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Original Research

Effects of nicotine exposure on clomiphene citrate induced rats: morphological and immunohistochemical analysis in the ovaries

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Summary

Objective: The most common use of ovulation induction in clomiphene citrate (CC) administered rats is to investigate whether there is any morphologically and immunohistochemically difference in nicotine exposure between rats not exposed to nicotine and with no CC. *Materials and Methods:* A total of 24 healthy rats were randomly divided into three groups: Group 1 was the group that received transdermal nicotine patched followed by clomiphene citrate. Group 2 was the only intraperitoneally CC applied group. Group 3 was the normal saline administered intraperitoneally group. On the fourth day of the cycle animals were sacrificed and bilateral salpingo-oophorectomy was performed. Sections were taken and stained with standard haematoxylin for histopathological examination. For immunohistochemical evaluation, sections were stained with Ki-67 and CD34. *Results:* There were no significant differences between the groups in terms of ovarian follicular number and types, and follicle-stroma immunohistochemical staining Ki-67 expression. There was also no significant difference in the thickness of granulosa cell between the groups. However, among the groups, CD34 expressions in group 1 were statistically less in the secondary follicle ($p = 0.000$), corpus luteum ($p = 0.012$), and more in the ovarian stroma ($p = 0.001$). *Conclusion:* In the CC-stimulated animal model, the authors did not observe that transdermal nicotine exposure was morphologically deleterious to the follicular count and types. They also could not detect the thickness of granulosa cell. Perhaps the effect on over-stimulation with CC may be less than expected, depending on the route and dose of nicotine administered.

Key words: Nicotine; Clomiphene citrate; Ki67 and CD34 expression; Ovulation induction.

Introduction

A healthy ovulation is critical in terms of health and fertility of women in the reproductive period. Whereas, in infertility which affects approximately 15% of the couples, one fourth of the problems is caused by the ovulatory factors [1-2]. Clomiphene citrate (CC) is usually used as the first choice for anovulation treatment for a long time. Moreover, CC is very inexpensive, easily accessible, and well-tolerated by patients [3-6].

There may be some confounding factors that affect success in the use of CC such as obesity, alcohol use, and smoking [7]. Smoking is seen in about 25-33% of women in the reproductive age [8, 9]. Active smoking may reduce natural conception by about 80% [10]. Cigarette contains over 4,000 chemicals and it is not clear which of these are particularly effective on ovulation [11, 12]. Furthermore, levels of these toxicants in target organs may be different from their serum levels [13, 14].

Nicotine, one of the most studied components of cigarette, partially inhibit follicular growth during all stages [15, 16]. In a study by Mailhes *et al.* with mouse oocytes, nicotine was reported to increase the rate of aneuploidy in the in vitro media and to cause premature separation of sister chromatids [17]. Liu *et al.* also showed high likelihood of aneuploidy due to deterioration of meiosis in bovine oocytes exposed to in vitro nicotine [18].

The effect of nicotine on steroidogenesis may be dose-dependent, such as on oxidative stress [16]. However, steroidogenesis is impaired due to granulosa cell apoptosis produced by nicotine rather than angiogenesis disruption and accordingly, the production of androgen and progesterone decreases [16, 19, 20]. Although previous studies have shown that smoking may inhibit ovulation because of the impaired angiogenesis [21], Bordel *et al.* demonstrated that nicotine did not disrupt neovascularization [16].

Some dysplastic changes may develop in the ovary that are a result of excessive proliferation and increased angiogenesis when CC is used for induction of ovulation [22]. In this context, both smoking and CC use can cause synergistic dysplastic changes in ovarian tissue and negatively affect follicular growth. Ki-67, the best proliferation marker which can detect these changes [23] and CD34 which is used as a panendothelial cell marker in the assessment of angiogenesis [24] may show the changes occurring in the

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ovaries due to both ovulation induction and smoking.

Based on this information, the objective of this study is to investigate whether there is any morphological or immunohistochemical differences between the rats administered CC exposed to nicotine, and the rats that were not given CC and not exposed to nicotine. In addition, the authors aimed to shed light on how follicular growth is affected by the complex association between smoking and ovulation induction.

Materials and Methods

This study was conducted at the animal laboratory of the Veterinary Faculty of Bezmialem Foundation University in 2016. The ethics committee of the same university approved the study in June 2016.

A total of 24 healthy rats were used. The reason for choosing this type of rats was that they have been used in similar previous studies [25, 26]. All animals weighed 200-250 grams on average and had regular four-day estrous cycles. The animals were kept at 12 hours light/dark cycle at 22°C and they had free access to food and water.

Rats were randomly divided into three groups. Group 1: The group which firstly received nicotine and then CC; a total of eight rats were shaved from the nuchal area and 1 mg/kg/day nicotine band was applied by changing every day for three consecutive cycles before the use of CC. As demonstrated in earlier acute toxicity studies, it has been reported that application of nicotine at this dose is non-toxic [26]. No nicotine-dependent side effects were observed in the rats. Then, rats were intraperitoneally injected with 1 mg/kg/day CC diluted in saline, starting from the first day of the cycle for 3 days. Group 2: The group which received CC only; again, eight rats were intraperitoneally administered 1 mg/kg/day CC from the first day of the cycle for three days. Group 3: Randomly selected eight rats were intraperitoneally given normal saline as 2.5 mL/kg/day in the first three days of the cycle for three days.

All rats were killed by 60 mg/kg xilazyne (100 mg/ml) following intraperitoneal injection of 2 ml/kg ketamine (50 mg/ml) on the forth day of the cycle. Each rat was laid on its back and bilateral salpingo-oophorectomy was performed. The ovaries were placed in a 10% formol solution and sent to the Medipol University Medical School, Pathology Laboratory for examination.

Ovaries of each material were sampled. The samples were embedded into paraffin blocks after tissue processing. The numbers of total primordial follicles (the follicle where the oocyte is surrounded by with the flattened granulosa cells), primary follicles (the follicle where the oocyte is surrounded by a single layer of granulosa cells), preantral-antral follicles (the follicle surrounded by two or more layers of granulosa cells and containing antral cavity) and the corpus luteum were noted on 4-micron thickness hematoxylin eosin stained sections under light microscope at ×200 magnification. The thicknesses of the granulosa cells in corpus luteum were measured in micrometers with microscope using NIS-elements program.

Four micrometer thick ovarian sections were immunohistochemically stained with CD34 and Ki67 antibodies using an automated device. Diaminobenzydine was used as a chromogen. The Ki67 immunoreactivity was expressed as percentage (%) in the area in follicles and stroma where it had the highest value. CD34 where the endothelial cells were stained were calculated by counting the vessels at 1 mm^2 in the secondary follicle, corpus luteum, and ovarian stroma.

Statistical analyses were performed using SPSS v20. Continuous variables are presented as mean ± standard deviation (SD) or median (range) while categorical variables were expressed as frequencies (%). Multi-group comparisons of continuous variables were performed by one-way ANOVA test, and Dunnett's post hoc test was run to confirm where the differences occurred between groups when it was shown an overall statistically significant difference in group means. A two tailed p-value < 0.05 was considered statistically significant.

Results

There was no statistically significant difference between the groups in terms of the number of follicular types and the thickness of the granulosa cells in the follicles. In the groups administered CC, the mean number of corpus luteum was higher than the groups which did not receive CC, the difference was not statistically significant ($p=0.093$) (Table1, Figures 1 and 2).

Immunohistochemical examination revealed that, although Ki67 expression was higher in the follicles than in the stroma; difference between the groups was not statistically significant (p = 0.68, p = 0.61; respectively) (Figure 3). CD34 expression in the secondary follicles was significantly different among all groups (p = 0.000). In the detailed analysis of the differences between the groups, CD34 expression was found to be significantly lower group 1 compared to both groups 2 (p = 0.008) and 3 (p = 0.000) (Figure 4). There were again statistically significant difference between the groups in terms of CD34 expression in the corpus luteum (p = 0.012). Although not statistically significant, CD34 expression in the corpus luteum was found to be lower in group 1 than in group 2 (p = 0.735). However, when compared with the group 3, CD34 expression in the corpus luteum was quite significantly lower in group-1 (p = 0.009) (Figure 5). In addition, differences between the groups in terms of CD34 expressions in the stroma had statistical significance (p = 0.001). Here also group 1 was the group which caused the difference. When groups 1 and 3 were compared, although the difference between them did not reach statistical significance, stroma showed higher CD34 expression in favour of group 1 (p = 0.572). However, the authors found that CD34 expression in the stroma was statistically significantly higher in group 1 compared to group 2 (p = 0.001) (Tables 1 and 2, Figure 6).

Discussion

In has been found in the CC-stimulated animal model that previous exposure to transdermal nicotine does not have a significant negative effect on the number of the follicular types in the ovary, and the thickness of the granulosa cells in corpus luteum was not changed by transdermal nicotine band application. When examined immunohistochemically,

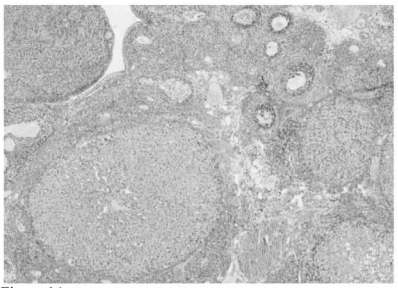


Figure-1A

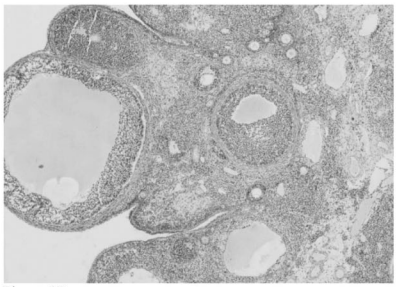


Figure-1B

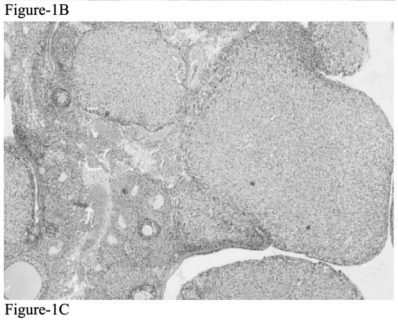


Figure-1C

Figure 1. — Ovarian sections showing follicles in group 3 (A), group 2 (B), and group 1 (C). (HE×40)

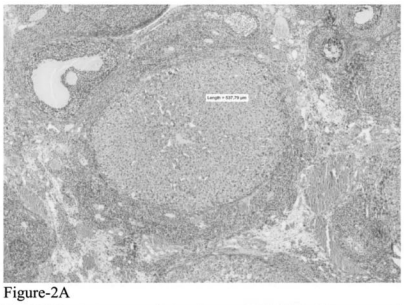


Figure-2A

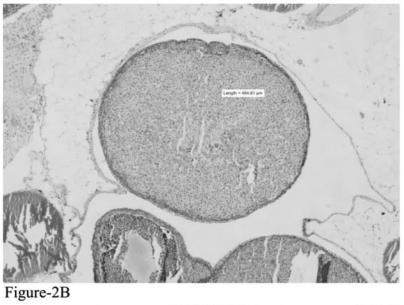


Figure-2B

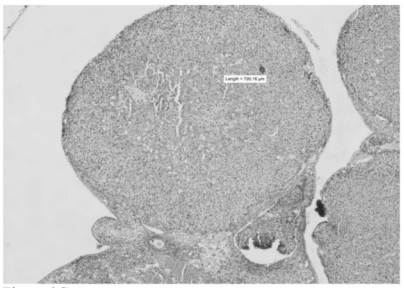


Figure-2C

Figure 2. — Granulosa cell thickness in group 3 (A), group 2 (B), and group 1 (C). (HE×40)

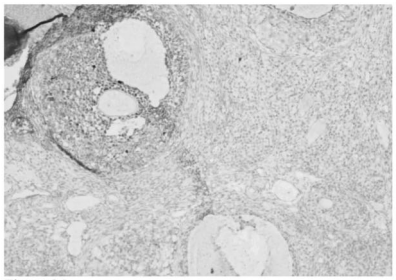
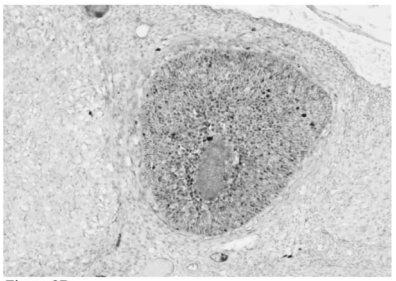


Figure-3A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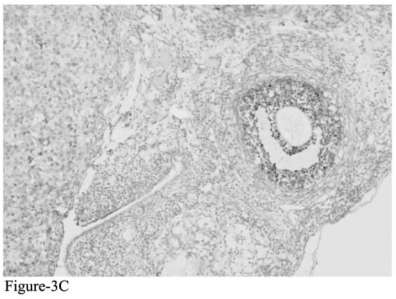
Figure-3C

Figure 3. — Ki67 expressions in follicles and stroma of the ovaries in group 3 (A), group 2 (B), and group 1 (C). (Ki67×100)

Table 1. — *Comparison of follicular and corpus luteum counts, the thicknesses of granulosa cells, and immunohistochemical findings among groups.*

	Group 1 (n=8)	Group 2 (n=8)	Group 3 (n=8)	*p* value
Primordial follicular count	5.2 (5± 3.05)	4.7 (4.5± 1.82)	4.5 (4± 2.32)	0.83
Primer follicular count	8.2 (8.5± 2.81)	9.3 (9± 3.88)	7.6 (7.5± 2.97)	0.56
Preantral follicular count	7.5 (8± 3.02)	9 (9± 3.77)	5.5 (4.5± 2.82)	0.12
Antral follicular count	8.6 (8.5± 4.10)	9.8 (9.5± 3.94)	9.5 (8.5± 4.17)	0.82
Corpus luteum count	23 (22.5± 6.67)	16.1 (14± 9.3)	15.5 (15.5± 4.98)	0.09
Granulosa thickness (µm)	548.12 (536.5± 98.47)	497.75 (513± 69.90)	573.87(546.5± 150.54)	0.39
Ki67 follicle (%)	4.5 (4± 1.41)	5.1 (5.5± 1.95)	5.1 (4.5± 1.55)	0.69
Ki67 stroma (%)	1 (1± 0.00)	1.1 (1± 0.35)	1.1 (1± 0.35)	0.61
CD34 seconder follicle (1 mm²)	0.87 (1±0.64)	2.1 (2±0.64)	2.8 (3±0.99)	0.00*
CD34 corpus luteum (1mm²)	8.5 (8±3.54)	9.5 (10±1.6)	13.2 (13±3.49)	0.01*
CD34 stroma (1mm²)	14.1 (14.5±5.54)	6.6 (6.5±1.4)	12.5 (12.5±2.39)	0.00*

* = $p \leq 0.05$ *statistically significant*

it has been determined that the use of nicotine band did not change the expression of Ki67 which is the known best proliferation marker. However, it has been found that with the use of transdermal nicotine band, expression of CD34 which is a panendothelial cell marker, is low in the secondary follicle and corpus luteum but high in the stroma.

The ovulation process, one of the most important stages of fertility, is highly sensitive to potentially toxic agents. Smoking may lead to a decrease in the number of ovarian follicles in smokers compared to non-smokers [27]. Dif-

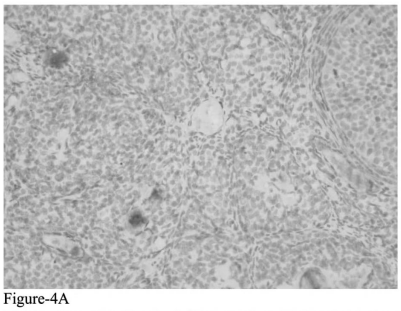
Figure-4A

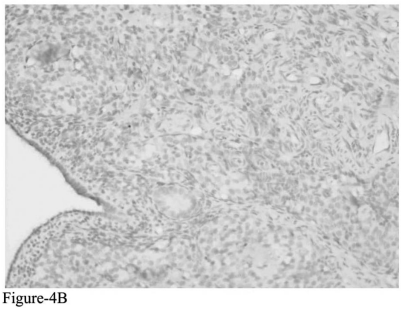
Figure-4B

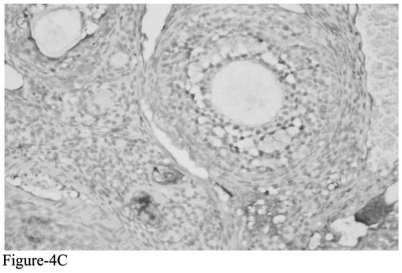
Figure-4C

Figure 4. — CD 34 staining in the secondary follicles of group 3 (A), group 2 (B), and group 1 (C). (CD34 ×200)

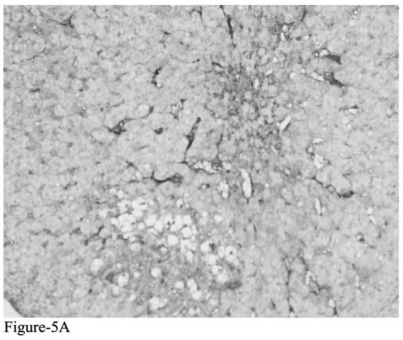
Figure-5A

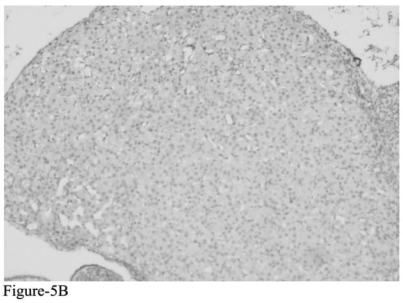
Figure-5B

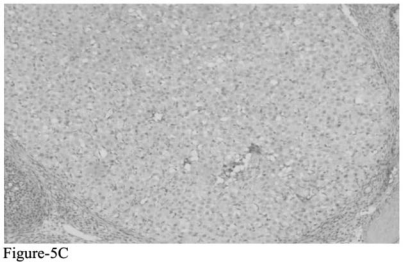
Figure-5C

Figure 5. — CD34 staining in the corpus luteum of group 3 (A), group 2 (B), and group 1 (C). (CD34×100)

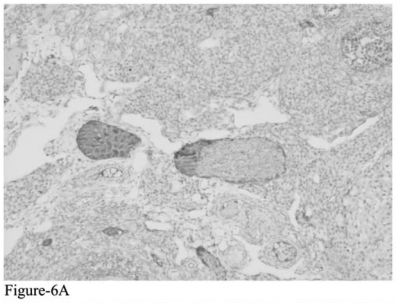
Figure-6A

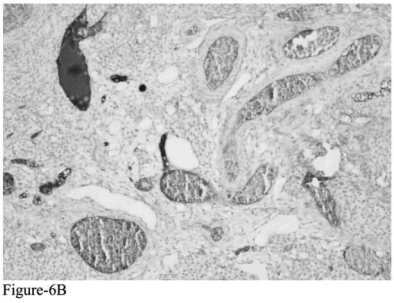
Figure-6B

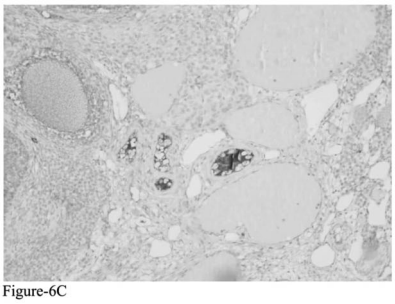
Figure-6C

Figure 6. — CD34 expression in the stroma of the ovaries in group 3 (A), group 2 (B), and group 1 (C). (CD34×100)

ferent animal studies supporting these findings have been conducted. Mohammadghasemi *et al.* examined follicle numbers by exposing mouse ovaries to nicotine and contrary to the present study, they reported that the number of follicles, especially other than primordial follicles, were greatly reduced by the effect of nicotine [28]. They noted that this situation might be a result of the apoptotic effect of on the granulosa cells, and thus its negative effect was seen on the more growing follicles. Likewise, Tuttle *et al.* showed that smoking causes follicular loss in the mouse ovaries [29]. However, unlike this information in the present study the authors determined that the number and types of follicles were not affected by nicotine exposure. Moreover, transdermal nicotine administration did not change the number of growing follicles affected by CC. This may be related to the dose and administration way that the present authors used in this study. In addition, since thousands of toxic substances (carbon monoxide, cadmium, benzapyrine, etc.) in the cigarette are not found in the nicotine bands, as reported by studies on nicotine replacement therapy, some of the harmful effects of cigarette smoking reported in the literature might not be observed in this study [30].

As we know, nicotine acetylcholine receptors (nAChR-2) have been identified in granulosa cells [31]. Both clinical and animal studies have reported that smoking has deleterious effects on granulosa cells through these receptors. Freour *et al.* found that active smoking disrupts granulosa cell function, reducing ovarian reserve and IVF outcomes [27]. Bordel *et al.* also showed in rats administered subcutaneous nicotine that dose-dependent apoptosis occurs in granulosa cells. They found that the involvement of anti-PCNA (proliferating cell nuclear antigen) antibody, which they used as an apoptotic cell parameter, was quite higher in rats given high doses of nicotine compared to the rats administered a low dose or no subcutaneous nicotine. Thus, they emphasized the fact that nicotine stimulates the granulosa cell apoptosis in a dose-dependent manner [16]. Although the present authors did not use any apoptotic marker in this study, they did not find a detrimental effect of nico-

Table 2. — *Post-hoc analysis of groups and variables.*

Dependant variable	Groups		Std. Error			95% CI		*p value*
Primordial follicular count	Group-3	Group-1	-0.75	⁊	1.229	-3.66	2.16	0.769
	Group-2	Group-1	-0.50	⁊	1.229	-3.41	2.41	0.888
Primer follicular count	Group-3	Group-1	-0.62	⁊	1.630	-4.48	3.23	0.899
	Group-2	Group-1	1.12	⁊	1.630	-2.73	4.98	0.717
Preantral follicular count	Group-3	Group-1	-2.00	⁊	1.618	-5.83	1.83	0.375
	Group-2	Group-1	1.50	⁊	1.618	-2.33	5.33	0.560
Antral follicular count	Group-3	Group-1	0.87	⁊	2.037	-3.95	5.70	0.876
	Group-2	Group-1	1.25	⁊	2.037	-3.57	6.07	0.767
Corpus luteum count	Group-3	Group-1	-7.50	⁊	3.607	-16.04	1.04	0.089
	Group-2	Group-1	-6.87	⁊	3.607	-15.42	1.67	0.124
Granulosa cell thickness (μm)	Group-3	Group-1	25.75	⁊	55.71	-106.30	157.80	0.858
	Group-2	Group-1	-50.37	⁊	55.71	-182.42	81.67	0.575
Ki67 follicle (%)	Group-3	Group-1	0.62	⁊	0.82	-1.34	2.59	0.675
	Group-2	Group-1	0.62	⁊	0.82	-1.34	2.59	0.675
Ki67 stroma (%)	Group-3	Group-1	0.12	⁊	0.14	-0.21	0.46	0.600
	Group-2	Group-1	0.12	⁊	0.14	-0.21	0.46	0.600
CD34 second follicle (1 mm²)	Group-3	Group-1	2.00	⁊	0.38	1.08	2.91	0.000*
	Group-2	Group-1	1.25	⁊	0.38	0.33	2.16	0.008*
CD34 corpus luteum (1 mm²)	Group-3	Group-1	4.74	⁊	1.50	1.17	8.32	0.009*
	Group-2	Group-1	1.00	⁊	1.50	-2.57	4.57	0.735
CD34 stroma (1 mm²)	Group-3	Group-1	-1.62	⁊	1.78	-5.86	2.61	0.572
	Group-2	Group-1	-7.50	⁊	1.78	-11.73	-3.26	0.001*

** = $p \leq 0.05$ statistically significant*

tine exposure in the thickness of granulosa cells in the corpus luteum in the rats they administered CC, possibly due to the dose and adminstration way of nicotine. Furthermore, contrary to previous studies, the present authors determined that the number of follicles and thickness of granulosa cells did not change also in rats with ovaries induced with CC alone. Chaube *et al.* reported that CC treatment caused atresia-like changes in rat ovaries. They indicated that this atresic effect caused the disruption of steroidogenesis in granulosa cells by CC [32]. Another study conducted by the same group one year later showed that atresic effects in granulosa cells were less when estradiol was co-administered with CC [33]. However, CC doses used by these authors in rats were 10 mg/kg in both studies, which is quite high compared to the dose used in the present study. In the present study, granulosa cells were not affected in both groups, possibly due to the dose we applied.

Ki67 antigen, one of the known most important markers to show cell proliferation, is expressed in all active phases of the cell cycle [23]. Therefore, it shows a good correlation with the mitotic indices of the cells [34]. In the present study, they examined this expression to investigate whether nicotine bands had any effect on cell proliferation. The authors were determined that nicotine did not affect Ki-67 expression in both the stroma and follicles of the ovary. However, Bordel *et al.* reported inhibition at every phase of follicular growth, especially in small-diameter follicles [16]. Although nicotine doses were the same with the present study, in that study subcutaneous administration way was preferred. The authors already reported in their studies that this administration method is not applicable for normal nicotine exposure and should be taken into account in interpreting the results [16]. In addition, Bordel *et al.* used PCNA as the marker of cell proliferation, different from the present. In the present study, Ki-67 expression, which the authors used as a proliferation marker, was not different between the groups, and might be due to the way the nicotine was administered. Interestingly, Ki67 expressions that were not statistically significant compared to the control group were determined in the present group-2. However, Lima *et al.* reported that Ki67 expressions after stimulation with CC were significantly higher than the control groups with more marked at the stromal levels [35]. Whereas, the dose and administration way of CC were the same in the present and their studies. But in their study, both carried out CC stimulation for two days and used 100 IU / kg hCG afterwards. Perhaps, rather than CC, what caused the changes was hCG effect or combined use of CC and hCG.

CD34 is a panendothelial cell marker specific to usually pre-existing vascular structures [34, 36]. Therefore, CD34 is of great importance in assessment of angiogenesis characterized by the formation of new capillaries in existing blood vessels. Since the physiological events such as folliculogenesis and corpus luteum formation in the female reproductive system are associated with angiogenesis, CD34 may provide important information of the ovary [37]. Smoking may have harmful effects by influencing the nAChR receptors on the endothelium and decreases the

numbers of vessels in the corpus luteum and follicles [38]. In the present study also CD34 expression was significantly decreased in the secondary follicle and corpus luteum of the rats which received transdermal nicotine compared to those did not receive it. The harmful effects of nicotine on normal folliculogenesis were also observed during CC induction. However, Bordel *et al.* reported that examining the follicular structures with microcirculation analyses after subcutaneous administration of nicotine at the same dose with that of the present authors, reported that this application had no significant effect on new vascular formation [16]. In fact, these different results might be due to the angiogenesis assessment method of that study that differed from the present. In addition, the present authors found that CD34 expression in the ovarian stroma was significantly higher in the rats given transdermal nicotine compared to those that did not receive it. As pointed out in previous studies, this supports the fact that it has a dose-dependent angiogenesis effect which is associated with microenvironment in the different regions of the ovary [16, 39].

In conclusion, it can be stated that the number of follicles was not affected, but the angiogenesis in the follicles was suppressed in the ovaries of the rats exposed to transdermal nicotine prior to over-stimulation with CC. However, the fact that thickness of granulosa cells in the corpus luteum was not affected by nicotine exposure may indicate that nicotine may have a more limited effect than expected during CC over-stimulation, perhaps due to the nicotine dose and the way it is administered. Finally, it can be stated that this study may shed a different light in explaining the complex relations between nicotine and ovarian function during stimulation.

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