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ORIGINAL ARTICLE

An animal model of effects of nicotine exposure on endometrial receptivity and embryo implantation in pregnancy

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ABSTRACT

Objective: This study aims at evaluating the endometrial receptivity in uterus of pregnant rats exposed to nicotine via examination of integrin expression by immunohistochemical effect.

Methods: In this study, 16 healthy pregnant rats were divided into two groups of control and study groups each comprising eight rats. The rats randomised to study group were given a certain amount of nicotine before and during the pregnancy. Integrin expression was detected in uterus of all rats by immunohistochemical staining. The effect of nicotine exposure on embryo implantation and the endometrial receptivity were immunohistochemically and pathologically evaluated.

Results: Comparison of both groups revealed no difference in living, viable foetuses. Intensity and universality of immunohistochemical staining of Integrin β3 for endometrial epithelium and endometrial stroma were detected to be identical between the groups.

Conclusion: No immunochemical effect was observed on integrin expression, which is a very important part of receptivity in an animal model created with pregnant rats that were transdermally exposed to nicotine. Our study demonstrated that the harmful effect of nicotine use before and pregnancy on implantation is limited at the level of integrin expression, in a dose-dependent manner and also by considering the method of administration.

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Endometrial receptivity; implantation; integrin; smoking; tobacco

Introduction

Endometrium is a very important and dynamic tissue in occurrence and continuation of pregnancy. There are numerous studies in the literature on endometrium that mostly examined its exposure in two groups as local and systemic factors [1]. The postovulatory phase, particularly days 6 and 10, is the window period where maximal endometrial receptivity takes place. During this period, stromal cells undergo pseudo-decidualisation reaction, while epithelial cells form a special structure where adhesion molecules play an active role [2,3]. Following the occurrence of pregnancy, the endometrial stroma required for pregnancy turns into the mesenchymal tissue with a specific epithelium [4].

Many molecular mediators that play an active role within such mechanisms were determined and studied in the literature, including adhesion molecules, cytokines, growth factors and lipids [5]. However, among these, the integrin β3 is differentiated as the most effective endometrial marker with the closest association with window phase. Integrins belong to the class of cell adhesion molecules and are endometrial markers that ensure connection with extracellular matrix, other cell adhesion molecules and matrix metalloproteinases [6]. Deficiency occurs in clinical conditions such as endometriosis, hydrosalpinges, polycystic ovary syndrome and reduces the endometrial receptivity [7]. In fact, reduced integrin secretion is one of the causes of unexplained infertility failures [8].

Despite increased public awareness regarding the adverse health effects of smoking, tobacco use remains prevalent in the home, public facilities, and the workplace. An estimated 27.2% of reproductive aged women smoke cigarettes, and the prevalence of smoking during pregnancy ranges between 15–30% [9]. Smoking while pregnant may result in serious outcomes that can cause many pregnancy complications including foetal death. However, many patients keep smoking despite the 4000 compounds and 600

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additives (e.g. carbon monoxide, cadmium, lead, benzene, nicotine, radioactive polonium-210) in the cigarette or are sometimes exposed to tobacco smoke through second-hand smoking before and during their pregnancy [10].

The potential effects of cigarette smoke compounds on the endometrium are not well known [11]. Nicotine, the major toxic ingredient of cigarette, is an alkaloid compound that is composed of pyridine and pyrrolidine ring and isolated from tobacco leaves. It was well-studied and demonstrated that it causes oxidative stress by stimulating the production of reactive oxygen species in the peripheral and central nervous system, thereby harms the critical structural form of brain as a neuroteratogen [12]. It causes harmful effects on placenta by disrupting the relationship between acetylcholine and its receptor. This has detrimental effects on blood flow, amniotic fluid and transfer of nutrients and is unfavourable for development of placental bed [13]. However, the effects of nicotine on endometrial receptivity and implantation are not clarified.

Cigarette exposure is known to negatively affect the embryo adherence to endometrium, causing early abortion [10]. We designed this study to examine whether the negative effects of cigarette on implantation are caused by any of its toxic ingredients or the main ingredient, nicotine. We aimed at investigating the effects of nicotine exposure on endometrium by exposing rats to nicotine patch. We believe that the possible results of this study will provide an insight into further studies regarding the use of nicotine patch both when planning and during the pregnancy in patients who are addicted to cigarette.

Materials and methods

Experimental animals and study design

A total of 16 healthy rats with a body weight of 200–250 g were taken from the Bezmialem Vakif University, Veterinary School, Animal Laboratory after obtaining the approval of ethics committee of the same university. Animals were kept in a room with a 12-h light and dark cycle at 22 °C with *ad libitum* access to food and water.

Rats were divided into two equal groups as control and transdermal nicotine exposure (TD-NIC-e) study group. Animals in TD-NIC-e group were shaved and exposed to transdermal nicotine at a dose of 1 mg/kg/day corresponding to a total of three cycles before pregnancy. Acute toxicity studies were not conducted as this dose was determined based on the dose used in the previous studies [14]. No nicotine-related side effect was observed in rats throughout the trial.

Transdermal patches were replaced daily. At the end of three cycles, oestrous female rats selected via the vaginal smear method were caged with male rats at a ratio of 1:1 overnight. The next morning, female rats were individually assessed, and the day of detection of the vaginal plug or sperm-positive smear was designated as first day of pregnancy. All rats get pregnant in both groups. TD-NIC-e were continued till 19th day of pregnancy. All rats were laparotomised under light ether anaesthesia on the 19th day of pregnancy. Both horns of the uterus were observed for the number of implantation sites, resorption and dead or alive foetuses. The observations of the TD-NIC-e study group were compared with those of control group.

Immunohistochemical (IHC) evaluation of endometrial epithelium and stroma

All preparations were stained using conventional streptavidin–biotin technique. All the 1.5 mm and 3 mm cores of tissue array specimens embedded in paraffin slice on coated slides were washed in xylene to remove the paraffin, rehydrated through serial dilutions of alcohol, followed by washings with a solution of PBS (pH 7.2). All subsequent washes were buffered via the same protocol. Treated sections were then placed in a citrate buffer (pH 6.0) and heated in a microwave for three 5 min sessions. The samples were then incubated with a rat monoclonal anti-integrin b3 antibody (EPR2417Y, ab75872, Abcam, Cambridge, UK, 1:150 dilution) for 60 min at 25 °C. The conventional biotin–streptavidin method (Thermo, Ultravision anti-Polyvalent HRP/DAB Kit TP-015-HD) was performed for signal development, and the cells were counterstained with haematoxylin. Positive controls were simultaneously obtained by staining tissues of the tonsil.

Statistical analysis

The data were analysed using the SPSS version 19 (SPSS Inc., Chicago, IL). The values were expressed as numbers and percentages. The categorical variables were compared using chi-square test. For permanent data, Kruskal–Wallis test and one-way analysis of variance (ANOVA) were used, and the results were expressed as median, minimum, maximum. $p < 0.05$ was considered statistically significant.

Results

The mean number of total and living foetuses were listed in Table 1. No difference was detected between

Table 1. Comparison of foetuses and living foetuses among groups.

	Control ($n = 8$)	TD-NIC-e (1 mg/kg) ($n = 8$)	p
Number of foetuses Median (min–max)	10 (5–13)	9 (4–12)	>0.05
Number of living foetuses Median (min–max)	8 (3–12)	8 (3–11)	>0.05

Table 2. Comparison of integrin β3 staining intensities and universalities endometrial stroma and epitelium of groups.

	Control ($n = 8$)	TD-NIC-e (1 mg/kg) ($n = 8$)	p
Endometrial epithelium			
Staining intensity			
Absent	0 (0.0%)	0 (0.6%)	>0.05
Light	3 (37.5%)	3 (37.5%)	
Dark	5 (62.5%)	5 (62.5%)	
Staining universality			
Absent	0 (0,0%)	0 (0.0%)	>0.05
≤50%	1 (12.5%)	2 (25%)	
>50%	7 (87.5%)	6 (75%)	
Endometrial stroma			
Staining intensity			
Absent	0 (00.0%)	1 (12.5%)	>0.05
Light	2 (25%)	2 (25%)	
Dark	6 (75%)	5 (62.5%)	
Staining universality			
Absent	0 (00.0%)	1 (12.5%)	>0.05
≤50%	3 (37.5%)	2 (25%)	
>50%	5 (62.5%)	5 (62.5%)	

the groups in terms of number of total and living foetuses (Table 1). Intensity and universality of immunohistochemical staining of Integrin β3 for endometrial epithelium and endometrial stroma were detected to be identical between the groups (Table 2, Figure 1).

Discussion

We conducted this study to determine whether the negative effects of cigarette on embryo implantation and continuation of pregnancy are caused by the mutagenic agents in the cigarette (polycyclic aromatic hydrocarbons, nitrosamines), other toxic ingredients (carbon monoxide, cadmium, benzo[a]pyrene, lead, mercury, etc.) or the main ingredient, nicotine and found that the use of transdermal nicotine patches before and during pregnancy caused no difference in implantation and continuation of pregnancy as compared to pregnant rats not administered nicotine patches.

Embryo implantation needs a properly prepared endometrium. Thus, the quality of endometrium plays a major role in occurrence and continuation of pregnancy. Despite the controversial results, the implantation rates are observed to be lower in smokers compared to nonsmokers [15]. In a study that

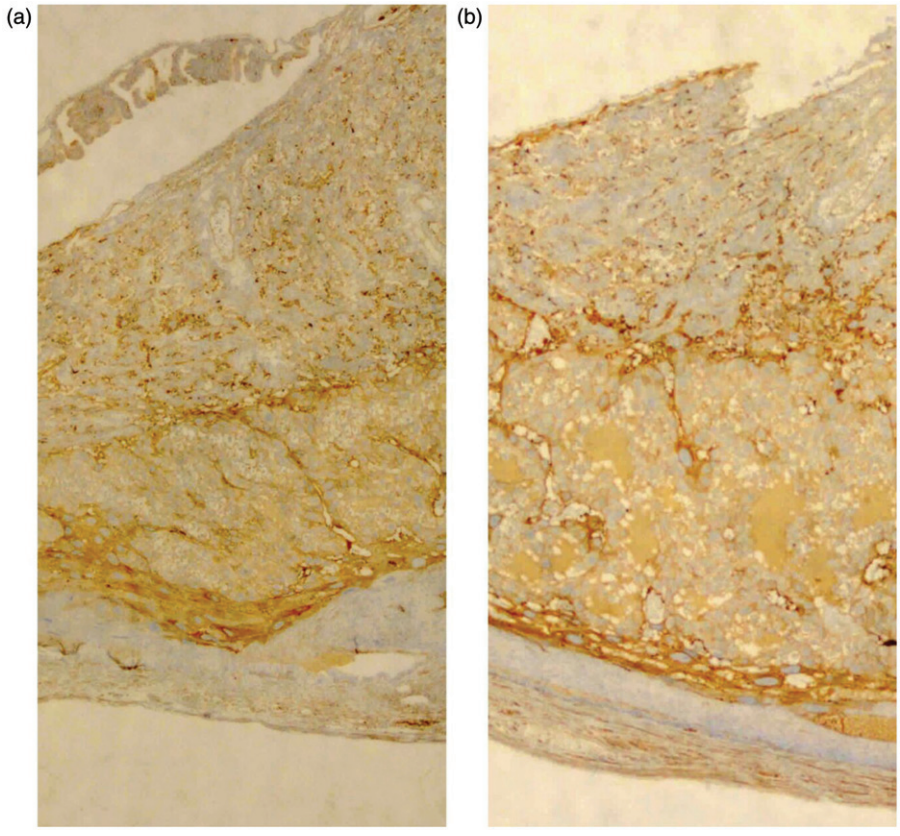

Figure 1. Immunohistochemical staining pictures of Integrin β3 in different tissues. Immunohistochemical staining pictures of control (a) and study (b) groups. Intensity and universality of Integrin β3 in endometrial epitelium and stroma seems to be identical.

evaluated *in vitro* fertilisation (IVF) cycles with 785 oocyte donors to more objectively assess the uterine receptivity, the patients smoking less than 10 cigarettes a day were defined as light smokers and the patients smoking more than 10 cigarettes a day as heavy smokers. A statistically significant decrease was observed in implantation in group of heavy smokers, while no difference was observed in group of light smokers compared to non-smokers [16]. Contradictory results were also obtained in studies on determination of cigarette exposure in self-reports from questionnaires conducted during IVF cycles and on metabolites of nicotine. In a study performed using creatinine-adjusted urinary cotinine metabolite in 921 IVF patients exposed to second-hand tobacco smoke, no significant difference could be found in implantation failure, fertilisation failure and spontaneous abortion as compared to group of patients not exposed to second-hand tobacco smoke [17]. Another study of the same authors that evaluated the concentrations of cotinine, the metabolite of nicotine with a longer half-life, in follicular fluid of 1909 non-smoker patients exposed to second-hand tobacco smoke showed a higher rate of implantation failure and decreased number of live births in the group of patients with a higher concentration of cotinine in follicular fluid [10]. In another study designed as a case–control study, the evaluation based on the plasma cotinine levels in IVF patients exposed to second-hand tobacco smoke revealed a higher rate of spontaneous abortion as compared to patients not exposed to second-hand tobacco smoke [18].

In general, the studies on cigarette exposure were conducted in IVF patients (who are completely different from the general population) and established based on the metabolites of nicotine examined in many tissues including implantation rates, pregnancy failure rates and follicular fluid [10]. These studies were too far from the specific evaluation of endometrial response and the natural course of pregnancy as well as an independent review of many active factors. To the best of our knowledge, this is the only animal model study that compared the effects (transdermal) of nicotine, the specific, most efficacious and mostly accused ingredient of cigarette, and Integrin β3 which directly evaluates the endometrial receptivity.

The most common undesirable pregnancy outcomes occurred following the tobacco use are intrauterine growth restriction and low-birthweight infant. The development of placenta should be completed and the pregnancy should be continued enough to allow large amount of blood flow for an exposure that is required for occurrence of these undesirable effects [11]. Therefore, the exposure durations are crucial during the design of studies. These effects usually do not occur during the period of first 6 days, which is considered to be the pre-/peri-implantation period in animal models using rats. In fact, in a study, the day of occurrence of these effects was found to be gestational day 9.75 on average [19]. In our study, the nicotine exposure was initiated 3 days before the pregnancy and continued till the 19th day of pregnancy. In addition, in nicotine studies using rats, nicotine as the investigational material was repeatedly administered via routes that might cause metabolic stress in pregnant dams and thereby, negatively affect the scientific results, such as intraperitoneal injections, osmotic mini pumps and jugular infusions [20]. The nicotine patches applied transdermally in our study ensured the near-natural evaluation of pregnant rats in their physiological environment. Following these standardisations, our study showed no significant negative effect of nicotine exposure with regard to Integrin β3.

Implantation failures and abortions arising from this tobacco-related toxic condition may be led by the other mechanisms. Decreased expression of epidermal growth factor receptor and decreased amount of E-cadherin are caused by the effects of pathway mediated by the toxic material, benzo(a)pyrene [21]. It was also demonstrated that the amounts of aryl hydrocarbon receptor (AhR) were also decreased, affecting the adhesion molecules that are essential for the communication of cells, and thereby, negatively affected the placentation and trophoblast invasion [22]. Maternal levels of oestriol, oestradiol, human chorionic gonadotropin (hcG), and human placental lactogen (hPL), the placental markers are observed in lower amounts in smokers as compared to non-smokers. Placental aromatase activity is also decreased in smokers and those with high amounts of nicotine and its metabolite, cotinine. On the other hand, the heavy metal, cadmium was documented as a placental toxin both in humans and in animal models [22–24]. Nicotine also inhibits regeneration of human embryonic stem cells and binding of active adhesion molecules [25]. Such events may compromise trophoblast invasion and thus, placentation and cause undesirable pregnancy outcomes.

Nicotine replacement therapy (NRT) is another treatment method for nicotine exposure as well as smoking. NRT in the form of gums, nasal sprays and transdermal patches, has been used in adult smoking cessation programmes in conjunction with behavioural support. Behavioural support alone can increase smoking cessation rates by up to 7% and the addition of pharmacotherapy increases this further by 1.5- to

2-fold [26]. NRT use in pregnant women is still questionable. A double-blind, placebo-controlled, prospective, randomised trial studied the effectiveness of transdermal nicotine patches in 12–24 weeks pregnant women who smoked 10 or more cigarettes before pregnancy and five or more cigarettes during pregnancy. This study was conducted in 1050 patients and found a significantly higher cigarette cessation rate after 4 weeks in transdermal group as 21.3% when compared to 11.7% in placebo group. However, at delivery, a higher rate was observed in group used transdermal nicotine patch, although not statistically significant (9.4% vs. 7.6%). Postpartum follow-up of infants was continued up to two years and no statistically significant difference was found with regard to development disorders including postnatal death [27,28]. Toxin exposure route and mode are as important as the toxin itself. The finding that salivary cotinine concentrations during transdermal replacement were lower in pregnant women than reported in smoking or non-smoking nonpregnant adults [29]. In this context, it has been proposed that episodic nicotine replacement may be advantageous in producing lower peak blood levels and more frequent recovery periods between foetal exposures [21]. Studies evaluating the levels and transfer of cotinine in placenta and amniotic fluid detected that the exposure was increased in the third trimester where transfer volume and permeability are increased [30]. The systemic and cardiovascular effect of transdermal nicotine is minimal compared to other administration routes and the plasma concentrations based on the body mass index are relatively low. Ultimately, all discussions and studies have always come to conclusion that the use of transdermal nicotine patches yields more favourable outcomes than smoking during pregnancy [31].

In conclusion, our study demonstrated that the harmful effect of nicotine use before and pregnancy on implantation is limited at the level of integrin expression, in a dose-dependent manner and also by considering the method of administration.

Consent

Written consent was obtained from the Bezmialem Vakif University Animal Experiments Local Ethic Committee.

Disclosure statement

We certify that we had no relationship with companies that may have a financial interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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