Toxicology Reports 8 (2021) 793–803

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Effects of ethanol and nicotine co-administration on follicular atresia and placental histo-morphology in the first-generation mice pups during intrauterine development and lactation periods

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ARTICLE INFO

Edited by Dr. A.M Tsatsaka

Keywords:
Ethanol
Nicotine
Follicular atresia
Placenta
First-generation mice

ABSTRACT

This study is evaluating the effects of ethanol and nicotine exposure during pregnancy and lactation on placenta histology and follicular atresia in the first-generation (f1) mice pups. The experimental groups were 5 groups of NMRI pregnant mice, including: control, vehicle (received normal saline) ethanol (3 g/kg/day, 20 % v/v intraperitoneally), nicotine (1 mg/kg/day, subcutaneously), and ethanol plus nicotine which received both. Pregnant animals in each group were then divided into two groups, one group for examining the placenta that was treated for 18 days and the other group for the ovary of one-day-old (PND1) and fifty-six-day-old (PND56) female offspring who were treated for 42 days (during intrauterine development and lactation). After the autopsy procedure, histopathological and morphometrical observations were done. Data revealed that the exposed mice had a significant change in the placenta morphometry and histology as well as a marked increase in the number of ovarian TUNEL positive cells on postnatal days 1 and 56. Therefore, maternal exposure to alcohol and nicotine during developmental and lactation periods could lead to changes in the placenta properties as well as an increase in the apoptotic ovarian follicles in f1 mice pups.

1. Introduction

Cigarette smoking and alcohol drinking have many harmful social, physical and mental, effects on the human societies. According to the World Health Organization (WHO) report, the prevalence of cigarette smoking is 47 % in men and 12 % in women, respectively and this prevalence is increasing among the females of childbearing age [1].

Pieces of evidence indicate that more than 4000 chemical compounds exist in the cigarette. Nicotine is one of the most powerful toxic components among them [2]. It can be absorbed into the body in a rate of 1−3 mg per cigarette, or about 20 mg per pack [3]. Nicotine has adverse effects on various organs specially, reproductive system. It can significantly reduce fertility through inducing apoptosis, increasing atretic follicles, and decreasing follicle reserve due to oxidative stress process [4].

In the early 7 weeks of intrauterine development, cigarette compounds especially nicotine can pass through the blood-placenta barrier and accumulate in embryonic structures [5] and cause sudden infant death syndrome, weight loss at birth, physical, cognitive and behavioral disorders in newborns [6,7]. Nicotine can be absorbed rapidly and completely by the mother's blood and then transmitted to the infant's body through breast milk [3].

In the USA, of the 40 % of pregnant women who consume alcohol, 3–5 % consume heavily in their pregnancy [8]. Ethanol (C_2H_5OH), that

Abbreviations: NMRI, Naval Medical Research Institute; PND, Postnatal day; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; WHO, World Health Organization; GD, gestation day; CTL, control; Veh, vehicle; i.p., intraperitoneally; s.c., subcutaneously; Ni, nicotine; cat.no, catalogue number; EtOH, ethanol; ELISA, enzyme-linked immunosorbent assay; AMH, Anti-Müllerian hormone; FSH, Follicle-stimulating hormone; H&E, Hematoxylin and Eosin; HCL, Hydrogen Chloride; PBS, Phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; SPSS, statistical package for the social sciences; ANOVA, analysis of variance; SEM, standard error of the mean; IUGR, intrauterine growth restriction; DNA, Deoxyribonucleic acid; BMP, Bone morphogenetic protein; E_2, Estradiol; OFR, ovarian follicular reservoir; OS, oxidative stress; nAChRs, nicotinic acetylcholine receptors; Bcl-2, B-cell lymphoma 2; BAX, BCL2 Associated X; ROS, reactive-oxygen-species; Ca^{2+}, calcium.

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https://doi.org/10.1016/j.toxrep.2021.03.033
Received 5 November 2020; Received in revised form 28 March 2021; Accepted 31 March 2021
Available online 2 April 2021

has been found in alcoholic drinks is the most famous type of alcohol, which is widely being used in various industries. The percentage of ethanol in alcoholic beverages is different and it's about 4–6% in beer, 7–15% in wine, and 40–95% in distilled beverages [9]. Furthermore, it is reported that traditional alcoholic beverages have a higher human safety level rather than bulk beverages produced by individuals [10].

Alcohol addiction causes functional reproductive disorders such as anovulation, luteal phase disorder, amenorrhea, early menopause, and increased risk of abortion in women [11]. Alcohol consumption in moderate doses has a negative effect on milk production and the level of prolactin and oxytocin balance in mothers. Ethanol could pass through the blood-milk-barrier [12] and impact the child.

Ethanol can also affect placental growth and function. It comes into the mother's blood and rapidly transmits into the placenta and fetus. The levels of alcohol in the fetus and maternal circulation are the same after 1 h [13].

Except for the only 300–400 eggs that are available for ovulation and fertilization, the other ovarian follicles will be degenerated through the apoptosis process from the middle of the fetal period to the end of the female fertility life [14].

As noted, before, some factors such as smoking and alcohol drinking can cause irrecoverable effects on ovarian development and exacerbate the natural degeneration process of the follicle. As regards the mother's lifestyle during pregnancy plays an important role in the newborn's life, therefore, the purpose of this study was to investigate the effects of alcohol and nicotine, alone and together during pregnancy and breast-feeding on placenta histomorphology and follicular atresia in first generation of mice offspring at two important periods of folliculogenesis: at birth and puberty.

2. Materials and methods

2.1. Animals

A total number of 20 male and 60 female NMRI mice (6–8 weeks, 25–30 gr weight) were used for breeding. All animals were maintained in conventional open-top polypropylene cages with wood shavings (3-4 mice/cage) in the animal house of Afzalipour School of Medicine under controlled lighting conditions (12 h light/dark cycle) and with free access to tap water and standard rodent chow at 21± 2 °C and 40–60 % relative humidity. All protocols approved by the institutional ethics committee of Kerman Medical University (approval number IR.KMU.REC.1397.145)

2.2. Experimental design

One male and three female mice were placed in each cage for overnight mating. The next morning, females were checked for vaginal plaque formation indicating, gestational day 0 (GD0). Pregnant mice were randomly divided into five experimental groups: in the control group (CTL) the animals were intact; in the vehicle group (Veh), the animals received normal saline)3 g/kg/day (intraperitoneally (IP) and (1 mg /kg/day) subcutaneously (SC). In the nicotine group (Ni), the animals received nicotine (cat.no. N3876, Sigma, USA) 1 mg/kg/day, SC [6,7]. In the ethanol group (EtOH), the animals received 20 % v/v ethanol (3 g/kg/day; diluted in physiological saline (IP) [15] and in the nicotine plus ethanol group (Ni + EtOH), the animals co-administered nicotine (1 mg/kg/day, SC) and ethanol (3 g/kg/day, IP). The treatment of pregnant mice started from GD0 and they divided into two subgroups randomly as follows:

1) Analysis of placenta: placenta of euthanized pregnant mice at 18th days of gestation (GD18, 18 days after starting of treatment) was removed and analyzed (n = 8).

2) Analysis of follicular atresia: offspring`s ovaries were evaluated at two critical times:

a) Immediately after birth (n = 8) (21 days of treatment which carried out during intrauterine development) and, b) onset of puberty (n = 8) (PND56; 42 days of treatment which carried out during intrauterine development and lactating periods) (Fig. 1).

2.3. Blood sampling and serum hormone assays

In PND56, the mice were anesthetized with ketamine 10 % plus Xylazine 2% (Alfasan Co, Holland) (80 mg/kg +15 mg/kg) [16]. Heart blood was gathered (at 8–9 A.M), centrifuged at 2500−2000 rpm for 10 min and then the blood plasma separated and stored at −20 °C until further hormonal analysis. Specific mice ELISA kits were used for measuring the levels of FSH (EAETBIOPHARMA, cat.no.CK-E20419, China), AMH (EAETBIOPHARMA, cat.no.CK-E90200, China), 17-β estradiol (EAETBIOPHARMA, cat.no.CK-E20381, China) hormones.

2.4. Ovarian morphometry

The weight of the animal body (on PND1 and 56) and the right ovary were recorded by a sensitive digital scale (A&D, GF-300, Japan) on PND56 in each group. Large and small ovarian diameters on PND56 were measured by a digital caliper (Mitushi PRO-CAL).

2.5. Histological examination

The left ovaries were fixed in 10 % formaldehyde (cat.no.222, Dr. Mojalali Industrial Chemical Complex Co, Iran), embedded in paraffin (cat.no. 1071581000, Merck, Germany), and then 5 μm sections prepared. One out of every ten section was selected for staining with hematoxylin and eosin (H&E) and was assessed under a light microscope (Olympus IX51, Japan) for morphological and pathological examination. A single expert investigator that was blinded to the treatment conducted the microscopy observations.

2.6. Follicular count and classification

After H&E staining, the follicles were counted and classified by types as following:

1 Primordial follicles: include an oocyte surrounded by a complete layer of squamous granulosa cells.
2 Primary follicle: an oocyte with a layer of cuboidal granulosa cells.
3 Secondary follicle: an oocyte with multiple layers of cuboidal granulosa cells, with no antrum.
4 Preantral follicle: follicles with emerging antral spaces.
5 Antral follicle: a central oocyte surrounded by a space filled with liquid and several layers of granulosa cells.
6 Preovulatory follicle: the largest follicles having a defined cumulus granulosa cell layer around the oocyte.
7 Atretic follicle: follicle with oocyte fragmentation, plasma membrane rupture, and granulosa cells dropped into the antrum [17].

2.7. TUNEL assay

The rate of apoptotic follicles was examined by TUNEL Assay (In Situ Cell Detection Kit, Fluorescein cat.no.11684795910 Roche, Germany). Briefly, the sections were deparaffinized, rehydrated. and after washing with PBS, were incubated in Proteinase (cat.no. P2308, Sigma, Germany) +Tris/HCL (cat.no. T5941, Sigma, USA) for 25 min (37 °C). Finally, after rising again with PBS, a mixture of 50 μl enzyme solution with 450 μl label solution was put on the samples for 1 h at 37 °C. Next, the nucleuses were stained with 4′,6-diamidino-2-phenylindole ((DAPI) (cat.no. D9542, Sigma, USA)). The stained sections were analyzed with a fluorescent Olympus IX71 microscope (Tokyo, Japan).

After observation, the pre-antral and antral follicles scored blindly, according to the numbers of apoptotic cells as explains below:

Score (1): without any TUNEL positive granulosa cells;

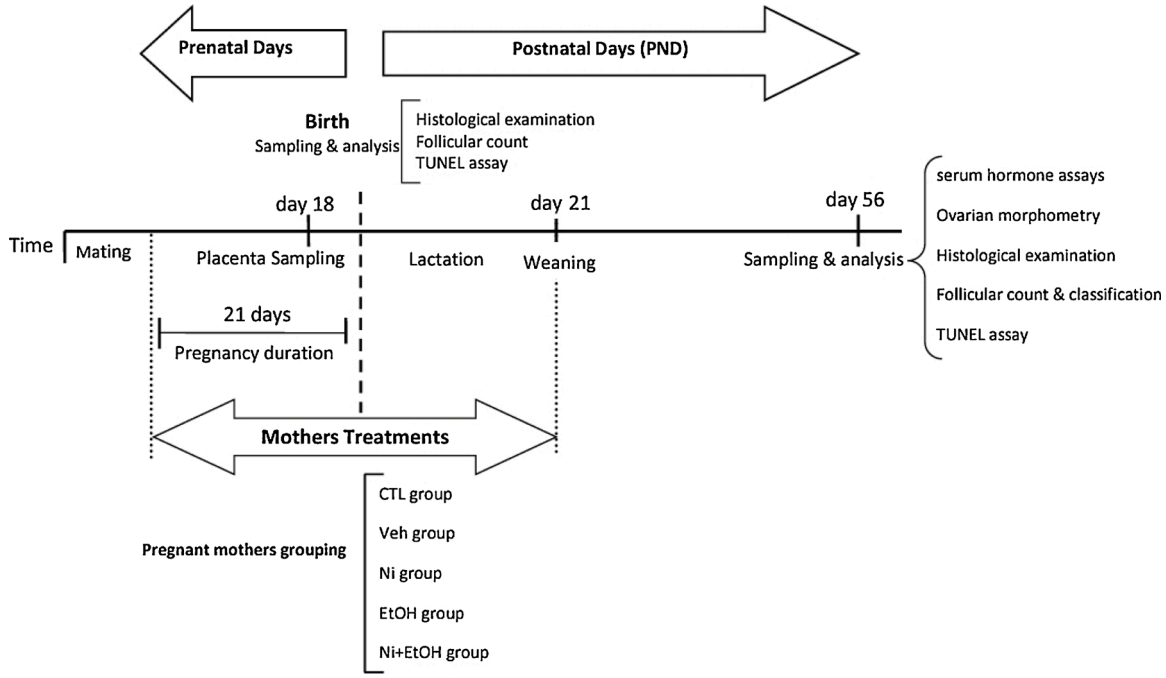

Fig. 1. Schematic representation of the experimental design in different groups of mothers and offspring. CTL group: control group; Veh group: vehicle group received normal saline; Ni group: nicotine group received 1 mg/kg/day nicotine, subcutaneously; EtOH group: ethanol group received 3 g/kg/day, 20 % v/v ethanol intraperitoneally; Ni + EtOH group: ethanol plus nicotine which received both.

Score (2): 0 < the number of positive TUNEL cells ≥ 2;
Score (3): 2 < the number of positive TUNEL cells≥ 3;
Score (4): 3 < The number of positive TUNEL cells≥ 4,
Score (5): the number of positive TUNEL cells≥ 5

For different TUNEL signal intensities, each score was attributed to different intensity as below:

score (1); no staining; score (2) = weak; score (3) = intermediate; score (4) = strong and score (5) = very strong.

In the end, the below formula was used for measuring the apoptosis prevalence as apoptotic index (AI):

$$AI = 0n_1 + 1n_2 + 2n_3 + 3n_4 + 4n_5;$$

n_1, n_2, n_3, n_4 and n_5 are the numbers of cells representing scores 1, 2, 3, 4 and 5, respectively. The apoptotic score determining the average number of apoptotic cells for every follicle type [18].

2.8. Placenta morphometric and histological examinations

Pregnant mice in GD18 were anesthetized with ketamine 10 % plus Xylazine 2% (Alfasan Co, Holland) (80 mg/kg +15 mg/kg) and sacrificed by cervical dislocation. Six fetuses and their placentas were randomly removed from the middle part of the uterus horn of each pregnant mouse.

Weight and diameter of placenta were recorded by digital scale and caliper, respectively. The placenta existence, atrophy, and size were recorded. In the next step, the placenta was fixed in 10 % formaldehyde and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin (H&E) and then examined under a light microscope (OlympusIX51, Japan). The thickness of the labyrinth zone, basal zone, and decidua basalis were measured blindly in placentas using Anix analysis software (shaheed Chamran University of Ahvaz, Iran).

2.9. Statistical analysis

Data were analyzed by SPSS software version 22. The normality of the data was tested by using One-sample Kolmogorov-Smirnov test. Data with normal distribution were analyzed by the analysis of variance (ANOVA) and Tukey post-hoc test and the Krus-kalwalis test was used for nonparametric data. Values were presented as mean ± SEM. In addition, $p \leq 0.05$, $p \leq 0.01$and $p \leq 0.001$ were considered statistically significant.

3. Results

3.1. Litter data

The number of delivered litters in each parturition, male to female ratio in the pups, and mortality in PND1 and 21 (End of lactating period) are summarized in Table 1.

There was no significant difference in the number of pups per litter and the sex ratio among the experimental groups. The mean rate of mortality in the Ni + EtOH group on PND1 was significantly higher than the control and ethanol groups ($p \leq 0.05$). On PND21, the mean rate of mortality in the Ni + EtOH group significantly increased in compare

Table 1
Effects of ethanol and nicotine co-administration on fetal parameters (n = 8).

Variables		CTL	Veh	Treatment groups		
				Ni	EiOH	Ni + EtOH
No. of pups/litter		7.5 ± 0.5	8.33 ± 0.8	7.5 ± 0.72	7.89 ± 0.51	8 ± 0.84
Male fetuses (Mean % ± SEM)		42.08 ± 6.13	54.96 ± 5.03	49.07 ± 5.67	46.6 ± 3.13	51.51 ± 5.03
Female fetuses (Mean% ± SEM) (%)		57.91 ± 6.13	43.65 ± 5.03	50.92 ± 5.67	53.4 ± 3.13	48.49 ± 5.03
Mortality	PND 1	0 ± 0	1.19 ± 1.19	0 ± 0	0 ± 0	28.78 ± 13.41[ad]
	PND 21	7.14 ± 3.71	12.99 ± 9.09	1.81 ± 1.78	8.8 ± 3.61	28.69 ± 7.39 [abcd]

The values are comparable in the same row, a: significant difference versus the control (CTL) group; b: significant difference versus the vehicle group (Veh); c: significant difference versus the nicotine-treated group (Ni); d: significant difference versus the ethanol-treated group (EtOH), ($p \leq 0.05$).

with the CTL ($p = 0.03$), Veh ($p = 0.05$), EtOH ($p = 0.04$) and Ni ($p = 0.002$) groups. The mice exposed to ethanol and nicotine, prenatally, had the lowest level of body weight rather than non-exposed counterparts on PND1 and 56 (Fig. 2).

3.2. Hormone analysis

As shown in Table 2, the serum level of FSH and 17-β estradiol (E_2) hormones in the experimental groups decreased non-significantly in comparison to the control and vehicle groups. There was no significant alteration in serum AMH concentration among studied groups (Table 2).

3.3. Ovarian morphometric analysis

Results depicted in Table 3 indicating that there is no statistically significant difference in the large and small ovarian diameters of the control and vehicle groups. However, prenatally exposure to nicotine and ethanol, simultaneously, could reduce the ovarian weight of the first-generation pups when compared to the control ($p \leq 0.01$) and EtOH groups ($p \leq 0.05$).

3.4. Histological findings on ovarian follicle numbers

Fig. 3 is representing the number of ovarian follicles, categorized based on mice offspring age in different groups ($n = 8$).

According to the results, the number of primordial follicles was reduced in the experimental groups on PND1 in comparison to the control and vehicle groups, although it did not reach a significant level (Fig. 4.a). Histological analysis revealed that the number of primordial follicles decreased significantly in the EtOH group in comparison to the Ni group ($p = 0.05$) on PND56, and increased in the Ni + EtOH group compared to the EtOH group ($P = 0.04$) group. The mean number of secondary follicles in both EtOH and Ni + EtOH groups significantly increased in comparison with the Ni group ($p = 0.02$), but the average of primary follicles had no difference among the groups. A significant reduction was observed in the mean number of pre-antral and antral follicles in the nicotine, ethanol, and Ni + EtOH treated groups compared to the control and vehicle groups ($p < 0.05$).

The number of pre-ovulatory follicles was significantly reduced in the ethanol group in comparison to the control ($p = 0.001$) and vehicle ($p = 0.03$) groups. The mean number of corpus luteum in the Ni + EtOH group was decreased in comparison with the control ($p = 0.0014$) and vehicle mice ($p = 0.01$). In the Ni + EtOH group, atretic follicles' number was raised markedly when compared to the control ($p = 0.04$) and vehicle ($p = 0.05$) animals (Fig. 4.b, c).

3.5. Evaluation of apoptosis

Fig. 6 is displaying the effects of nicotine and ethanol on the granulosa cells' apoptosis in the ovaries of offspring($n = 8$). The data revealed that the levels of TUNEL positive cells in the granulosa cells and

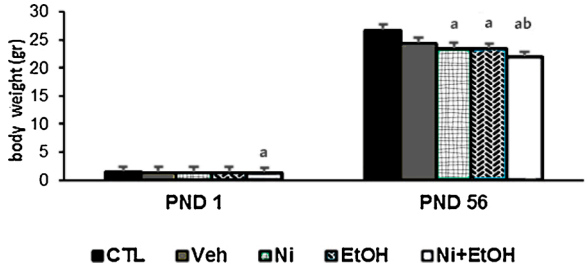

Fig. 2. Body weights on PND 1 and 56 in the control, vehicle, and experimental groups. Values expressed in mean SEM, a: Significant difference versus the control group; b: Significant difference versus the vehicle group, ($p \leq 0.05$).

Table 2
Serum levels of AMH, FSH and 17-β estradiol in female offspring mice in different groups ($n = 8$).

variables	CTL	Veh	Treatment groups		
			Ni	EtOH	Ni + EtOH
AMH (ng/mL)	11.76 ± 3.41	8.32 ± 0.99	9.98 ± 0.84	6.72 ± 0.42	7.01 ± 1.52
FSH (mIu/mL)	39.5 ± 5.59	38.62 ± 2.60	33.87 ± 3.30	25.75 ± 2.28	27.25 ± 3.62
17-β estradiol (ng/L)	53.37 ± 5.91	73.5 ± 16.51	50.62 ± 8.32	31.37 ± 6.66	32.62 ± 6.26

The values are expressed as (mean ± SEM).

Table 3
Comparison of the ovarian weight and diameters in different groups ($n = 8$).

variables	CTL	Veh	Treatment groups		
			Ni	EtOH	Ni + EtOH
Ovaries weight (mg)	4 ± 0.6	3.2 ± 0.5	2.7 ± 0.3	3.3 ± 0.3	2.3 ± 0.3 [ad]
Large ovaries diameter (mm)	2.44 ± 0.09	2.22 ± 0.14	2.49 ± 0.05	2.32 ± 0.07	2.21 ± 0.08
Small ovaries diameter (mm)	1.47 ± 0.06	1.45 ± 0.08	1.51 ± 0.07	1.43 ± 0.05	1.3 ± 0.15

The values are expressed as (mean ± SEM) and comparable in the same row, a: significant difference versus control group ($p \leq 0.01$); d: significant difference versus ethanol-treated group (EtOH) ($p \leq 0.05$).

the inner layers of pre-antral and antral follicles were much greater than theca layers. (Fig. 7).

TUNEL analysis of the ovaries of PND1 revealed that the average number of TUNEL positive cells in the treated groups was significantly rise ($p \leq 0.05$) in comparison with the control and vehicle groups. (Figs. 5 and 6a).

The number of the TUNEL positive cells in the pre-antral and antral follicles was recorded as weak; intermediate; strong and very strong on the PND56. The results represented that the number of pre-antral follicles with strong apoptotic signals between the vehicle and nicotine-treated mice was significantly different ($p \leq 0.05$). In addition, the number of pre-antral follicles with intense apoptotic signals increased in all of the treated groups ($p < 0.01$). However, no difference was observed in the number of pre-antral follicles with weak or intermediate apoptotic signals (Fig. 6. b). The number of antral follicles with very strong TUNEL signals' intensity in the EtOH and Ni + EtOH groups ($p \leq 0.05$) was significantly higher than non-exposed groups (Fig. 6.c). Ultimately, mean score of apoptosis in the pre-antral and antral follicles were evaluated in the apoptotic index formula, which was described before.

The average apoptotic index in the pre-antral follicles had a significant enhancement in the nicotine ($p \leq 0.05$, versus control and vehicle), ethanol, and Ni + EtOH exposed mice ($p \leq 0.01$ versus control and $p \leq 0.05$ versus vehicle). Apoptotic index in the antral follicle was increased greatly in the EtOH group compared to the control group, and also in the Ni + EtOH group compared to the vehicle ($p \leq 0.05$) (Fig. 6.d).

3.6. The placental/fetal weight ratio and placental morphology

The effects of ethanol and nicotine on fetal and placental weight on the GD18 showed a ratio (W_p/W_f) in Table 4. This ratio was significantly decreased in the EtOH group compared to the control and vehicle ($p \leq 0.05$) groups and especially a reduction was observed in the Ni + EtOH group compared to the vehicle ($p \leq 0.05$) and EtOH ($p \leq 0.01$) groups.

The placental size in the experimental groups was macroscopically smaller than control. There was no difference in the placental thickness

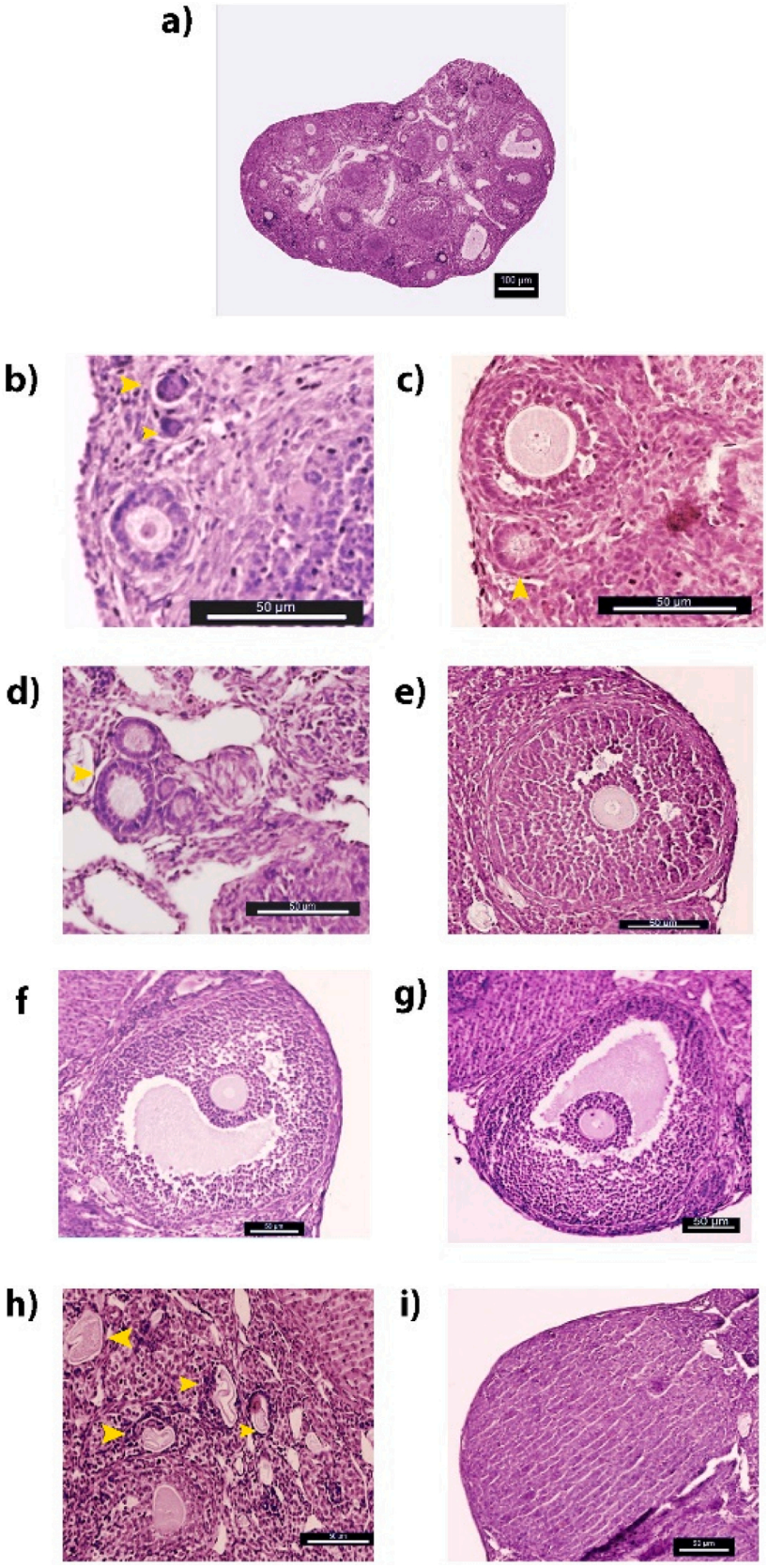

Fig. 3. Representative photomicrographs of PND56 H&E-stained ovaries.
a) mouse ovary has follicles at all stages of development (×64); b) primordial follicles (yellow arrow heads); c) primary follicle (yellow arrowhead); d) secondary follicle (yellow arrowhead); e) pre-antral follicle; f) antral follicle; g) pre-ovulatory follicle; h) atretic follicles (yellow arrowheads); i) corpus luteum. In pictures, e-g magnification is ×200 and in b–d, h magnification is ×320. In all pictures scale bar is 50 µm.

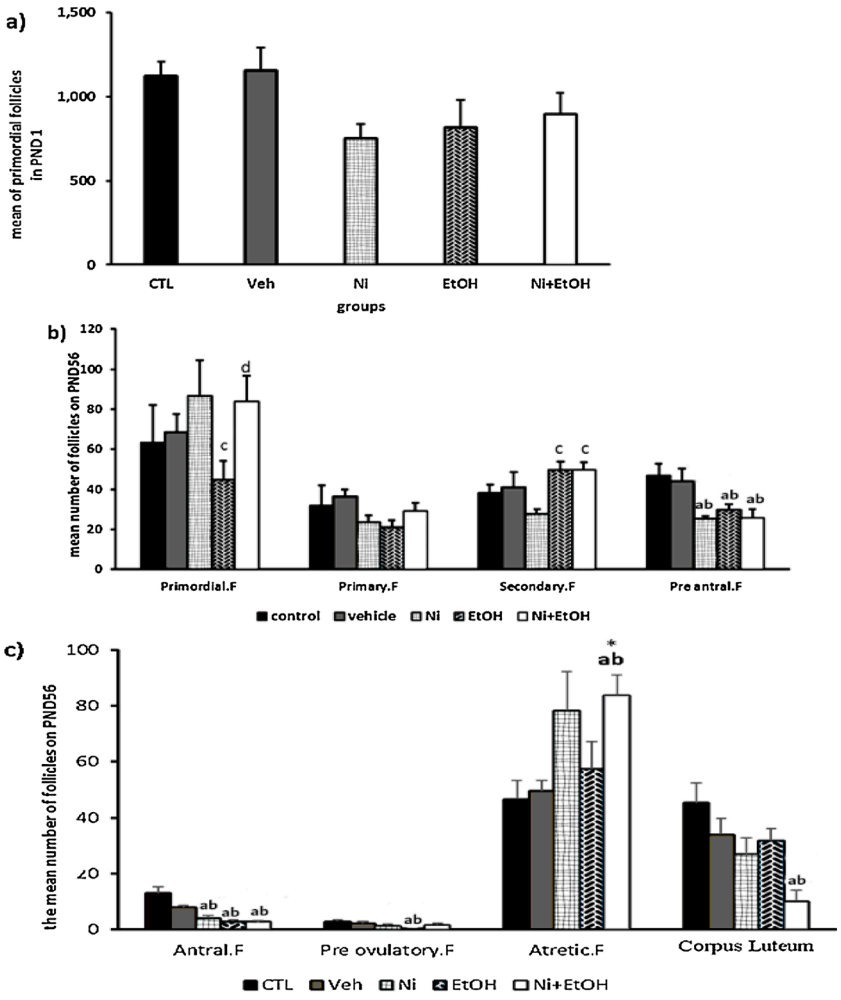

Fig. 4. Effects of co-administration of ethanol and nicotine on folliculogenesis at PND1 and 56. a) the average number of primordial follicles on PND1 in different groups; no difference was saw; b) Distribution (mean ± SEM) of growing follicles (primordial, primary, secondary, and pre-antral) at PND 56 in different groups; c) Proportions (mean ± SEM) of Graafian (antral and pre-ovulatory), atretic follicles and corpus luteum in the ovary on PND 56.
a: Significant difference versus Control group; b: Significant difference versus Vehicle group; c: Significant difference versus nicotine-treated group (Ni); d: Significant difference versus ethanol-treated group (EtOH), ($p \leq 0.05$).

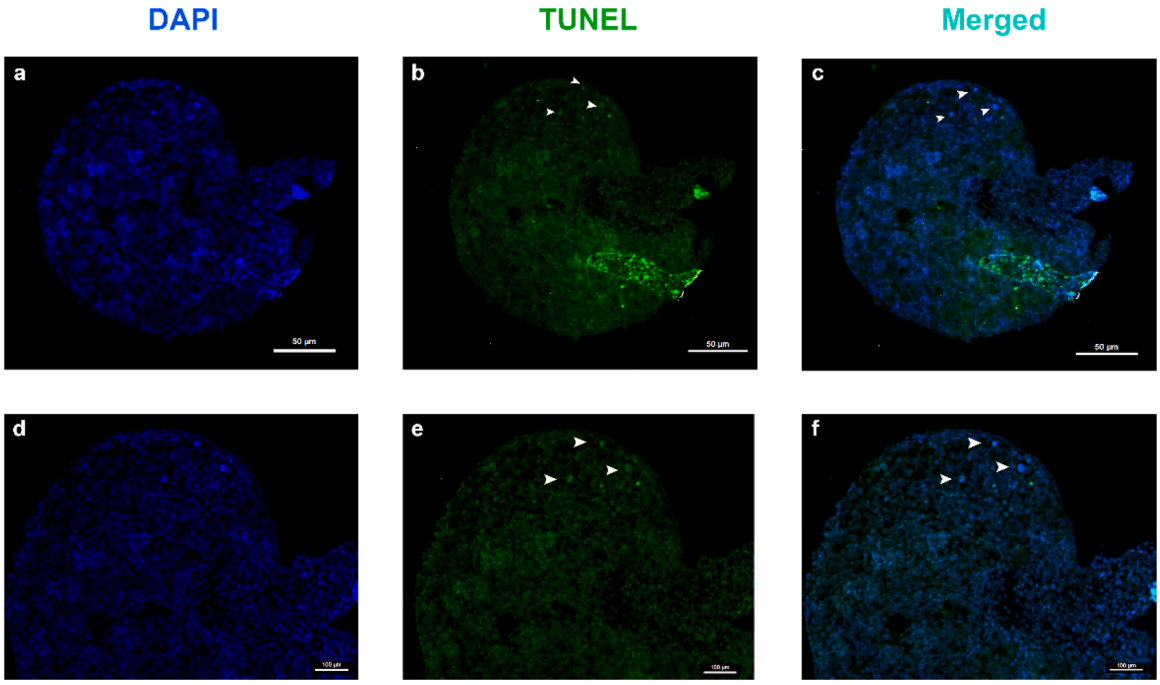

Fig. 5. TUNEL staining of ovary tissue in PND1. TUNEL-positive cells labeled green (b, e), and nuclei labeled blue (a, d) (DAPI). Light yellow arrowheads point TUNEL positive granulosa cells (b, c, e, f), magnification in parts a-c = 320x, scale bars = 50 μm, and magnification in parts d-f = 640, scale bars = 100 μm.

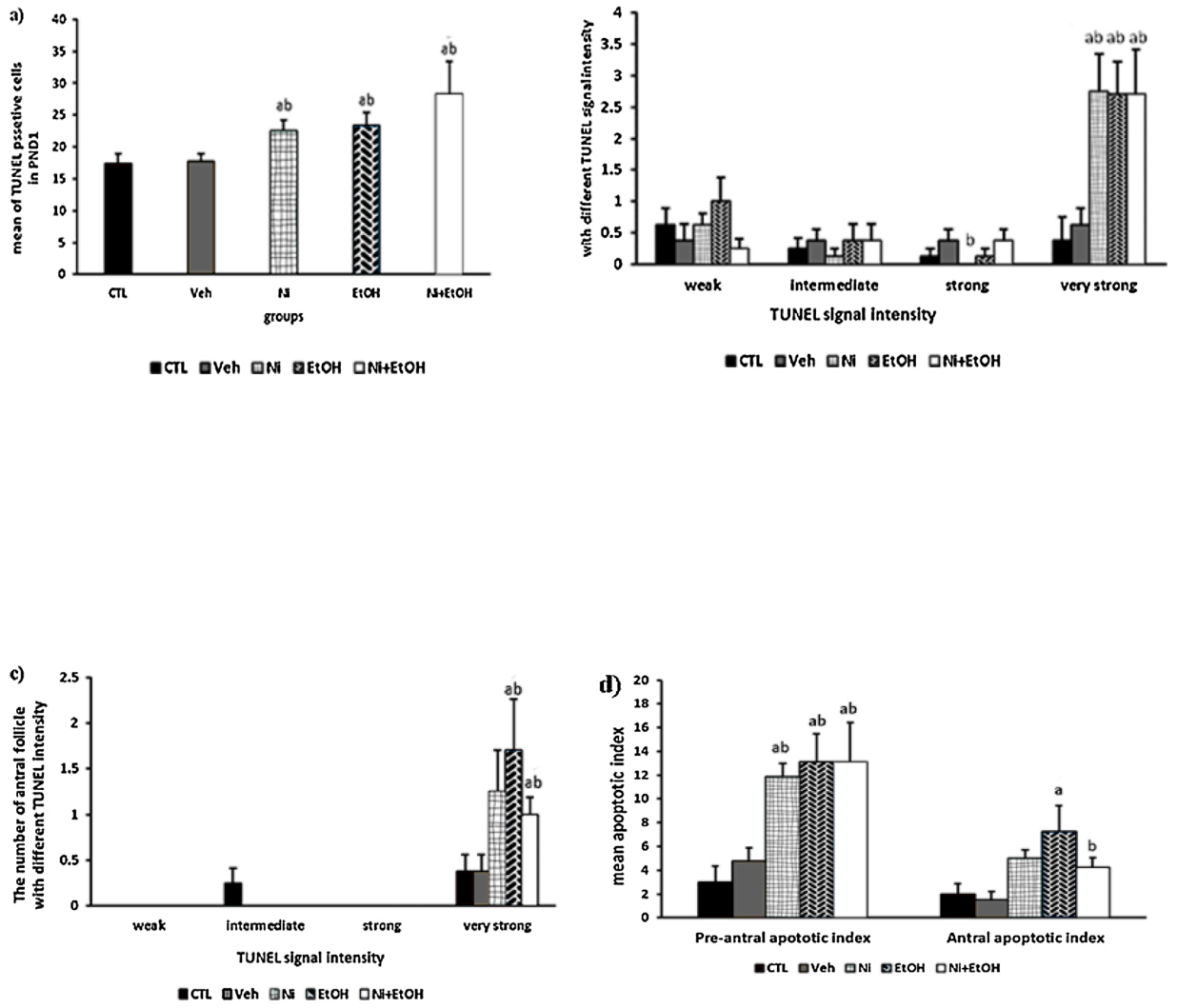

Fig. 6. Effects of ethanol and nicotine on granulosa cells apoptosis on PND1 and 56. a) is showing the mean number of TUNEL positive cells in PND1. b, c) are representing the average number of pre-antral and antral follicles with different TUNEL signaling intensity. d) mean apoptotic index are shown in different groups, in all chart values expressed in mean ± SEM, a: significant difference versus control group; b: Significant difference versus Vehicle group; ($p \leq 0.05$).

among all groups. The placental diameter decreased significantly in the nicotine-treated animals compared to the control ($p \leq 0.05$) and vehicle ($p \leq 0.001$); in the EtOH group compared to the control and vehicle ($p \leq 0.001$) and nicotine ($p \leq 0.05$) groups, also in the Ni + EtOH group in comparison to the control ($p \leq 0.01$) and vehicle ($p \leq 0.001$) groups.

Macroscopic observation revealed that the shape and color of the collected placentas from the control and vehicle animals were normal with no macroscopic lesions. However, placenta abnormality was observed in all of the experimental groups; an abnormal shape, dark color, white spots, and peripheral atrophic rim were seen especially in the placenta of the Ni + EtOH group. (Fig. 8).

3.7. Histological observation of placenta

Microscopic evaluation of the placenta sections revealed that thickness of labyrinth, basal zones, and decidua basalis significantly diminished in the experimental groups compared to the control and vehicle groups (Fig. 9) as follow:

3.7.1. Decidua basalis

The decidua basalis thickness significantly reduced in all of the treated groups compared to the control group ($p \leq 0.001$). A remarkable diminish in the thickness of decidua basalis was observed in the nicotine-treated ($p \leq 0.001$) and ethanol-treated ($p \leq 0.05$) animals (Fig. 9).

3.7.2. Basal zone

In the nicotine-treated mice, the thickness of the basal zone significantly decreased compared to the control ($p \leq 0.01$) and vehicle ($p \leq 0.001$). A significant difference was observed in the basal zone thickness of the ethanol-treated group in comparison with the nicotine ($p \leq 0.05$), and the Ni + EtOH ($p \leq 0.05$) groups (Fig. 9).

3.7.3. Labyrinth zone

A significant reduction of labyrinth zone thickness was observed in the ethanol and nicotine-ethanol treated groups in comparison with the control group ($p \leq 0.05$) (Fig. 9).

4. Discussion

Although several investigators have studied the adverse effects of maternal smoking and alcohol consumption on fertility and pregnancy outcomes, reports focused on the effects of co-administration of alcohol and nicotine on placenta histomorphology and follicular atresia during intrauterine development and lactation in first-generation pups at the end of these two important periods of follicular atresia are few.

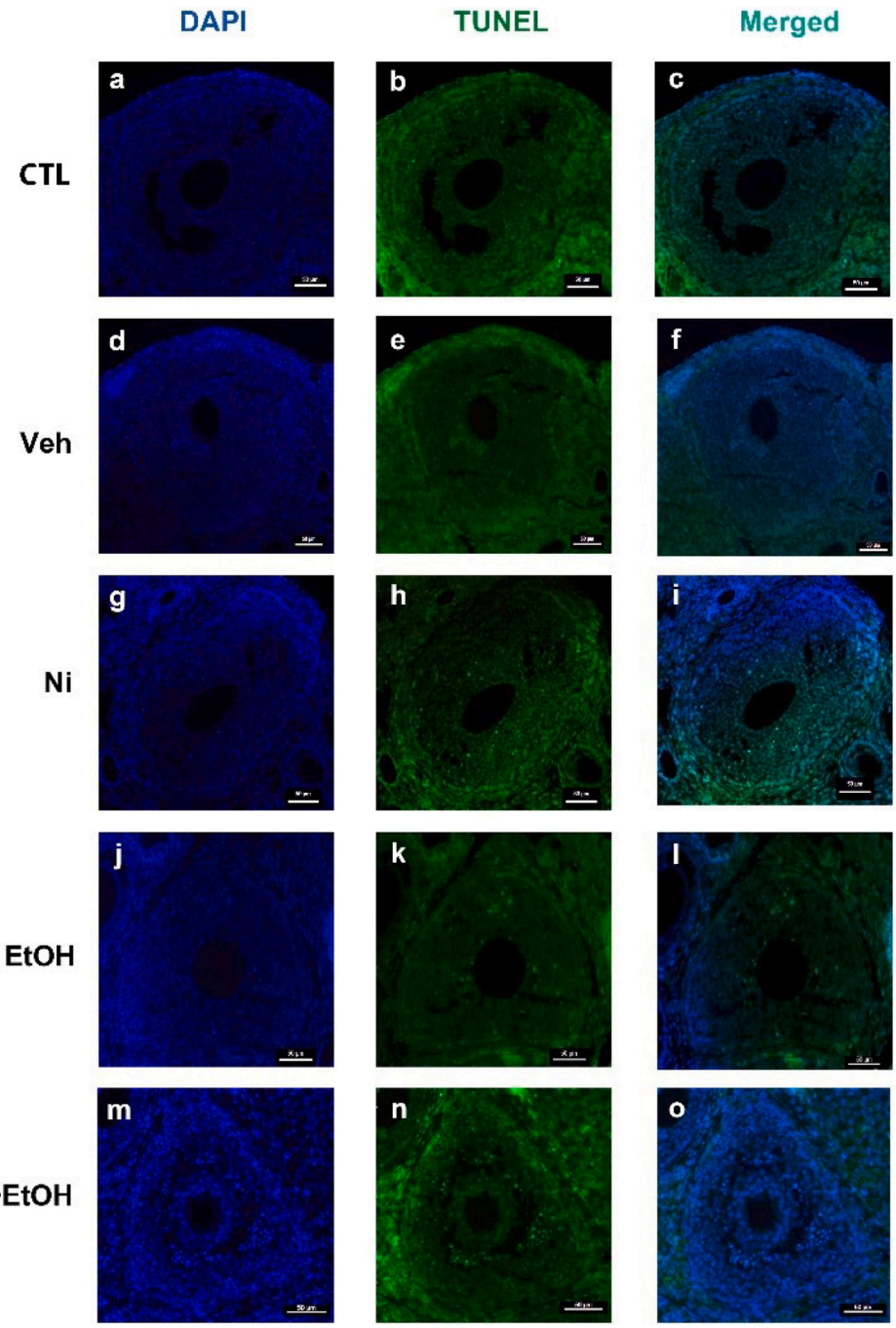

Fig. 7. The photograph is representing immunofluorescence staining in ovary section on PND56. TUNEL positive granulosa cell (shiny green) and counterstained with DAPI (blue), magnification = 320, and scale bars = 50 μm.

Folliculogenesis begins near the middle of intrauterine life (5th month of the human gestational period). After that, the majority of follicles degenerate by apoptosis until birth, so at the time of birth, only 1 million out of 7 million follicles remain. Then again many of the follicles will be lost and the number of follicles reaches 300,000–400,000 [19]. The ovarian follicular reserve determines the length of fertility and the health condition of females. The research question is whether, in addition to endogenous factors for ovarian depletion of follicles, what is the role of exogenous factors such as smoking and alcohol drinking on the ovarian follicle reservoir especially in the fore-mention important times (intrauterine development and lactating periods). To achieve these goals, hormonal, histological, and immunohisto-chemical assessments were done to detect the effects of co-administration of ethanol and nicotine on the placenta and follicular reserve.

Reduced placental blood flow and the hypoxia that happens afterward is a suggested hypothesis in the mortality of animals exposed to nicotine and nicotine plus alcohol. In addition, ethanol and nicotine could have adverse effects on lactational efficiency and maternal behavior [20]. The results of the present study illustrated that ethanol and nicotine alone and together caused the loss of body weight both at birth and puberty. Studies showed that nicotine mediated most of the smoking effects on body weight. Nicotine's effects on the nervous system lead to suppress appetite and decrease food intake. During smoking in the pregnancy period, nicotine transfers to the placenta and aggregate in the fetal blood and amniotic fluid. Carbon-monoxide also diffuses into the fetal circulation and leads to fetal hypoxia and then intrauterine

Table 4
The effects of ethanol and nicotine on placenta parameters in the GD18 (n = 6).

variables	CTL	Veh	Treatment groups		
			Ni	EtOH	Ni + EtOH
W_p / W_f (gr)	0.12 ± 0.004	0.13 ± 0.005	0.12 ± 0.005	0.13 ± 0.005 ^{ac}	0.11 ± 0.003 ^{bd}
Placenta diameter (mm)	7.97 ± 0.09	8.8 ± 0.09	7.47 ± 0.13 ^{ab}	7.02 ± 0.14 ^{abc}	7.42 ± 0.08 ^{ab}
Placenta thickness (mm)	2.1 ± 0.04	2.18 ± 0.04	2.01 ± 0.04	2.14 ± 0.07	2.02 ± 0.05

The values are expressed as (mean ± SEM) and comparable in the same row. a: significant difference versus the control (CTL) group; b: significant difference versus the vehicle group (Veh); c: significant difference versus the nicotine-treated group (Ni); d: significant difference versus the ethanol-treated group (EtOH), ($p \leq 0.05$).

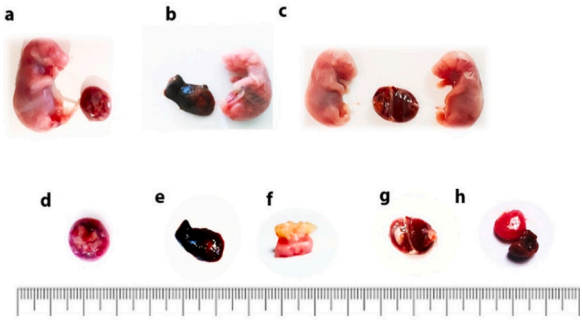

Fig. 8. Gross appearance of placenta of the control (a, d) and the treated groups (b, c, e-h). The placenta in the treated groups was macroscopically smaller and more irregular than that of the control and vehicle groups. Also, adhesion of two placentas together was observed. The ruler in the figure is graduated in millimeters.

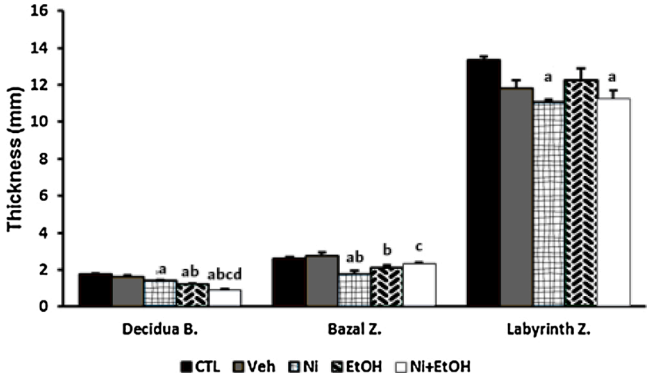

Fig. 9. The thickness of the decidua basalis, basal zone, and labyrinth zone in different groups. Each value represents mean ± SEM. a: significant difference compared to the control group; b: significant difference compared to the vehicle group; c: significant difference compared to the nicotine-treated group (Ni); d: significant difference compared to the ethanol-treated group (EtOH), ($p \leq 0.05$).

growth restriction (IUGR) [21]. Prenatal alcohol exposure inhibits cell proliferation, so postnatal growth potential reduces and the fetus can't reach normal body weight [22,23]. During lactation, the nicotine and alcohol present in breast milk can transmit to infant and child and may impair the sucking reflex at birth, so it makes breast-feeding a challenge. Thus, the inevitable feeding affects the growth of the infant [21,24]. In the present study, we observed a reduction in ovarian weight in the Ni + EtOH group. Previous studies have indicated that nicotine and ethanol lead to estrogen suppression and consequently ovarian weight reduction [25,26].

In this study, the levels of hormones were decreased insignificantly in the treated groups. Ethanol and nicotine inhibited the release of gonadotropins from the pituitary gland and suppressed gonadotropin-stimulated ovarian steroidogenesis, so the levels of FSH, AMH, E_2 were reduced, and then ovarian reserve impressed. Based on other studies' results, when FSH expression is suppressed, the rapid atresia of ovulatory sized follicles (pre-ovulatory follicles) is evident [4,27,28]. A prior study indicated that ethanol decreases the synthesis of nitricoxide synthase and activity of nitric oxide, also suppresses the circulating level of E_2, which all cause a harmful effect on ovarian activities [29].

In this study, we observed that the number of primordial cells reduced in the treated groups on PND1, although it was not significant. TUNEL assays also revealed a significant increase in the number of apoptotic cells at this age in all treated groups. Therefore, it is likely that smoking and alcohol drinking during pregnancy decreased the ovarian follicular reservoir (OFR) due to increased apoptosis. In utero alcohol exposure is well known to be teratogenic, causing developmental aberrations, but how alcohol causes neonatal ovarian dysfunction is currently unclear [30]. Maternal smoking during pregnancy has direct effects on the ovary of developing fetuses that will appear later in the reproductive lifetime. Fetal and neonatal exposure to nicotine may destructively affect hypothalamic–pituitary–ovarian function, alters steroidogenesis, and increases apoptosis in the ovarian cell via oxidative stress (OS), so impairs offspring reproductive performance [31–33]. Our follicular count on PND56 indicated that all of the follicles were impressed except primary follicles in treatment groups. Graafian follicles were more impressive than other follicles, which may be due to the fact that granulosa cells are more affected by ethanol and nicotine and on the other hand the number of these cells in large follicles is much more than small follicles. Furthermore, ovarian volume and the number of antral follicles in the early follicular phase were important to measure the ovarian reserve condition and determine fertility [26,30]. In the present study, a significant rise in the number of atretic follicles and the apoptotic index was observed in the nicotine + ethanol treated group. Researchers demonstrated that ethanol and nicotine induce apoptosis in granulosa cells by several mechanisms. One of these mechanisms induced by nicotine is nicotinic acetylcholine receptors (nAChRs) activation, nAChR-2, and -7 express in granulosa cells. Nicotine induces apoptosis by altering the bcl-2/bax ratio and inducing activation of the caspase-3 pathway in growing and developing follicles via nAChRs [32]. The deleterious effects of the co-administration of alcohol and nicotine to disrupt the balance between ovarian production and antioxidant capacity can also be attributed because these agents are potentially capable of producing free radicals, ROS (reactive-oxygen-species), systemic and follicular fluid OS. Intra-follicular OS can induce apoptosis of follicular granulosa cells via damaging cell membrane, mitochondria, and finally DNA fragmentation (detected by TUNEL assay), so leads to disruption of folliculogenesis process and follicle loss [34–39].

A significant decrease in the number of corpora lutea in the nicotine + ethanol-treated group indicated a reduced ovulation rate which leads to follicular atresia. The main metabolite of nicotine is cotinine, which was detected in follicular fluid. A study revealed that cotinine involves in the suppression of granulosa–luteal cell performance, causing corpus luteal apoptosis by a reduction in progesterone synthesis [40]. Another study also indicated that ethanol causes a decrease in the formation of corpora lutea or cause its absence because growing follicles never became mature. Thus they could not ovulate and form a new corpus luteum [41].

Placenta has an important role in many regulatory functions for pregnancy maintenance during the fetal period. Based on the results of the present study, both cigarettes and alcohol have adverse effects on the placenta and fetus during pregnancy. In the present study, ethanol and nicotine increased placental/fetal weight ratio and placental size. According to experimental investigations, a reduction in placental weight

is macroscopically considered as a small placenta. Several direct placental damages such as mitotic suppression, degeneration and/or necrosis of trophoblasts, apoptosis prevent placental development, therefore leading to a small placenta. Furthermore, mitotic suppression and apoptosis of trophoblasts in the labyrinth zone cause a reduction in placenta size and intrauterine growth retardation with or without some abnormalities [42]. In our research, we observed changes in the placental layers in treatment groups, which indicated the adverse effects of ethanol and nicotine on the placenta. The decidua basalis thickness reduced significantly in treated groups in comparison to the control group. The thickness of the basal zone decreased significantly in the nicotine-treated group when compared to the control, also an insignificant decrease was observed in the ethanol and Ni + EtOH-treated groups. Significant reduction of labyrinth zone thickness was observed in the nicotine and nicotine + ethanol-treated groups in comparison with the control group. Based on the results, our hypothesis is that nicotine was more destructive than ethanol in the Ni + EtOH group. Nicotine and its metabolites can quickly pass through placental-blood-barrier as early as 7 weeks gestation and accumulate in fetal compartments and amniotic fluid [5]. Nicotine causes a reduction in vascularization and cell proliferation in the placental labyrinth zone via raising its hypoxic environment. All of these events may damage trophoblast invasion and fetomaternal circulation [43]. Nicotine acts via nAChRs, which are widely expressed on all placental cells and vessels. When nicotine crosses through the placenta, binds to a-subunit of nAChRs, and activates them, then nAChRs increase intracellular calcium $[Ca^{2+}]_i$ that leads to intracellular signaling disconnection, apoptotic pathways activation, or organelle actions disruption which causes ROS production and then OS happens [44–46]. Also nicotine could change the expression of some placental microRNAs [47] which play several roles in many biological processes such as regulation of trophoblast differentiation, emigration, invasion, programmed cell death and angiogenesis [48]. Researches demonstrated that ethanol causes a decrease in the size of the zone and degenerative alterations in both labyrinthine and basal zones also interrupt blood flow [49]. Ethanol also causes damages in mitochondrial function, thus leads to OS, DNA impairment, lipid peroxidation, and uncontrolled expression of prolactin family hormones in placental trophoblasts [50].

5. Conclusions

According to the present study data, the co-administration of ethanol and nicotine during intrauterine development and lactation periods leads to significant placental damage and a decrease in the number of ovarian follicles. The reduced ovarian follicular reserve is likely to result in early-menopause in addition to decreased fertility.

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Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The present work was granted by the Kerman Neuroscience Research Center, Kerman, Iran [number: 95000490].

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This work was supported by Kerman University of Medical Sciences, Kerman, Iran (95000490).

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