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Original Article

Caffeic Acid Phenethyl Ester Reduces the Adverse Effects of Nicotine on the Endometrium

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Abstract

Background: Tobacco smoke contains various toxins that negatively affect the human reproductive system. Caffeic acid phenethyl ester (CAPE), a potent antioxidant, has protective effects on the reproductive system against oxygen-free radicals, methotrexate, and pesticides. Herein, the effect of CAPE on some key markers of endometrial receptivity has been evaluated.

Methods: A cross-sectional study was conducted during 2018-2019 in the Department of Clinical Biochemistry, School of Medicine, Fasa University of Medical Sciences (Fasa, Iran). Primary endometrial cells were divided into five groups, namely control, nicotine, CAPE, vehicle, and nicotine+CAPE. Real-time polymerase chain reaction (PCR) and methylation-specific PCR were performed to evaluate gene expressions and methylation, respectively. Appropriate doses of CAPE and nicotine were determined using the MTT assay. Data were analyzed using SPSS software (version 16.0) with a one-way analysis of variance. $P<0.01$ was considered statistically significant. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method.

Results: Treatment of cells with nicotine significantly reduced the expression of C-X-C motif chemokine ligand 12 (*CXCL12*), fibroblast growth factor 2 (*FGF2*), and vascular endothelial growth factor A (*VEGF-A*) genes ($P<0.0001$). However, the expression levels increased significantly when treated with nicotine+CAPE ($P<0.0001$). Despite the reduced *CXCL12* gene expression in cells treated with nicotine, *CXCL12* was unmethylated in all study groups, indicating that the methylation status of the *CXCL12* gene was not affected by nicotine or CAPE.

Conclusion: CAPE can be a suitable agent to protect female smokers from the harmful effects of nicotine.

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Keywords ● Nicotine ● Endometrium ● Caffeic acid phenethyl ester

What's Known

- Nicotine significantly reduces estrogen receptor and progesterone receptor mRNA expression in primary human endometrial cells, while it increases the expression of vascular endothelial growth factor (VEGF) mRNA.

What's New

- Caffeic acid phenethyl ester significantly reduced the adverse effect of nicotine by increasing the expression of *VEGF-A*, C-X-C motif chemokine ligand 12 (*CXCL12*), and fibroblast growth factor 2 (*FGF2*) genes.

Introduction

Female infertility is clinically defined as the failure to achieve a pregnancy after one year of unprotected intercourse.[1] Almost 186 million individuals around the world suffer from infertility for various reasons.[2] Smoking is a preventable risk factor

for various complications during pregnancy that can adversely affect maternal and fetal health. These complications include premature delivery, intrauterine growth restriction, placenta previa, placental abruption, premature rupture of membranes, and perinatal mortality.[3] Tobacco smoke contains various toxins and carcinogens that negatively affect the reproductive system. It has been reported that female smokers are 1.6 times more likely to run the risk of infertility than non-smokers.[4] Smoking lowers the success rate of *in vitro* fertilization (IVF) due to inadequate uterine receptivity and reduced endometrial thickness. It is also suggested that tobacco smoke has a detrimental influence on implantation and pregnancy rates during assisted reproduction therapy.[5, 6]

Smoking reduces the expression of C-X-C motif chemokine ligand 12 (*CXCL12*) and fibroblast growth factor 2 (*FGF2*) genes.[5] *CXCL12* gene is highly expressed in the placenta tissue and plays an important role in several processes, such as placentation and implantation.[7, 8] Several studies conducted on rodents, sheep, and buffaloes showed that *FGF2* promotes implantation, trophoblastic cell migration, and embryogenesis.[9-11] *FGF2* activates the adhesion of primary human endometrial epithelial cells to extracellular matrix components by stimulating ERK1/2 phosphorylation, thus communicating with the trophectoderm in the blastocyst.[12] Vascular endothelial growth factor A (*VEGF-A*) is another uterine receptivity marker found in the uterine fluid, which regulates the implantation of blastocysts, increases the mitotic activity of endometrium cells, elevates blastocyst development, and improves adhesion of uterine epithelial cells.[13, 14] Therefore, *FGF2*, *VEGF-A*, and *CXCL12* are the three important maternal-embryonic junction axis and uterine receptivity markers.[13, 15, 16] Maternal smoking increases *VEGF-A* expression in the placenta and cytotrophoblasts.[17] In addition, nicotine, as a toxin, can directly act on the placenta causing placental dysfunction. In a previous study, we found that nicotine significantly reduced the expression of estrogen receptor (ER) and progesterone receptor (PR) mRNA in primary human endometrial cells while increasing the expression of VEGF mRNA.[18] Another study reported that endometrial angiogenesis is regulated by progesterone receptor type B (PR-B).[19] On the other hand, caffeic acid phenethyl ester (CAPE), a potent antioxidant, is shown to have protective effects on the reproductive organs against oxygen-free radical molecules, methotrexate, and pesticides.[20] CAPE is a nuclear factor kappa B (NF_KB) inhibitor, anti-cancer, anti-inflammatory, immune modulator, and blood pressure reducer. CAPE is also described as a DNA methyltransferase inhibitor.[21]

Despite an increasing number of studies on the harmful effects of smoking and nicotine exposure on the reproductive system, women continue to smoke; probably due to nicotine dependence. In addition, maternal smoking lowers the success rate of IVF and may even negatively affect infertility treatments.[22, 23] The present study, therefore, aimed to investigate the impact of nicotine on the expression of *CXCL12*, *VEGF-A*, and *FGF2* genes and assess the protective effects of CAPE against nicotine.

Materials and Methods

A cross-sectional study was conducted from October 2018 to March 2019 in the Department of Clinical Biochemistry, School of Medicine, Fasa University of Medical Sciences (Fasa, Iran). A total of five healthy women aged 20-40 years (mean=28.83±3.1 years) were included in the study. Tissue samples were obtained from Shiraz Research Center after surgery. The inclusion criterion was women with regular menstruation. The exclusion criteria were the use of hormonal contraceptives within three months prior to the study, a history of cancer, tobacco smoking, alcohol consumption, and use of illicit drugs.

The study was approved by the Ethics Committee of Fasa University of Medical Sciences, Fasa, Iran (IR.FUMS.REC.1397.170). Written informed consent was obtained from the participants before tissue samples were collected.

Chemicals

Penicillin/streptomycin solution, trypsin-ethylenediaminetetraacetic acid (EDTA), Dulbecco's modified eagle medium/nutrient mixture F12 (DMEM/F12), and fetal bovine serum (FBS) were purchased from Gibco BRL (Paisley, UK). cDNA synthesis kit was purchased from Yekta Tajhiz Azma (Tehran, Iran). CAPE was obtained from Cayman Chemical (Michigan, USA). Nicotine was purchased from Sigma-Aldrich (Dorset, UK). RealQ Plus 2x Master Mix Green with high Rox™ was purchased from Ampliqon (Odense M, Denmark). The RNA extraction kit (RNX-plus solution for total RNA isolation) was purchased from SinaClon (Tehran, Iran). A human methylated and unmethylated DNA set was purchased from Zymo Research (Irvine, CA, USA). Hydroquinone and sodium bisulfite were obtained from Sigma-Aldrich (Dorset, UK).

Endometrial Stem Cell Extraction and Cell Culture

In line with a previous study, the power analysis approach was used to calculate the sample size.[18] Afirin and colleagues suggested that the acceptable range of degrees of freedom (DF) for the error term in the analysis of variance (ANOVA) should be between 10 to 20. Accordingly, we defined five study groups (i.e., DF=N-K=[(5×3)-5]=10) and performed the tests in triplicate.

Endometrial biopsies were obtained under sterile conditions from five fertile women in their childbearing years with regular menstrual cycles. The biopsy of normal tissues was validated using conventional histological evaluation. Human endometrial stromal cells were identified and isolated as described previously.[24] Briefly, endometrial tissue was sliced into smaller pieces (2 to 3 mm) under sterile conditions, then incubated with 1 mg/mL of collagenase type I for 30 min at 37 °C. After 5 min centrifugation at 1200 RPM, endometrial cells were added to DMEM/F12 and cultured with 10% FBS and 1% penicillin/streptomycin solution under controlled conditions of 37 °C and then incubated with 5% carbon dioxide. The culture medium was passaged three times a week and used for treatments when reached about 80% confluence. The five defined study groups were the control group (untreated), treated with nicotine (10-6 µM) for 24 h, treated with CAPE (4 µg/mL) for 24 h, treated with 10-6 µM nicotine+CAPE for 24 h, and treated with ethanol 1% as the vehicle. For each, the media was discarded, and the cells were treated with CAPE (4 µg/mL) for another 24 h. All experiments were carried out using cells at 80% confluence in passage numbers 3-5.

Cell Viability Assay

The MTT assay was performed according to previous studies.[18, 25, 26] After 24 h treatment, 100 µL of MTT solution (0.5 mg/mL in PBS) was added to the cells in each well of the 96-well plates. The plates were incubated at 37 °C for 4 h, and the MTT solution was then removed. DMSO was then added to dissolve the dye. The absorbance of the samples was measured at 570-620 nm. The appropriate treatment dose for CAPE and nicotine was 4 µg/mL and 10^{-5} M, respectively.

Real-time Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted using RNX plus solution according to the manufacturer's instructions. cDNA was synthesized with 400 ng of the total RNA using a first-strand cDNA synthesis kit for reverse transcriptase. The RT-PCR was performed using the RealQ Plus 2x Master Mix Green with high Rox™. The PCR for *VEGF-A*, *FGF2*, and *CXCL12* was performed in 40 cycles at 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 25 s. Then, the gene expression levels were computed using the $2^{-\Delta\Delta CT}$ method.[27] All tests were carried out in triplicate. The sequence of primers is presented in table 1. Finally, β-actin was used as the housekeeping gene.

DNA Extraction and Methylation-specific PCR

The genomic DNA was obtained from primary human endometrial stromal cells using a DNA extraction kit (Pars Tous Biotech, Tehran, Iran) according to the manufacturer's instructions and then treated with sodium bisulfate. Methylation-specific PCR (MSP) uses bisulfite modification to convert unmethylated cytosine to uracil. The modified DNA is then used to specifically amplify methylated or unmethylated DNA by MSP. The sequence of primers used for amplification of the promoter region of each of the three genes was *CXCL12*-M: forward 5'-GGAGTTTGAGAAGGTTAAAGGTC-3', reverse 5'-TTAACGAAAAATAAAAATACGACG AT-3' and for *CXCL12*-UM: forward 5'-GAGTTTGAGAAGGTTAAAGGTTGG-3' and reverse 5'-TAACAAAAAATAAAAATACAACAA T-3'. The product size for *CXCL12*-M and *CXCL12*-UM were bp242 and bp241, respectively. The PCR reactions were performed in a 50 µL reaction volume containing 25 pmol of each primer (sense and antisense), 0.2 mmol/L dNTPs, and 80 ng bisulfite-modified DNA in 1×PCR buffer provided using Taq enzyme (Poland). The reaction mixture was denatured at 95 °C for 10 min,

Table 1: The sequence of *FGF2*, *CXCL12*, VEGF, and β-actin primers used for the real-time polymerase chain reaction

Name	Primer Sequence (5'→3')
FGF2	Forward: 5′ CGACCCTCACATCAAGCTACAA 3′ Reverse: 5′ CCAGGTAACGGTTAGCACACACT 3′
CXCL12	Forward: 5'-GTCAAGCATCTCAAAATTCTCAACAC-3' Reverse: 5'-CACTTTAGCTTCGGGTCAATGC-3'
VEGF-A	Forward: 5'- CTGGAGTGTGTGCCCACTGA-3' Reverse: 5'-TCCTATGTGCTGGCCTTGGT-3'
β-actin	Forward: 5'-GCCTTTGCCGATCCGC-3' Reverse: 5'-GCCGTAGCCGTTGTCG-3'

FGF2: Fibroblast growth factor 2; *CXCL12*: C-X-C motif chemokine ligand 12; VEGF: Vascular endothelial growth factor

added with 1.5 U Taq polymerase, and then amplified in 40 cycles, each consisting of 30 s denaturation at 95 °C, 45 s annealing at 61 °C, and 45 s polymerization at 72 °C, followed by a single 10 min extension at 72 °C. A human methylated and unmethylated DNA set was used as a positive and negative control for the methylated and unmethylated gene. The PCR products were analyzed on 2.5% agarose gel.

Statistical Analysis

Data were analyzed using SPSS software (version 16.0) with one-way ANOVA. P<0.01 was considered statistically significant. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method. Data were expressed as mean±SD of the triplicate experiments.

Results

Real-time PCR

The results of RT-PCR showed that treatment of endometrial stromal cells with nicotine (10-5 µM) for 24 h significantly reduced the expression of *CXCL12*, *FGF2*, and *VEGF-A* genes (P<0.0001) (figure 1). The expression of *CXCL12*, *FGF2*, and *VEGF-A* genes was significantly increased in the nicotine+CAPE group (P<0.0001). This means that CAPE can reduce the adverse effects of nicotine in endometrial stromal cells.

Methylation-specific PCR

We evaluated the methylation status of *CXCL12* in the promoter region of (493 to +68) in primary human endometrial stromal cells. The results of methylation showed that the *CXCL12* gene was unmethylated in all groups of cells treated with CAPE, nicotine, and their combination (figure 2).

Discussion

We found that nicotine significantly reduced the expression of *VEGF-A*, *FGF2*, and *CXCL12* genes; indicating the detrimental effect of tobacco smoking on endometrial receptivity. There was no difference in the methylation status of the *CXCL12* gene before and after treatment with nicotine.

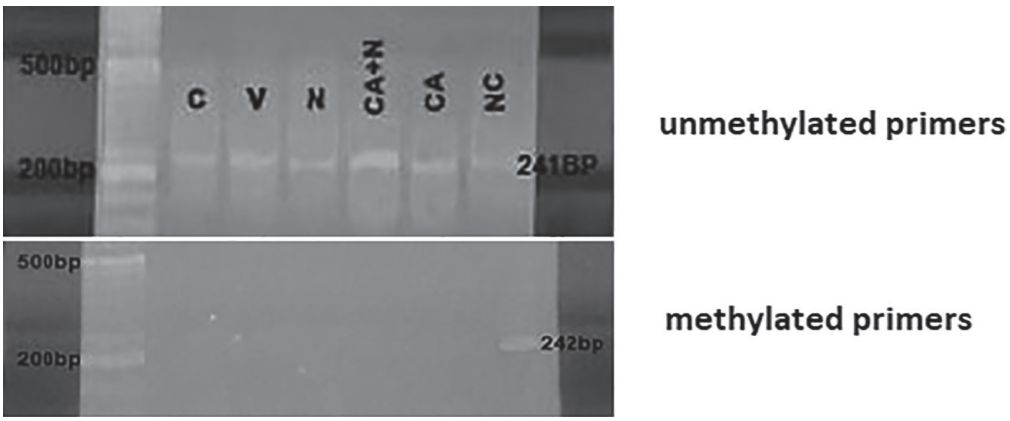

Figure 1: Real-time PCR of *CXCL12*, *FGF2*, and *VEGF-A* genes is illustrated. *P<0.0001; **P<0.0001

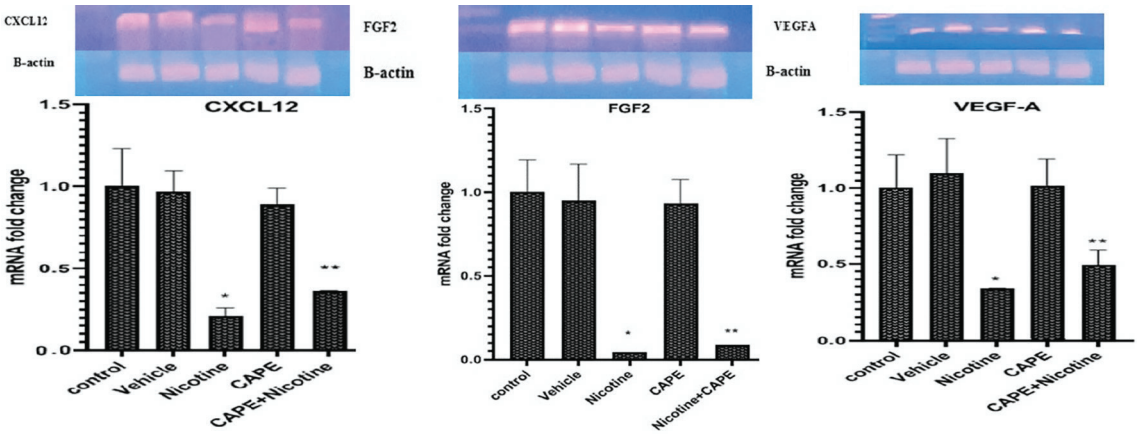

Figure 2: The result of methylation-specific PCR for the *CXCL12* gene. C: Untreated cells as control; V: Vehicle (cells treated with ethanol 1%); N: Cells treated with nicotine; CAPE+N: Cells treated with CAPE and nicotine; CAPE: Cells treated with CAPE; NC: Negative control; PC: Positive control; Positive and negative controls were used to ensure the accuracy of the assay.

CAPE is shown to induce S- and G2/M-phase cell cycle arrests and trigger apoptosis in human cervical cancer cells by upregulating the expression of the E2 factor family of transcription factors 1.[28] Assumpcao and colleagues introduced propolis as a new source of DNA methyltransferase inhibitor and found that CAPE had the highest interaction with DNA methyltransferase-1.[21] Various mechanisms for different activities of CAPE are suggested, including upregulation of the ERK1/2-CREB signaling pathway, expression of heme oxygenase-1, and inhibition of NF-κB signals.[29] In line with our results, Chen and colleagues showed that CAPE can improve the homing and engraftment of hematopoietic stem/progenitor cells in bone marrow by upregulating the expression of *VEGF-A*, stromal cell-derived factor 1α (SDF-1α), and hypoxia-inducible factor-1α (HIF-1α) genes.[29] Khademi and colleagues demonstrated that nicotine administration decreased antioxidant defense markers in endometrial stromal cells and induced higher MDA levels in a dose-dependent manner.[30] CAPE acts as a protective agent through its antioxidant and anti-inflammatory effects. Guney and colleagues showed that CAPE improved fluoride-induced apoptosis and oxidative stress in rat endometrium. They also reported that co-administration of CAPE and fluoride treatments significantly decreased MDA levels and increased SOD and CAT activity in endometrial tissue compared to fluoride alone.[31]

Several studies showed that *CXCL12* and *FGF2* play an important role in endometrium receptivity, implantation, and maintenance of pregnancy.[7, 32-34] *CXCL12* stimulates trophoblast invasion and tissue angiogenesis after binding to its receptor (CXCR4).[35] In contrast, nicotine downregulates *CXCL12* expression and inhibits trophoblast invasion.[36] These findings were in line with our results that exposure to nicotine can reduce the expression of *CXCL12* and *FGF2* genes. However, despite the reduced *CXCL12* gene expression in cells treated with nicotine, the *CXCL12* gene was unmethylated in all cells treated with CAPE, nicotine, or a combination. This means that the methylation status of *CXCL12* gene was not affected by either of these two substances. Puspasari and colleagues showed the therapeutic effect of propolis on traumatic ulcers caused by diabetes mellitus. They found that propolis increased the expression of *FGF2* and fibroblasts in traumatic ulcer healing processes in diabetic rats.[37] Another study showed that CAPE has an anti-cancer effect on breast cancer, inhibits cell migration, and can promote cancer chemoprevention.[38] Overall, we have demonstrated the benefits of CAPE in protecting against the harmful effects of nicotine on human endometrial.

As the main limitation of the study, due to financial constraints, we did not examine the effect of other markers on embryo implantation and fertility. Further studies are recommended to assess the beneficial effects of CAPE on the human reproductive system.

Conclusion

Nicotine reduced the expression of *VEGF-A*, *CXCL12*, and *FGF2* genes in human primary endometrial cells. In contrast, CAPE significantly reduced the adverse effect of nicotine by increasing the expression of these genes. CAPE can be an beneficial agent to protect female smokers from the harmful effects of nicotine.

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Authors' Contribution

Both authors have contributed to the study conception and design, material preparation, data collection and analysis, and drafting and revision of the manuscript. They have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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