

<b>Original Article</b>	<b>Effect of Nicotine Injection and its Stoppage on Reproduction in Female Rats</b>  <i>Manal M.S. El-Meligy<sup>1</sup>, Randa H. Abdel Hady<sup>2</sup>, Amal R. Abdel Samaei<sup>1</sup> and Heba M. Saad Eldien<sup>3</sup></i>  <i><sup>1</sup>Human Anatomy and Embryology Department, <sup>2</sup>Forensic Medicine and Clinical Toxicology Department and <sup>3</sup>Histology Department, Faculty of Medicine, Assiut University</i>
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### ABSTRACT

**Background:** There is a clear support for an association between smoking and decreased female fertility. The substantial harmful effects of cigarette smoking on fecundity and reproduction have become apparent but not generally appreciated.

**Aim of the Work:** The present work aimed to study the effect of nicotine on some reproductive organs and the morphological abnormalities of the offspring of the nicotine-treated mothers.

**Material and Methods:** The experimental female rats aged three-month old were divided into two main groups: Group A (nonpregnant rats) and group B (pregnant rats). Group A was subdivided into three subgroups eight rats each: (I) Saline-treated control subgroup that was injected with 0.9% saline subcutaneously daily for 3 weeks, (II) Nicotine-treated subgroup that was injected subcutaneously daily with nicotine in a dose of 0.4mg/100gm body weight which is equivalent to the amount of nicotine passing to the blood of the heavy smoker for 3 weeks and (III) a third subgroup, that was injected with nicotine in the same dose and for the same duration then left for 2 months without injection. Group B was subdivided into two subgroups eight rats each: (I) Control subgroup which was injected with 0.9% saline subcutaneously daily from the day 6 to the day 20 of pregnancy and (II) Nicotine-treated subgroup that was injected with nicotine in the same dose used in group (A) from the day 6 to the day 20 of pregnancy. The nonpregnant animals of subgroups A-I, II were sacrificed at the end of 3 weeks, whereas those of subgroup A-III were sacrificed 2 months later. The uterine and ovarian specimens were fixed in 10% formaline solution for histopathological examination. The pregnant rats of subgroups B-I, II were sacrificed on the 20<sup>th</sup> day of gestation, and the fetuses were extracted, examined by the naked eye and some of them were processed for Alizarin red staining for skeletal examination. The placenta was fixed in 10% formaline solution for histopathological and immunohistochemical examinations. Statistical analysis for evaluation of the effect of nicotine on pregnancy outcome and fetal growth was done.

**Results:** As regards the histological changes in the ovary, nicotine caused retardation in the follicular growth, a decrease in healthy follicles and an increase in atretic and cystic follicles. Apoptotic granulosa cells were clearly observed. Fatty degenerative changes in corpora lutea were noticed. When nicotine injection was stopped, there was an improvement in the follicular growth with less degenerative changes in corpora lutea but they did not reach the control level. In the uterus, nicotine caused reduction in the endometrial and myometrial thickness and marked reduction in endometrial glands. When nicotine injection was stopped, although the endometrium and myometrium looked more or less similar to the control, the endometrial glands were reduced in some animals. Several placental degenerative changes were observed under nicotine treatment. The fetuses of nicotine-treated mothers showed several abnormalities including reduction in body size and weight, wrinkled skin, mis-shaped head, kyphosis, reduced neck region, micromelia, spina bifida and meningo-encephalocele. Also, the skeletal examination of Alizarin red-stained fetuses of nicotine-treated mothers revealed retarded ossification of axial and appendicular skeleton. The statistical analysis showed marked reduction in pregnancy outcome, fetal weight and length under nicotine treatment.

**Corresponding Author:** Dr. Manal El-Meligy, Human Anatomy and Embryology Department, Faculty of Medicine, Assiut University, Assiut, Egypt, Email: manal\_el-meligy65@hotmail.com Mobile: 0106807880.

**Key Words:** Nicotine, uterus, ovary, placenta, pregnancy outcome.

## INTRODUCTION

Cigarette smoke is a complex mixture of toxic chemicals including nicotine, carbon monoxide, and several recognized carcinogens and mutagens (Stedman, 1968; Rogers, 2009). These toxicants are absorbed through the pulmonary vasculature and transported via the bloodstream causing cytotoxicity, genotoxicity, and tumorigenicity throughout the body (Stillman, et al. 1986; St Clair et al., 1994; Baroni et al., 2010). Nicotine is metabolized primarily by the liver, and to a lesser extent, the lung and kidney, with the primary metabolite being cotinine (Kyerematen et al., 1990a&b). In addition to the deleterious effects on cardiovascular and pulmonary physiology, cigarette smoking affects the reproductive system and imposes a number of unique risks specific to women, as smoking has been found to be associated with infertility, ectopic pregnancy (Saraiya et al., 1998), spontaneous abortion (Kendrick et al., 1996), menstrual abnormalities and early onset of menopause (Midgett & Baron, 1990). Interestingly, women who smoke have decreased risks of breast cancer (Vessey et al., 1983) and endometrial cancer (Cramer et al., 1986). Because these phenomena are all estrogen dependent, it has been suggested that smoking has antiestrogenic effects (Baron et al., 1990). In fact, female smokers have significantly lower levels of estradiol, estrone and estrone during the luteal phase of menstrual cycles and tend to have lower levels of these estrogens during the follicular phase compared to nonsmokers (MacMahon et al., 1982). Treatment of rats with nicotine was associated with a decrease in estrogen-dependent parameters including uterine weight and diameter, and thickness of the myometrium and endometrium (Patil et al., 1999). Also, nicotine and M-nicotine (a nicotine metabolite) can induce a sort of luteal insufficiency by inhibiting progesterone release, probably through modulation of the prostaglandin system (Miceli et al., 2005).

Newnham et al. (1990), Wen et al. (1990), Ashfaq et al. (2003), Vaglenova et al. (2004), Ronco et al. (2006) and Jauniaux and Burton (2007) reported that active and passive maternal smoking caused a damaging effect in every trimester of human pregnancy. Cigarette smoke contains scores of toxins which exert a direct effect on the placental and fetal cell proliferation and differentiation and can explain the increased risk of miscarriage, fetal growth restriction, stillbirth, preterm birth, placental abruption, increased

perinatal morbidity, and childhood cognitive and behavioral deficits. All these changes were associated with extensive loss of trophoblasts by apoptosis

Although many adverse effects of cigarette smoking on reproduction have become clear, the findings of previous studies have largely been contradictory. So, the aim of the present work was to declare the possible mechanisms by which nicotine can interfere with female fertility and affect pregnancy outcome, and whether its stoppage has an improving effect or not.

## MATERIAL AND METHODS

A total number of 40 adult female albino Wistar rats aged 3 month old were used in this study. The rats were obtained from the Animal House, Assiut University. They were housed in stainless steel cages under 12 h light/dark cycle at 25 C° and allowed water and food (laboratory chow) ad libitum. The experiments reported here were approved by Faculty of Medicine, Assiut University, Ethics Committee. The animals were divided into two main groups: (A & B), group A consisted of 24 non pregnant rats and group B consisted of 16 pregnant rats.

### Group A (nonpregnant rats) was subdivided into three subgroups, 8 rats each:

- Subgroup A-I: was the control subgroup injected with 1 ml of 0.9% saline subcutaneously daily for 3 weeks.
- Subgroup A-II: was the nicotine treated subgroup injected with nicotine in a dose of 0.4mg/100 gm body weight subcutaneously daily for 3 weeks. This dose was equivalent to the amount of nicotine passing to the blood of the heavy smoker (Aydos, et al. 2001). Nicotine (liquid) was purchased from Fluka Bio Chemika (Switzerland).
- Subgroup A-III in which nicotine injection was stopped for 2 months after treatment with the same dose used in subgroup A II for 3 weeks.

### Group B (pregnant rats) was subdivided into two subgroups, 8 rats each:

- Subgroup B-I: was the control subgroup injected with 0.9% saline subcutaneously daily from the day 6-20 of pregnancy.

- Subgroup B-II: was the nicotine-treated subgroup injected with nicotine in the same dose used in group (A) from the day 6-20 of pregnancy.

Nonpregnant animals of subgroups A-I & II were sacrificed at the end of the third week, whereas those of subgroup A-III were sacrificed 2 months later. Uterine and ovarian (in the diestrus phase) specimens were fixed in 10% formaline solution for histopathological examination using Haematoxylin and Eosin stains.

Pregnant rats of subgroups B-I & II were sacrificed on the 20<sup>th</sup> day of gestation, after being anaesthetized by ether early in the morning. The number of living and dead fetuses, as indicated by their movement following a gentle pressure, were recorded before opening either uterine horn (Wilson, 1965). The weight, length and general morphology of fetuses were examined. The placentas were kept in 10% formaline solution for histopathological examination using Haematoxylin and Eosin stains for general histological examination, Masson's trichrome stain for collagen fibres and periodic acid Schiff stain for polysaccharide (Carleton, 1980). Two-thirds of the fetuses were fixed in Bouin's solution for external and visceral examinations. Transverse sections were made in the offspring of the control and nicotine-treated mothers to investigate the presence of any fetal abnormalities that could be apparent by naked eye.

The other third of fetuses was eviscerated and preserved in 95% alcohol for skeletal examination using Alizarin red stain (Wilson, 1965).

Statistical analysis was done to evaluate pregnancy outcome and fetal growth in both the control and nicotine-treated subgroups using statistical package for social sciences version II (SSPS).

## RESULTS

### Histological results:

**Group (A):** Examination of the ovary of subgroup A I animals revealed that the cortex was occupied by follicles in various stages of development and multiple corpora lutea (Figs. 1,2,3). Examination of the ovary of

subgroup A II animals revealed multiple cystic and degenerated follicles in various stages of degeneration. Collapsed follicles were observed on the surface of the ovary (Figs. 4,5). Multiple apoptotic cells were noticed among the follicular cells, with degenerated ova in some growing follicles (Figs. 6,7). Multiple degenerated granulosa cells with vacuolated cytoplasm and dense nuclei were also present (Figs. 8,9). Irregularity in the capsule of some corpora lutea was observed with many pale vacuolated cells immediately beneath the capsule where some of these cells appeared non-nucleated (Figs. 10,11). As regard subgroup A-III, there was an increase in healthy follicles and corpora lutea and reduction in degenerated follicles and corpora lutea to some extent which did not reach the control level (Fig. 12). Mild degenerative changes were observed in the ovum, corona radiata and granulosa cells of some animals (Fig. 13). In some corpora lutea, granulosa and theca lutein cells appeared with dense nuclei and slightly dark cytoplasm, while other cells appeared with pale vacuolated cytoplasm (Fig. 14).

Examination of the uterus of subgroup A-I animals revealed normal histological structure of the endometrium (glands and surrounding stroma), myometrium and perimetrium (Fig. 15). Examination of the uterus of subgroup A-II animals showed a marked reduction in the thickness of both endometrium and myometrium with an observable reduction in the endometrial glands (Fig. 16). In subgroup A-III, endometrium and myometrium appeared more or less similar to those of the control. However, fewer endometrial glands were observed as compared to the control (Fig. 17).

**Group (B):** Examination of the placenta of subgroup B-I animals (the basal plate by Haematoxylin and Eosin stains) revealed the maternal part with normal decidual cells and multiple maternal blood spaces that were separated by fetal trophoblasts (Fig. 18). Multiple branching chorionic villi had blood vessels inside their cores with intact endothelial lining (Fig. 19). A moderate amount of collagen fibres in the core of villi and in the subtrophoblastic membranes was observed on using Masson's trichrome stain (Fig. 20). Positive periodic acid Schiff reactions were observed in the basement membranes of the trophoblasts and those of blood capillaries (Fig. 21).

Examination of the placenta of subgroup B-II animals revealed separation of the decidual cells from the basal plate and also from the trophoblastic cell columns. Necrotic as well as apoptotic changes were observed in the decidual cells and multiple degenerated cell columns were present (Fig. 22). Wide maternal blood spaces surrounded by degenerated decidual cells were also observed (Fig. 23). Apoptotic signs were seen in the decidual cells in the form of fragmentation of the nucleus. Margination of the nuclear chromatin was associated with cytoplasmic blebbing (Figs. 24,25). Multiple degenerated chorionic villi with a relatively small sized blood vessel in their cores as well as degenerated cell columns with an amorphous matrix were observed (Figs. 26,27). Discontinuation in the syncytiotrophoblast that lead to free connection between the maternal and fetal blood was present in some animals (Fig. 28). Masson's trichrome stain demonstrated an increase in the collagen fibres in the core of the villi and in the trophoblastic basement membrane (Fig. 29). A Highly positive periodic acid Schiff reaction was seen in the basement membranes of both the trophoblasts and blood capillaries, so they appeared thicker than those of the control placenta (Fig. 30).

**Naked-eye examination:**

- The fetuses of nicotine-treated mothers showed a wide range of abnormalities including marked reduction in body size, wrinkled skin, malformed head, meningo-encephalocele (a), spina bifida (failure of fusion of the neural arches) (b), depressed frontal area of the head (c), micromelia (short limbs) (d), kyphosis (increased curvature of the vertebral column at

the thoracic region) (e) (Fig. 31) and haemorrhage (Fig. 32).

- The cross sections taken in fetuses of nicotine-treated mothers revealed spina bifida in some animals (Fig. 33).

**Skeletal examination:**

- Alizarin red-stained fetuses of the control mothers revealed the presence of the ossification centres of the metacarpals, metatarsals, ribs and vertebral column (Fig. 34).
- Ailzarin red-stained fetuses of nicotine-treated mothers revealed partial ossification of the ribs and impaired ossification of the vertebral column and metatarsal bones (Figs. 35,36).

**Statistical Results:**

- The pregnancy outcome of nicotine-treated mothers showed a marked reduction in comparison to the control mothers and the difference was found to be highly significant (Table 1).
- As regards fetal body weight, it was found that fetuses of nicotine-treated mothers were lighter than those of the control mothers. Statistically, the difference in fetal body weight was found to be highly significant (Table 2).
- As regards fetal crown-rumb length, it was found that fetuses of nicotine-treated mothers were shorter than those of the control mothers. Statistically, this difference was reported to be highly significant (Table 2).

**Table 1:** Effects of nicotine on pregnancy outcome.

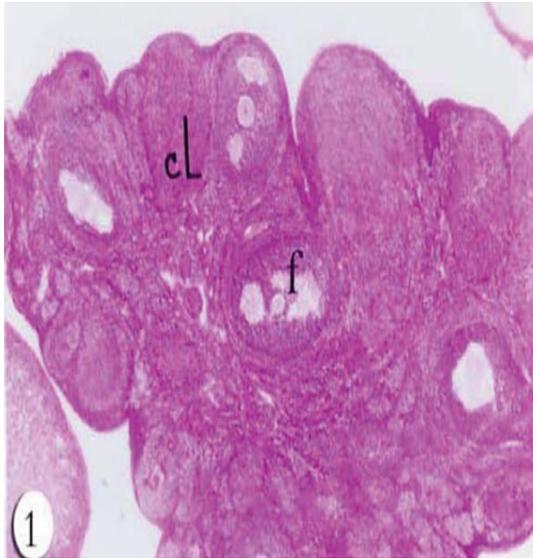
Group	Mean SD	t	Sig. (2-tailed)	Correlation (r)
Pair (1) * Number of fetuses of control animals – * Number of fetuses of nicotine-treated animals	4.2500 ± 3.1053**	3.871	0.006	1.000 -
Pair (2) * Number of living fetuses of control animals – * Number of living fetuses of nicotine-treated animals	5.3750 ± 2.1998**	6.911	0.000	0.980 -

P<0.05

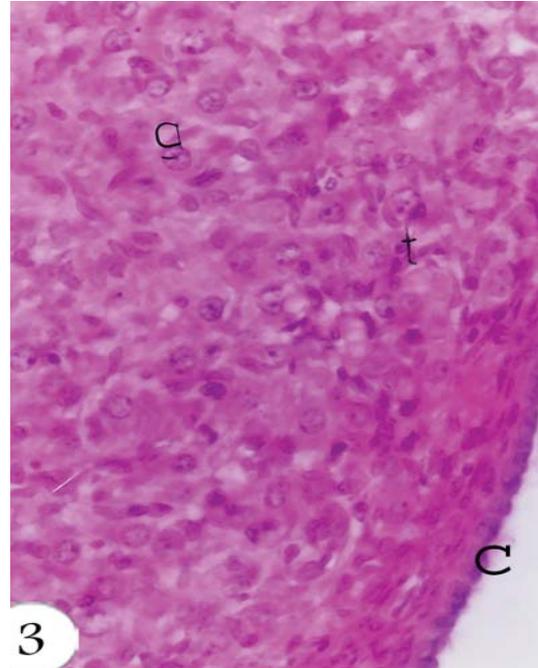
SD = standard deviation

**Table 2:** Influence of nicotine on fetal growth.

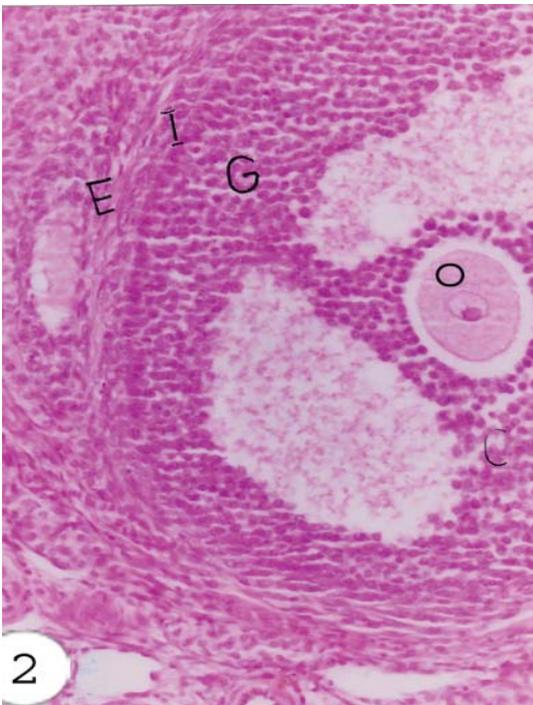
Group	Fetal weight (gm) (X ± SD)	Fetal crown-rump length (cm)
Control	1.24 ± 3.3 E-02	2.28 ± 0.11
Treatment (nicotine)	0.25** ± 5.23 E-02	1.16** ± 0.10



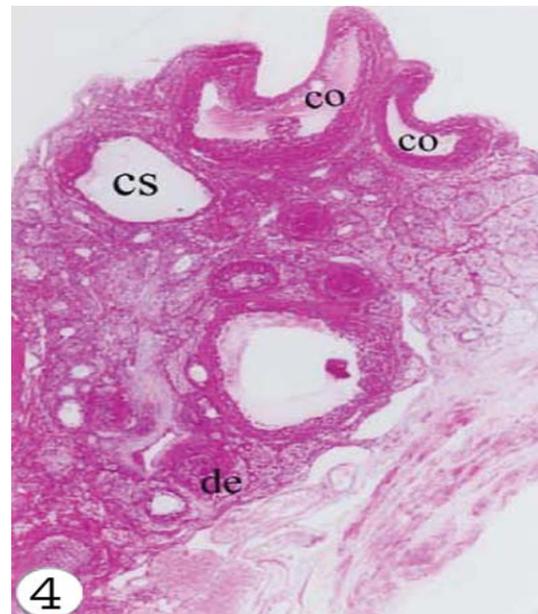
**Fig. 1:** A photomicrograph of the ovary of a control animal (subgroup A-I) showing the cortex occupied by follicles (f) in various stages of development and multiple corpora lutea (CL).  
Hx. & E.; X40



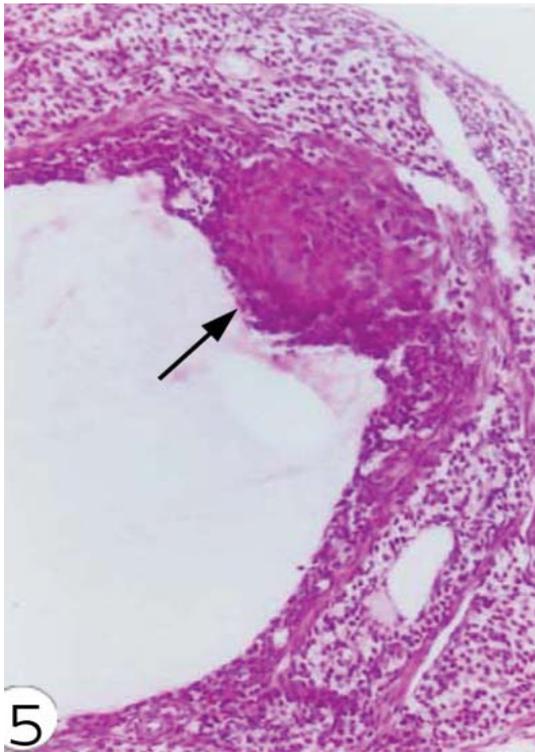
**Fig. 3:** A photomicrograph of a magnified part of a corpus luteum in the ovary of subgroup A-I animals showing the surrounding connective tissue capsule (c), granulosa lutein (g) and theca lutein cells (t).  
Hx. & E.; X400



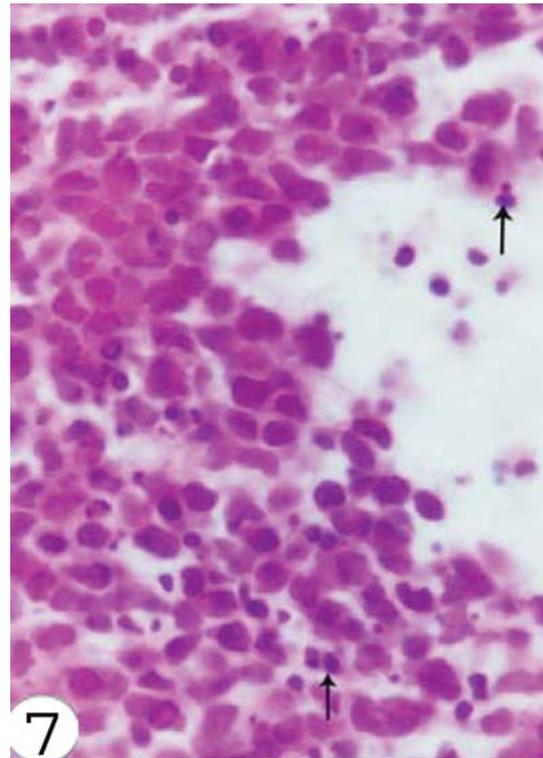
**Fig. 2:** A photomicrograph of a magnified part of a growing follicle in the ovary of subgroup A-I animals showing the ovum (O) and granulosa cells (G) that are resting on the basement membrane. Notice theca interna cells (I) and theca externa cells (E).  
Hx. & E.; X200



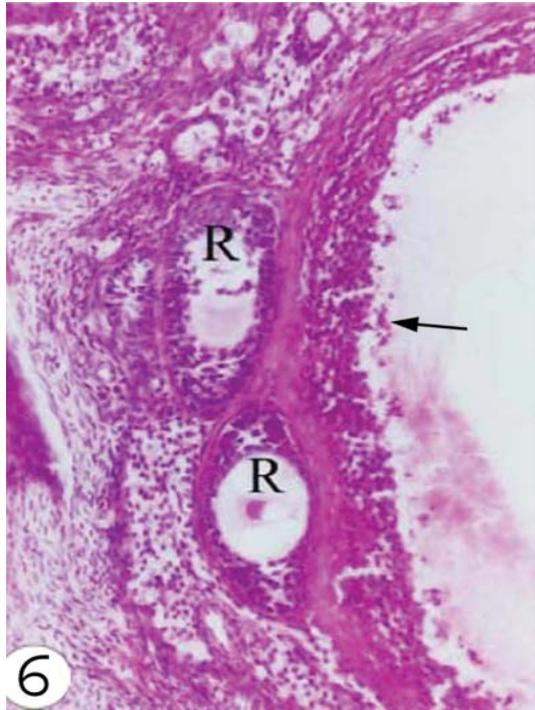
**Fig. 4:** A photomicrograph of the ovary of a nicotine-treated animal (subgroup A-II), showing multiple cystic follicles (cs) and degenerated follicles (de) in various stages of degeneration. Notice two collapsed follicles (co) on the surface of the ovary.  
Hx. & E.; X 40



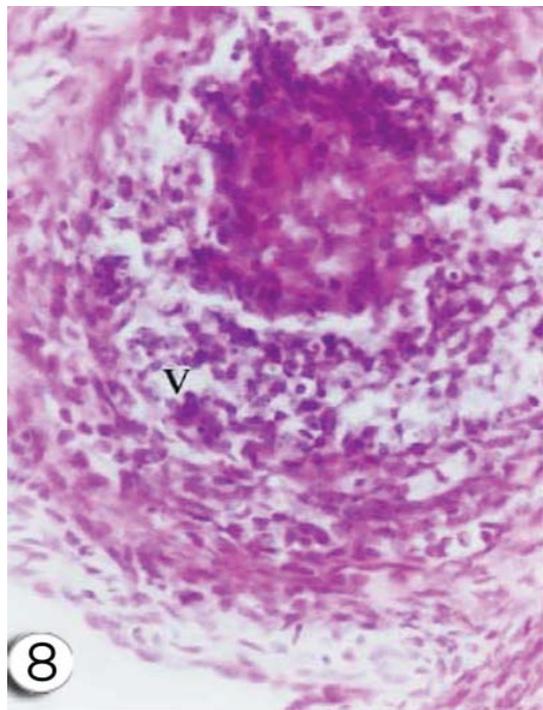
**Fig. 5:** A photomicrograph of a magnified part of subgroup A-II ovary showing a large cystic follicle with localized thickening at one side which is formed of multiple dark degenerated granulosa cells (↑). Hx. & E.; X 200



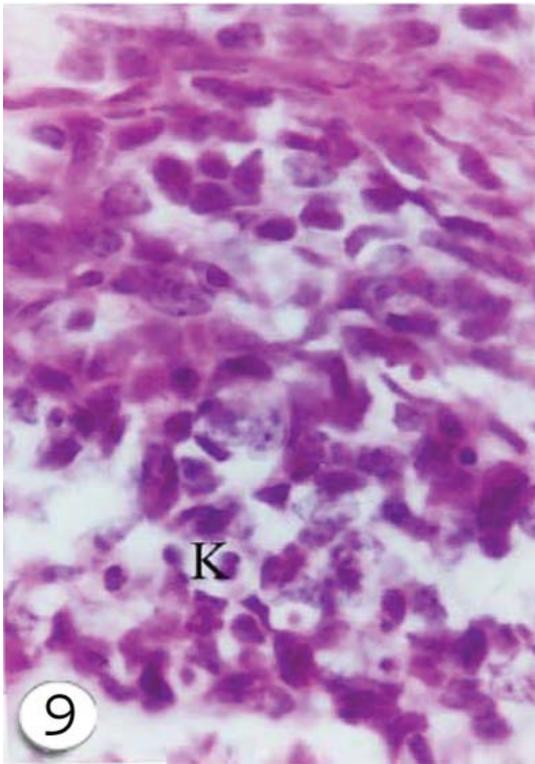
**Fig. 7:** A photomicrograph of a magnified part of subgroup A-II ovary showing a part of the follicular wall with multiple apoptotic cells (↑) between the surrounding dark cells. Hx. & E.; X 1000



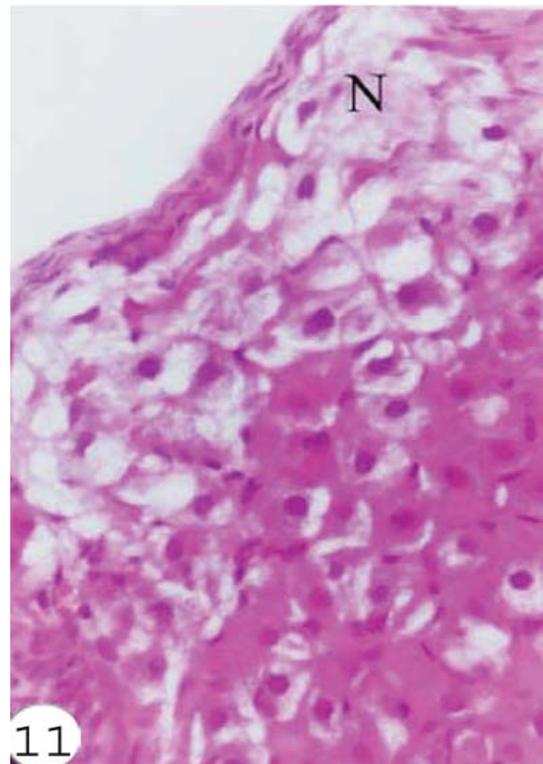
**Fig. 6:** A photomicrograph of a magnified part of subgroup A-II ovary showing a part of cystic follicle with detached apoptotic cells (↑) towards the lumen. Notice the two adjacent follicles with degenerated ova (R) and surrounded by degenerated follicular cells. Hx. & E.; X 200



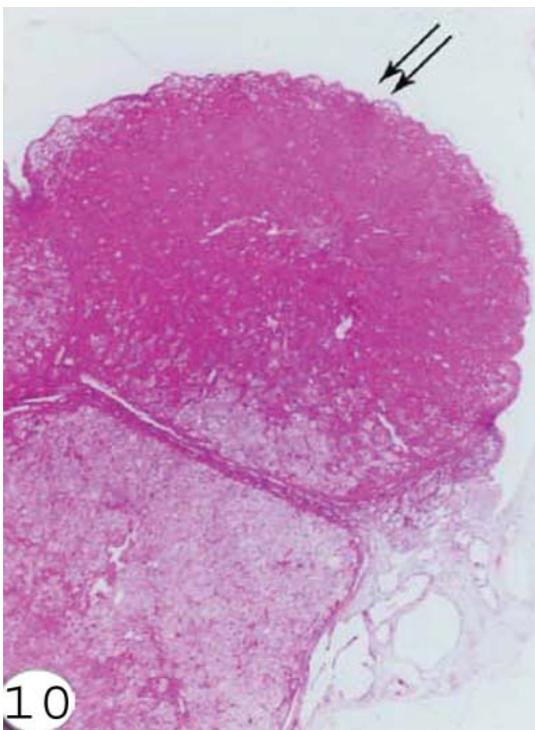
**Fig. 8:** A photomicrograph of a magnified part of subgroup A-II ovary showing an atretic follicle with no observable ovum. Multiple vacuoles (v) of degenerated granulosa cells are present between the surrounding dark cells. Hx. & E.; X 400



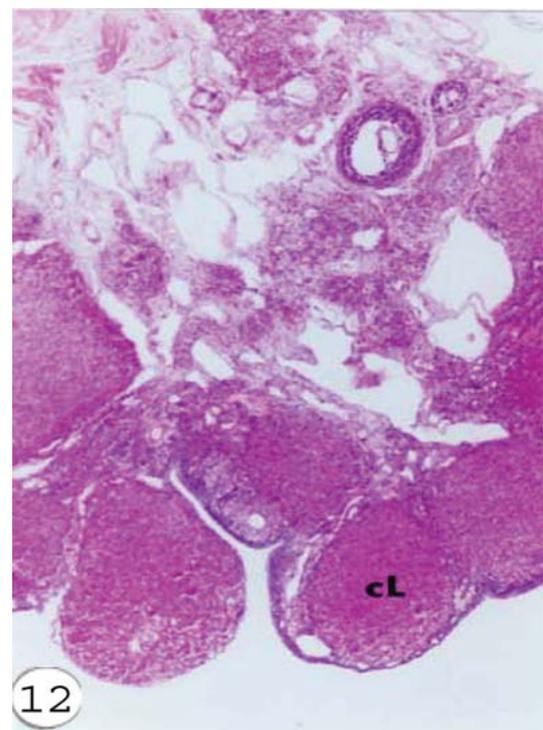
**Fig. 9:** A photomicrograph of a magnified part of the previous view showing condensed nuclei that are surrounded by vacuolated cytoplasm (K). Hx. & E.; X 1000



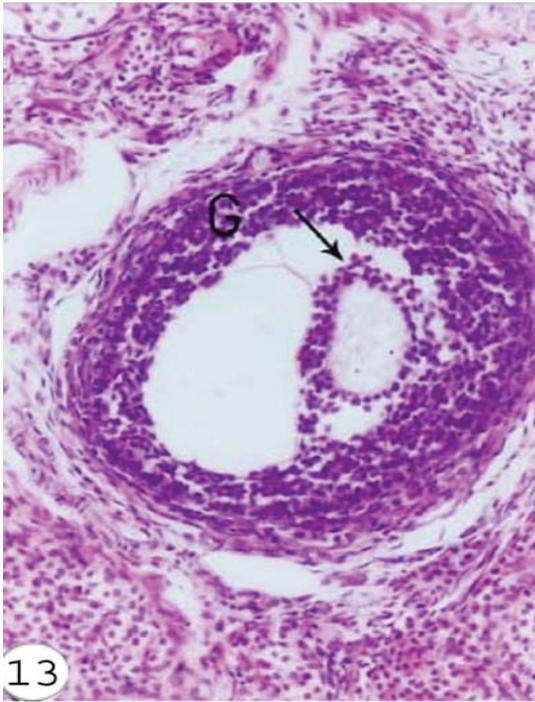
**Fig. 11:** A photomicrograph of a magnified part of subgroup A-II ovary showing a part of corpus luteum with a large group of pale vacuolated cells immediately beneath the capsule; some of these cells appear anucleated (N). Hx. & E.; X 400



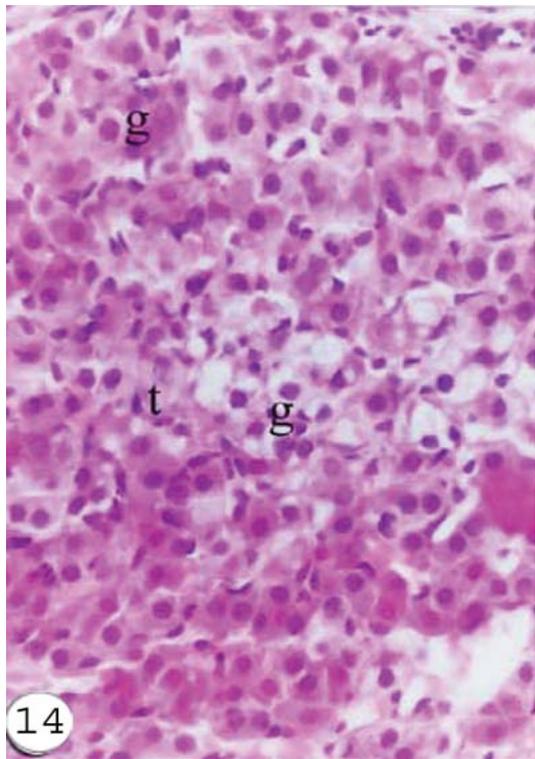
**Fig. 10:** A photomicrograph of subgroup A-II ovary showing multiple corpora lutea with marked irregularity in their capsules (↑↑). Hx. & E.; X 40



**Fig. 12:** A photomicrograph of the ovary after stoppage of nicotine injection (subgroup A-III) showing multiple more or less healthy follicles and corpora lutea (CL). No cystic or collapsed follicles are observed. Hx. & E.; X 40



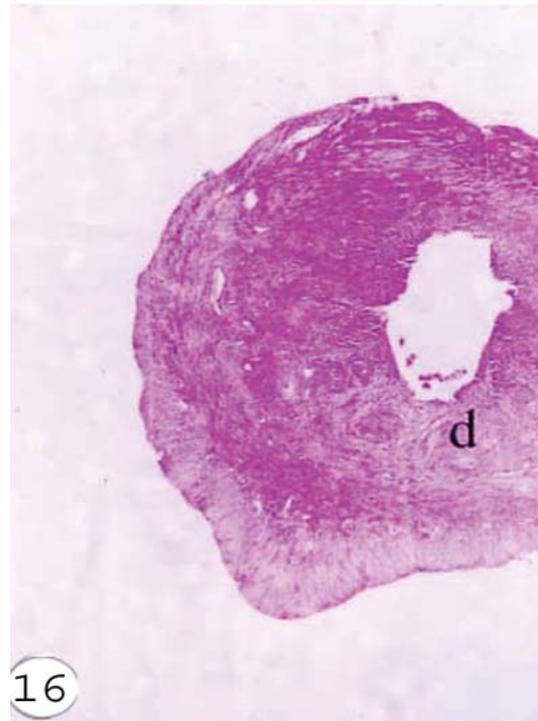
**Fig. 13:** A photomicrograph of a magnified part of subgroup A-III ovary showing a well rounded secondary follicle with a degenerated ovum. The corona radiata (↑) and granulosa cells (G) look dense in comparison to those of the control. Hx. & E.; X 200



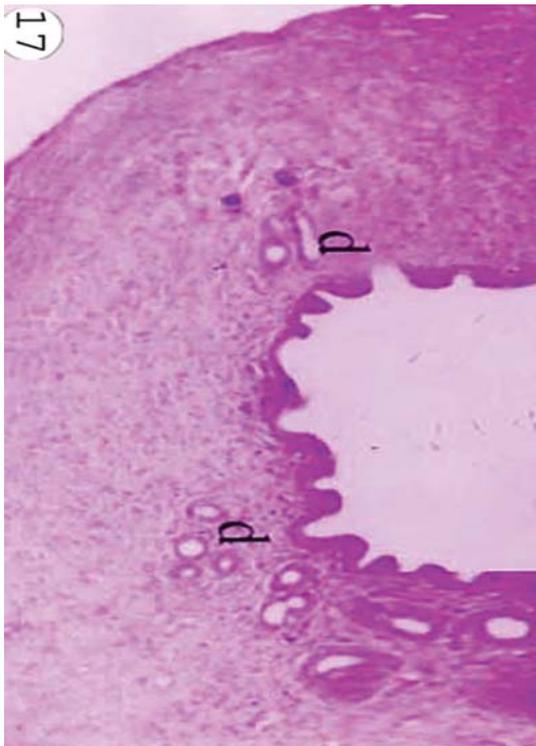
**Fig. 14:** A photomicrograph of a magnified part of subgroup A-III ovary showing a part of the corpus luteum with few pale vacuolated cells. Granulosa and theca lutein cells have dense (g,t) nuclei and slightly dark cytoplasm. Hx. & E.; X 400



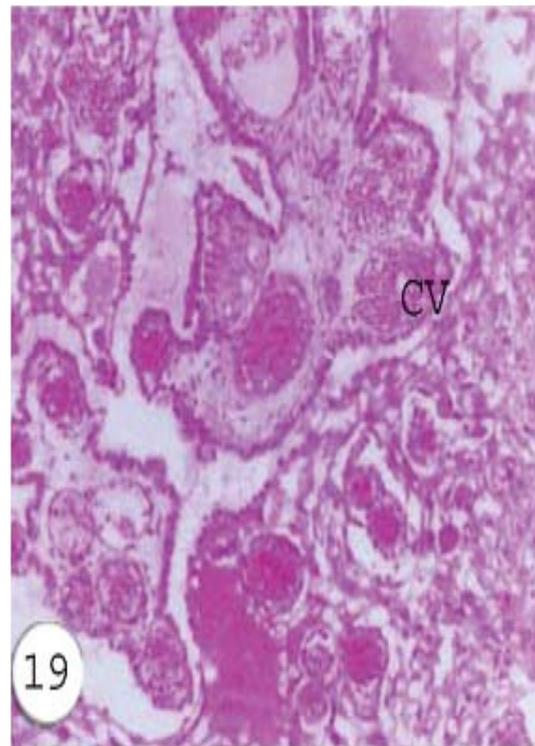
**Fig. 15:** A photomicrograph of the uterus of subgroup A-I animals showing the endometrial glands (d) and surrounding stroma, myometrium with its blood vessels (BV) and perimetrium. Hx. & E.; X 40



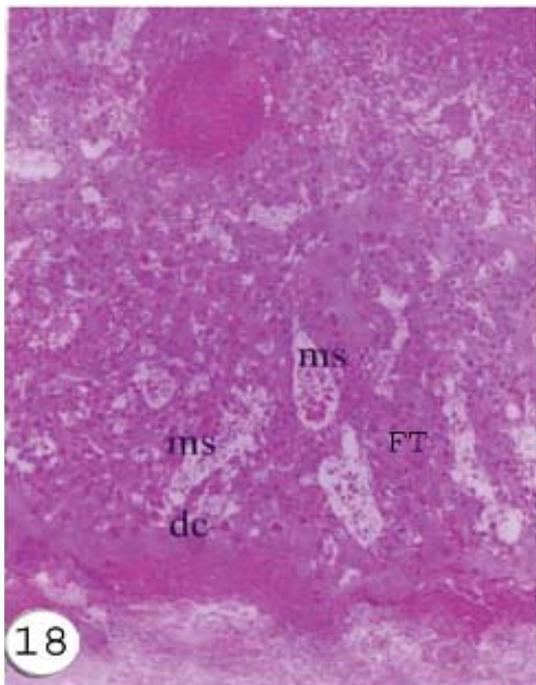
**Fig. 16:** A photomicrograph of the uterus of subgroup A-II animals showing a marked reduction in the thickness of both endometrium and myometrium with an observable reduction in the endometrial glands (d). Hx. & E.; X 40



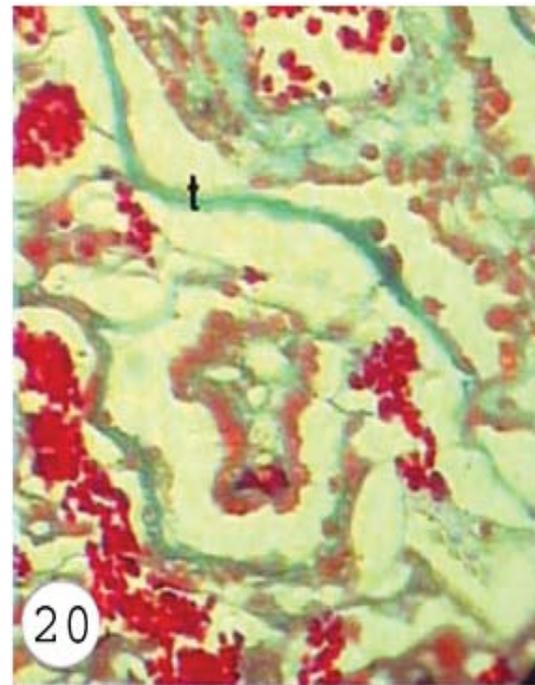
**Fig. 17:** A photomicrograph of the uterus of subgroup A-III animals showing that the endometrium and myometrium are more or less similar to those of control, however, the glands (d) appear fewer in some parts of the endometrium. Hx. & E.; X 40



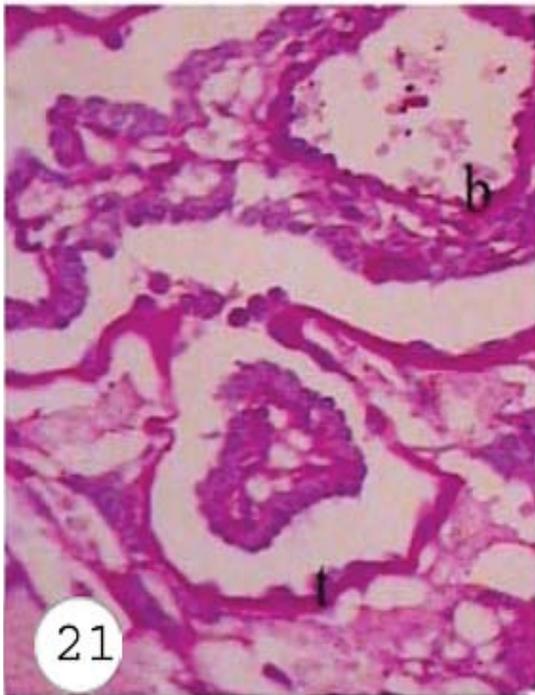
**Fig. 19:** A photomicrograph of the placenta of subgroup B-I animals showing multiple floating branching chorionic villi (CV). Hx. & E.; X 100



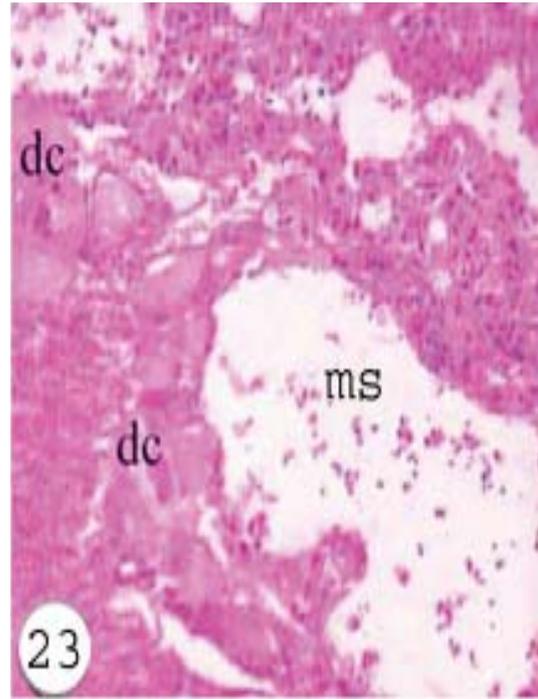
**Fig. 18:** A photomicrograph of the basal plate of the control placenta (subgroup B-I) which constitutes the maternal part showing decidal cells(dc), multiple maternal blood spaces(ms) separated by fetal trophoblast cells (FT). Hx. & E.; X 40



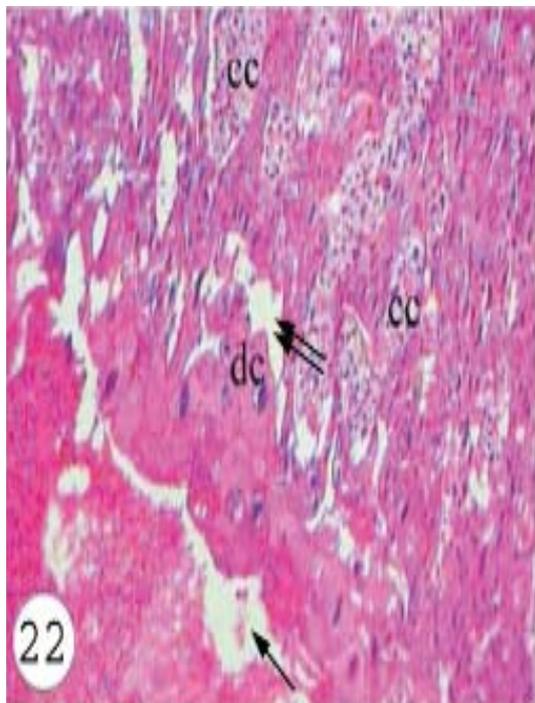
**Fig. 20:** A photomicrograph of the placenta of subgroup B-I animals showing the collagen fibres in the core of the villi and in the trophoblastic basement membrane (t). Masson's trichrome; X400



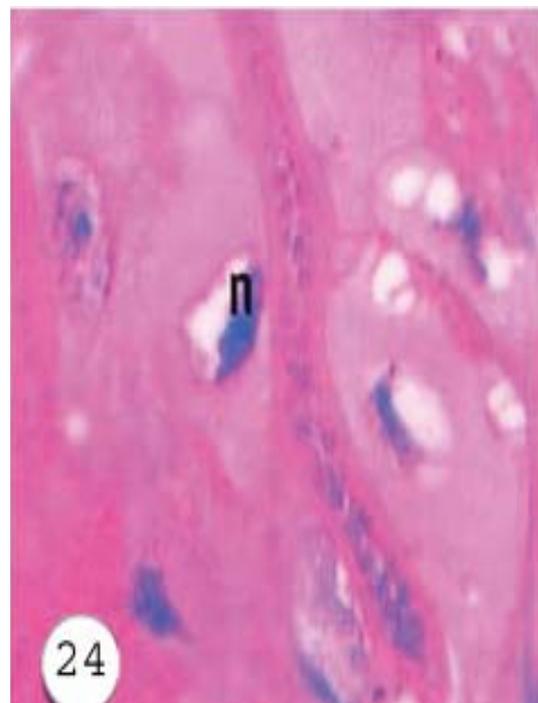
**Fig. 21:** A photomicrograph of the placenta of subgroup B-I animals showing a positive reaction in the basement membranes of the trophoblasts (t) and the blood capillaries(b).  
PAS; X400



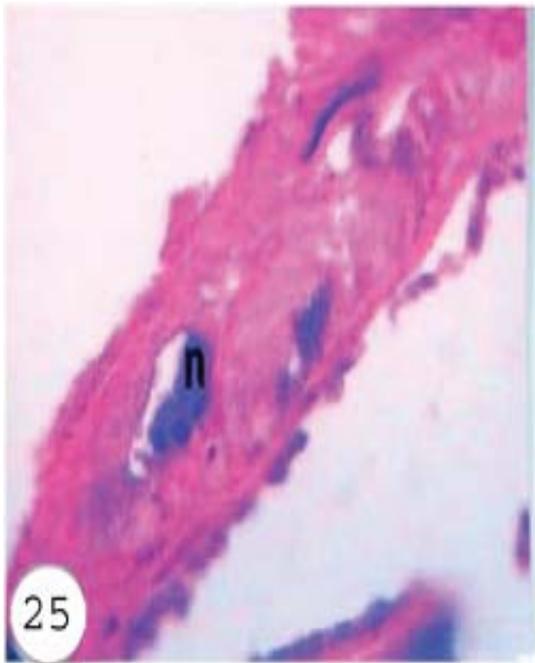
**Fig. 23:** A photomicrograph of the placenta of subgroup B-II animals showing maternal blood spaces (ms) that are surrounded by degenerated decidual cells (dc).  
Hx. & E.; X 40



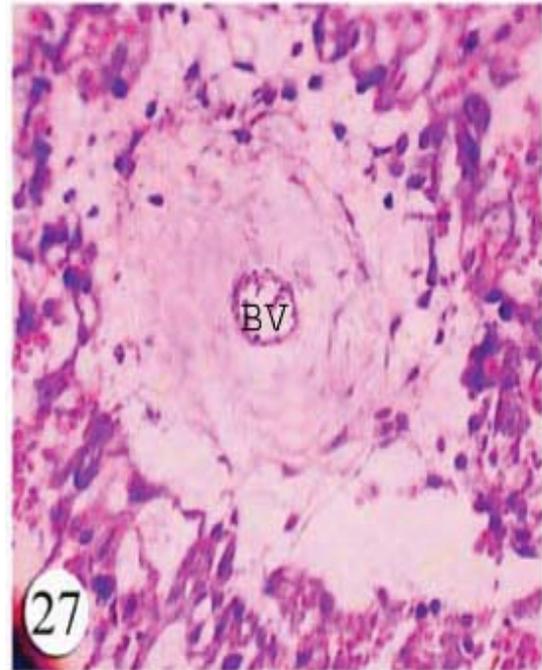
**Fig. 22:** A photomicrograph of the placenta of the nicotine-treated animals (subgroup B-II) showing a separation of the decidual cells from the basal plate (↑) and also from the trophoblastic cell columns (↑↑). Necrotic as well as apoptotic changes are observed in the decidual cells (dc). Notice multiple degenerated cell columns (cc).  
Hx. & E.; X 40



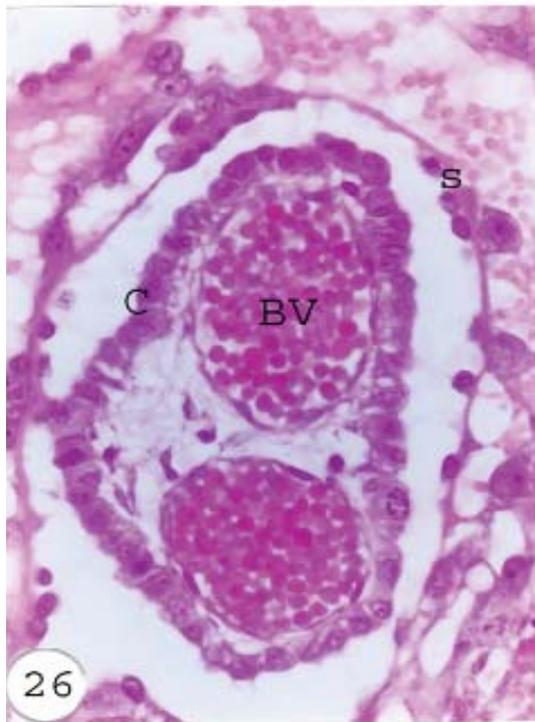
**Fig. 24:** A photomicrograph of a magnified view of decidual cells of the subgroup B-II animals showing margination of the nuclear chromatin (n) associated with cytoplasmic blebbing  
Hx. & E.; X 1000



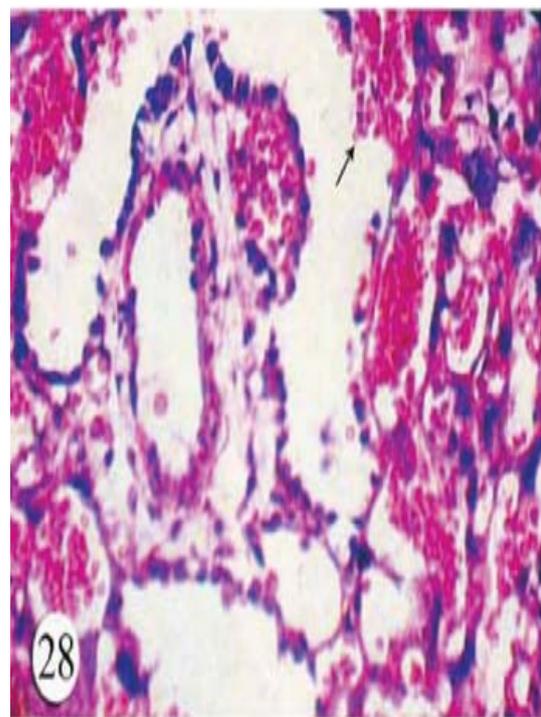
**Fig. 25:** A photomicrograph of a magnified view of decidual cells of the subgroup B-II animals showing fragmented nuclei into multiple dense rounded globules (n). Hx. & E.; X 1000



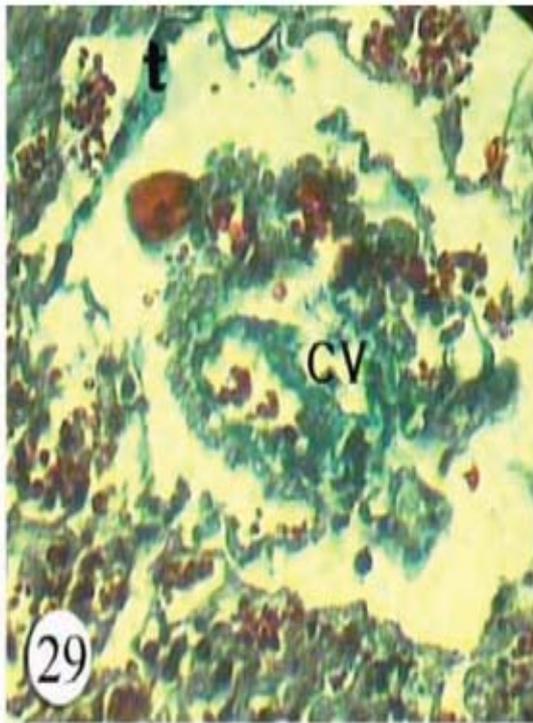
**Fig. 27:** A photomicrograph of a magnified part of a degenerated chorionic villus of the subgroup B-II placenta showing a relatively small vessel (BV) in the core and no observable cyto- or syncytiotrophoblast. Hx. & E.; X 400



**Fig. 26:** A photomicrograph of a magnified part of a chorionic villus in the placenta of subgroup B-I animals showing fetal blood vessel (BV) in the connective tissue core. Cytotrophoblasts (C) and syncytiotrophoblasts (s) are clearly observed. Hx. & E.; X 400



**Fig. 28:** A photomicrograph of a magnified chorionic villus of subgroup B-II animals showing a discontinuation in the syncytiotrophoblast (↑) with free connection between the maternal and fetal blood. Hx. & E.; X 400



**Fig. 29:** A photomicrograph of the placenta of subgroup B-II animals showing an increase in the collagen fibres in the core of the villi (cv) and in the trophoblastic basement membrane (t). Masson's trichrome; X400



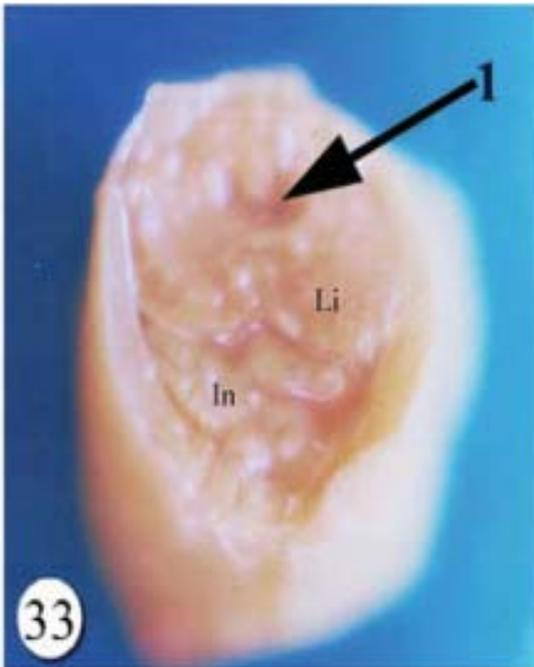
**Fig. 31:** A lateral view photograph of fetuses of control (C) and nicotine-treated (N) mothers. The fetus of nicotine treated mother shows several congenital anomalies including marked reduction in body size, malformed head, meningoencephalocele (a), spina bifida (b), depressed frontal area of the head (c), micromelia (d) and kyphosis (e).



**Fig. 30:** A photomicrograph of the placenta of subgroup B-II animals showing a highly positive reaction in the basement membrane of both the trophoblasts (t) and the blood capillaries (b). PAS; X400



**Fig. 32:** A lateral view photograph of a nicotine-treated subgroup offspring showing an area of subcutaneous hemorrhage (↑).



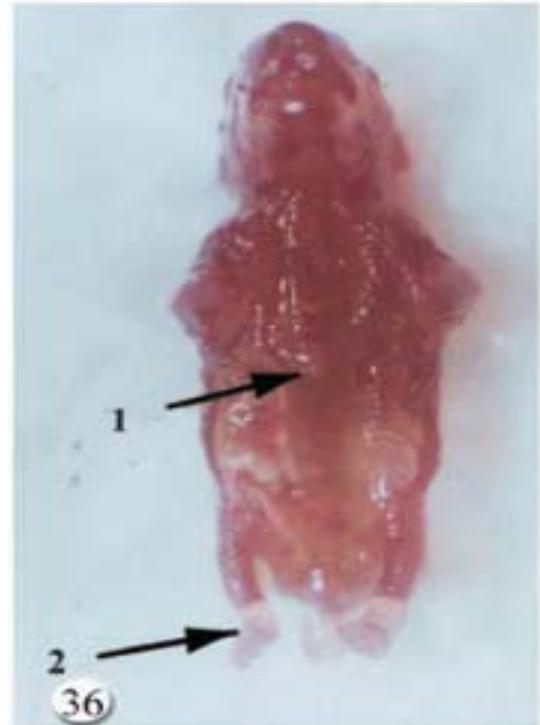
**Fig. 33:** A photograph of a transverse section in the abdominal region of a nicotine-treated subgroup offspring showing an abnormal spinal cord; spina bifida with some degrees of rachischisis (failure of fusion of several vertebral arches) and myeloschisis (failure of closure of neural folds) (1). (Note): the liver (Li) and the intestine (In).



**Fig. 35:** A photograph of the lateral view of a nicotine-treated subgroup offspring showing partial ossification of ribs (1). Alizarin red stain



**Fig. 34:** A photograph of the ventral view of a control subgroup offspring showing the ossification centres of the metacarpals (1), metatarsals (2), ribs (3) and vertebral column (4). Alizarin red stain



**Fig. 36:** A photograph of the ventral view of a nicotine-treated subgroup offspring showing impaired ossification of vertebral column (1), and metatarsal bones (2). Alizarin red stain

## DISCUSSION

The present study revealed multiple cystic, degenerated and collapsed follicles with multiple apoptotic cells among the follicular cells in the ovary of nicotine-treated animals. These results coincide with those reported by *Yildiz et al. (1998)* who observed disturbance in the follicular cellular membrane integrity and suggested that the membrane damage may be due to free radical generation. However, *Bordel et al. (2006)* reported a dose-dependent inhibition in the follicular growth and an increased apoptotic cell death specially with a high dose of nicotine in Syrian golden hamsters.

The present work also showed reduced thickness of granulosa cell layers in nicotine-treated animals in comparison to the control. In accordance with these results, *Gocze and Freeman (2000)* observed that cigarette smoke alkaloids inhibited progesterone production and cell growth of cultured MA-10 Leydig tumor cells. The retarded follicular growth may be attributable to a general cytotoxic effect of nicotine (a toxic alkaloid), its constituent cadmium (a heavy metal) or its metabolite cotinine.

This suggestion agrees with that of *Zenzen (2000)* who found that cotinine incorporated into ovarian granulosa–lutein cells and compromised the developmental potential of follicles. Benzopyrene resulting from cigarette combustion binds to DNA, forming adducts. Smoking-related adducts were detectable in ovarian granulosa–lutein cells, oocytes, spermatozoa and pre-implantation embryos (*Zenzen, 2000*). These adducts may explain the retardation in the oocyte growth and so the degenerative changes in the ova seen in the present study. However, other factors may be responsible for the retardation in the follicular growth, for instance, nicotine induces inflammatory reaction and so the free radicals are released after neutrophils activation. These suggestions are in agreement with the work of *Hellermann et al. (2002)* who noticed the release of pro-inflammatory cytokines in the human bronchial epithelial cells upon cigarette smoke stimulation, and the work of *Jay et al. (1986)* who mentioned that nicotine-induced exacerbation of neutrophil superoxide anion production may be involved with the enhanced risk of cardiovascular and pulmonary diseases. Similarly, *Helen et al. (2003)* supported these suggestions

and found that the S-allyl cysteine sulfoxide (SACS) isolated from garlic (*Allium sativum* L.) can combat the nicotine-induced peroxidative damage in rats.

In the present study, the observed degenerative changes and necrosis were much more prominent than apoptosis that may indicate a toxic and/or an ischemic form of cell death in addition to a programmed cell death (apoptosis). The observations of *Motejlek et al. (2006)* are in accordance with the present suggestion since they found that benzopyrene, cadmium, or cotinine might have a role in the reduction of blood circulation after exposure to smoke, which could lead to a decreased quantity of mature oocytes. They added that nicotine might be responsible for the impairment in the vascular endothelial growth factor A which is considered to be one of the substances able to increase ovarian blood circulation, and therefore improves oocyte maturation.

In addition, several authors reported necrosis as a form of death in different tissues after nicotine exposure as in chick ciliary ganglion neurons (*Pugh & Margiotta, 2000*), human coronary endothelial cells (*Hakki et al., 2002*), spinal cord neurons (*Garrido et al., 2003*) and in rat cardiac tissue (*Suzuki et al., 2003*).

In nicotine-treated animals, the present study demonstrated pale vacuolated cytoplasm in the follicular cells and in the luteal cells of the corpus luteum. This may indicate a large amount of accumulated lipids that have dissolved during the preparation of the slides for haematoxyline and Eosin staining that may reflect an impairment in the steroidogenesis. In harmony with these results, *Patil et al. (1999)* found an increase in the ovarian cholesterol level in nicotine-treated rats. Others reported that the antiestrogenic effects of nicotine may be exerted indirectly by acting at the level of the theca interna to decrease androgen biosynthesis (*Sanders et al., 2002*), or at the level of granulosa cell aromatase that is responsible for the conversion of androstenedione to estradiol (*Barbieri et al., 1986*).

In contrary to the previous results, *Bódis et al. (1997)* suggested that nicotine-free base augments estradiol secretion and inhibits progesterone secretion by human granulosa cells in a dose-dependent manner. This increase in the

estradiol secretion may have a suppressive effect on the progesterone produced by the follicular cells. These suggestions are supported by the work of *Fortune and Hansel (1979)* who noticed that high intrafollicular concentrations of estradiol may inhibit follicular progesterone production in vivo before the LH surge. So, nicotine and M-nicotine (a metabolite of nicotine) can induce a sort of luteal insufficiency by inhibiting progesterone release, probably through modulation of the prostaglandin system (*Miceli et al., 2005*).

In the present work, there was reduced thickness of the endometrium and myometrium and decreased number of the endometrial glands. These observations may be due to the direct toxicity of nicotine or its related metabolites. This may explain the degenerative and atrophic changes observed specially in the uterine epithelium and glands. In accordance with these findings, *Bao et al. (2002)* reported that the uterine enzymes involved in estrogen metabolism were not induced by benzo (a) pyrene (a cigarette smoke related hydrocarbon) in the endometrial cells. Also, it has been found that smoking is associated with decreased incidence of uterine fibroids, endometriosis and uterine cancer, which may reflect inhibitory effects of smoke constituents on uterine cell proliferation and extracellular matrix interactions. In addition, the increased miscarriage rate observed among mothers who smoke might be related to direct adverse effects of nicotine, cadmium and polyaromatic hydrocarbons on trophoblast invasion and proliferation (*Shiverick & Salafia, 1999*).

The observed increased amount of collagen in chorionic villi and increased thickness of subtrophoblastic as well as fetal capillary basement membranes in the present work reflect the degenerative changes that may result from nicotine itself and/or its metabolites and lead to a noticeable retardation in the fetal growth and low birth weight. This agrees with the suggestion of *Ashfaq et al. (2003)* who reported that these degenerative changes affected the functional component of an organ by reducing its nutritive and excretory functions.

The present study also showed degenerative and necrotic changes in the trophoblast cells of nicotine-treated animals. The predominant degenerated cells may be a result of the toxemic effect of nicotine that interferes with the

connection of the chorionic villi with the uterine wall leading to impairment of the placental barrier and intrauterine fetal growth; suggestions that coincide with those of *Ashfaq et al. (2003)* and *Zdravkovic et al. (2006)*.

The present work revealed irregular thickening of the basement membrane of both the trophoblasts and the blood capillaries, degeneration of the villous capillary endothelial cells, and reduced diameter of the fetal capillaries. These changes may have a role in the impairment of the fetomaternal exchange of gases and nutrients and so, fetal hypoxaemia. These suggestions are in accordance with those of *Larsen et al. (2002)* who found that the significant increase of the trophoblast volume in the mothers who smoked cigarettes was associated with a significant reduction in the lengths of villous capillaries. Also, the volume density of the fetal vessels in the terminal villi of the smoker's placenta was shown to be decreased and so, the exchange area of the smoker's placenta (*Van Der Velde et al., 1983 & 1985; Bush et al., 2000*).

However, other authors reported that some of the fetal changes cannot be explained on this basis and that these may possibly be due either to cadmium toxicity or to accumulation of polycyclic aromatic hydrocarbons in the placenta and/or in the fetal tissues (*Van Der Veen & Fox, 1982*). *Zenzes et al. (1997)* supported the latter opinion as they detected immunohistochemically cotinine and benzo (a) pyrene in the embryos as well as in granulosa-lutein cells of the ovaries of women who had IVF and were exposed to cigarette smoke. The authors postulated that benzo (a) pyrene was a potent mutagen and carcinogen. In agreement with these results, *Jauniaux and Burton (2007)* reported that the anatomical changes in the smoker's placenta were associated from early pregnancy with changes in placental enzymatic and synthetic functions. In particular, nicotine depresses active amino acid uptake by human placental villi and trophoblast invasion, and cadmium decreases the expression and activity of 11 beta- dehydrogenase type 2 which is causally linked to fetal growth retardation. They added that maternal smoking also dysregulates trophoblast expression of molecules that govern cellular responses to oxygen tension.

The observed perforation in the syncytium in the present study and so connection between

fetal and maternal blood as a result of syncytial degenerative and atrophic changes may expose the fetus to toxic substances in the maternal blood. This suggestion disagrees with that of *Demir et al. (1994)* who mentioned that focal syncytial necrosis was associated with degenerated cytoplasmic organelles, abnormalities of microvilli, decreased syncytial pinocytotic activity that might lead to impairment of placental barrier, and these morphological changes were likely to compromise, rather than assist transplacental oxygen transfer.

The present work pointed out that stoppage of nicotine injection lead to some improvement in the ovarian and uterine tissues as they became more or less similar to those of the control animals. This comes in accordance with the observations of *ELAdlali (2000)* and *Galal et al. (2005)* who suggested that complete recovery might occur if the period of stoppage was prolonged or accompanied with an adjuvant antioxidant therapy. On the other hand, *Riesenfeld and Oliva (1988)* reported that the toxic effects of nicotine were irreversible and persisting for the entire life as did infertility.

This work revealed a serious influence of nicotine on women's reproductive ability. This may be due to the adverse effect of nicotine on several of the crucial steps within the reproductive process required for achieving a pregnancy or its maintenance, as this study showed the deleterious effect of nicotine on the female reproductive organs represented by the ovary and uterus. Also, its detrimental effect on the placenta cannot be ignored. This suggestion agrees with those of *Newnham et al. (1990)*, *Wen et al. (1990)*, *Economides and Braithwaite (1994)*, *Cnattingius and Nordstrom (1996)*, *Nelson et al. (1999)* and *Gruslin et al. (2001)* who stated that maternal smoking is associated with spontaneous abortion, reduced fetal growth, prematurity, and increased perinatal mortality and morbidity. Also, this is in accordance with *Zdravkovic et al. (2005)* who suggested that tobacco constituents exerted direct effects on cytotrophoblast proliferation and differentiation; a finding that explains the mechanisms by which smoking negatively affects human pregnancy outcome.

Similar observations were reported by *Rostand et al. (1990)* in humans, who found that maternal smoking was detrimental to the very early embryo, and even if smoking was stopped, the

effects would persist at least for some days and there was no immediate catch-up growth. Also, *Pauly et al. (2004)* suggested persistent general dependent changes in the behavior of the newborn after giving oral nicotine to pregnant mice.

Moreover, *Kharrazi et al. (2004)* concluded that environmental tobacco smoke exposure in pregnant women adversely affected pregnancy by increasing fetal mortality and preterm delivery at higher exposure levels, and lead to fetal growth retardation across all levels of exposure. In addition, the results of the study of *Ng et al. (2006)* demonstrated that inhalation exposure of pregnant mice to a low dose of mainstream cigarette smoke shortened gestation and altered hormone secretory patterns, which were important for maintaining pregnancy.

The present study showed a significant reduction of the fetal growth parameters in the subgroup of nicotine-treated mothers. This may be attributed to the interference of nicotine with the fetal nutrition through its serious effect on the placenta. This comes in accordance with the suggestion of *Kulal et al. (2001)* who stated that nicotine could adversely affect uterine and placental blood flow by causing constriction of the blood vessels.

Also, *Van Der Veen and Fox (1982)* as well as *Shivericka and Salafia (1999)* stated that retardation of fetal growth could be due to ultrastructural changes of the placenta that are believed to contribute to reduced placental nutrient and oxygen transfer. *Ronco et al. (2006)* showed that metallothionein -2 isoform was specifically induced in smokers' placentas. This could be involved in placental cadmium and zinc retention that could contribute to reduce that transference of zinc to the fetus. This in turn, may be associated with detrimental effects on fetal growth.

*Jauniaux and Burton (2007)* stated that in the fetus smoking is associated with fetal growth retardation and, as in the placenta, with alterations in protein metabolism and enzyme activity. These alterations seem to be the result of a direct toxic effect on the fetal cells or an indirect effect through damage to, and/or functional disturbances of the placenta.

In addition, *Kalinka et al. (2005)* found that tobacco smoke exposure was a significant factor inducing increased resistance of umbilical blood

flow. They stated that this could be one of the main mechanisms leading to decreased birth weight and fetal biparietal diameter observed among infants with prenatal exposure to tobacco smoke.

The present work revealed a variety of congenital malformations including wrinkled skin, malformed head, kyphosis, shortened neck region, micromelia, spina bifida and meningo-encephalocele in fetuses of nicotine-treated mothers. This may reflect the severe detrimental effect of nicotine and/or its metabolites on the developing embryo. This suggestion agrees with that of *Saad et al. (1990)* who concluded that nicotine had a serious effect on general growth and development as well as on palatogenesis of mice. Also, *Rostand et al. (1990)* and *Shaw et al. (2009)* found a large number of craniofacial characteristics in the infants of heavy smokers. A great link between cigarette smoke and teratogenicity was found by *Reckzeh et al. (1975)*, *Nelson et al. (1999)* and *Baroni et al. (2010)*. They found also a dose-dependent reduction in fetal body weight, sizes, lengths and biparietal diameters. *Gartner et al. (1997)* suggested that nicotine interferes with both palatal and mesenchymal components in utero affecting greatly the tongue development. Furthermore, the studies of *Vaglenova et al. (2004)* and *Rogers (2009)* demonstrated that prenatal nicotine exposure produced significant long-term developmental and behavioral teratogenic effects. In particular, smoking interferes strongly with the fetal brain and pancreas biological parameters and induces chromosomal instability, which is associated with an increased risk of cancer, especially childhood malignancies (*Jauniaux & Burton, 2007*).

There is an increasing evidence that points to a role for oxidative stress in toxicity by nicotine entailing major body organs including the lung, cardiovascular system, central nervous system, liver, kidney, testis, ovary, pancreas and esophagus (*Kovacic & Cooksy, 2005*). On the other hand, the results of the work of *Seller and Bnait (1995)* indicated that tobacco smoke was not a potent teratogen in the mouse, but that it might have minor effects in those individuals genetically predisposed to an abnormality. Also, *Daeninck et al. (1991)* reported that following exposure to nicotine during early gestation, dysmorphogenesis was not observed.

The present work revealed the major deleterious effect of nicotine on the central nervous system development. This supports the suggestion of the presence of a great relationship between maternal smoking and fetal congenital central nervous system malformations as was shown by *To and Tang (1999)* who concluded that maternal smoking might be associated with particular vascular patterns of damage to the developing brain that could predispose to a hydranencephalic malformation. Moreover, *Dempsey and Benowitz (2001)* indicated that nicotine adversely affected the developing fetal central nervous system, and that nicotine effects on the brain might be involved in the pathophysiology of sudden infant death syndrome (SIDS).

Also, *Qiao et al. (2005)* reported that nicotine elicited oxidative damage to developing neural cells both in vitro and in vivo; a mechanism that explains some of the neurodevelopmental endpoints that are common to that agent.

*Slotkin et al. (1999)* suggested that during a critical developmental period, nicotine exposure produced stimulation of cholinergic receptors in target cells and provided signals that influenced cell replication and differentiation. They added that nicotine might induce mitotic arrest in brain cells possessing high concentrations of nicotinic cholinergic receptors.

Furthermore, it was indicated that maternal exposure to nicotine might induced significant neurobehavioral deficits, a decrease in the surviving neurons and an increased expression of glial fibrillary acidic protein in the cerebellum and hippocampus of the offspring in the postnatal period in addition to chromosomal instability in the brain. So, it is suggested that nicotine exposure may produce long-term neuropathological alterations in the offspring (*Mattson et al., 2002; Abdel-Rahman et al., 2005; Huizink and Mulder, 2006; Jauniaux & Burton, 2007; Paz et al., 2007*).

Regarding the effect of nicotine on the fetal skeletal system, the present work showed an impaired ossification of the axial and appendicular skeleton. This may be due to a toxic effect of nicotine on the development of bone ossification centres. This suggestion agrees with that of

*Seller and Bnait (1995)* who found reduction in the number of skeletal ossification centres, and reported a developmental delay with a modest increase in the frequency of open spina bifida and exencephaly. Also, *Paulson et al. (1994)* concluded that high dose resulted in significant growth retardation and decreased ossification.

In addition, *Nelson et al. (1999)* and *Carmines et al. (2003)* concluded that maternal passive smoking during pregnancy produces widespread ossification abnormalities regardless of the dose. That results supported epidemiologic data on developmental toxicity of passive smoking.

In contrary to the above-mentioned research results, a multivariate analysis as regard smoking and the occurrence of congenital malformations and spontaneous abortions was done by *Hemminki et al. (1983)*. They found that the possibility for the smoker's child to be born with central nervous system defects, oral cleft, or musculoskeletal malformations was statistically non-significant. That controversy in the results of the different researches on nicotine teratogenic effects may be due to the differences in the doses and routes of administration of nicotine. Also, it may be due to variation in the genetic predisposition and sensitivity to nicotine.

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## تأثير حقن النيكوتين وإيقافه على الإنجاب في إناث الجرذان

منال المليجي<sup>١</sup>، رندة عبد الهادي<sup>٢</sup>، أمل عبد السميع<sup>٣</sup>، هبه سعد الدين

قسم التشريح الأدمي وعلم الأجنة<sup>١</sup>، قسم الطب الشرعي والسموم الإكلينيكية<sup>٢</sup>، قسم الهستولوجي<sup>٣</sup>  
كلية الطب – جامعة أسيوط

### ملخص البحث

توجد علاقة وثيقة بين التدخين ونقص الخصوبة في الإناث عموماً. ولكن تفسير هذه العلاقة لم يتم توضيحه بالكامل بعد. لذلك اهتمت هذه الدراسة بتأثير النيكوتين على بعض الأعضاء التناسلية، بالإضافة إلى التغيرات الشكلية في الأجنة للأمهات التي تمت معالجتها بالنيكوتين. وقد قسمت الحيوانات (إناث الجرذان) البالغة من العمر ثلاثة أشهر إلى مجموعتين أساسيتين:

- مجموعة (أ) وتشمل الجرذان غير الحوامل، وقسمت هذه المجموعة إلى ثلاث مجموعات فرعية:

١- المجموعة الضابطة وتم حقنها بمحلول ملحي بتركيز ٠.٩% تحت الجلد يومياً لمدة ثلاثة أسابيع.

٢- المجموعة التي تم حقنها تحت الجلد يومياً بالنيكوتين بجرعة ٠.٤ مجم/١٠٠ جم من وزن الجسم لمدة ثلاثة أسابيع.

٣- المجموعة التي تم حقنها بالنيكوتين بنفس الجرعة والمدة السابقتين، ثم تركت لمدة شهرين بدون حقن.

- مجموعة (ب) وتشمل الجرذان الحوامل وتم تقسيمها إلى مجموعتين فرعيتين:

١- المجموعة الضابطة وحقنت بمحلول الملح فقط من اليوم السادس إلى اليوم العشرين من الحمل.

٢- المجموعة التي حقنت بالنيكوتين بنفس الجرعة المستخدمة في المجموعة (أ) من اليوم السادس إلى اليوم العشرين من الحمل.

وقد أخذت عينات من المبيضين والرحم من المجموعتين الفرعيتين (أ١) و (أ٢) عند الأسبوع الثالث، بينما تم الحصول عليها من المجموعة الفرعية (أ٣) بعد ذلك بشهرين وجهزت للفحص المجهرى.

أما بالنسبة للمجموعة الثانية (ب) فقد تم استخراج الأجنة في اليوم العشرين من الحمل، وتم فحصهم بالعين المجردة، وبعضهم تم تجهيزه لفحص العظام باستخدام صبغة الأليزارين الحمراء. كما تم تجهيز المشيمة أيضاً للفحص المجهرى. وقد تم عمل تحليل احصائي لتقييم تأثير النيكوتين على ناتج الحمل والنمو الجنيني.

وقد أظهرت النتائج أن النيكوتين أدى إلى تأخر في نمو الحويصلات الموجودة في المبايض، وزيادة في تلك المتهاكلة، ونقص ملحوظ في السليمة منها، كما حدث تهالك في الأجسام الصفراء. ومع انقطاع النيكوتين حدث تحسن ولكنه لم يصل إلى المجموعة الضابطة.

أما بالنسبة للرحم فقد لوحظ نقص في سمك الطبقتين الداخلية والوسطى من جدار الرحم، وكذلك الغدد الموجودة في الطبقة الداخلية. وقد حدث تحسن مع انقطاع النيكوتين ولكنه لم يصل أيضاً إلى المجموعة الضابطة. كما لوحظ حدوث تهالك في تركيب المشيمة عند الخضوع للعلاج بالنيكوتين.

وقد أظهرت أجنة الأمهات التي تم حقنها بالنيكوتين العديد من التشوهات الخلقية والتي شملت: صغر في الحجم والوزن، تجاعيد في الجلد، تشوه في عظام الجمجمة وتقوس في الظهر وانكماش في منطقة العنق والأطراف، بالإضافة إلى حدوث صدع في العمود الفقري مصحوباً بفتق في النسيج السحائي والمخى، وتأخر في التعظم.

كما أظهر التحليل الإحصائي إنخفاضاً ملحوظاً في ناتج الحمل، ووزن وطول الأجنة عند خضوع الأمهات للعلاج بالنيكوتين.