Original paper

Effects of nicotine exposure during gestation on uterus and ovary of female WISTAR rats

abstract:

With more than 7000 chemical compounds, cigarette smoke; represents a major environmental risk to the health of the mother and her fetus. Nicotine is the main alkaloid of tobacco and is responsible for its addiction. The aim of our study is to assess the effects of nicotine on the uterus and the ovary of pregnant female rats. WISTAR rats with regular estrous cycle were divided into two equal groups; a control group and a group receiving 1mg/kg of nicotine daily from day 1 to day 19 of gestation. The rats were anaesthetized, their uteri and ovaries were removed, weighed and histologically examined. Serum estrogen and progesterone determinations were also performed. Our results showed that nicotine induced endometrial atrophy, follicular degeneration and a significant decrease in uterine weight and estrogen levels. Through these results, we suggest that gestational exposure of rats to nicotine affects steroidogenesis resulting in endometrial damage and follicular degeneration.

Key words: Nicotine, rats, ovary, uterus, histology, hormones

Introduction:

The combustion of tobacco generates many toxic substances for the body including tar, toxic gases such as carbon monoxide, and heavy metals (cadmium, mercury, lead, ...). Indeed, cigarette smoke contains up to 7,000 chemical compounds including 70 carcinogenic agents (Anses 2020).

In 2020, the World Healch organazation (WHO) predicted that 36.7% of men and 7.8% of women worldwide will be smokers (WHO, 2015).

Tobacco use during critical developmental periods such as pregnancy and lactation is associated with various health problems. Several studies have shown that smoking cigarettes during pregnancy could raise the risk of prematurity, low birth weight, impaired child growth, and higher infant mortality (Pinheiro, Moura et al. 2015) (Marufu, Ahankari et al. 2015) (Liu, Wang et al. 2014). It is also associated with high risk of congenital malformations and functional disorders (Leppée, Culig et al. 2012).

Additionally, women smokers face a greater risk of poor embryonic implantation and spontaneous loss of a pregnancy. The mechanisms involved in these possible outcomes are complex and variables e.g defect in endometrial maturation, alteration of physiological angiogenesis, disruption of trophoblastic invasion and placental development, alteration of uterine hemodynamics, and a defect in myometrial relaxation. Although, the effects of smoking on reproductive functions are modulated by many factors such as intensity, duration time and type of exposure, which must be taken into account when interpreting the tobbaco toxicological studies results (Dechanet, Brunet et al. 2011).

Data available from epidemiological studies have shown that smoking has an impact on several hormone-dependent disorders in women. Smoking increases the risk of early menopause, reduces the risk of endometrial cancer, and probably increases the risk of osteoporotic fractures. Although data are limited, it appears that women smokers are more likely to suffer from menstrual irregularity and early menopausal symptoms but not uterine

fibroids and endometriosis which are less commonly observed in smokers (Baron, La Vecchia et al. 1990).

Pregnancy offers a unique opportunity for women to stop smoking. Up to 45% of women who smoke quit during their pregnancy. However, the use of electronic nicotine delivery systems (ENDS) in pregnant women remains an emerging issue. In 2015, it has been reported that 7.4% of the united states women were using e-cigarettes; 40% of them did not realize that ENDS contained nicotine and would be addictive (Scherman, Tolosa et al. 2018).

Recent studies on adverse health effects have associated e-cigarette use with an increased risk of lung damage. In addition to the direct impact on lung health, previous studies in laboratory animals have shown that the offspring of mothers exposed to e-cigarettes may increase the risk of impaired neurodevelopment, decreased lung development, reduced rump length and fetal weight and increased oxidative stress and inflammation (Regan and Pereira 2021).

The chemical profiles of e-cigarette aertosols are almost entirely different from that of cigarette smoke. Nicotine is one of the only chemicals consistently found in both forms of smoke (Tsai, Byun et al. 2020).

Nicotine is the major alkaloid of tobacco. It is well thought that nicotine is resposable of addiction to cigarettes by creating a satisfying and pleasurable feeling after smoking. In fact, nicotine has deleterious effects on some vital visceral organs with observations similar to those reported in women smokers (BOLARINWA 2009).

Nicotine and its metabolite cotinine have been found in the follicular fluid. The ovaries retain the compounds from cigarette smoke, creating a toxic environment for the follicles and inducing increased oxidative stress, abnormal intercellular signaling crosstalk, impaired meiosis and activation of apoptosis pathways (Dechanet, Anahory et al. 2011).

Nicotine affects human fertility in several ways including inhibition of aromatase enzyme activity in uterine granulosa cells, reduced urinary and plasma estradiol levels, and increased prevalence of early menopause with high rates of *in vitro* fertilization (IVF) failure (Totonchi, Miladpour et al. 2016) (a, et al. 2002).

Therfore, we suggested to study the effects of nicotine during gestation. The main purpose of our study was to investigate if the physiological variations which occur during gestation would accentuate the reprotoxic effect of nicotine.

Materials and methods:

Animals:

Experiments used two months old female WISTAR rats weighting 260±5 g at arrival and with normal estrous cycle, from the Algerian pasteur institute (1 rue docteur Laveran Alger). Animals were housed individually and exposed to a 12 h light/dark cycle with ad libitum access to food and water. All experiments were conducted according to the published guidelines of the care and use of laboratory animals in research.

Drug:

(-) NICOTINE PESTANAL ,analtical standard (3050 Spruce Street , Sigma Aldrich, St. Louis, MO 63103, USA) was administered by gavage at 1 mg / kg / day(Carolyn E. Cesta1 2009). This dose was chosen to mimic the nicotine concentrations resulting from the consumption of 1-2 packs of cigarettes, used in previous studies conducted on laboratory rats (Laule, Wing et al. 2017) (A. C. Holloway 2006).

Protocol :

Animals were divided into two groups; an experimental group that received nicotine from day 1 to day 19 of gestation and a control (Roguski, Sharp et al. 2014) . Daily vaginal smears stained with 1% methylene blue were examined for at least two consecutive estrous cycles before the experiment to confirm that all the female rats used in the study had a regular oestrus cycle (Blake, Scaramuzzi et al. 1972) (Krinke 2000).

Females were housed with males overnight (two females /one male). The next day, sperm-positive vaginal smears were used to confirm mating. Female rats were considred in day one of gestation (D1) if their vaginal smears contained sperm cells. On day 19 of gestation, females were anesthetized with chloroform (Mantella, Kent et al. 2013) and dissected. The fertility index was then calculated (number of pregnant females / number of coupled animals × 100) (Camargo, Leite et al. 2014).

The ovary and uterus were removed, weighed and fixed immediately in 9% formalin for histological examination (BOLARINWA1 2009) . Blood was collected by cardiac puncture. Samples were centrifuged for 15 minutes at 16 100 ×g and sera were separated and stored at -80°C until analysis (Halder, Trauth et al. 2016).

Histological and serological analyses:

One uterus and one ovary from each animal were fixed in 9% formalin for 48 hours. Paraffin embedded tissue were then prepared and stained with hematoxylin and eosin, and the histo-morphology of the uterus and the ovaries was examined under light microscope (Halder, Trauth et al. 2016), The identification of the different types of follicles was based on the classification of Pedersen and Peters (1968)(PETERS 1968).

Serum estrogen and progesterone determination was performed using COBAS e411 analyzer.

Statistical analysis:

Data were analyzed using SPSS version 26 software (IBM corporation and its licensors 1989.2019) (Akpak, Cekmez et al. 2017). Organs weight, serum estrogen and progesterone levels from experimental and control groups were statistically analyzed using the student's t-test.

Results:

Vaginal smears and fertility index:

Rats from both control and experimental groups showed regular estrous cycles; represented by four phases: pro-estrus, estrus, metoestrus and di-estrus (Figure 1). In addition, the fertility index was 100% for both groups.

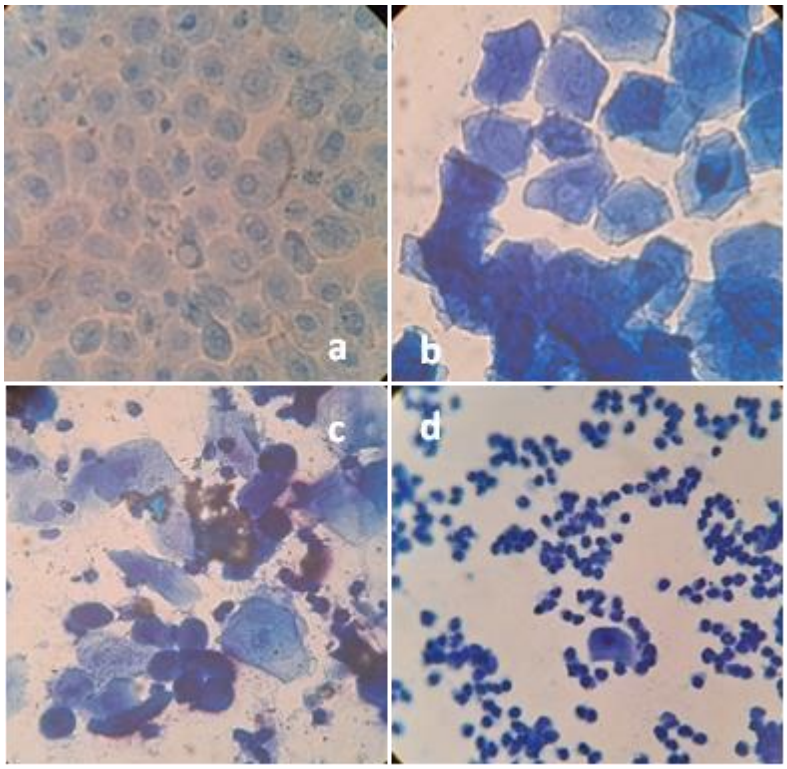


Figure 1: Vaginal smears of rats representing the four phases of an estrous cycle: preparatory, estrus, metaestrus, and dioestrus (G400).

Effect of nicotine on genital organs relative weight:

absolute ovarian weight of nicotine-intoxicated rats showed no significant difference compared to controls, whereas relative uterine weight was significantly decreased in rats of the nicotine-exposed group compared to controls ($P<0.05$) (Table 01).

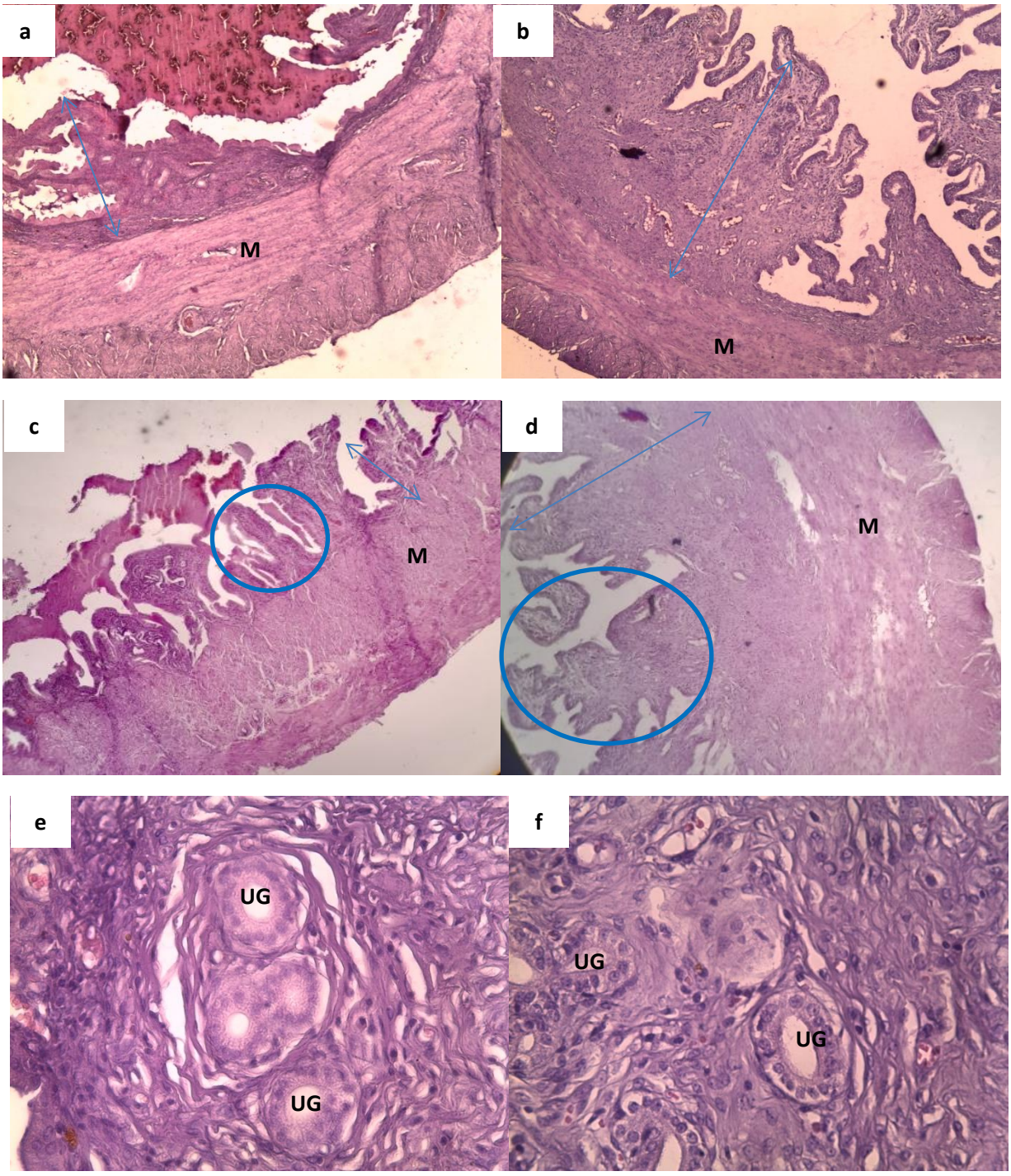
Table 1: Ovary and uterus main weights of nicotine-exposed and control rats

Group	**Ovary weight (g)**	**Uterus weight (g)**
Nicotine exposed group	0.124± 0.018	1.711± 0.089
Controls	0.120± 0.010	1.930± 0.102

Effect of nicotine on uteri and ovaries histology :

Microscopic analysis of histological sections of the controls uteri showed that endometrial decidualization was uniform along the entire length of the uterine body, whereas the degree of decidualization was considerably reduced and uterine villi were less developed in nicotine-exposed female rats (Figure2 a,c), (Figure2 b,d).

The myometrium development was reduced in nicotine-treated rats compared to controls (Figure 2 a, b, c and d), while vascularity (Figure 2 g and h) and uterine glands aspects were almost similar in the two groups (Figure 2 e and f).



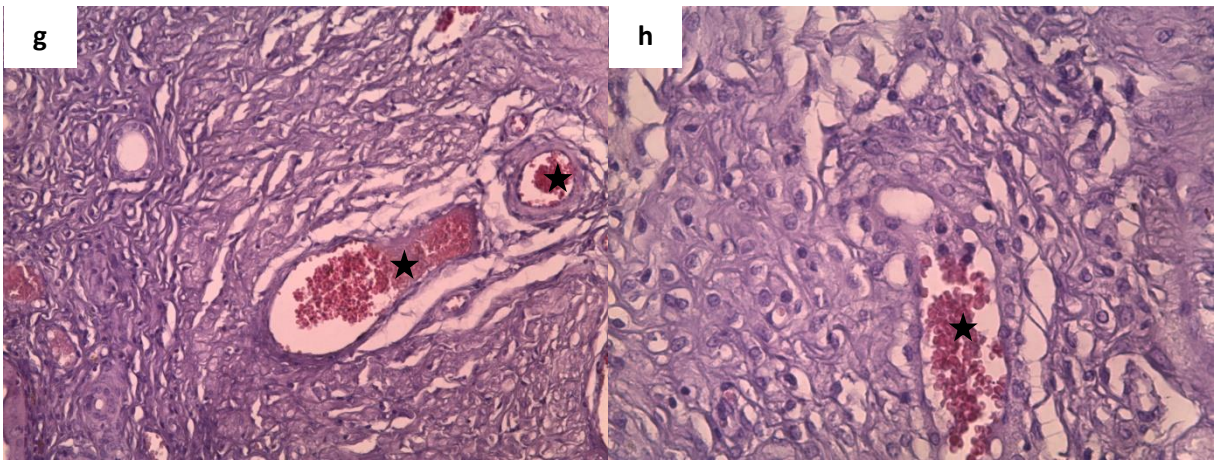
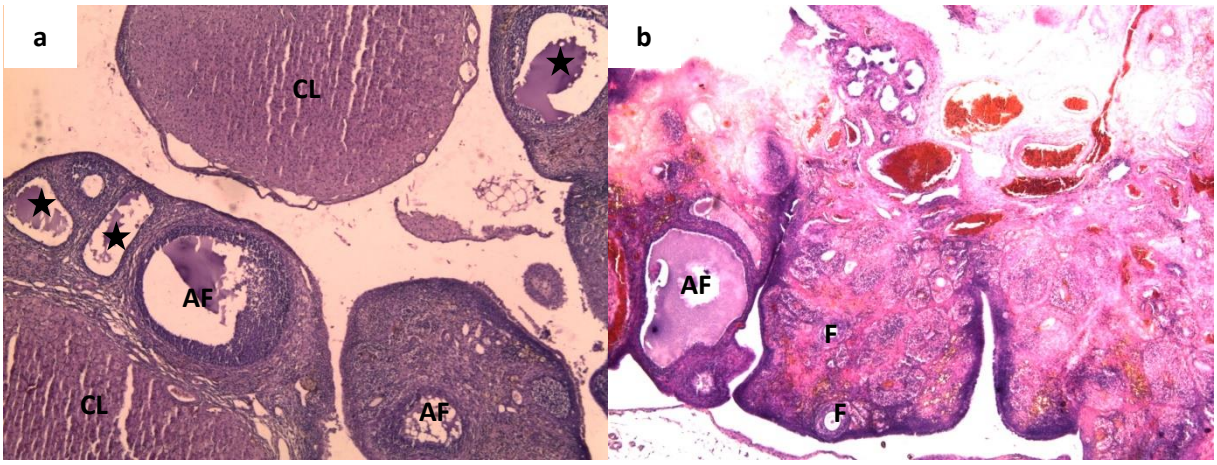


Figure 2 : Uteri histological changes induced by nicotine exposure during gestation in female rats

Endometrial decidualization in nicotine-exposed females (a) were reduced compared to controls (b) (G x10). Endometrium developpement was also reduced in nicotine-exposed rats (c) compared to controls (d), M: myometrium, (Gx40). Histological sections of the endometrium showed no changes in uterine glands (UG). Bewteen nicotine-exposed rats (e) and controls (f)., Vascularity was also unchanged in nicotine exposed rats (g) compared to controls (h); Star: blood vessels (G x40).

Histological analysis of the ovaries from controls showed several follicles in different stages of development (Figure 3 b, d and f)) with numerous gestational corpora lutea. However, ovaries sections from the nicotine-treated rats (Figure 3, h) showed significant follicular degeneration associated with fibrin deposition (Figure 3 e, g), the presence of numerous atresic follicles and cysts (Figure 3 a, c and e) an decreased number of gestational corpora lutea compared to controls.



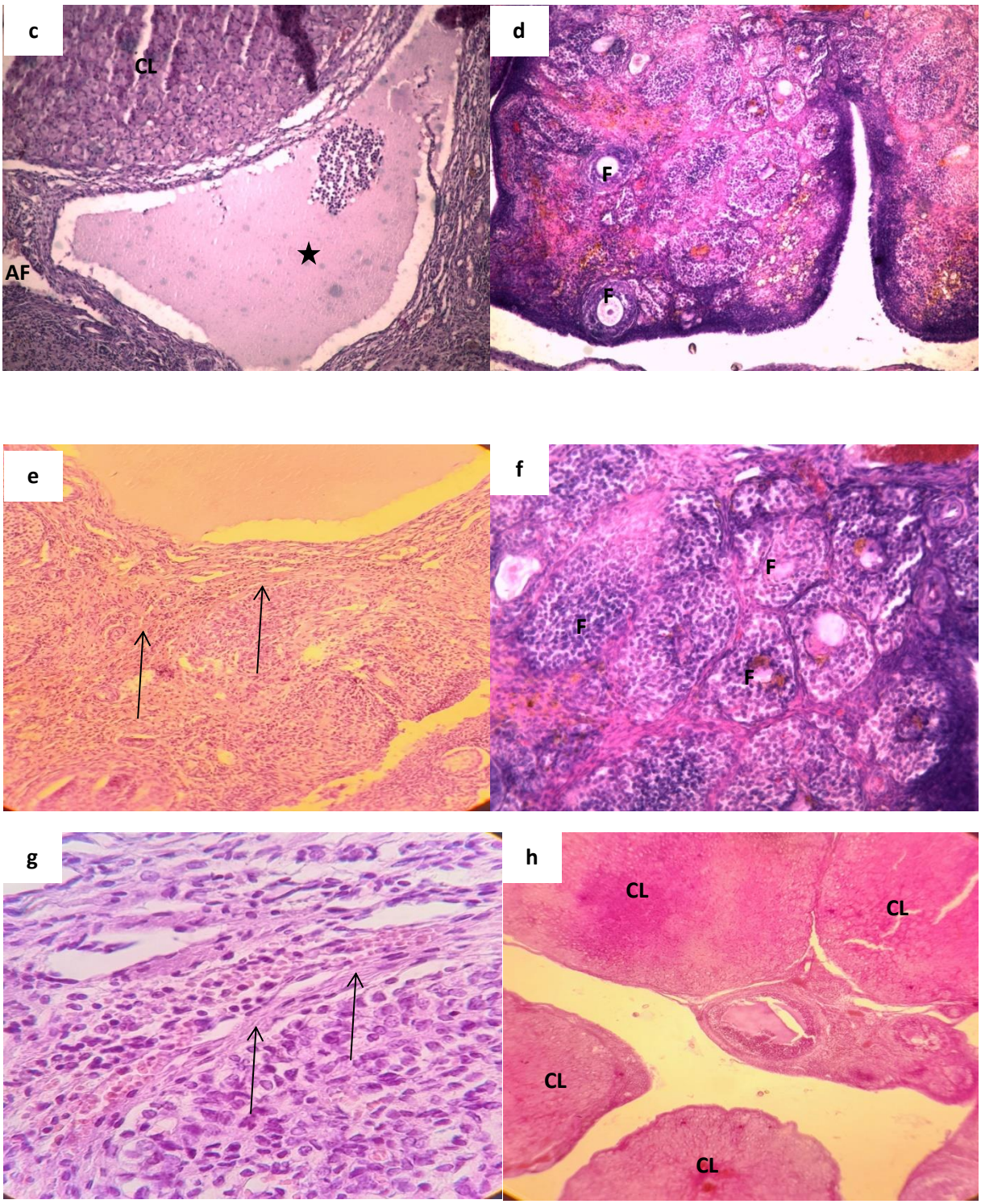


Figure 3: Effetcs of nicotine exposure during gestation on the ovarian histological structure in female rats H&E stain (G×40)

(a,c,e,g): strong presence of atresic follicles (AF), follicular cysts (Stars) and fibrin deposition (arrows) compared to controls (b,d,f,h) where we noted numerous developing follicles (F), more corpus luteum (CL) and less atresic follicles.

Sex hormone levels:

Serum progesterone levels showed no significant diffrences bewteen nicotine-exposed group and controls, whereas estrogen levels were significantly decreased in nicotine-exposed rats ($p<0.05$) (Table 02).

Table 2: Serum progesterone and estrogen levels of nicotine-exposed rats and controls

Groupe	Progesterone in ng/ml	estrogen in pg/ml
Nicotine-exposed group	67.72± 8.80	22.84± 7.36
controls	69.98± 1.66	40.72± 1.30

Discussion :

We used an animal model to investigate the toxic effects of nicotine, the major addictive component of all forms of tobacco, on the structure and the functions of uterus and ovary during gestation.

In this study, gestational exposure to nicotine did not affect the relative weight of the rat ovary. This observation is supported by Siti Norashikin,Ghosh et al.2014. However, it may depend on the duration of exposure. Longer nicotine administration periods of 30 to 60 days induced significant reduction in ovarian weight in exposed females (BOLARINWA 2009).

On the other hand, the uterus appears to be more sensitive to nicotine exposure. In our study, we found that gestational exposure to nicotine significantly decreased the uterus relative weight in intoxicated rats compared to controls. Similarly, other researchers reported that gestational exposure during the first third of gestation affects uterine weight (Adeyemi, Oyeyipo et al. 2018). Nicotine induced the same effect in pseudo-pregnant (Siti Norashikin, Ghosh et al. 2014). The uterine weight decrease could result from a direct action of nicotine on this organ independently of the fetuses number. BOLARINWA (2009) also linked the effects of nicotine on the uterine wieght to the exposure duration. In his study, BOLARINWA noted that nicotine administration for 30 days did not reduce uterine weight, whereas after 60 days of nicotine treatment, uterine weight decreased significantly.

Nicotine negative effects not did not spare the uterine endometrium. Our results showed that gestational exposure to nicotine negativly affects the uterine endometrium by reducing its decidualization. These histological changes were also observed in the uterus of female rats treated with nicotine during the first third of gestation (Adeyemi, Oyeyipo et al. 2018) , and in pseudo-gestant rats intoxicated by nicotine (Siti Norashikin, Ghosh et al. 2014). Although, other researchers have found no toxic effects of nicotine on the endometrial epithelium and stroma in female rats before and during gestation (Akpak, Cekmez et al. 2017). Development and maintenance of uterine endometrium decidualization are mainly controlled by ovarian estrogen and progesterone, which are regulated by hypothalamic and anterior pituitary hormones (Siti Norashikin, Ghosh et al. 2014). We suggest that nicotine affects the endometrium by disturbing the production of steroid hormones involved in the uterine endometrial decidualization process. Indeed, in nicotine-exposed rats supplemented with progesterone, decidualization was inceased compared to the nicotine-treated group (Siti Norashikin, Ghosh et al. 2014).

In addition, our study showed that gestational exposure to nicotine increased follicular degeneration and atresia and the formation of follicular cyst in the ovaries of nicotine-exposed rats. Previous studies also reported that female rats intoxicated with nicotine for thirty days showed atrophied ovaries and intense follicular atresia associated with the formation of

follicular cycts (Camargo, Leite et al. 2014). Other researchers have reported that exposure of female rats to nicotine for four weeks induced only follicular atresia and reduced the number of developing follicles (SH. ROSHANKHAH1 and F. KARIMI 2 2017). Nicotine has also been shown to significantly reduced the number of pre-antral and antral follicles and the concentration of estradiol and increased the follicular atresia (Fahimeh Mohammadghasemi 1 and Saadat 2012). According to (Bordel, Laschke et al. 2006) and (BOLARINWA 2009), nicotine inhibits the growth of ovarian follicles by inducing apoptosis of granulosa cells, which may result in negative effects on fertility. In our study, although serum progesterone levels showed a slight non-significant decrease in nicotine-exposed rats, this decrease seems to be involved in the histological alterations mentioned above, taking into account the regenerative effect of progesterone treatment on decidualization (Siti Norashikin, Ghosh et al. 2014). (Adeyemi, Oyeyipo et al. 2018 showed that the decrease in serum sexual hormones levels in nicotine-exposed rats during gestation was related to ovarian damage. We also found that serum estrogen levels were significantly decreased in nicotine-exposed rats compared to controls. This observation is supported by the work of (Yang, Shi et al. 2014) and (Adeyemi, Oyeyipo et al. 2018) who reported that gestational exposure of female rats to nicotine causes a significant decrease in serum estrogen levels. According to (Ruan and Mueck 2015), nicotine affects steroidogenesis by decreasing aromatase activity in granulosa cells and peripheral tissues. Normal follicular development and the physiological changes of the endometrium during gestation are directly related to the action of estrogen. This implies that estrogens disturbance, whatever the cause, can be considered as the main reason of the uterine and ovarian histopathological alterations induced by nicotine during gestation.

Epidemiological evidence has shown that cigarette smoking is involved in several estrogen-related disorders in women including increased risk of early menopause (Baron, La Vecchia et al. 1990). Animal studies revealed a toxic effect of cigarette smoke on the ovary, affecting follicular development and decreasing the sexual hormones (estrogens) levels (Marom-Haham and Shulman 2016). Through our study, we can confirm that nicotine alone can also induce considerable negative effects on the reproductive organs involved in gestation. The oviarian and the uterine alterations were associated with a decrease in serum levels of sexual hormones, mainly estrogens, which could be attributed to granulosa cell apoptosis or aromatization defect. Those may also result from a direct cytotoxic action of nicotine on endocrine cells. Nicotine-induced hormonal disrubance may be one of the major mechanisms of nicotine toxicity on the ovary and uterus during gestation. Although, we was not able to determine other possible biochemical, biophysical and genotoxic mechanisms that may better explain nicotine toxicity during gestation.

Conclusion

Although the adverse effects of tobacco on the reproductive organs during gestation are well known, those of nicotine remain controversial. Our results showed that gestational exposure to nicotine has negative impact on reproductive function in the female WISTAR rat and affects both steroidogenesis and synthesis of progesterone. Gestational exposure to nicotine results in histological changes affecting the uterus and ovary; characterized by follicular atresia and uterine endometrial damage. Nicotine decreases ovarian reserve and interferes with steroid hormone synthesis. Our study may contribute to better understanding of the outcomes of gestational exposure to nicotine and provides information on possible mechanisms by which this exposure may contribute to reduced fertility in smokers pregnant women.

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