells of developing follicles with increasing drug dose. It was found that methyl parathion exposure may cause infertility. More further work to determine whether primordial follicles will be affected or not after long-term chronic exposure to methyl parathion are needed.

Conflict of interest

The Authors declare that they have no conflict of interests.

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