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Original Article

Effect of nicotine exposure during gestation on neonatal rat ovaries

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Abstract: The purpose of present study was to investigate effects of nicotine on follicular development in the neonatal rats. In this study, 14 adult Sprague-Dawley rats weighing 230-260 g (±10 g) were used as experimental animal. After detecting pregnancies with vaginal smear method, pregnant rats were two equal groups were separated.The pregnant females were treated with the nicotine for 21 days and litters were sacrificed at first day of birth. The ovaries of litters were processed through routine paraffin embedding method, serially sectioned by microtome, stained with hematoxylin-Eosin and immunohistochemical technique investigated by light microscope.In the ovary of group treated nicotine, Preventing follicular development was observed in granular degeneration and luteal cells. It was also observed in inflammation and bleeding vessels. Immunohistochemical localization of vimentin was restricted to the granulosa cells and stromal area. Nicotine treatment group in granular cells and luteal cells in E-cadherin expression was weak. We concluded that nicotine might affect the cellular junctions in the ovarian follicular development. The high intensity in the staining with vimentin observed in the granulosa cells of the follicles is probably due to functional changes that occur during the process of cystogenesis.

Keywords: Nicotine, neonatal rat, e-cadherin, vimentin, VEGF

Introduction

Smoking during pregnancy is one the most important problems in public health globally that causes different harmful outcomes such as intrauterine growth retardation, cardiovascular diseases, and abortion in fetus accompanied with some complications in mothers [1, 2]. Nicotine passes from placenta and enters in fetus circulation and causes an increase in vascular resistance and vasopressin that result in umbilical vasoconstriction [3, 4]. Paulson et al. [5], demonstrated a direct relationship between nicotine dose and decrease of the fetal weight, number of resorptions and malformations, embryotoxicity and intrauterine growth retardation. Cigarette smoking is thought to affect female fertility via a number of alterations in ovarian function, including abnormal steroidogenesis, depleted ovarian reserves and increased frequency of oocyte chromosomal abnormalities [6, 7]. Data from animal studies suggest that nicotine exposure may be a critical component in the development of adverse reproductive effects in the offspring of women who smoke. In rat the ovarian development begins before birth and among the initial and the main changes, the formation of follicles is noted [8]. In our animal model, nicotine exposure during fetal and neonatal development resulted in reduced fertility, dysregulation of ovarian steroidogenesis, and altered follicle dynamics in female offspring [9]. Vimentin filaments are more broadly distributed among tissues such as in the cells of the mesenchymal origin, certain other non-epithelial cells of various other tissues [10-13].

Material and method

The study protocol was approved by the Animal Research Committee of Dicle University, Turkey. 14 adult Sprague-Dawley rats weighing 230-260 g (±10 g) were used as experimental ani-

Table 1. Comparison between vascular and follicular diameter

	Control group (n=7)	Nicotine group 2 (n=7)	P (MWU)
	mean (SD) median (SEM) min-max		
Vascular diameter (μ)	7.28 (0.75)	10.0 (0.97)	0.001
	7.40 (0.23)	10.1 (0.30)	
	6.03-8.34	7.90-11.0	
Follicular diameter (μ)	15.4 (1.29)	11.6 (1.16)	0.001
	15.8 (0.41)	11.7 (0.36)	
	13.1-17.3	9.41-13.4	

SD: Standard Deviation, SEM: Standard Error of the Mean, p: Significance, MWU: Mann-Whitney U Test, Min: Minimum, Max: Maximum, μ: Micrometer.

mal. The animals were group- housed (7 per cage) under standard conditions in the Animal Health and Research Center of Dicle University. The animals were fed ad libitum with water and standard laboratory animal diet, under the care of trained wardens. After detecting pregnancies with vaginal smear method, pregnant rats were two equal groups were separated. The rats of experimental group (n=7) were nicotinized systemically with nicotine sulphate (Sigma, Aldrich), 2 mg/kg subcutaneously, daily in period of 21 days. The rats of group control (n=7) was used as control and did not receive NIC, but were maintained in similar environment and food. The pregnant females were treated with the nicotine for 21 days and litters were sacrificed at first day of birth.

Histological examination

The ovaries of litters were examined for histopathological changes. The samples were placed in 10% formaldehyde and dehydrated in 70-100% ethanol series. They were then placed in paraffin baths at 58°C for paraffin inclusion. Sections of 4-6 *μ*m were prepared from paraffin blocks using a rotary microtome. These sections were then stained with Hematoxylin-Eosin (H-E) and photographed using an Nikon microscope.

Immunohistochemical staining

Antigen retrieval process was performed in citrate buffer solution (pH=6.0) two times first 7 minutes, later 5 minutes boiled in microwave oven at 700 W. They were allowed to cool to room temperature for 30 minutes and washed in distilled water for 5 minutes two times. Endogenous peroxidase activity was blocked in 0.1% Hydrogen peroxide for 15 minutes. Ultra V block (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 10 minutes prior to the application of primary antibodies (E-cad antibody and Vimentin mouse monoclonal, 1/200, Santa Cruz) for overnight. Secondary antibody (Histostain-Plus Kit, Invitrogen, Carlsbad, CA) was applied for 20 minutes. Slides then were exposed to streptavidin-peroxidase for 20 minutes. Diaminobenzidine (DAB, Invitrogen, Carlsbad) was used as a chromogen. Control slides were prepared as mentioned above but omitting the primary antibodies. After counterstaining with Hematoxylin, washing in tap water for 5 minutes and in distilled water for 2 × 5 minutes, the slides were mounted.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences for Windows (version 15.0, SPSS Inc.,Chicago, IL, USA). The Mann-Whitney U test was used for the statistics as indicated, test and results were expressed as mean ± SD. *P* values below 0.05 were considered to indicate statistical significance.

Result and discussion

Nicotine group and the control group were compared in terms of follicles diameter and vessel diameter (**Table 1**).

In the control group, histological analysis of the ovaries fetus showed ovarian oocytes were distributed in the form of a ring. The ovaries of the neonates from the control group were coated by a well-developed germinal epithelium consisting of cubic cells over a thin layer of dense connective tissue forming the tunica albuginea. primordial follicles, and the large follicles observed contained a single layer of granular cells in neonatal rats at postnatal day 1 (**Figure 1A**). In the ovary of group treated nicotine, Preventing follicular development was observed in granular degeneration and luteal cells. It was also observed in inflammation and bleeding vessels (**Figure 1B**).

Some other studies have reported the effects of prenatal exposure of rats to nicotine and its

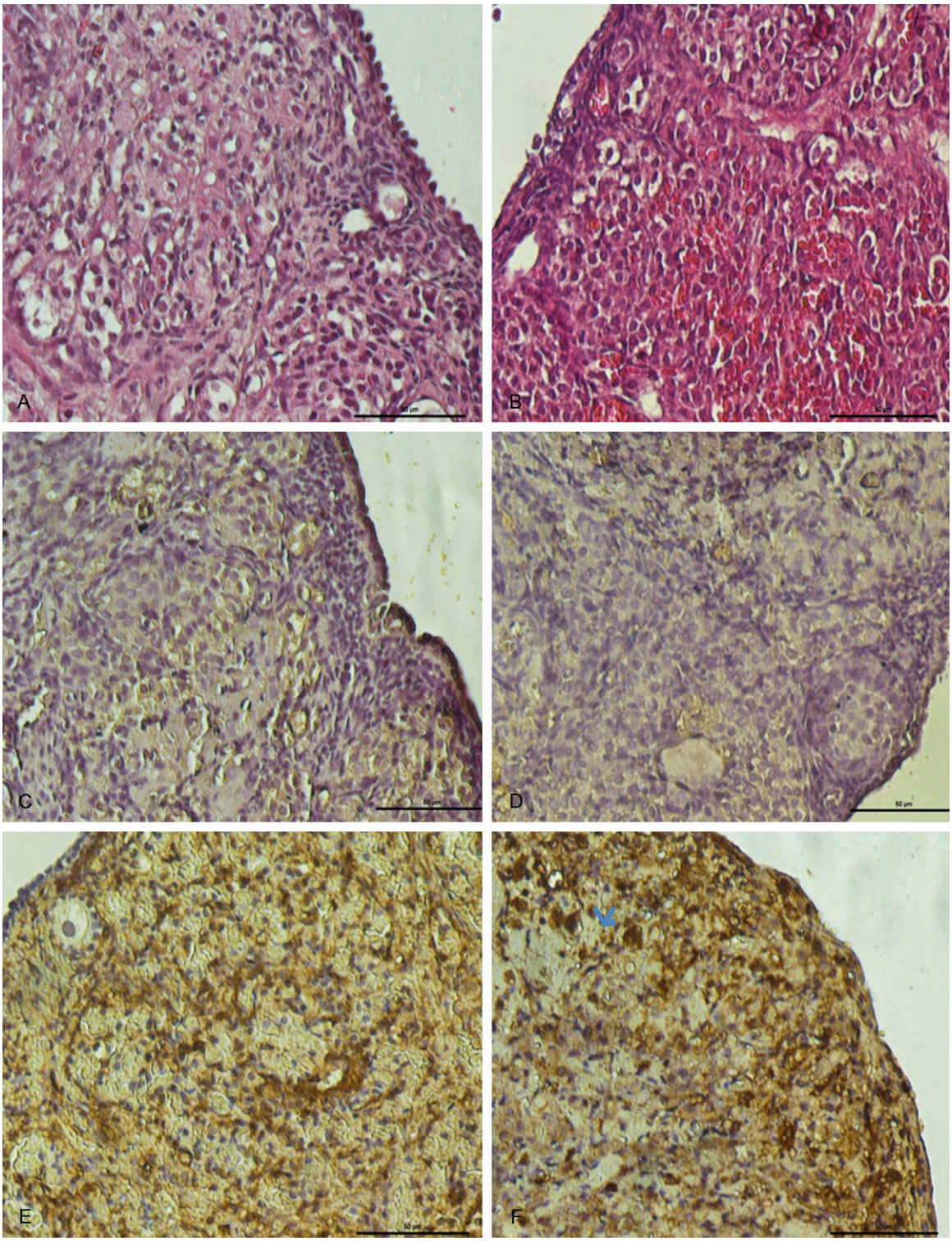

Figure 1. Comparisons of figures in groups. A. Control group, The normal appearance of germinal epithelium, follicular cells. and oocytes H-E staining Bar 50 μm. B. Nicotine group, Degeneration of the germinal epithelium and granular cells, In blood vessels between the stromal cells, hemorrhage and distributed free erythrocytes H-E staining Bar 50 μm. C. Control group At postnatal day 1, The cells in the germinal epithelium and primary follicles expression of E-cadherin positive E-cadherin immunstaining Bar 50 μm. D. Nicotine group At postnatal day 1, E-cadherin immunoreactivities were markedly localized to the oocytes (arrows). but E-cadherin immunoreactivity was weak. E. Control group, positive VEGF expression in granulosa and luteal cells Vimentin immunstaining Bar 50 μm. F. Nicotine group, positive Vimentin expression in inflammatory cells around the degenerative follicles (arrow).

associated effects on cardiovascular system such as tachycardia, arrhythmia, ischemia, and atherosclerosis [14-17]. In adult humans, cotinine, the metabolite of nicotine, has been detected in the follicular fluid of women who smoke [18, 19] demonstrating that nicotine has access to the ovary and the developed follicles. In pregnant women who smoke or use NRT, nicotine crosses the placenta, concentrates in fetal blood and amniotic fluid, and is detectable in breast milk during lactation resulting in both fetal and neonatal exposure to nicotine.

Negi G, Kumar A and Sharma SS, reported that prenatal application of nicotine causes arterial hypertension of embryo, reduction of oxygen saturation of the blood, and reduction of palpitation in the embryo [20]. In vivo and in vitro studies have clearly shown that nicotine alone can have adverse effects on adult ovarian function, including an increased number of atretic follicles, reduced ovarian and uterine weights, and irregularities in the estrous cycle [21, 22]. The same results were found by Sami MM, Abdel Hadii RH, Abdel Samaei AR, Saad Eldien HM [23]. In examining the effect of nicotine on pregnant female rats at the end of the pregnancy, which observed a decrease in the number of uterine glands and the thickness of the endometrium and myometrium. Nicotine treatment group in granular cells and luteal cells in E-cadherin expression was weak.It was observed that weaken the connections between the cells. Vimentin is a type of intermediate filament and the major cytoskeletal component of mesenchymal cells including ovarian granulosa cells that support oocyte growth and development [24].

Vimentin has been localized in the granulosa cells of healthy. Immunohistochemical localization of vimentin was restricted to the granulosa cells and luteal cells The high intensity in the staining with vimentin observed in the granulosa cells of the follicles is probably due to functional changes that occur during the process of cystogenesis (**Figure 1D**). E-cadherin (E-cad) is a cell-cell adhesion transmembrane molecule. It plays important roles in cell adhesion and morphogenesis [25]. In addition, in the wound re-epithelialization mechanims, the involvement of E-cadherin especially in controlling cellular polarity [26], differentiation, growth and migration is crucial [27]. In our study we observed that E-cadherin expression was significantly decreased in nicotine group, as compared to non-treated group (**Figure 1F**). We concluded that NIC might affect the cellular junctions in the ovarian follicular development.

Disclosure of conflict of interest

None.

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