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EXPERIMENTAL STUDY

The dosage-dependent prenatal caffeine exposure adversely affects levels of integrin αVβ3 and MMP-9 in a rat model of embryo implantation

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ABSTRACT
OBJECTIVE: In the pregnancy period, it is recommended to limit the consumption of caffeine. However, the mechanisms of caffeine effect during pregnancy are not fully known. In our study, we aimed to investigate the effect of prenatal caffeine consumption on the embryonic implantation in rats as well as shed light on the relationship between the molecules and implantation stages.
MATERIALS AND METHODS: Forty-five Wistar albino pregnant rats were randomly divided into 3 main groups, namely into control, low-dose and high-dose groups, representing the dose-dependent effects of caffeine. Each main group was divided into 3 subgroups, namely those to be sacrified on days 4 (pre-implantation), 5 (peri-implantation) and 6 (post-implantation) . Different doses of caffeine were given on consecutive days, starting from day 1 of pregnancy up to the day of euthanasia. The implantation sites were investigated with the use of hematoxylin & eosin, Masson trichrome and immunostaining of VEGF, MMP-9, integrin αVβ3, mucin-1 and HB-EGF.
RESULT: Prenatal caffeine consumption in rats resulted in a dose-dependent decrease in the number of implantation sites. It has been shown that the immunoreactivity of integrin αVβ3 and MMP-9 underwent a change.
CONCLUSION: It has been shown that the levels of integrin αVβ3 and MMP-9 were decreased by prenatal caffeine consumption in rats, which resulted in a decrease in embryo implantation in a dose-dependent manner, especially in the high-dose group *(Fig. 5, Ref. 36)*. Text in PDF *www.elis.sk*
KEY WORDS: caffeine, embryo implantation, integrin, MMP-9.

Introduction

Implantation is a process of interaction between the embryo and endometrium, which must take place in a short window of time. In this process, the embryo approaches the surface of endometrium, tight connections are established, and the embryo invades the endometrium (1, 2, 3, 4). The implantation window is also defined as a period between days 20 and 24 of the 28-day menstrual cycle or 6-10 days after ovulation (5). Morphological and functional characteristics of both embryo and endometrium are subject to change as a result of the signal exchanged between the embryo and endometrium (6). The implantation is examined in 3 stages. In stage 1, referred to as apposition, the embryo approaches the surface of endometrium (7, 8). Just before the apposition, the mucin coating of endometrium (anti-adhesive molecule) will be removed (9). In this process, an increase in adhesion molecules (integrin, selectin, cadherin, Ig superfamily) is observed on the surface of endometrium (10). In stage 2, referred to as adhesion, the adhesion molecules establish tight junctions between the endometrium and embryo. Simultaneously, a process of decidualization of the endometrium begins (11). In stage 3, referred to as invasion, the embryo invades the endometrium by MMP secreted from the endometrium and trophoblast cells (12). Decidualization is the difference of endometrial stromal cells to decidual cells. VEGF secreted by the endometrium plays a stimulating role in this process. Both morphological and functional properties of decidual cells are subject to change. Morphologically, they are polygonal cells with euchromatic nuclei, and they store lipid and glycogen in the cytoplasm. Functionally, they secrete cytokines as food source for the embryo and to prevent maternal rejection of the embryo (13).

Caffeine is found in many foods and drinks in our daily lives. For example, coffee, chocolate energy drink, coca-cola, green tea, etc (14). One of the causes of infertility with no underlying pathology is high consumption of caffeine. Evidence also supports the fact that a daily consumption of up to 300 mg caffeine/ /day in healthy pregnant women is associated with a general lack

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of adverse reproductive and developmental effects (15). It is recommended to limit the use of caffeine during pregnancy by reducing it below 300 mg per day. It is reported that a moderate amount of caffeine taken daily does not have any adverse effects on the fetus, but the recommended caffeine amount is 125 mg/ /day (16).

We wondered why it is recommended to limit caffeine consumption during pregnancy, and what particular mechanism results in pathology. In literature, caffeine has been shown to reduce embryo implantation in rats, but the mechanism of this condition has not been adequately studied so far. In our study, we evaluated immunohistochemically the level of five important markers of implantation to explain the etiology of the effect of prenatal caffeine consumption on the embryo implantation.

Materials and methods

Animals

Experimental procedures were approved by the Dokuz Eylul University Local Ethical Committee of Animal Experiments (Protocol No. 44/2017). This study was done in March 2018 at Experimental Animal Laboratory, Dokuz Eylul University.

In this study, a total of forty-five Wistar albino female rats obtained from the Research Unit of Dokuz Eylul University, Faculty of Medicine were used. The strain of Wistar albino was chosen due to its availability in Experimental Animal Laboratory of Dokuz Eylul University, Faculty of Medicine, as well as because of its compatibility with published studies. All subjects were housed in cages situated in a standard animal room at 20–22 °C room temperature. 50–60 % relative humidity, and 12/12-hour dark/light periods. They were fed *ad libitum* with tap water that was left to stand, and standard pellet feed in Experimental Animal Laboratories until the end of the experiment.

Experimental design

Our study is designed as a prospective experimental study. Virgin females were mated with males overnight, and the day of the morning (08:00 AM) finding of the vaginal plug was designated as day 1 of pregnancy. After vaginal plugs had been identified, forty-five pregnant rats were divided randomly into three groups (17), namely into control group (n = 15), low-dose group (n = 15), and high-dose group (HD; n = 15).

The rats in the low-dose group were treated with 30 mg/kg caffein (SigmaAldrich, No. C0750). The rats in the high-dose group were treated with 120 mg/kg caffeine intraperitoneally (18). We planned to sacrifice the rats on three consecutive days to observe the change in the implantation process. Pregnant rats were sacrificed on day 4, 5 or 6 of pregnancy (at 09:00 AM). Thus 9 subgroups were formed (n = 5 in each subgroup), namely those to be sacrificed on day 4, 5 or 6, i.e. during the pre-implantation, peri-implantation or post-implantation period, respectively (19). The rats were euthanized 10 min after tail-vein injections of 0.1 ml 1 % Chicago blue dye (Sigma-Aldrich, USA) in saline to detect implantation sites. The uterus of each animal was flushed separately with sterile saline (20).

Drug administration

Previous studies have demonstrated that the effects of caffeine are dependent on its dosage (23, 24). According to the dose conversion standard between rats and humans (human: rats = 1:6.17, i.e. according to the body surface area) (21), a a dose of 30 mg/ /kg/day of caffeine (equivalent to the low dose in this study) in rats approximates the consumption of 1.5–2 cups of coffee a day for people (a cup of coffee contains 100–150 mg of caffeine on average). Although the intake of 120 mg/kg/day of caffeine (equivalent to the high dose in this study) was higher than routine exposure, the concentration did not reach the reported clinical toxic dose (~ 80 μg/ml) (22).

Vaginal plug-positive rats were treated with caffeine (Sigma-Aldrich, No. C0750) by oral gavage during days 1–6 of pregnancy and sacrificed to detect embryo implantation on day 4, 5, or 6 of pregnancy. Caffeine was administered once per day, namely at 8:30 AM at a dose of 30 mg/kg body weight (BW) (in the low-dose group) or 120 mg/kg BW (in the high-dose group).

Hematoxylin-eosin staining

After caffeine treatment, the pregnant rats were sacrificed on day 4, 5 or 6 (at 09:00 AM) and implantation sites were subsequently fixed in 10 % formaldehyde solution for 48 h. The fixed implantation sites were embedded in paraffin and cut into sections of 5 μm in thickness. These sections were later stained with hematoxylin solution for 5 min and eosin solution for 10 s.

Immunohistochemistry

Immunohistochemistry was performed as previously described (25). Uteri from day 4, 5 or 6 were fixed in 10 % formaldehyde, and examination of implantation sites was performed in 5-μm thick, paraffin-embedded sections by using antibody against VEGF (Cat. No: bs-1665R, Bioss-USA), MMP-9 (Cat. No: bs-4593R, Bioss-USA), αVβ3 integrin (Cat. No: bs-1310R, Bioss-USA), mucin-1 (MUC-1) (Cat. No: bs-1497R, Bioss-USA), and HB-EGF (Cat. No: bs-3576R, Bioss-USA; 1:100 dilution). The hematoxylin solution was further used for nuclear staining. The images were analyzed by using a computer-assisted image analyzer system consisting of a microscope (BX51; Olympus, Tokyo, Japan), and the images were transferred into the computer using a digital video camera (DP71; Olympus).

Immunohistochemical staining was evaluated by a semiquantitative method. The staining was classified as strong (+++, 3), moderate (++, 2), weak (+, 1), or ambiguous (−, 0). Two histologists inspected the slides (26).

Statistical analysis

Since the data obtained from the study were inconsistent with normal distribution, we used non-parametrical statistical methods. The values are presented as mean ± SD. The Kruskal-Wallis test was used for comparing nine groups. The differences between the two groups were examined with the Mann-Whitney U-test. Values of $p < 0.05$ were accepted as statistically significant.

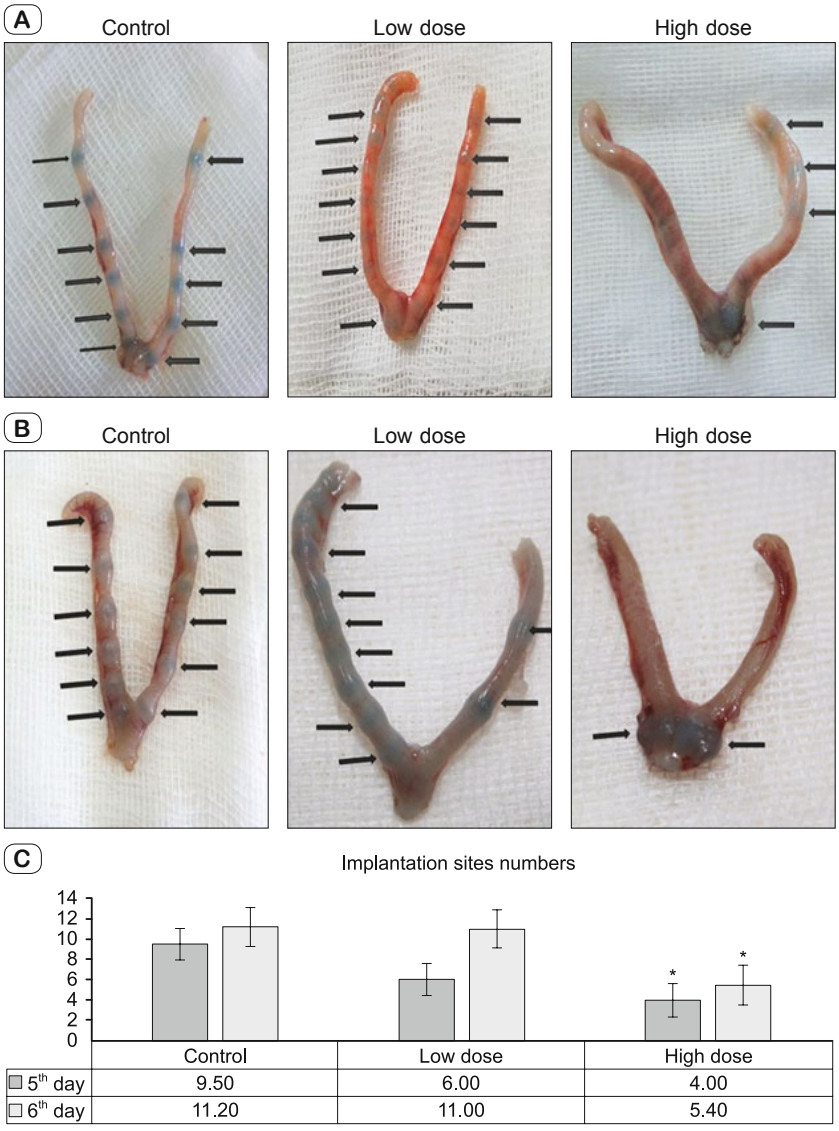

Fig. 1. Morphology and numbers of implantation sites in rat uterus on days 5 and 6 of pregnancy. A. Implantation sites on day 5 of gestation (D5) in rat uteri B. Implantation sites on day 6 (D6) of gestation in rat uteri C. Statistical analysis of total implantation site numbers. The high-dose group score is significantly lower than that of the control and low-dose groups (* p < 0.05, respectively).

Results

Evaluation of implantation site numbers

The implantation sites were not observed on day 4 because embryo implantation did not take place. On day 5, the mean implantation site number was 9.5 in the control group, while the numbers in the low-dose and high-dose groups were 6 and 4, respectively (Fig. 1A). On day 6, the mean implantation site number was 11.2 in the control group, 11 in the low-dose group, and 5.4 in the high-dose caffeine group (Fig. 1B). Statistically, we demonstrated that the dose-dependent caffeine consumption in the prenatal period reduced the number of implantation sites (Fig. 1C).

Hematoxylin & eosin staining

On day 4 of pregnancy, all 3 groups were differentiated into three layers, namely outermost layer of the uterus, middle layer, i.e. myometrium, and the innermost layer, i.e. endometrium. The prismatic epithelium covering the lumen was observed as a single layer.

When the 5th day of pregnancy was investigated, the initiation of decidualization in the control group was found to be similar. to that in the low-dose group. In the high-dose group, some areas had hemorrhage in the endometrium.

When the 6th day of pregnancy was investigated, it was observed that the decidualization that had started on day 5 of pregnancy increased in both the control group and low-dose group. As for the high-dose group, hemorrhage sites in the endometrium were observed in some experiments. It was observed that the narrowing of the lumen that was observed in the control group on the 6th day was not sufficiently developed in the high-dose group (Fig. 2A).

Masson trichrome staining

When we examined the uterus stained with Masson trichrome which is a collagen fiber dye, the collagen fibers were stained blue in the endometrial stroma on day 4 of pregnancy. Other structures stained in red were observed as smooth muscles in the myometrium layer, uterine epithelium and gland epithelium.

It was determined that on the 5th day of pregnancy, the control group and low-dose group started to show signs of collagen fibers being pushed aside, but this situation was thinner in the high-dose group. Unlike the collagen fibers in the control group and low-dose group on day 6 of pregnancy, those in the high-dose group were not completely pushed aside toward the myometrium and more collagen fibers were observed in the endometrial stroma. Hemorrhages stained yellow were also observed in the stroma (Fig. 2B).

Immunohistochemistry

MUC-1, HB-EGF and VEGF immunoreactivity calculated on consecutive days (4, 5 and 6) for the control, low-dose and high-dose groups (in lumen epithelium, gland epithelium, and endometrial stroma), was not significantly different (Figs 3A, 3B, 3C, 3D, 4A and 4B).

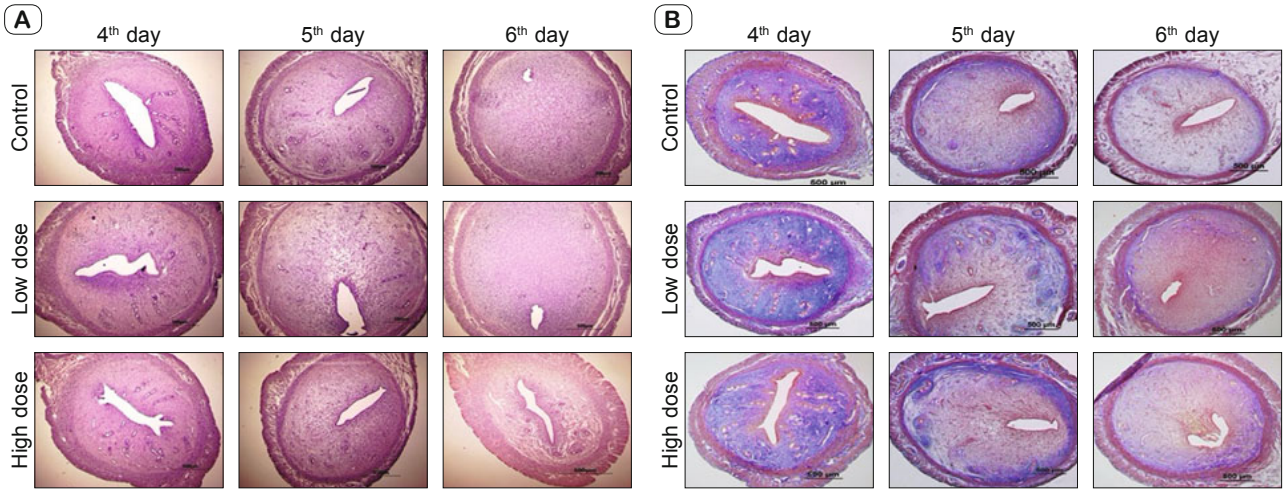

Fig. 2. A. Representative H&E staining of implantation site on days 4, 5 and 6 of pregnancy (4×). B. Representative Masson – trichrome staining of implantation site on days 4, 5 and 6 of pregnancy (4×).

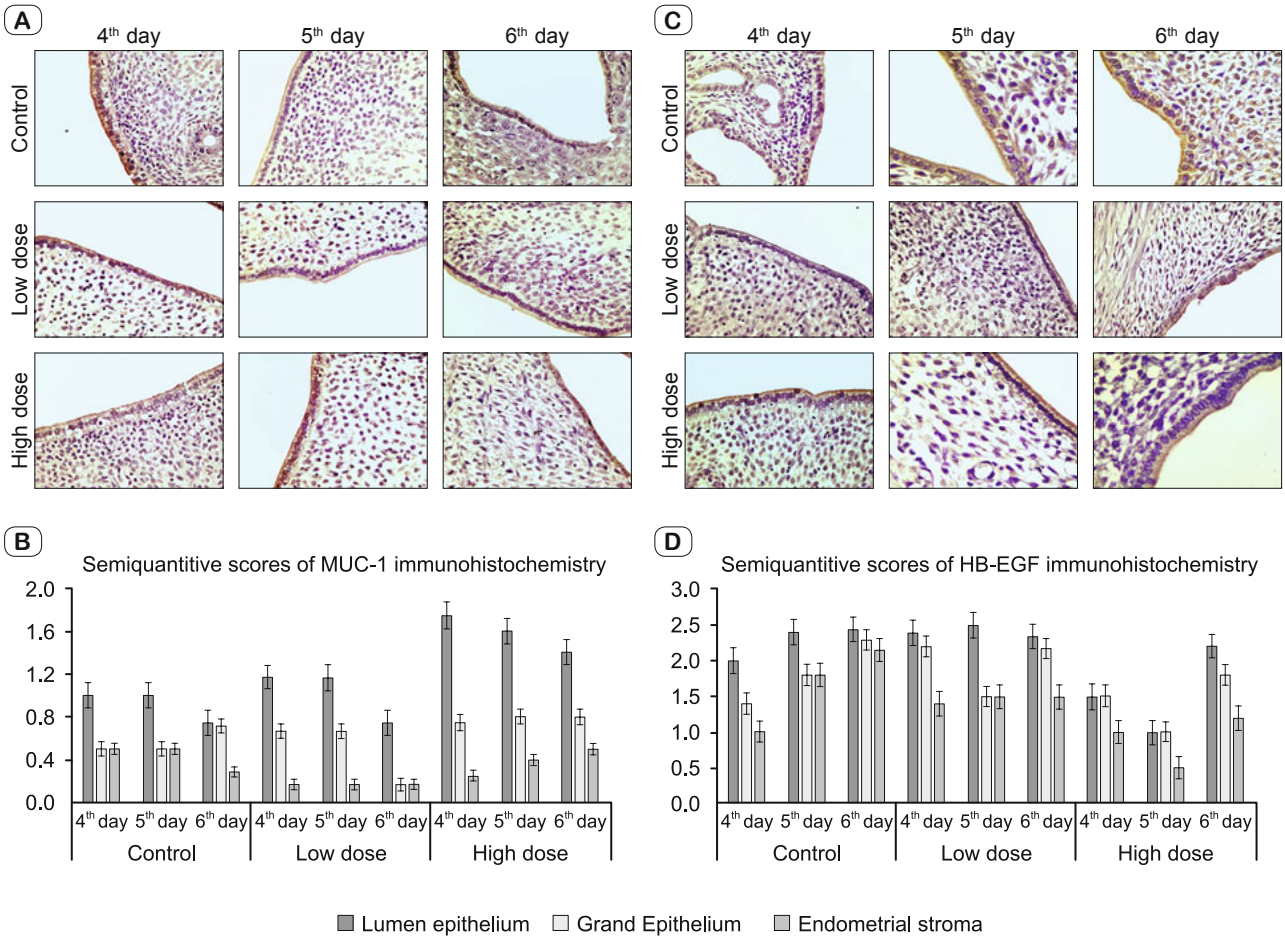

Fig. 3. MUC-1 (A) and HB-EGF (C) immunoreactivity results (x40). B. Semiquantitative scores of MUC-1 immunohistochemistry. D. Semiquantitative scores of HB-EGF immunohistochemistry. The control, low-dose and high-dose group scores are not significantly different in MUC-1 and HB-EGF. MUC-1 = mucin-1, HB-EGF = heparin-binding epidermal growth factor.

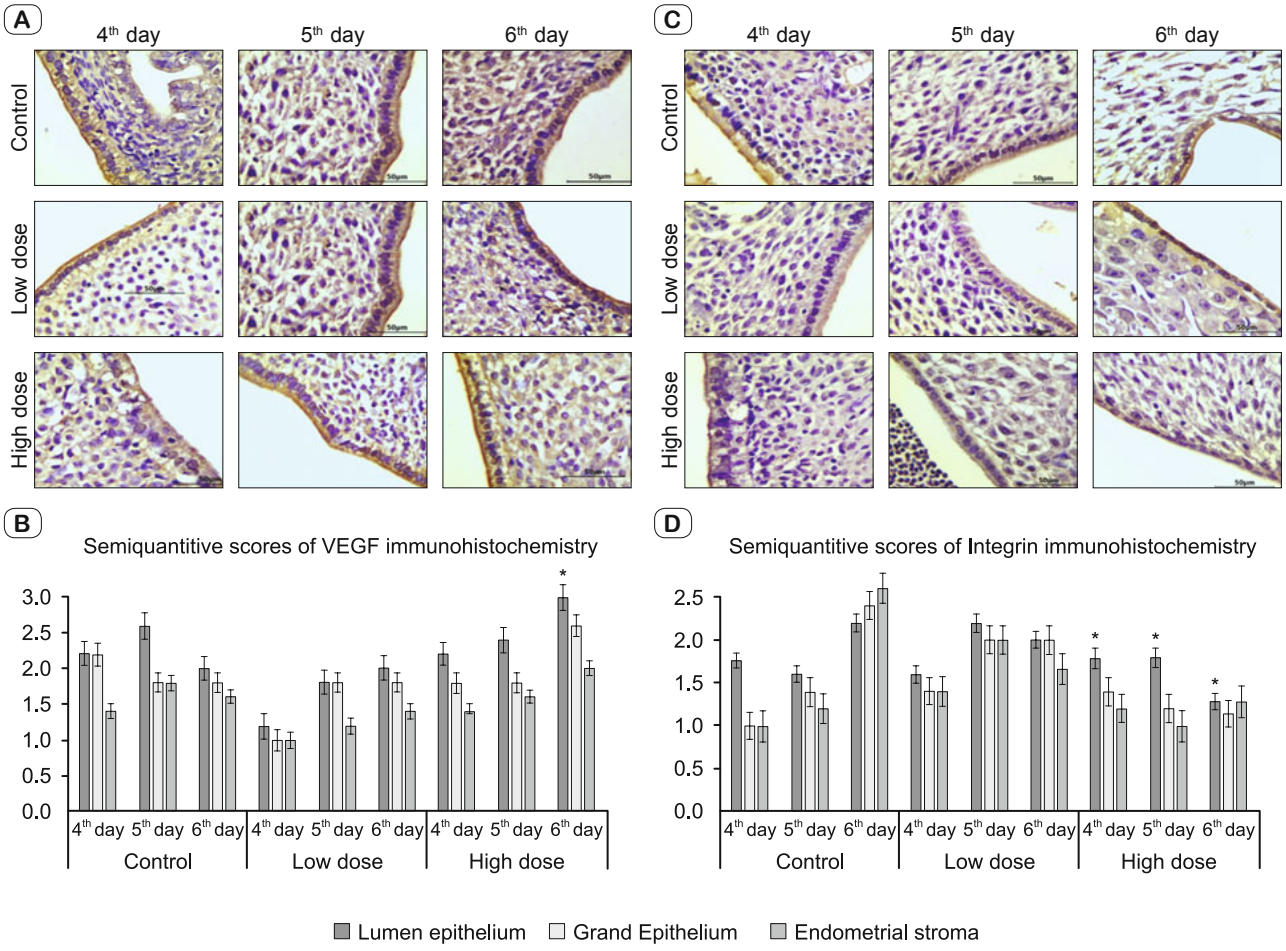

Fig. 4. VEGF (A) and integrin αVβ3 (C) immunoreactivity results (x40). B. Semiquantitative scores of VEGF immunohistochemistry. The control, low-dose and high-dose group scores are not significantly different D. Semiquantitative scores of integrin αVβ3 immunohistochemistry. The high-dose group score is significantly higher than that in the control and low-dose groups (* p < 0.05, respectively). VEGF = vascular endothelial growth factor.

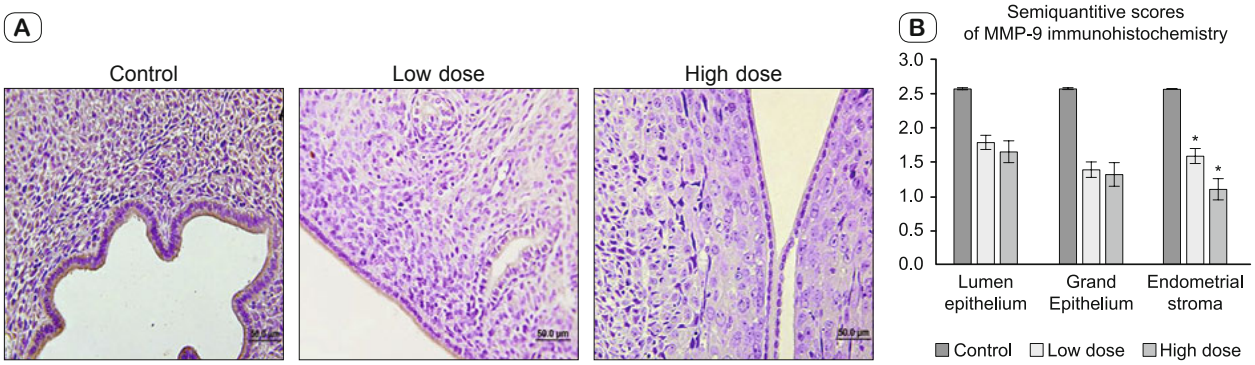

Fig. 5. MMP-9 (A) immunoreactivity results (x40). B. Semiquantitative scores of MMP-9 immunohistochemistry. The high-dose group score is significantly higher than in the control and low-dose groups (* p < 0.05, respectively). MMP-9 = matrix metallaproteinase-9.

Integrin αVβ3 immunoreactivity was calculated on consecutive days (4, 5, 6) for the control, low-dose and high-dose groups (in luminal epithelium, glandular epithelium, and endometrial stroma), and it was significantly higher in the high-dose group ($p < 0.05$) (Figs 4C and 4D).

MMP-9 immunoreactivity was calculated on day 6 for the control, low-dose and high-dose groups (in luminal epithelium, glandular epithelium, and endometrial stroma), and it was significantly higher in the high-dose group ($p < 0.05$) (Figs 5A and 5B).

Discussion

The prenatal period starts with fertilization and ends with birth. In rats, the embryo implantation occurs on the 5th day of gestation. In our study, the caffeine administration was initiated on day 1 of gestation and carried out until day 6, which means that it was consumed during preimplantation and implantation periods. We are referring to this period as 'prenatal' in order to be in line with the term used in literature (27).

We examined the dose-dependent effects of caffeine, which is included in many foods and drinks consumed in our daily lives, on the implantation process of the embryo. In our study, we used low (30 mg/kg, oral gavage) and high doses of caffein (120 mg/kg, oral gavage) and compared the effects of these two dosages. Similarly, Yadegari et al (2016) administered 150 mg/ /kg/day caffeine to rats by intraperitoneal route on 5 consecutive days, starting with day 1 of pregnancy, and found out that prenatal high-dose caffeine consumption reduced the number of implantation sites as shown in tissues harvested on day 7 of pregnancy (i.e. on the day that the rats were sacrificed) (27). In our study, we also demonstrated the negative effects of prenatal caffeine consumption on embryo implantation, and emphasized the importance of limited consumption of caffeine in pregnancy. Many published studies conducted on experimental animals have shown that prenatal caffeine consumption has a negative effect on implantation singly by investigating the number of implantation sites (27, 28). The particular mechanisms of action, or pathways leading to this effect have not been yet explained at the molecular level.

Our study was aimed at revealing the mechanism of action of caffeine on implantation. Therefore, we evaluated the relationship between dose-dependent caffeine consumption during the prenatal period in rats and particular molecules taking part in implantation stages (VEGF, HB-EGF, MUC-1, αVβ3 integrin, MMP-9), which we examined by means of immunohistochemical methods. We have shown that the reduction in the levels of αVβ3 integrin and MMP-9 molecules plays a role in the molecular mechanism leading to this negative effect.

Jingjing Qian et al (2018) showed that prenatal low and high oral/intraperitoneal doses of caffein consumption in mice (5 mg/ /kg and 30 mg/kg, respectively) adversely affect the embryonic development and uterine receptivity, as well as observed a change in expression of COX-2, MUC-1, LIF, Ltf, and Areg, which play a role in implantation (29). In our study, we observed that the implantation was not affected in the low-dose group (30 mg/kg, oral gavage) but the implantation decreased in the high-dose group (120 mg/kg, oral gavage). In addition, there was no change in MUC-1 immunoreactivity between the groups.,

In the literature, there are different studies investigating the relationship between caffeine, integrin αVβ3 and MMP-9. Cheng et al investigated the effect of caffeine on invasion in glioblastoma, i.e. the anti-cancer effect. It was determined that mRNA and protein expression of matrix metalloproteinase-1 tissue inhibitor (TIMP-1) was increased and MMP-2 decreased in caffeine-treated animals. Ki67, p-p38, phosphorylated extracellularly regulated protein kinases (p-ERK), and membranous integrins β1 and β3 decreased with caffeine. In addition, caffeine reduced the tumor size, cathepsin B and Ki67 expression in an animal model. Caffeine promoted the anti-cancer potential in glioma treatment by reducing the invasion of glioma cells via ROCK-cathepsin B/FAK/ /ERK signaling. It was emphasized that the mechanism of action of caffeine is based on the decrease in integrin and MMP-9 (30). These data support the results of our study.

It is known that the structure that first meets the embryo in the endometrial epithelium is the barrier of anti-adhesion molecules. By destroying the natural barrier created by anti-adhesion molecules during implantation, the epithelial adhesion molecules should facilitate the binding (31). MUC-1 is the most remarkable among the mucins. It forms a delicate balance between adhesion and anti-adhesion forces in cells. Some studies on mice show that while MUC-1 is strongly expressed in epithelial cells, it is downregulated in surface epithelium throughout the implantation phase. Based on these findings, it is thought that this decrease in MUC-1 expression is caused by the signals produced by the blastocyst (32). In our study, an intense MUC-1 immunoreaction was observed in the luminal endometrial epithelium in all groups, especially on the 4th day (Fig. 3A). A strong release of MUC-1 was considered to be an indicator of a barrier that protects the epithelium prior to implantation. On the 6th day, the observed decrease in epithelial expression may be aimed at facilitating the implantation. In their study of MUC-1, Hoffman et al observed its high immunoreactivity on the preimplantation day and low immunoreactivity on the implantation day (33). In our results, the decrease in MUC-1 immunoreactivity on consecutive days supported the notion that MUC-1 disappeared during the implantation of trophoblast when the adhesion molecules in the endometrial epithelium entered the binding phase.

Yuan et al provided evidence that HB-EGF was a potential mediator of endometrium-embryo communication within the implantation site (34). Similarly, Xien et al showed that HB-EGF mutant mice had failure of pregnancy (35). We also recorded that HB-EGF immunoreactivity on the 5th day (peri-implantation) was higher than that on the 4th day (preimplantation) in the control group, but in the high-dose group, the HB-EGF immunoreactivity on the 5th day is not higher than that on the 4th day, on the contrary, HB-EGF immunoreactivity on the 4th day is higher than that on the 5th day. HB-EGF might have played a role in the reduction of implantation in the high-dose group.

VEGF is very important to uterine vascular permeability and angiogenesis during implantation. In our study, the most intense VEGF immunoreactivity was observed in the control and low-dose groups, especially in the luminal epithelium on the 5th day, whereas the highest concentration was observed on the 6th day in the high-dose group. In addition, VEGF immunoreactivity was stained more intensely in the high-dose group as compared to other groups. We attributed this result to angiogenesis-enhancing effect of caffeine in general. In another study, it was emphasized that caffeine reduces angiogenesis dose-dependently while thrombospondin-1 (TSP-1) plays a role in this effect (36).

In literature, there are no studies examining the effects of caffeine consumed in the prenatal period on the expression of the implantation markers that we selected in the endometrium. The present study may be considered as a limited preliminary assessment, but extensive studies should be conducted on the deterioration of molecular mechanisms of other markers that play an important role in implantation. In the research, the methods enabling to obtain more specific data such as Q-PCR and Western Blot should also be used, more markers should be examined, and greater emphasis should be placed on molecular mechanisms that can reveal how these markers trigger or suppress each other.

Conclusion

This is the first study that investigates the mechanism of the effect of dose-dependent prenatal caffeine consumption in rats on the implantation process by examining VEGF, HB-EGF, MUC-1, αVβ3 integrin and MMP-9 molecules by immunohistochemical techniques.

In our study, the findings in the low-dose caffeine group were consistent with those observed in the control group while no pathological condition was recorded. In the high-dose group, the number of implantation sites decreased significantly. We have shown that the reduction in αVβ3 integrin and MMP-9 molecules plays a role in the molecular mechanism leading to this negative effect.

As a result, our data show that caffeine consumption in the early pregnancy impairs endometrial receptivity and adversely affects embryo implantation. In our study, we concluded that the duration of caffeine consumption, and restriction of caffeine intake for women planning to become pregnant are especially important during the pre-implantation period.

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