4

Environmental Electromagnetic Field and Female Fertility

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1. Introduction

Human beings are unavoidably exposed to ambient electromagnetic fields (EMFs) generated by every instrument that uses electricity. Electromagnetic fields are made by the combination of an electric field and a magnetic field and is propagated in a wave like manner. Thus sometimes called electromagnetic waves and or electromagnetic radiation. Under such condition the indoor and outdoor environment we live are bombarded by electromagnetic waves/radiations produced by home appliances; such as computer, television , mobile phone, industrial instruments; such as power transmission lines railway stations, medical and diagnostic tools; such as MRI, physiotherapy equipments and so on. The EMF which we are encountered everywhere, have a non ionizing nature and has two distinct parts; electrical and magnetic. The electrical part is generated by a voltage gradient and measured in volts. The magnetic part is produced by current flow and is measured in tesla. Therefore in any field, we are exposed to an electrical voltage gradient and a magnetic field. The electrical field is due to the difference between the voltage of the electricity used by the device and earth. The magnetic field is proportional to the current flowing through the device. Both types of field would induce biological effects, but magnetic field is more damaging because it penetrates living tissue more easily. Magnetic fields as low as around one microtesla can produce biological effects (Goldsworthy 2007; Sage et al. 2007; Jokela et al. 2004). The effect of EMF on human health vary widely depending on the frequency and intensity of the fields. Extremely low frequencies (ELF) such as those from home appliances are more potent than higher frequencies of radio waves (Genuis 2007; Zymslony 2007; Torregrossa 2005). According to WHO, EMFs of all frequencies represent one of the most common and fastest growing environmental influences, about which anxiety and speculation are spreading. All populations are now exposed to varying degree of EMF, and the levels will continue to increase as technology advances. It is obvious that in almost all societies the ambient EMFs are encountered everywhere and cause unavoidable exposure. There are body of information regarding adverse effects of EMF, especially chronic exposure to EMF (Wu 2008; Marek 2004; Adey 1993). Thus the concern about the public health hazards of EMFs has highly increased.

Based on the functional and or structural disorders it is shown that in a biological system, EMF may harm any organ. Epidemiological studies suggest a possible link between EMF exposures and clinically recognized medical disorders such as leukemia, brain cancer,

breast cancer, kidney cancer and or cardiovascular disease (Kovacic and Pozos 2006; NRC 1996; UNEP/WHO/IRPA 1987). An ultra-structural study on rats shows that long term exposure to EMF could result in lymphatic organ disturbances and consequently weakening of immune system (Mohammadnejad et al. 2010). Some authors have reviewed risks of EMF exposure on reproduction and demonstrated that experimental exposure to EMF in laboratory animals has several adverse effects (Djeridane et al. 2008; Ozguner et al. 2005; Chung et al. 2005; Ahmed et al. 2002; elbetieha et al. 2002; Cheronff et al. 1992). There are numerous studies showing that EMF exposure of male rat/mice affects testicular architecture , spermatogenesis, sperm motility, leydig cell reduction, increased apoptosis of germ cells and in general subfertility and or infertility (Khaki et al. 2006; Lee et al. 2004; Soleimani Rad and Katebi 1997; Devita et al. 1995; Lokmatova 1993). In human and animal studies, it has been reported that female exposure to EMF cause some adverse effects and reviewed the potential effect of EMFs on infertility, implantation rate, number of living fetuses, sex ratio, miscarriages, premature births, growth retardation, low birth weight, congenital malformations and prenatal deaths (Roshangar and Soleimani Rad 2007; Lahijani et al. 2007; Feychting et al. 2005; Chiang et al. 1995; Huuskonen et al. 1993; Juutilainen et al. 1993; Mc Govern et al. 1990).

Although the epidemiological studies on the effect of EMF exposure in human, are not conclusive but the experimental findings on the effect of EMF on different organs would act as a corner stone for exploring the complicated topic of EMF effect on reproduction. As several organs are involved in the fertility of females and their well being are necessary for fertility, the present chapter will deal with the effect of EMF on three major female reproductive organs i.e. ovary, fallopian tubes and uterus.

2. Methods

In these series of experiments the Wistar rats are used as an experimental model and the studies were approved by the ethical committee of Tabriz University of Medical Sciences. For producing EMF, an EMF generating apparatus was designed in the Department of the Histology and Emberyology. The apparatus uses 220 V and 50 HZ alternative current and could generate up to 5 milli tesla EMF. In the presented studies, the adult female rats were exposed to 3 milli tesla, 4 hours/day for 2 months. After the experimental period the rats were sacrificed and their reproductive organs including; ovaries, uteri, and uterine tubes were dissected apart. Half of the samples were fixed in 10% formalin and paraffin-embedded sections were used for light microscopy and immunohistochemical studies. TUNEL techniques were used for detection of apoptosis and Ki-67 technique for proliferation assay. In both cases toluidine blue was used for counterstaining.

The other half of the samples were fixed in 2% glutaraldehyde and resin embedded ultra thin sections were used for electron microscopy.

3. Results

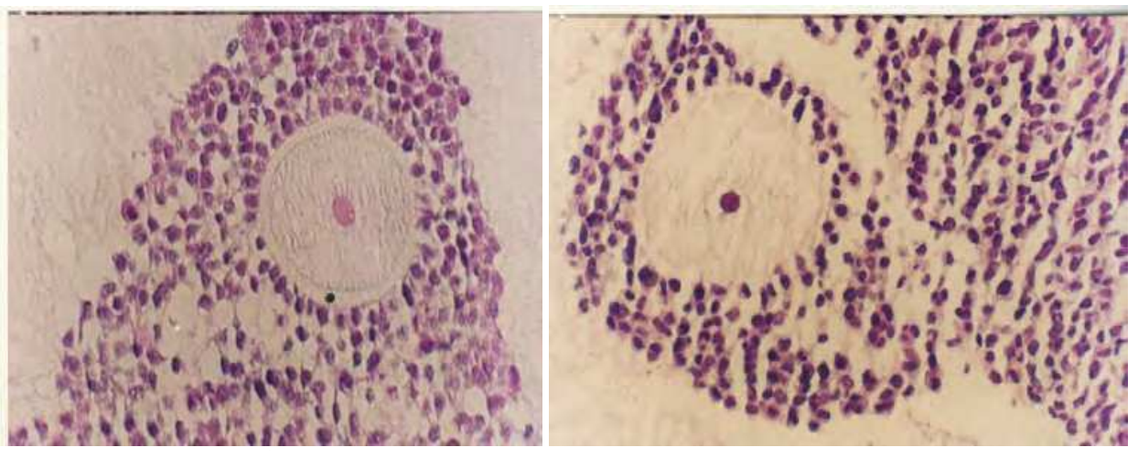
3.1 Ovarian effect of EMF exposure

Ovary is an organ involved in follicular development and germ cell production. In other word, the ovary provides a proper environment for oogenesis. Any disturbances in ovarian

function may lead to the folliculogenesis disorder and or ceasing of ovulation. Several factors including hormonal disturbances, changes in ovarian stroma and or any factor that affect oocyte maturation would affect oogenesis.

3.1.1 Light microscopic studies

Light microscopy showed that, in control group, oocyte had a euchromatic nucleus and was encompassed with a homogenous zona pellucida, and well organized corona radiate and granulosa cells (Figure 1-A). In experimental group, the oocyte had a condensed nucleus so that it appeared small and darkly stained (Figure 1B). The cytoplasm of the oocytes were condensed and surrounding zona pellucida had changed and appeared narrower than that in control group (Figure 1B). The cells in granulosa and corona radiata layers were disorganized and contained dense nuclei (Figure 1B).



A B

Fig. 1. Photomicrographs of ovarian sections from rat ovaries. A-from control group, showing part of a graffian follicle with oocyte and cumulus. B-from EMF-exposed group, showing an oocyte with condensed nucleus, surrounded with a faint zona pellucida and an irregularly arranged cumulus and corona radiata. H&E staining. 300X.

Ovarian stroma both in cortex and medulla contained several macrophages wich was rarely seen in control group and their number was higher in cortex than the medulla (Figure 2). Morphometric studies revealed that the number of ovarian follicles, in different stages of development, were higher in EMF-exposed group, but the number of corpora lutea were fewer than the control group. Atretic follicles in the EMF-exposed group were numerous than the control group.

3.1.2 Electron microscopic studies

Ultra structural studies revealed that granulosa cells in control group, were regularly arranged and the corona radiate layer was composed of columnar cells that were attached to eachother by intercellular junctions. Their microvilli were penetrated into zona pellucid and could be recognized in it. The cytoplasm of coronal cells contained different organels including spherical or ovoid mitochondria with limited cristae (Figure 3).

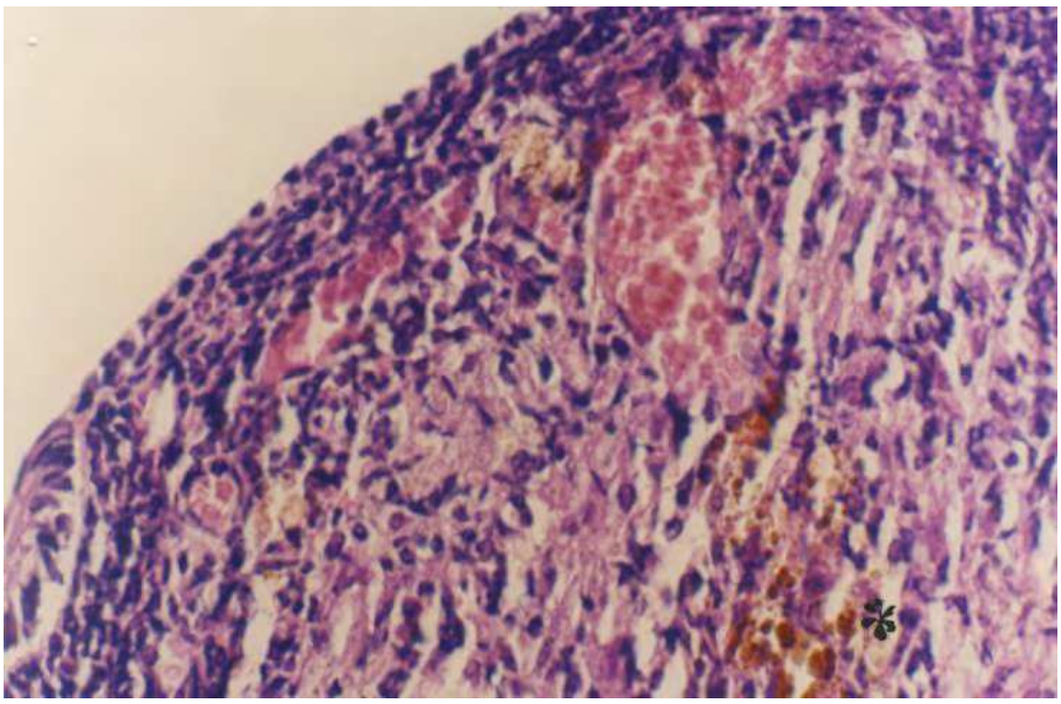


Fig. 2. Photomicrograph of a section of an ovary from EMF-exposed rat. Showing several macrophages with brownish residual bodies (*). H&E staining. 200X.

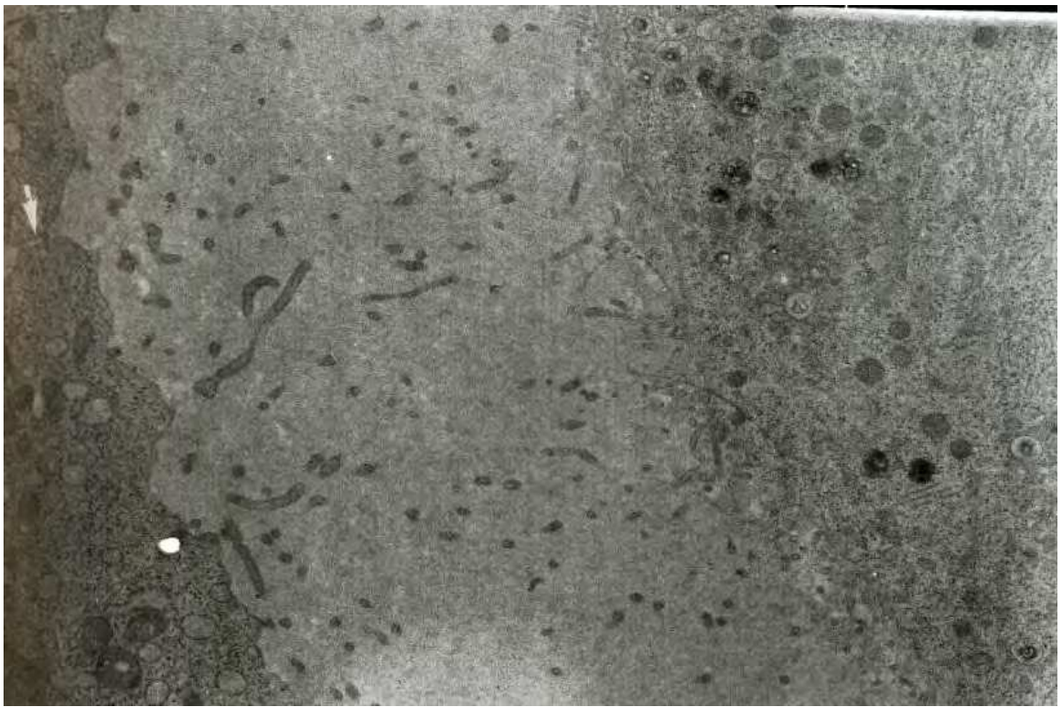


Fig. 3. Electron micrograph of developing follicle from a control rat ovary. Note the thickness of zona pellucida and sections of microvilli from oocyte and granulosa cells in it. cellular junction between granulosa cells is obvious (arrow). 15000X.

Cytoplasm of oocytes in this group contained numerous lamellae, scattered organells including round or ovoid mitochondriae with short and limited crista. The endoplasmic reticulum were poorly developed (Figure 4). The nuclei of oocytes were euchromatic and contained an obvious nucleolus. In the vicinity of granulosa cell layer the capillary-rich theca interna and outer to it the collagen-rich theca externa were present.

Ovary also contained several corpora lutei composed of large and round cells with a prominent nuclei and nucleoli. The luteal cells were pale and contained numerous mitochondria, an extensive rough endoplasmic reticulum, smooth endoplasmic reticulum and Golgi apparatus. The cells also contained numerous free ribosomes, lipid droplets and a few number of lysosomes and multivesicular bodies (Figure 5).

In EMF-exposed group, the zona pellucida was narrower than in control, 3.24±0.25 μm VS 4.47±0.42 μm, and the difference was significant ($p<0.001$). The number of microvilli profiles per unit area in this group was fewer than in control, 4.13±0.83 VS 9.8±0.56, which was statistically significant ($p<0.001$) and (Figure 7).The coronal cells were shrunken, separated from zona pellucid and lost contact from eachother (Figure 6).

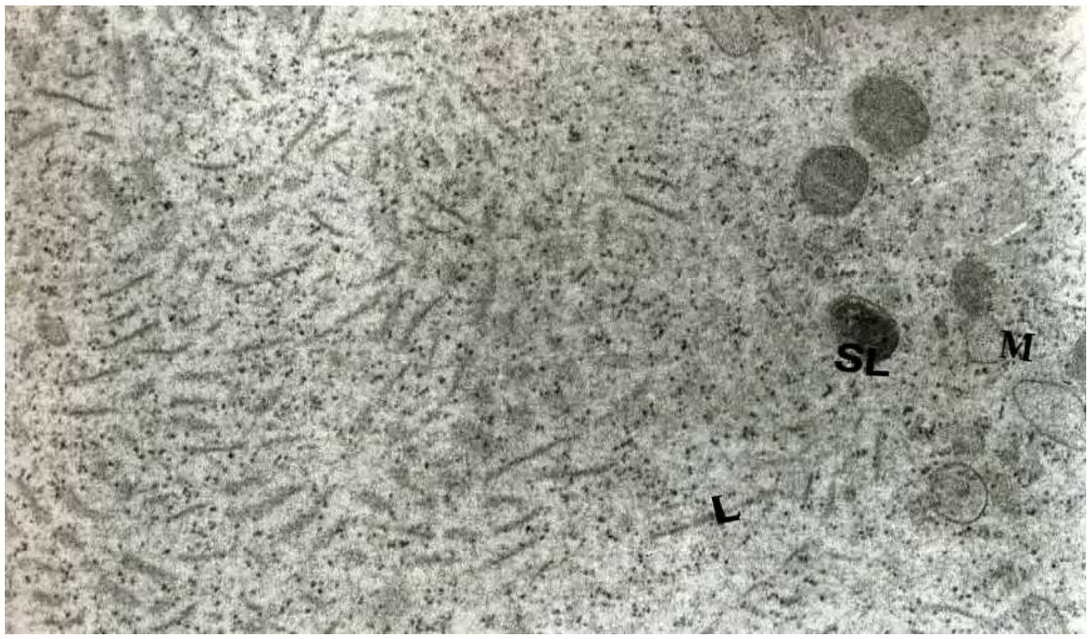


Fig. 4. Electron micrograph of cytoplasm of oocyte from a control rat ovary. Note mitochondria (M), lamellae (L), and secondry lysosome (SL). 15000 X.

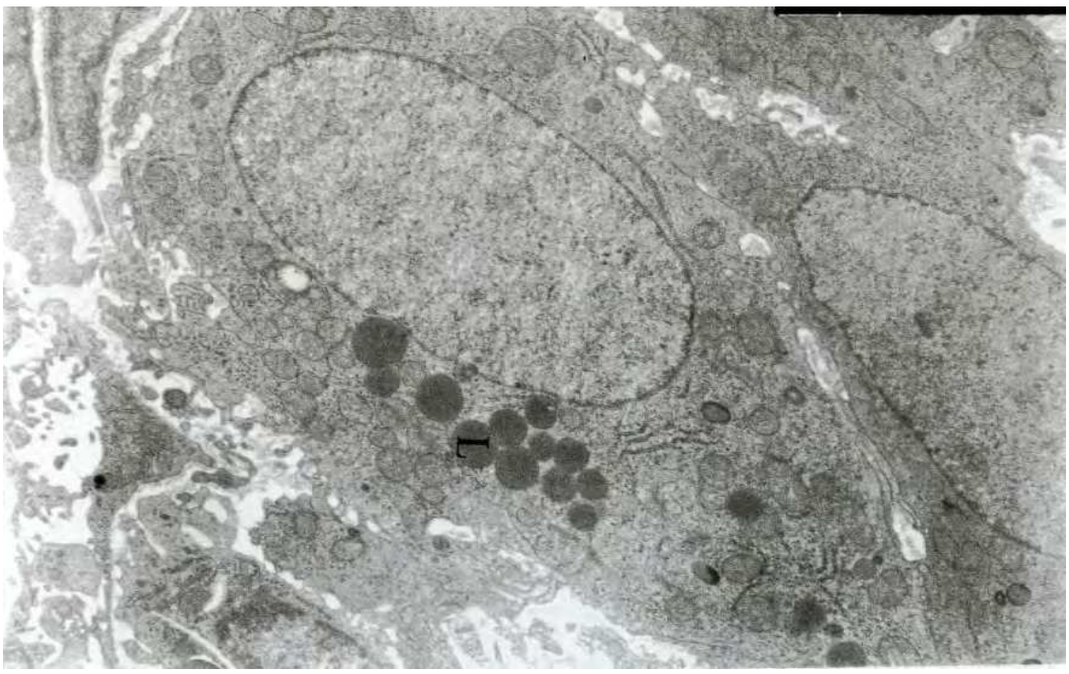


Fig. 5. Electron micrograph of corpus luteum from a control rat ovary. Note normal organels and some lipid droplets (L). 5000X.

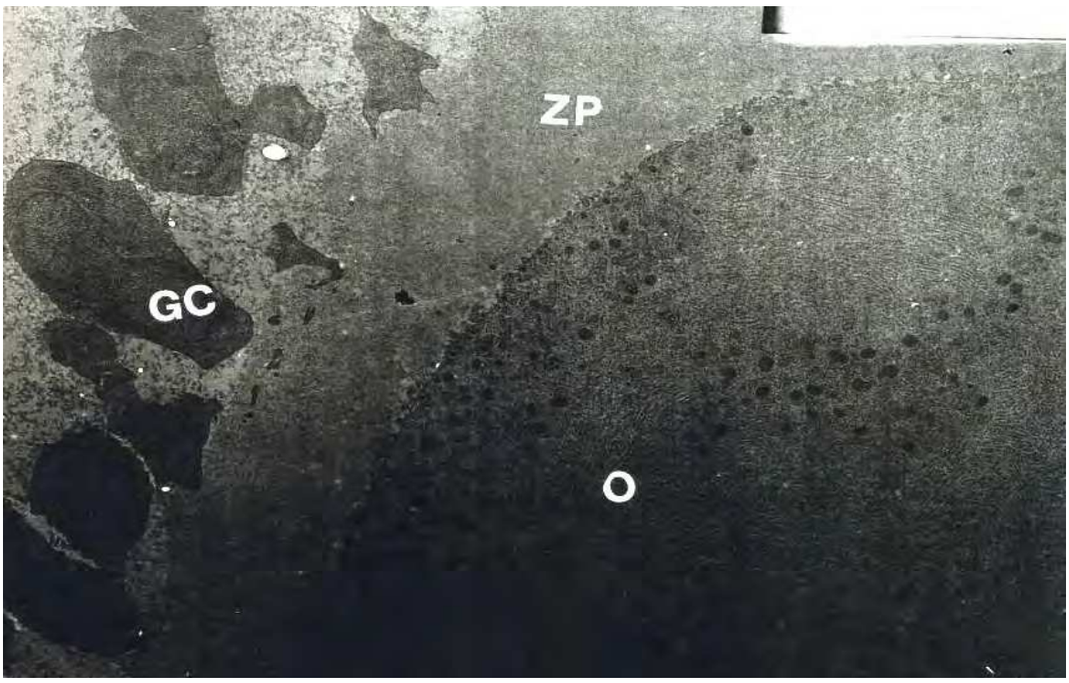


Fig. 6. Electron micrograph of an oocyte with zona pellucida and granulosa cells from EMF-exposed rat. Note condensed granulosa cells that lost contact from zona pellucida and neighboring cells (GC). Zona pellucid (ZP), and oocyte (O). 3000X.

The cytoplasm of oocytes showed fewer organell clusters and contained higher number of lamellae. The rough endoplasmic reticulum were poorly developed, mitochondria were smaller and numerous fat droplets were present in comparison to control group (Figure 7). The nuclei had irregular counter and nucleolus were condensed (Figures 8).

Numerous granulosa cells near the antrum had typical characteristics of apoptotic cells. They had condensed and cresent like nuclei and were separated from neighboring cells (Figure 9). The granulosa cells had also irregular basal lamina and in some follicles were broken. The thecal cells, in comparison to control group, had more condensed nuclei and contained; several lipid droplets, autophagic granules, apoptotic bodies, ruptured mitochondria, dilated nuclear membrane and cytoplasmic vesiculation (Figure 9). Similar changes were observed in the stromal cells from EMF-exposed group. In addition, several macrophages containing apoptotic bodies and multivesicular bodies were also present (Figure 10). These cells were mainly located near the blood vessels. The cells with cresent like nuclei, which is the characteristics of apoptotic cells, were present among stromal cells.

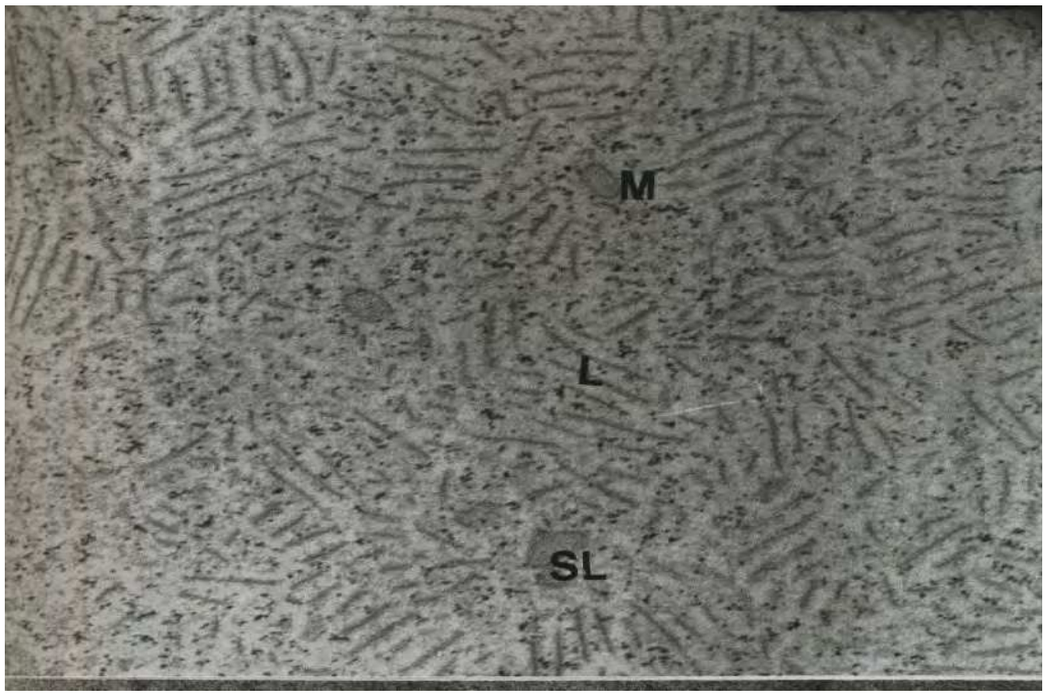


Fig. 7. Electron micrograph of cytoplasm of oocyte from EMF-exposed rat. Secondary lysosome (SL), lamellae (L), and mitochondria (M). 15000 X.

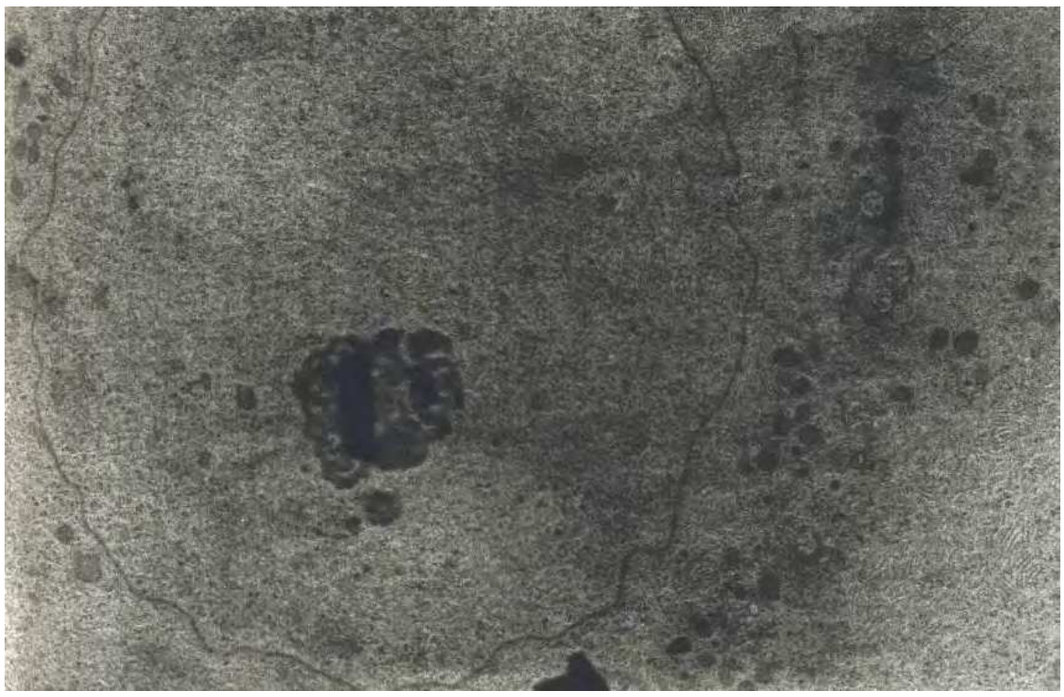


Fig. 8. Electron micrograph of oocyte nucleus and cytoplasm from an EMF-exposed rat. Note nucleus and nucleoulus. 10000 X.

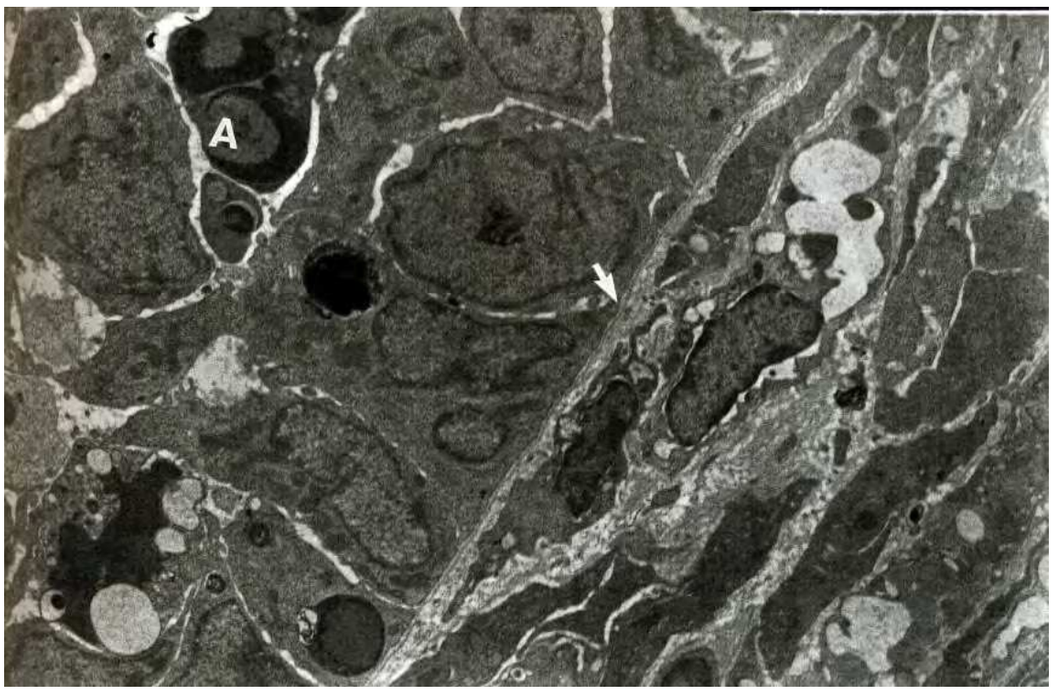


Fig. 9. Electron micrograph of a developing follicle from EMF-exposed rat. Apoptotic cell (A), basal lamina (arrow). 3000X.

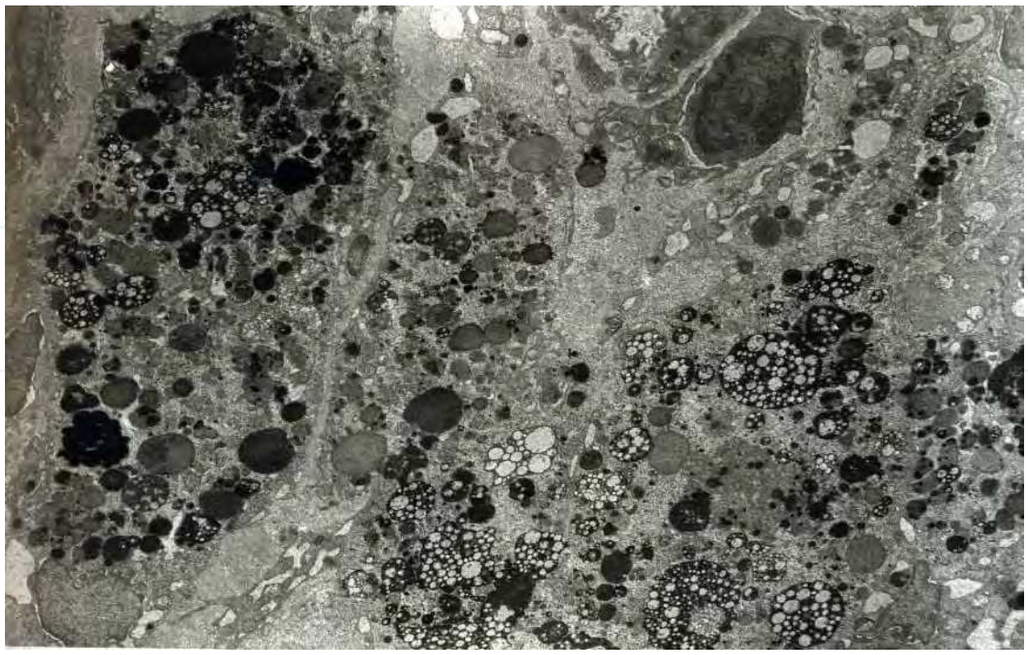


Fig. 10. Electron micrograph of ovarian stroma from an EMF-exposed rat. Note necrotic cells, and macrophages containing multivesicular and apoptotic bodies. 3700X.

3.1.3 Immunohistochemical studies

Immunohistochemical studies was performed on paraffin sections using TUNEL technique for detection of apoptosis and Ki-67 technique for proliferation assay. Both TUNEL positive and Ki-67 positive cells were distinct from nonpositive cells by their brownish color. In control group TUNEL positive cells were fewer and was limited to granulosa cells near the antrum of atretic follicles (Figure 11). Proliferative cells (Ki-67 positive cells), in this group, were observed in granulose layer close to the basement membrane.

In experimental group, exposed to EMF, apoptotic cells were mainly found near the antrum but also were present among cells close to basement membrane (Figure 12). TUNEL positive cells were not observed in thecal layers but were observed in ovarian stroma. The corpora lutea in the EMF-exposed group also contained numerous TUNEL positive cells. The result from apoptotic cells indicate that EMF exposure induces apoptosis not only in granulose cells but also in other parts of the ovary. Regarding Ki-67 assay, the number of Ki-67 positive cells in granulosa layer from EMF-exposed group were obviously fewer than that in control group meaning that EMF exposure inhibits proliferation.

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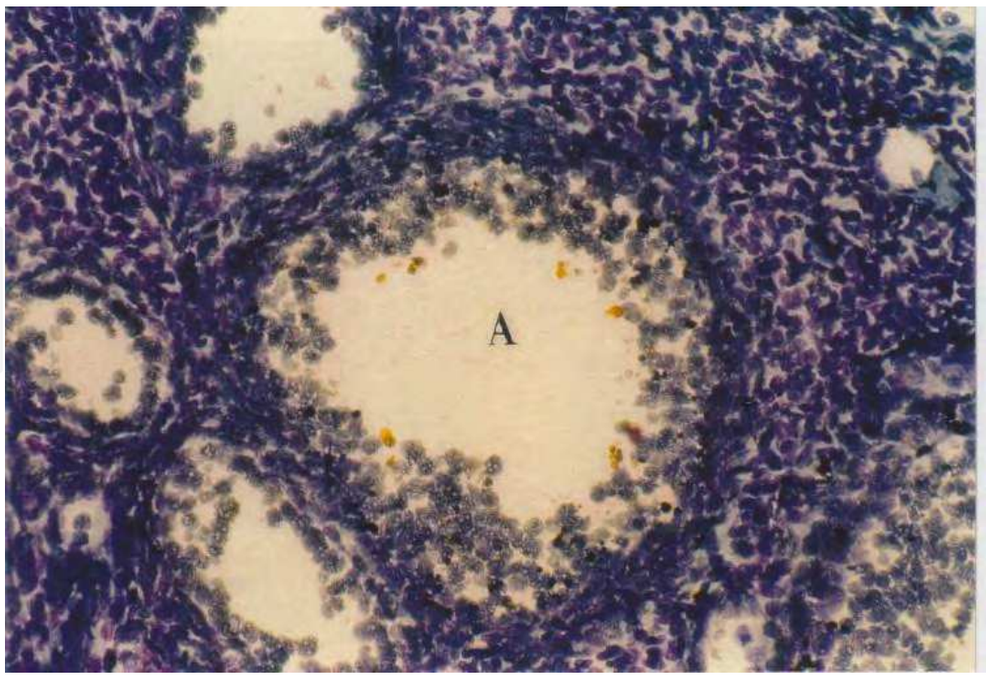

Fig. 11. Photomicrograph of a section from control rat ovary. An antral follicle (A), containing apoptotic cells with golden brown color at luminal surface. TUNEL method, counterstained with toluidine blue. 300X.

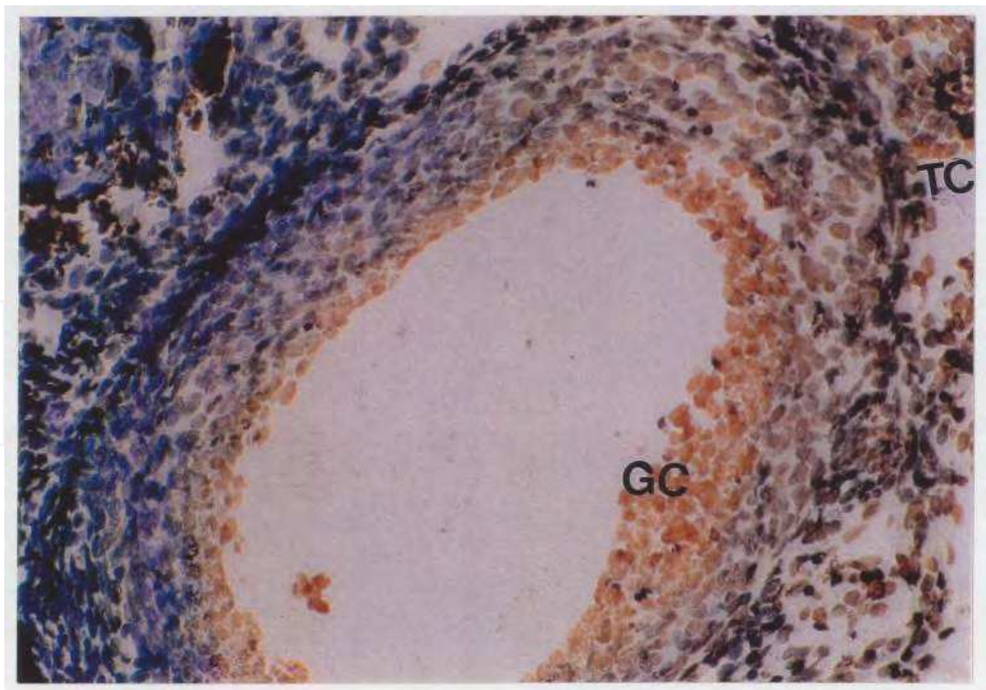

Fig. 12. Photomicrograph of an antral follicle from EMF-exposed rat ovary. Note numerous apoptotic cells with golden brown color among granulosa cells (GC), at luminal surface. Techa interna (TC). TUNEL method, counterstained with toluidine blue. 700X.

3.2 Effect of EMF on uterus

Different phases of the uterus is recognized according to the morphological characteristics of endometrium. The study was carried out in the proliferative phase. The phase was primarily selected based on estrous cycle and after sacrificing of the animals the morphological characteristics of the endometrium was used as the second criterion, otherwise the case was excluded from the study.

3.2.1 Light microscopic studies

In the control group, the endometrium is lined with simple columnar epithelium with nucleus located basally and contained limited uterine glands (Figure 13). In EMF-exposed group, the endometrial thickness was reduced and epithelial cells were smaller, shorter and had condensed nuclei (Figure 14). The difference between two groups was statistically significant ($p<0.01$). In experimental group, the cells in the stroma of the endometrium were smaller, contained condensed nuclei and blood vessels were less extensive. However, myometrium and perimetrium were similar in both groups.

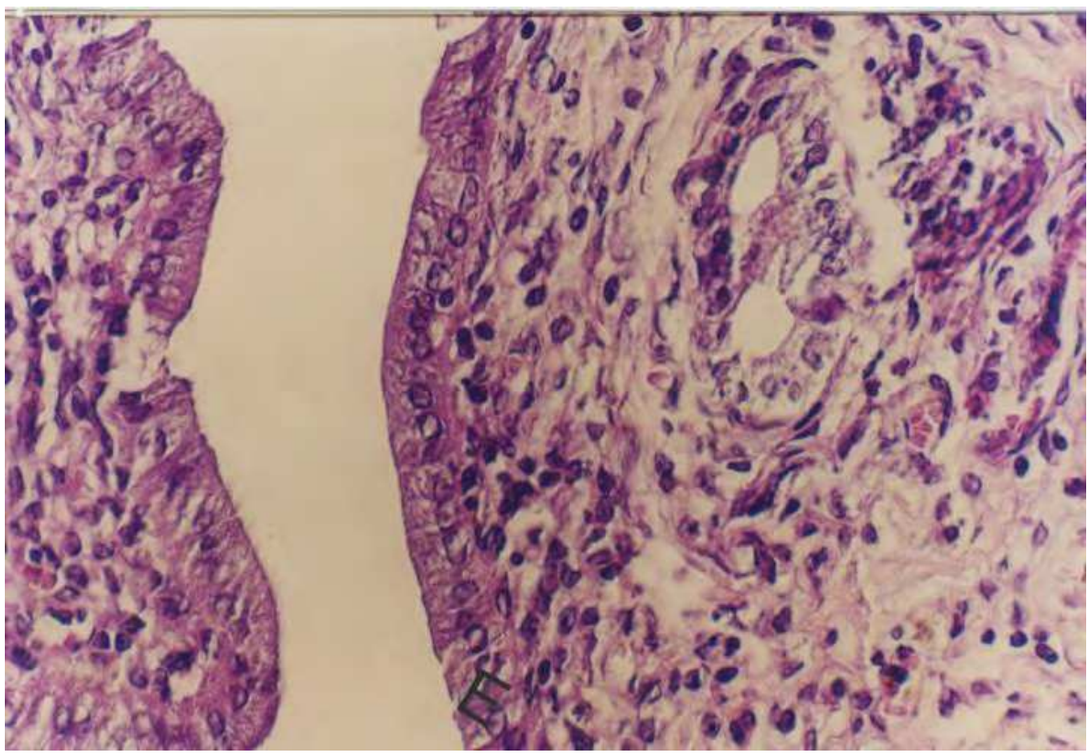

Fig. 13. Photomicrograph of uterine endometrium from a control rat. Note, columnar cells of endometrial lining (E). H &E staining, 200X.

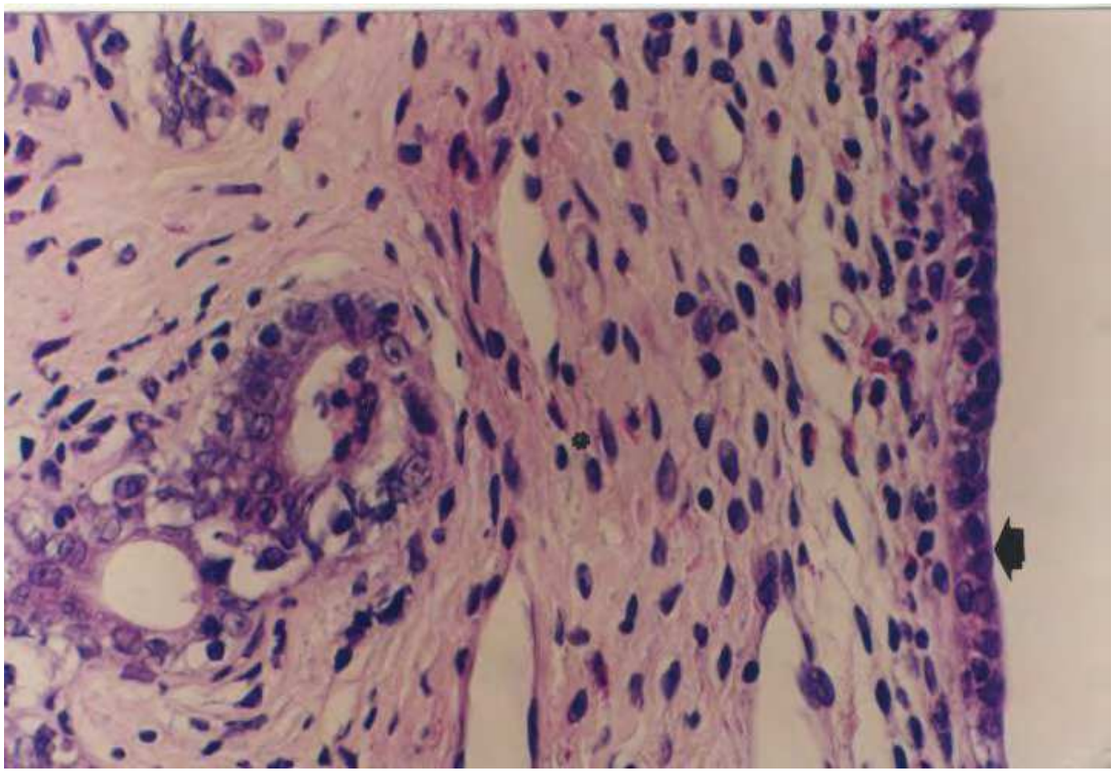

Fig. 14. Photomicrograph of uterine endometrium from an EMF-exposed rat. Note flattened endometrial epithelium (arrow head). H&E staining, 200X.

Stereological studies showed that the V/v of the nucleus to cytoplasm and axial ratio of the nuclei in the epithelial cells from experimental group was significantly lower than the control group ($p<0.01$).

3.2.2 Electron microscopic studies

Ultrastructural studies revealed that epithelial lining of the endometrium consist of two types of cell; ciliated and nonciliated cells. The ciliated cells are randomly scattered among the nonciliated cells. The nonciliated cells undergo morphological changes during different uterine phases. Both cell types contain euchromatic nuclei, a relatively well developed rough endoplasmic reticulum and Golgi apparatus, some apical secretory granules and some fat droplets. The cells prosses small and slender mitochondria. The cells at their lateral interface close to the apex show junctional complex, desmosomes and in some cases an interdigitation attaches the neighbor cells together. In addition to changes that observed with light microscope the electron microscopy showed that in experimental group the nuclear heterochromatin was increased and their mitochondria were condenser and sometimes ruptured in comparison to control group. Furthermore, the secretory granules were dispersed in the cytoplasm while in the control group they were localized to apical area. The rouph endoplasmic epithelium in experimental group was dilated and had a cystic appearance. The number of microvilli in experimental group was reduced in comparison to control group (Figures 15 and 16).

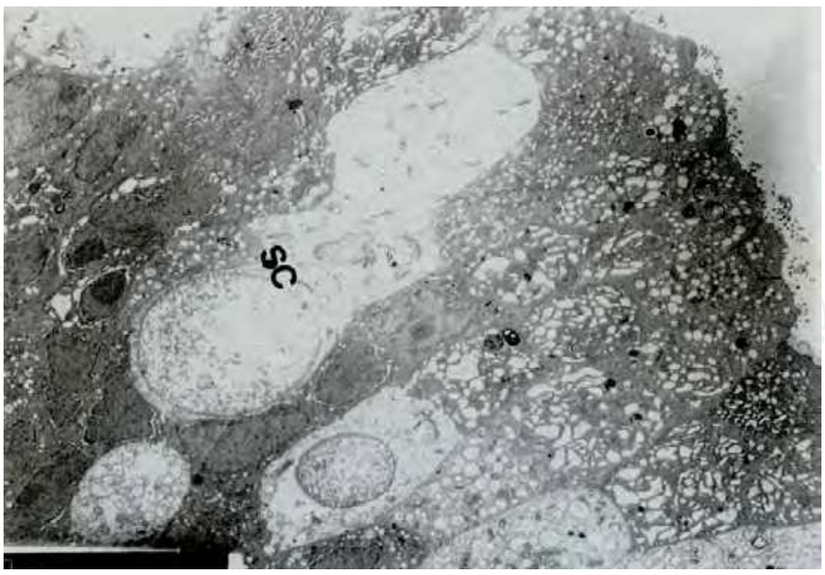

Fig. 15. Electron micrograph of uterine endometrial epithelium from control rat. Secretory cell (SC), ciliated cell (CC). 3500X.

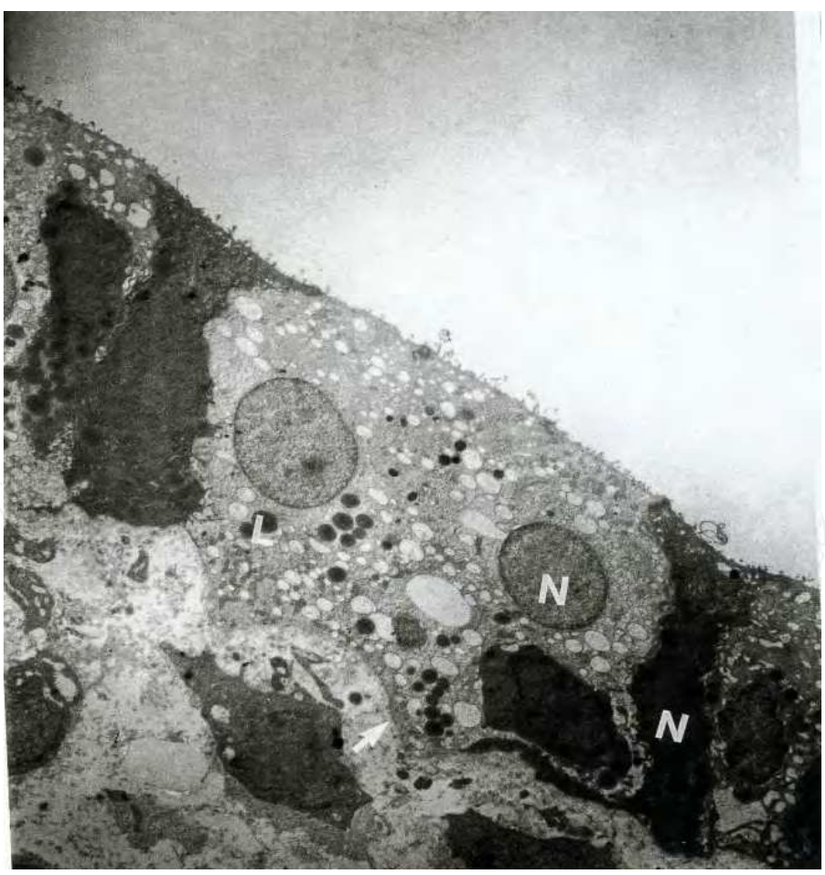

Fig. 16. Electron micrograph of uterine endometrial epithelium from EMF-exposed rat. Nucleus (N), lipid droplet (L), and irregular basal lamina (arrow). 3500X.

3.2.3 Immunohistochemical studies

Detection of apoptotic cells using TUNEL reaction technique showed that in control group the luminal epithelium appeared regular with some glycogen deposition but had almost no sign of apoptosis (Figure 17). In experimental group, the epithelium appeared irregular and there were several apoptotic cells in the luminal and glandular epithelium (Figure 18).

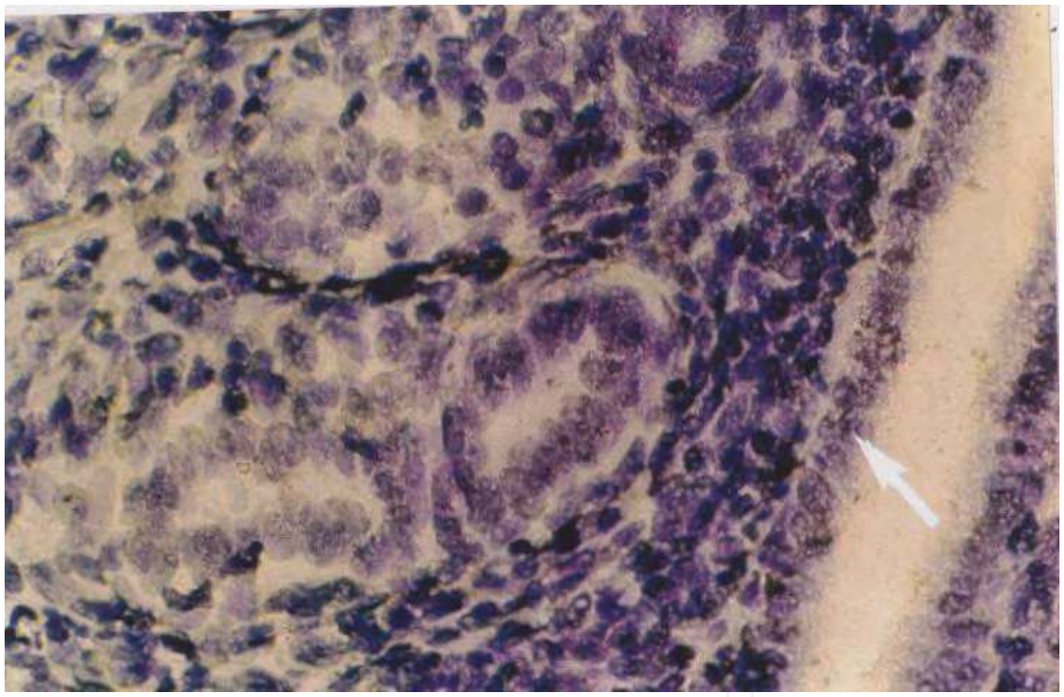

Fig. 17. Photomicrograph of a uterine section from control rat. Luminal epithelium (arrow), the uterine glands are seen. TUNEL method, counterstained with toluidine blue. 700X.

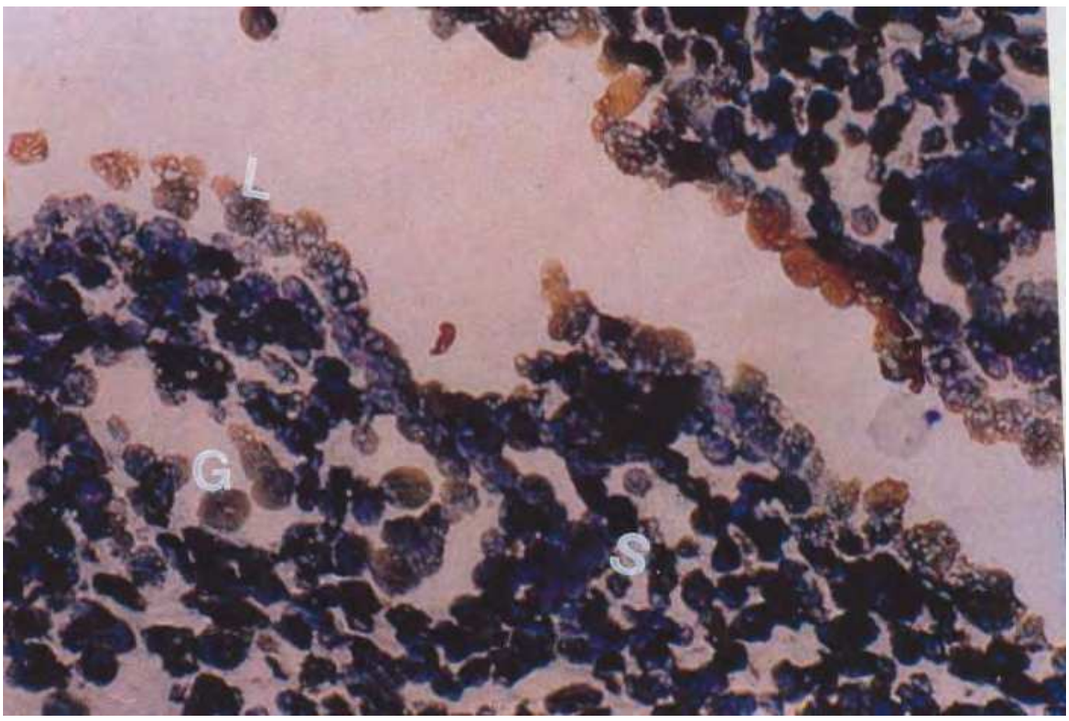

Fig. 18. Photomicrograph of uterine luminal epithelium from EMF-exposed rat. Note numerous TUNEL positive cells stained as golden brown. Luminal face (L), uterine gland (G), and uterine stroma (S). TUNEL method, counterstained with toluidine blue. 700X.

3.3 Effect of EMF on uterine tubes

The uterine tubes act as the site of fertilization and have a critical role in the conduction of zygote to the uterine cavity. Any changes in the structure and or function of uterine tubes would result in tubal pregnancies. The aim of the present study is evaluating the effect of EMF on uterine tubes by examining histological and morphological features using light and electron microscopy and immunohistochemical techniques.

3.3.1 Light microscopic studies

Light microscopic studies of uterine tubes from control group showed that: uterine tubes are lined with ciliated simple columnar epithelium and their cilia formed a smooth and orderly arranged ribbon at luminal face. The nuclei of epithelial cells were light and euchromatic. The epithelium was rested on a highly vascularized loose connective tissue (Figure 19). In the EMF-exposed group, epithelial cells were low columnar and mostly had lost their cilia. The nuclei of the epithelial cells were condensed and heterochromatic (Figure 20). Morphometric studies, based on final magnification, showed that the height of epithelial cells were decreased after EMF exposure, it was 2.77±0.19 mm VS 2.83±0.46 mm (Figure 21), the difference between two groups is significant ($p<0.05$).

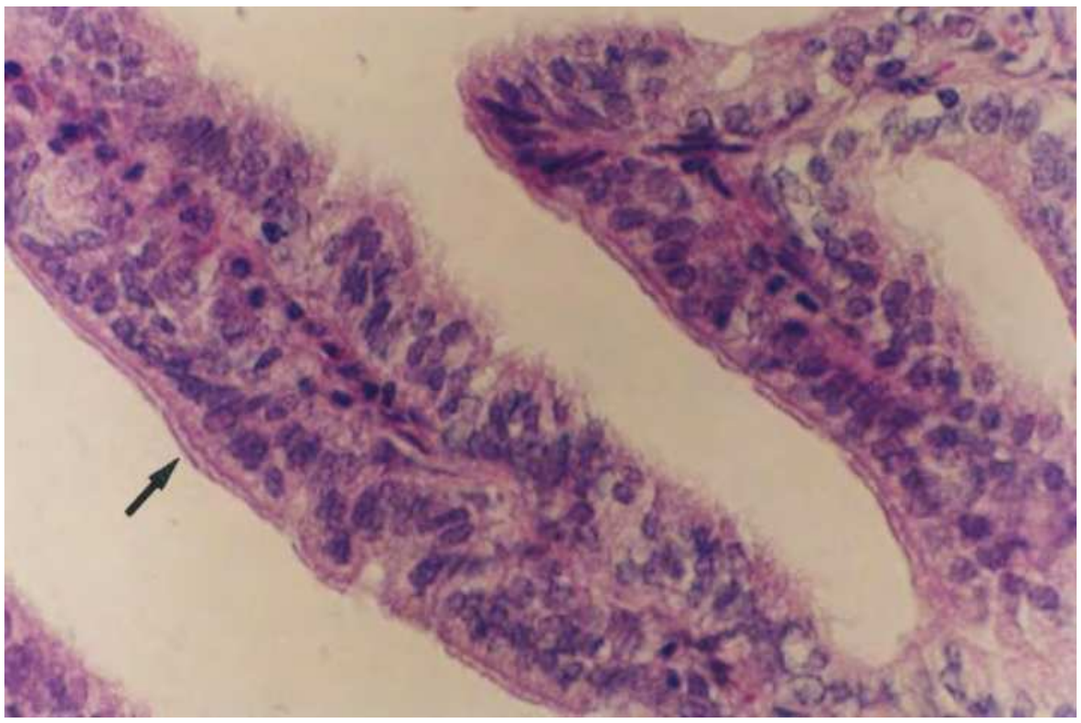

Fig. 19. Photomicrograph of uterine tube from control rat. Note epithelium with a ribbon-like cilia at luminal face (arrow). H&E staining. 600X.

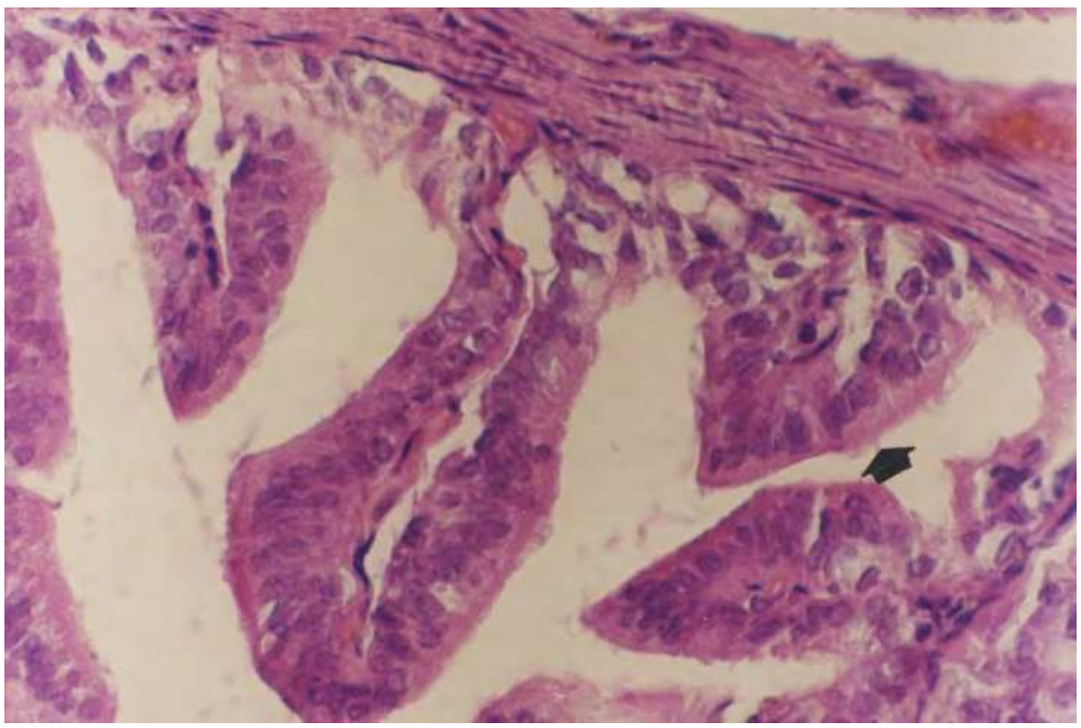

Fig. 20. Photomicrograph of a section of uterine tube from EMF-exposed rat. Note loss of cilia in epithelial cells, scattered cilia are left (arrow head). H&E staining. 600X.

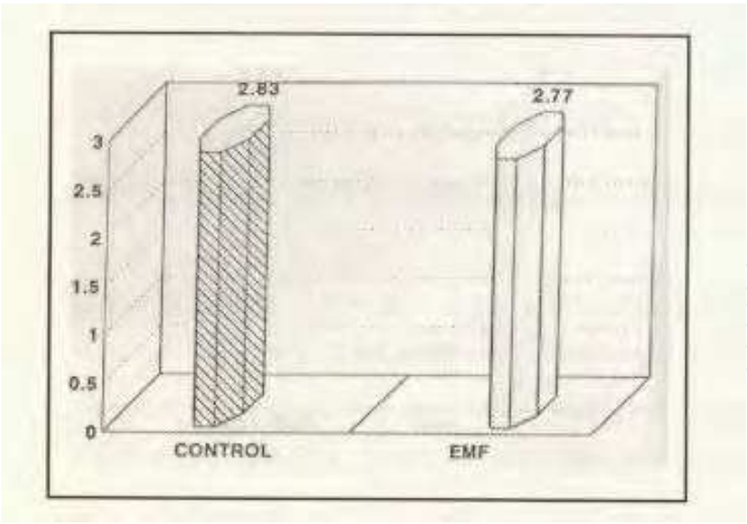

Fig. 21. A diagram showing the height of epithelial cells in uterine tubes (as mm) in control and EMF-exposed groups.

3.3.2 Electron microscopic studies

Electron microscopy showed clearly the ciliated and secretory cells in the epithelial lining of the uterine tubes. In the control group, the ciliated cells had a basal or central ellipsoid nuclei with a prominent nucleolus. The cytoplasm contained poorly developed organells. The apical cell surface bears numerous cilia. The secretory or non ciliated cells had a central elongated nuclei with a prominent nucleolus. The organells were well developed. Membrane bound granules were located at apical cytoplasm. The epithelial cells were held together by junctional complex and rested on a basal lamia (Figure 22).

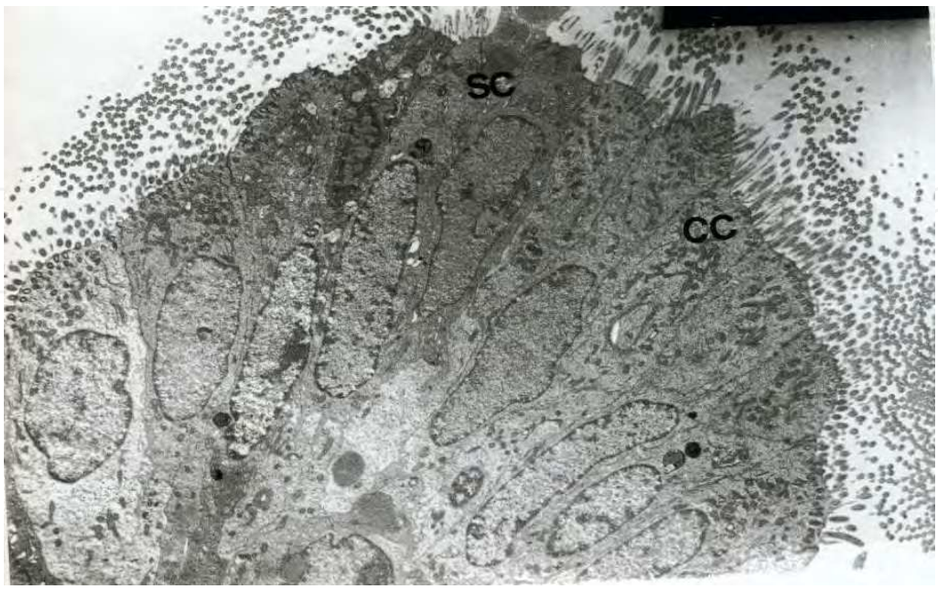

Fig. 22. Electron micrograph from uterine tube epithelium in control rat. Secretory cell (SC), ciliated cell(CC). 3000X.

In the EMF-exposed group, the nuclei were condensed, the apical cilia were apparently reduced and organells were sparsely distributed through the cytoplasm. Slight pyknosis, increased peripheral chromatin condensation and some degree of cytoplasmic condensation was observable. The most remarkable feature of the uterine tubes in this group was the presence of cells with preapoptotic characteristics i.e. flattened and cystic endoplasmic reticulum and nuclear condensation (Figure 23).

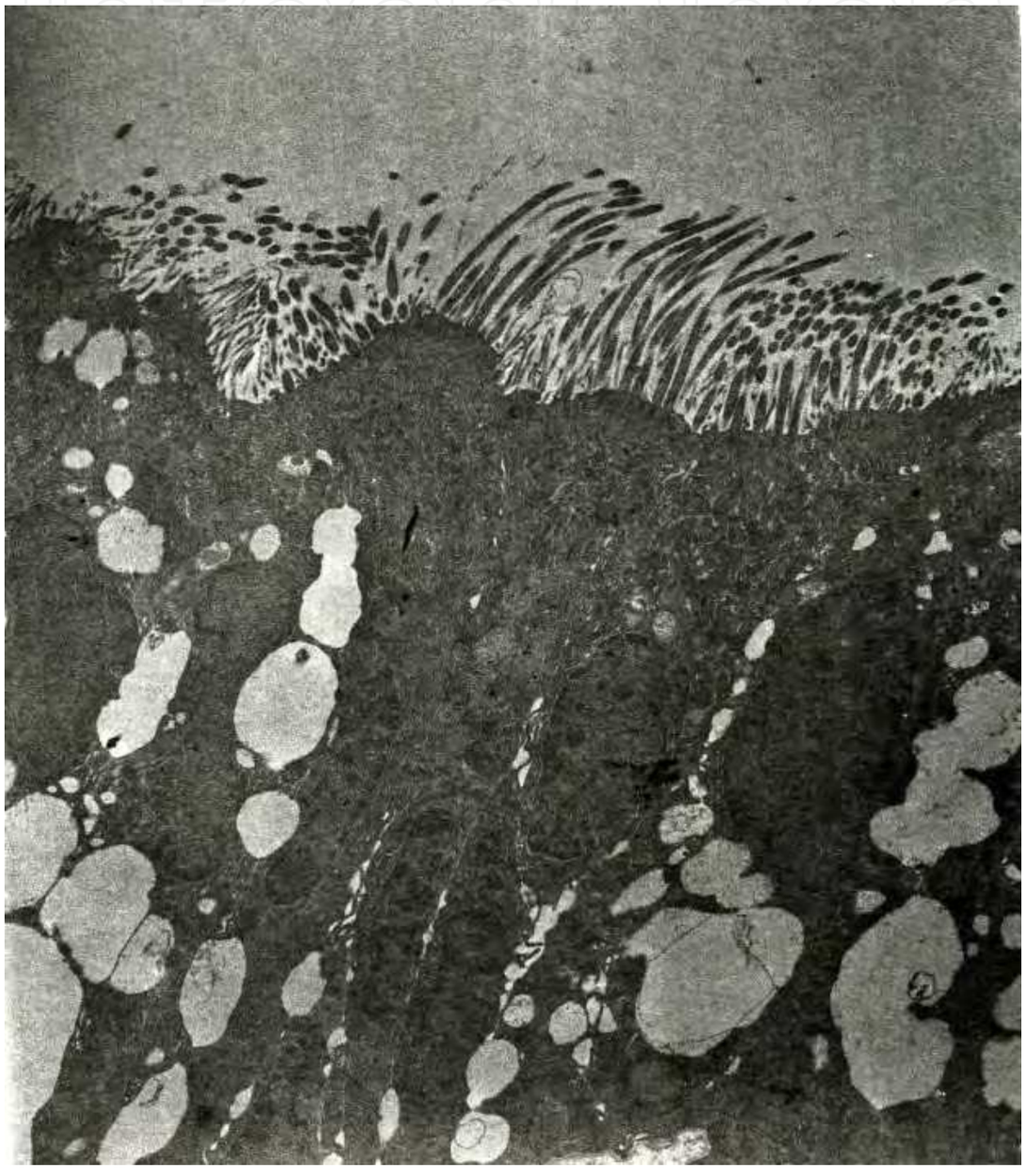

Fig. 23. Electron micrograph from uterine tube in EMF-exposed rat. Note decreased ciliar number and presence of intercellular spaces and cystic rER. 3500X.

3.3.3 Immunohistochemical studies

Immunohistochemical studies were restricted to TUNEL technique for detection of apoptotic cells. In the control group, with TUNEL assay, apoptotic cells was neither observed in the lining epithelium nor in the subepithelial layer (Figure 24). In the EMF-exposed group, there were numerous apoptotic cells in the lining epithelium. Apoptosis was observed both in secretory and ciliated cell types. Very few apoptotic cells was also present in the subepithelial layer (Figure 25).

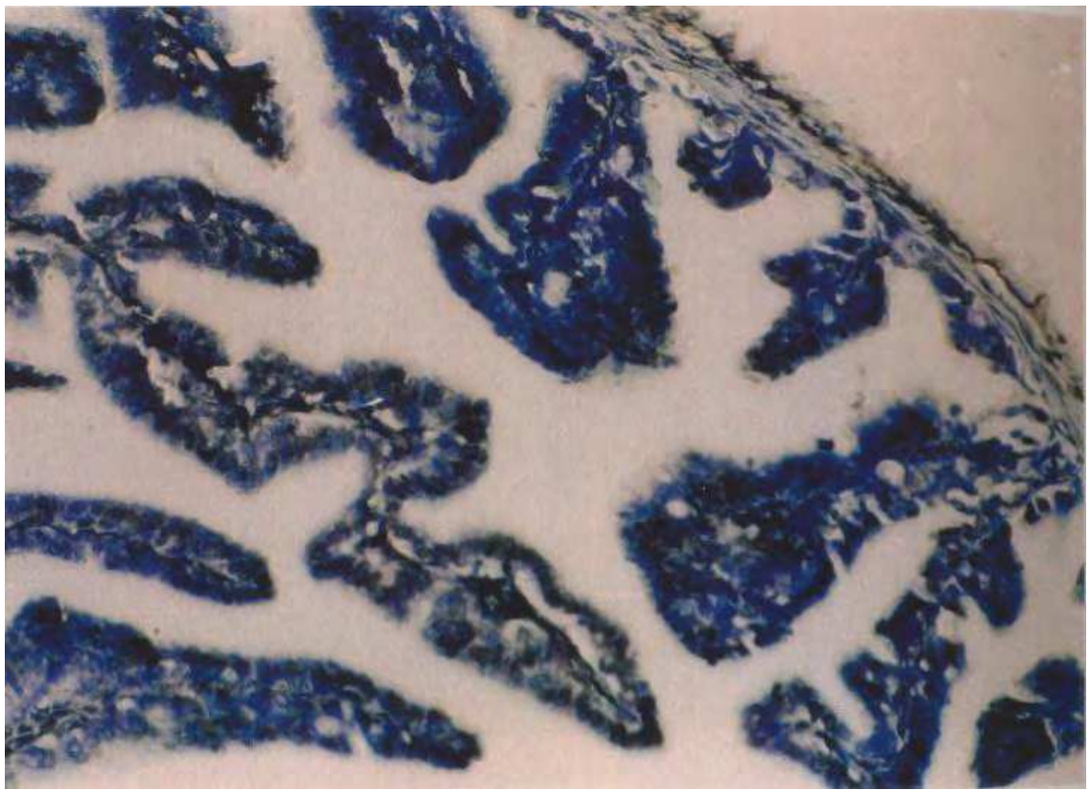

Fig. 24. Photomicrograph of a section from uterine tube in control rat. TUNEL method, counterstained with toluidine blue. 360X.

Fig. 25. Photomicrograph from uterine tube in EMF-exposed rat. Note apoptotic cells with golden brown color at luminal face (arrow). TUNEL method, counterstained with toluidine blue. 360X.

4. Discussion

This section is going to deal : 1) The morphological effects of EMF exposure on the genital organs including ovary, uterus, and Fallopian tubes using light and electron microscopy. 2) A possible role of apoptosis in the mediation of EMF-induced alterations using immunohistochemical techniques. To make it easier to explain, each organ is discussed separately, following the above theme, and made a general conclusion at the end.

4.1 EMF-induced morphological changes and apoptosis in ovary

The result of present study showed that EMF-exposure increased degenerative changes in the ovarian follicles. The results obtained from TEM studies have revealed that oocytes became shrunken, and the zona pellucida appeared narrower in the EMF exposed group in comparison to control group (Figs 1, 3, 4, 7). It was also shown that the number of microvilli in oocytes and coronal cells were decreased in experimental group. It is known that the microvilli of oocytes and granulosa cells are in contact, within the zona pellucida, by gap junction and are involved in oocyte nutrition (Martin et al. 2001; Takeo and Hokano 1995; Gondos 1982; Vazquez and Sotello 1967). The alterations produced by EMF could either be the result of initiation of apoptosis in the follicular cells or as a result of apoptosis in the oocytes themselves.

Comparison of oocyte degeneration with apoptosis since Wyllie et al. (Wyllie 1980) first described the morphological characteristics of physiological cell death (apoptosis) few studies have described the ultrastructure of the atretic oocyte and none have examined this in pubertal or adult animals. Most reviews of ovarian follicular atresia focused on changes in granulosa cells or equate the entire process with apoptosis (Tilly 1998; Kaipia and Hsueh 1996). Biochemical analysis of atretic follicles that have measured DNA integrity (188-189)or increases in cell death – related mRNA levels, including bax and Fas, fasL (Hsuhe et al. 1994; Mori et al. 1997), have confirmed that apoptosis is occurring in antral ovarian follicles. Because the oocyts is a very small component of these large follicles, such measurements most probably reflect the status of granulosa cells. Due to these restrictions, microscopic examination is required to study the process of atresia in oocytes in situ. Because ultrastructural characterization is a reliable method for the classification of cell death as apoptosis (Payne et al. 1995).

Alterations in oocytes from EMF-exposed ovaries mainly in rat atretic f'ollicles included: loss of both granulosa cell and oocyte microvilli from the zona pellucida, changes in cytoplasmic organelles such as lamellar condensation and shirinkage of oocytes. Loss of microvilli and cytoplasmic condensation do resemble apoptosis, but other events differed from those associated with traditional apoptosis. For example, the mitochondria do not maintain their characteristic appearance during, early stages of atresia, as normally occur in apoptosis. While, cytoplasmic condensation, which is reflected by an increase in electron density at the ultrastructural level, observed in degenerating oocytes. In support of the findings of the present study, condensed chromatin was never observed in oocytes of atretic follicles by other investigators (Devine 2000). These comparisons suggest that there are more differences than similarities between physiological oocyte cell death and apoptosis. Other reports attempting to identify the mechanism of oocyte death have not discussed the possibility of alternative, nonapoptotic, types of physiological death (Perez et al. 1999). Early ultrastructural studies occurred before apoptosis was characterized (Vazquez and Sotello 1967; Franchi and Mandl 1962). More recent studies using ovulated oocytes failed to prove definitively that apoptosis was the mechanism of oocyte death (Perez et al. 1999; Van et al. 1998; Phillips et al. 1992). Therefore, it seems likely that oocytes in postnatal rats have unique cell-death triggers, signal transduction pathways, and clearance mechanisms as compared with other cell types. Such flexibility has not been described for traditional apoptosis.

4.2 The unique aspects of oocytes

The unique nature of the oocyte relative to other cell types may be the cause for its unusual manner of cell death. Oocytes can remain arrested in meiosis for years, are surrounded by an acellular zona pellucida, are nonproliferating, and are known to rely on surrounding granulosa cells for survival (Hirshfield 1991). Apoptosis is an active process thought to protect the rest of an organism from an aberrant cell. Meiotic oocyte may not be required to undergo apoptosis, because they pose no threat of excessive proliferation and tumor formation. Overall, the results presented here support that oocyte loss in atretic follicles of postnatal rats can be morphologically distinguished from the two more widely described mechanisms of cell death, necrosis, and apoptosis. While it is generally accepted that granulosa cells are lost by apoptosis, the ability of the oocyte to undergo apoptosis is still in

question. Based on ultrastructural criteria traditionally associated with apoptosis (Wyllie et al. 1972), oocyte death should be assigned to a different class of physiological cell death. Such variations in the mechanisms of cell death are becoming more widely accepted (Chernoff et al. 1992) and will be the subject of future investigations.

In the present study, we characterized the degeneration of rat ovarian tissue after the exposure to EMF, cell death of the interstitial tissue in ovary was shown to be apoptotic by morphological criteria with TEM. Taken together, our results suggest that apoptosis play a critical role in the degeneration of in situ ovarian cortical and interstitial tissue, after the exposure to EMF.

Some follicles with intact oocytes contain several layer of granulosa cells and fail to form follicles consisting of multiple layers of granulosa cells. We hypothesized that this lack of increase in granulosa cell number is due to either a lack of granulosa cell proliferation or to an increase in granulosa cell apoptosis. In contrast, ovary demonstrated large number of TUNEL-positive granulosa cells (Fig. 12), suggesting that most of the granulosa cells especially near the lumen are apoptotic cells. It appears that soon after oocyte degeneration, granulosa cell begins to undergo apoptosis in the follicles of the EMF-exposed ovary.

In summary, EMF-induced changes in ovary may interfere with oogenesis, fertility, and is an indication of the cytotoxic effect of EMF on maturation of oocytes.

In EMF-exposed rats, granulosa cells have a nucleus with condensed chromatin, apoptotic bodies and several autophagic vacuoles. The presence of granulosa cells with condensed nuclei and their separation from zona pellucida and neighbouring cells, corresponds with the characteristic of apoptotic cells and constitutes the classical land mark of follicular atresia (Hardwick 2004; Tilly et al. 1992; Hurwitz and Adashi 1991). While the proportion of granulosa cells with condensed nuclei was low in control group, this was evidenced by few number of apoptotic cells. This study demonstrates induction of programmed cell death by EMF and suggest a role for EMF in increasing of follicular atresia in rat ovary.

Based on the sequential ultrastructural observations of this study as well as previous works, the following model is proposed to explain the initiation of apoptosis in ovaries. Alterations occur in the granulosa cells include; nuclear condensation, apoptotic body formation and blebbing of the cytoplasm (present study and Peluso et al. 1977). As the follicle enters into the apoptotic changes, pyknotic nuclei, apoptotic bodies and numerous autophagic vacuoles develop. The autophagic vacuoles are associated with the granulosa cells close to the basal lamina. The fact that these vacuoles contain acid phosphatase activity indicates that they are lysosomal in origin (Elfont et al. 1977) and in part responsible for the deterioration of the granulosa cell layer. Autophagic vacuoles are also observed in the thecal layers, and interstitial tissue particulary associated with the thecal cells and interstitial cells near the blood vessels. The deterioration of both granulosa and thecal cell layers is also enhanced by invasion of macrophages which occurs after many of the granulosa cells have undergone apoptosis. From all morphological standpoints cytoplasmic bodies apparently corresponding to phagolysosomes, these large cells have been identified as macrophages. It has been proposed that such cells induce the invasion of macrophages (Byskov 1974) possibly by releasing a chemotactic factor (Gaytan et al. 1998). Regarding the mechanism whereby macrophages promote granulosa cell change it is postulated that macrophages have the capacity to produce oxidative products such as nitric oxide (Bredt et al. 1994), superoxide radicals, and hydrogen

peroxide (Sugino et al. 1996); and in addition, macrophage-derived cytokines such as transforming growth factor and have been found to induce apoptosis in ovarian cells (Foghi et al. 1996). The present study describe not only the ultrastracture of follicle in rat ovaries but also the increased degenerative changes within follicle committed to undergo apoptosis after EMF-exposure (Roshangar and Soleimani Rad, 2007). In support of this idea several studies reported (Roshangar and Soleimani Rad 2001; Kaipia et al. 1997; Hughes and Gorospe 1991; Zamboni 1972) increased of macrophages after the EMF exposure. It is believed that the cell fragments produced by apoptosis are phagocytosed by macrophages. These macrophages do not release cytokines that would initiate an inflammatory response (Andrew et al. 1998).Thus the granulosa and thecal cells would be destroyed, leaving only fibroblasts and other connective tissue elements to represent the follicular wall, thereby transforming the follicle into a cystic follicle. It has been reported that macrophages were involved in the apoptotic process (Leonardo and Skeel 1980). This clearly indicates that increased number of macrophages in experimental group (Soleimani Rad and Roshangar 2000) potentially increases cell damages. Using rat ovaries, the present study confirms that the follicular granulosa cells undergo apoptosis after the EMF exposure on the basis of their microscopic features, i.e., condensation of nuclear chromatin to the margin of the nucleus, and presence of apoptotic bodies, both changes being characteristic of apoptosis (Ker et al. 1972), even more evidence is occurring in parallel with the above- mentioned morphological changes. As far as we aware, the fate of the dying granulosa cells in the ovarian follicles is unclear.

In the present study in rats, we demonstrated that large amount apoptotic and internalized granulosa cells and their fragments. The factors responsible for this basic difference are as yet unknown. In summary, this model is proposed to foster and more clearly focus future research on the mechanisms of follicular apoptosis after the EMF exposure.

Our results demonstrate that a remarkable proportion of oocytes in the rat ovary degenerate during the EMF exposure by the mechanism of apoptosis. This is already evident at experimental period, with a high number of apoptotic oocytes and increasing of macrophages and autophagic vacuoles in some occasional granulosa cells, and several lipid droplets in thecal and luteal cells. Previous TEM studies (Takeo and Hokano 1995) suggested that the process of apoptosis of the ingested cells was assumed to progress through the following steps. The nuclei of ingested cells underdog degenerative changes of successive karyopyknosis, karyorrhexis and karyolysis. The nuclear envelope and the two layers of cell membranes separating the ingested cell from the phagocytic cell were destroyed, and finally, a phagocytic vacuole was formed within the phagocytic cell.

The molecular mechanisms underlying apoptosis are poorly understood at this time. However, there are several models of apoptotic initiation that are now accepted. Apoptosis has been found to be induced via the stimulation of several different cell surface receptors in association with caspase activation. For example, the CD95(APO-Ifas) receptor ligand system is a critical mediator of several physiological and pathophysiological processes, including homeostasis of the peripheral lymphoid compartment and CTL - mediated target cell killing . Upon cross-linking by ligand or agonist antibody, the fas receptor initiates a signal transduction cascade which leads to caspase-dependent programmed cell death. The simplest way to observe this phenomenon in vitro is to use a cell permeant DNA-staining fluorescent dye such as Hoechst 33342, which allows a striking visualization of the chromatin condensation (Gartner and Hiatt 2001).

Apoptosis is over 20 times faster than mitosis. Seeing of dying cells in vivo are therefore rare. Apoptotic cells are engulfed and degraded by neighboring cells without a trace. For cell homeostasis to be maintained, a balance between the increase (by differentiation from precursors and by proliferation) and decrease (by further differentiation and cell death) in the number of a cell population has to be neatly balanced. If mitosis proceed without cell death, an 80-year-old person would have 2 tons of bone marrow and lymph nodes, and a gut 16 Km long.

Apoptotic death can be triggered by a wide variety of stimuli, and not all cells necessarily will die in response to the same stimulus. Among the more studied death stimuli is DNA damage (by irradiation or drugs used for cancer chemotherapy), which in many cells leads to apoptotic death via a pathway dependent on p53. Some hormones such as corticosteroids lead to death in particular cells (e.g., thymocytes), although other cell types may be stimulated. Some cell types express Fas, a surface protein which initiates an intracellular death signal in response to cross-linking. In other cases cells appear to have a default death pathway which must be actively blocked by a survival factor in order to allow cell survival, a survival factor normally binds to its cell surface receptor. When the survival factor is removed, the default apoptotic death program is triggered (Andrew et al. 1998).

Biochemical correlates of these morphological features have emerged during the subsequent years of study of this phenomenon. The first and most dramatic is DNA fragmentation, which was described by Brocklehurst 1996; McLauchlan 1981; and Wyllie 1980. When DNA from apoptotically dying cells was subjected to agarose gel electrophoresis, ladders with – 200 bp repeats were observed, corresponding histone protection in the nucleosomes of native chromatin. Subsequent pulsed field gel techniques have revealed earlier DNA cleavage patterns into larger fragments. Since even a few double stranded DNA breaks will render the cell unable to undergo mitosis successfully, such DNA fragmentation can be regarded as a biochemical definition of death. However, in some apoptotic systems (e.g., Fas killing of tumor cells) artificially enucleated cells lacking a nucleus still die, showing that the nucleus is not always necessary for apoptotic cell death.

The changes in the apoptotic cell which trigger phagocytosis by non-activated macrophages have been investigated by several groups. Macrophages appear to recognize apoptotic cells via several different recognition systems, which seem to recognize recognition used preferentially by different macrophage subpopulations. There is good evidence that apoptotic cells lose the normal phospholipid asymmetry in their plasma membrane, as manifested by the exposure of normally inward-facing phosphatidyl serine on the external face of the bilayer. Macrophages can recognize this exposed lipid head group via an unknown receptor, triggering phagocytosis.

Another biochemical landmark of apoptotic death which increasingly appears general is the activation of caspases, which are cysteine proteases related to *ced-3,* the "death gene" of the nematode *Caenorhabditis elegans.* caspases seem to be widely expressed in an inactive proenzyme form in most cells. Their proteolytic activity is characterized by their unusual ability to cleave proteins at aspartic acid residues, although different caspases have different fine specificities involving recognition of neighboring amino acids. Active caspases can often activate other pro-caspases, allowing initiation of a protease cascade. While several protein substrates have been shown to be cleaved by caspases during apoptotic death, the

functionally important substrates are not yet clearly defined. Persuasive evidence that these proteases are involved in most examples of apoptotic cell death has come from the ability of specific caspase inhibitors to block cell death, as well as the demonstration that knockout mice lacking caspase 3, 8 and 9 fail to complete normal embryonic development. A critical issue is how caspases become initially activated, which seems to be an irreversible commitment towards death. It seems that aggregation of some pro-caspases (those with large pro- domains) allows them to become autoactivated. Recent experiments make it clear that mitochondria are involved in one major pathway involving activation of pro- caspase-9. Other experiments show that ligands crosslinking death receptors such as Fas trigger formation of a cytoplasmic complex in which pro-caspase-8 is aggregated and activated. In both cases these initiator caspases in turn activate a cascade of other pro-caspases leading to death (Andrew et al. 1998).

While there is much to be learned about the molecular pathways leading to apoptotic cell death, it is increasingly clear that cell death is a normal part of normal biological processes. This had not been appreciated until relatively recently, and our understanding of such death, and our ability to manipulate it, could allow therapeutic intervention in major diseases such as cancer, heart disease, stroke, AIDS, autoimmunity, degenerative diseases, and others.

We can only speculate how our EMF would fit into one of these models for induction of apoptosis. One such model is apoptotic initiation by intracellular perturbation. Examples of this model are ionizing radiation and chemotherapeutic ionizing agents, which cause DNA damage and initiation of apoptosis. An electromagnetic field could have a similar effect (Norman et al. 1997).

Additionally, it has been shown that the overexpression of c-myc portion leads to apoptosis. It is interesting that EMFs have been shown to increase specifically transcription of c-myc in several cell lines (Lin et al 1998). This is just one of the numerous possible mechanisms that could be inducing apoptosis. Another possibility is EMF-inducing apoptosis is mediated through the production of free radicals. It is shown that EMF exposure may result in production of free radicals (Lucia et al. 2004; Brocklehurst and McLauchlan 1996; Grundler et al. 1992; Alexander 1954). It is also shown that addition of antioxidants, such as vitamine E reduces EMF-induced changes *in vivo* and *in vitro* (Mohammadnejad and Soleimani Rad 2010). On the other hand free radicals as an inducer of apoptosis is also established (Formica and Silvestri 2004).

The findings with TEM about apoptosis-induced by EMF is confirmed using TUNEL assay. TUNEL positive cells are localized in granulosa layer, thecal cells, luteal cells and interstitial cells. The localization of apoptotic cells are well correlate with TEM studies. It is proposed that combining other methods such as microscopic evaluation of morphological changes with TUNEL POD test can substantiate the specificity of results.

Although the mechanisms underlying follicular atresia are not well known at this time, DNA damage, which can be initiated by oxidative free radicals, has been proposed as a possible mechanism that leads to the activation of the apoptotic cascade in atretic follicles (Gougeon 1996). Macrophages have the capacity to produce oxidative products such as nitric oxide (Bredt and Synder 1994), superoxide radicals, and hydrogen peroxide (Sugino et al. 1996). Macrophage-derived cytokines such as transforming growth factor α induce apoptosis in ovarian cells (Foghi et al. 1997).

In addition to the ovarian follicles the apoptotic status and nuclear condensation is observed in corpora lutea and ovarian stroma. These findings was also confirmed with the presence of TUNEL positive cells. Ultrastractural characteristics of apoptotic cells in the corpora lutea in this study is similar to those reported by Shikone et. al. 1996. Another finding in the present study was the presence of numerous macrophages in the stroma and granulosa layer in EMF exposed rats. Since the presence of macrophages in the granulosa layer of atretic follicles and the degenerating corpora lutea of rats was demonstrated by Bulmer (Bulmer 1964), macrophages have been thought to scavenge degenerated cells in the ovary (Lauber et al 2004; Anderson et al. 2003).

In addition to apoptotic changes induced by EMF exposure the other ultrastractural changes include mitochondrial disruption, condensation and or their cristae disappearance, rER dilatation and in some occasion, cytoplasmic membrane dissolution. All these alterations are the sign of cell damage and necrosis rather than apoptosis. The rational explanation would be that: EMF induces both apoptosis and necrosis depending on its strength and cell types. For example typical degenerative changes were never observed in oocytes, while it occurred in many granulosa cells. The cytotoxic effect of EMF could be attributed to its production of local heat and free radicals. This hypothesis is evidenced by the studies have shown that EMF could produce heat and free radicals (Dandrea et al. 2003; Grundler et al. 1992; Alexander and Charlesby 1954).

In this regards, it has been shown that electromagnetic fields from power lines, household currents and video display terminals, microwaves, and ultrasound have also been studied with regard to their reproductive risks. Biological plausibility plays an even more important role in their evaluation than with ionizing radiation. EMF has the capacity to produce hyperthermia, which is a proven reproductive toxin. Numerous animal experiments have demonstrated that intrauterine exposures to hypertheimia from microwaves and ultrasound and EMFs can produce malformations, growth retardation, and embryonic loss. But the usual population exposures to EMFs are below the exposures that result in hyperthermia. Furthermore, those mechanisms that are involved in reproductive toxicity, such as cytotoxicity and abnormal differentiation and cell migration do not occur at the population exposures to these agents. Evaluations of pregnancy loss from intrauterine exposures to environmental toxicants presents special problems, especially if it is the only reproductive effect being evaluated. Many studies have ignored the basic concepts of reproductive toxicology and the biological plausibility of their findings. Investigators should be cautious about biological Investigator epidemiological studies dealing with pregnancy loss without concurrent collecting other reproductive endpoints. Studies evaluating multiple reproductive endpoints have markers to assist them in determining the validity of the fertility loss data (Byene 1999).

In summary, the present data suggest that the currently applied EMF levels under certain circumstances might induce biological effects. Results indicate that the genetic constitution of cells determined by loss of P53 function can influence EMF related cellular responses. Whereas wild-type cells were insensitive. It remains to be elucidated, whether EMF induced changes of expression levels of regulatory genes may be compensated or normalized, or would result in sustained biological effects in vivo. Further studies are needed to analyze the whole transcription of EMF exposed cells by genomics technologies, such as cDNA microchips or serial analysis of gene expression (SAGE), because of conflicting epidemiological data on human EMF exposure (Repacholi 1998).

For biological effects of EMF, non thermal mechanisms are also proposed. These include: 1) Free radical formation. 2) Removal of Ca ions from membranes and and making them more likely to tear and keak. 3) DNAase leaking through the lysosomal membrane and DNA damage. 4) Leakage of Ca ions into the cytosol and acting as metabolic stimulant (Goldsworthy 2007). With regard to the effect of' free radicals, it has been proposed that free radical could affect membrane integrity, produce DNA damage, and protein structures (Alexander and Charlesby 1954). This type of changes, would result in cell damage and induction of apoptosis (Formica and Silvestri 2004).

No conclusion can be drawn for electromagnetic fields and radiofrequencies because of lack of data, but there is no convincing evidence today that EMFs of the sort pregnant women or potential fathers meet in occupational or daily life exposures do any harm to the human reproductive process (Cheronff et al. 1992).

Additionally, it has been hypothesized that electromagnetic fields initially affect cells at their surface, since these low energy fields cannot directly access the cell interior because of the high resistance of the cell plasma membrane (Luben et al. 1982). The proteins that span the width of the plasma membrane therefore, have been hypothesized to act as potential sensors of ELF electromagnetic so that their actions may be transmitted to intracellular enzymes and organelles (Adey 1990).

Moreover, transformed cells and normal ones show different electrical characteristics as extensively documented by several scientists (Capko et al. 1996; Shulyakovskaya et al. 1993; Goller et al. 1986). These results led to the hypothesis that EMF of more than 1 mT, may through their effect on motion of charged matter, have a selective action on cell signaling which influences cell survival mechanisms in transformed cell, inhibiting their growth and differentiation (Tofani 1999). Also EMF have been shown to affect different aspects of biomolecular synthesis in cell, including the kinetics of DNA, RNA, and protein production (Libof 1985). Increased DNA and proteoglycan synthesis have been observed in chondrocytes (Rodan et al. 1978). In fibroblasts, low – intensity electric and magnetic fields altered collagen and proteoglycan synthesis (Farndale and Murray 1985). A complex range of effects was observed depending on the exact magnetic field configurations.

Gap junctions are specialized areas of the plasma membranes between two contiguous cells where a" pore" is formed that allows for the passage of small molecules between cells (Dean et al. 2002; Loewenstein 1979). These gap junctions are composed of proteins called connexins which have extracellular regions that attach to other connexin proteins of a contiguous cell as well as having intramembranous and cytoplasmic domains. Thus connexin proteins could also be targets of electromagnetic fields. Gap junctions have been ultrastructurally described in bone (Doty 1981). They occur among osteoblasts and osteocytes (Takahashi 2002; Boone and Tsang 1997). It has been noted that gap junctions can be regulated by change in cellular Ca^{2+} concentration. Micromolar concentration of Ca^{2+} have been demonstrated to decrease gap junction intercellular communication in a variety of tissues including cardiac muscle and liver (Li et al. 1999; Hertzberg et al. 1981). Thus it would be important to understand if changes in intracellular Ca^{2+} metabolism that may occur with exposure to ELF magnetic fields would be related to alterations in gap junction dependent intercellular communication (Luben 1991).

Research on the effects of electromagnetic fields on cells has known to alter some important physiological pathways. Of note are ionic conductances with such species as calcium ion (Ca^{2+}) which is a known interacellular messenger in cell functions such as proliferation and intercellular communication. Specifically, with respect to extremely low-frequency (EMF) (<300 Hz) magnetic fields, Ca^{2+}, uptake into lymphocytes was shown to increase (Lednev 1991). Walleczek (1992), summarized the effect of ELF magnetic fields, showing that such fields could either increase or decrease Ca^{2+} uptake into lymphocytes, depending on the time of exposure, the frequency and shape of the signal, and concomitant induced electric field intensity.

Mechanisms of interaction of ELF fields have been reviewed by Blank (1995), NRC (1996), Tenforde et al. (1996), and Valberg et al. (1997). A well-known mechanism of interaction of exposure of biological tissues to ELF fields is the induction of time- varying electric currents and fields. At sufficiently high levels, these can produce direct stimulation of excitable cells such as nerve and muscle cells. At the cellular level, the interaction induces voltages across the membranes of cells sufficient to stimulate nerves to conduct or muscles to contract. This mechanism accounts for the ability of humans and animals to perceive electric currents in their bodies and to experience electric shocks.

Our results have also been demonstrated the junctional changes that occur after EMF-exposure (Figs. 7, 9). Moreover, it is postulated that developmental exposure to EMF may reduce oocyte differentiation and diminish folliculogenesis at earlier stages of oocyte and follicular nest formation. Both of which could result in decreasing of ovarian reservoir and thus the individual will be prone to subfertility in adulthood.

4.3 EMF-Induced ultrastractural changes and apoptosis in uterus and fallopian tubes

Other findings in the present study are the effects of EMF on the lining epithelium of endometrium, uterine glands and Fallopian tubes. Ultrastractural results from EMF exposed rats revealed that the height of epithelial and glandular cells both in uterus and Fallopian tubes were reduced, indicating metabolic activity of cells were decreased. On the other hand, there were condensation and cilliary loss, which could be considered as pre apoptotic changes.

This postulation was confirmed by apoptosis assay, using TUNEL reaction technique which was revealed apoptotic cells (TUNEL positive cells) in both endometrial surface and glandular epithelium of uterus and covering epithelium of Fallopian tubes. Ultrastractural changes corresponding to cytotoxic effect of EMF were also observed. These changes include; accumulation of numerous fat droplets in the secretory cells, presence of secondary lysosomes and morphological changes of mitochondria.

As we know, endometrial surface epithelium plays a key role in blastocyst implantation and an implantation window is required for the process of implantation to begin (Marti et al. 2001; Otasuki 2001). Additionally, increasing of secretion is usually occurs in preimplantation stage (Marti et al. 2001). Any changes in the amount and or nature of secretory substance would obviously affect implantation process. Similarly, the activity of ciliated and nonciliated cells in the covering of Fallopian tubes are very important factor for transport and early development of preimplant embryo. The structural changes produced by EMF could affect both development and transport of early embryo.

There is no doubt that cell damages are the basis of all disorders occurs after EMF exposure. In support of our findings Sandra et. al. (Sandra et al. 2000) suggested that EMF-exposure might impair mammalian female reproductive potentiality by reducing the capacity of the follicles to reach a developmental stage that is an essential pre requisite for reproductive success.

The acceleration of cell damage with EMF-exposure in reproductive organs in rathas previously been reported (Roshangar and Soleimani Rad 2002; Soleimani Rad and Roshangar 2000; Byskov 1974). To elucidate the mechanisms underlying the acceleration of cell damage (Armstrong et al. 2001; Chun et al. 1996), in the present study, the earlier step of cell damage on the cellular membrane integrity, mitochondrial features, appearance of apoptotic bodies, nucleus condensation, and lipid droplet accumulation is investigated with EM and TUNEL assay. Each of these end points showed a parallel correlation with apoptosis when the animals exposed to EMF for long time.

These findings may explain the acceleration and increasing of apoptotic process by the conditioning dose. Contrary to the acceleration of apoptosis shown in the present study, in some types of cells, such as malignant cells (Ohnishi et al. 2002) and mouse spleen cells (Takahashi et al. 2002). As a possible mechanism, the attenuation of P53 response has been postulated (Dean et al. 2002; Doty 1981). In cellular responses to ionizing radiation, P53 plays very important roles. It regulates DNA repair, which leads cells to die. The partial involvement of P53 in the regulation of apoptosis is also suggested (Perez et al. 1999; Vousden 2005).

The recent evidence pointing to the role of caspases in activating DNA degradation (Green and Reed 1998; Liu et al 1997) suggest that in order for ovarian cells to complete the apoptotic program they must contain caspase-3, and an endogenous nuclear DNAase. Boone et.al. (Boone and Yan 1995) demonstrated that granulosa and luteal cells contain endogenous nuclear DNAase, and they hypothesized that these cells would therefore only require a signal to activate this enzyme in order to degrade their DNA in an apoptotic fashion (Laun et al. 2000; Boone and Tsang 1997).

4.4 Conclusion

In conclusion, we have shown that EMF-exposure causes a large proportion of oocytes in the rat ovary to degenerate by a mechanism similar to apoptosis. This is evident in the EMF-exposed group with a large number of degenerative oocytes. Other findings of the study are: an increased number of macrophages; autophagic vacuoles in some granulosa cells; and appearance of several lipid droplets in thecal and luteal cells. The present study has also shown the increased number of macrophages not only in the corpora lutea but also in the growing follicles in the EMF-exposed group. Based on our TEM, we have proposed a model that can explain the initiation of apoptosis by EMF-exposure in ovaries.

The aim of this work was to monitor the reproductive effect of exposure to a magnetic field in rat. Taken together, our results suggest that apoptosis plays a critical role in the degeneration of ovarian cortical tissue, luminal epithelium, glandular epithelium and stromal cells in uterus and luminal epithelium in fallopian tube. The present EMF- exposure model can be used when striving to find ways to improve the viability of ovarian tissue in

order to grow follicles for subsequent IVF treatment, and or to protect reproductive organs from EMF effect. Regarding the human effect of EMF, the public concern about EMFs is motivated mainly by the fact that they are ubiquitous and nobody can totally avoid this type of exposure. The available epidemiologic studies all have limitations that prevent to draw clear-cut conclusions on the effects of EMFs on human reproduction.

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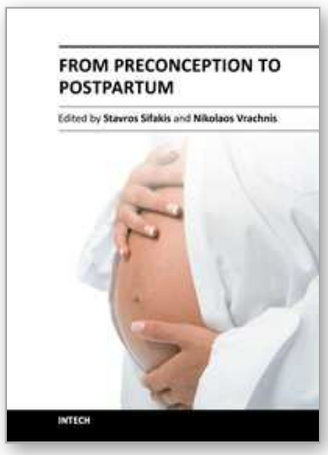

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