Adverse effects of nicotine on endometrium receptivity markers: and protective effect of caffeic acid phenyl ester

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Abstract

Background

nicotine adversely affects the female reproductive system and changes the methylation pattern of some genes in the placenta. In contrast, caffeic acid phenylethyl ester) CAPE (, as a potent antioxidant, has protective effects against the harmful effects of oxygen free radical molecules, methotrexate, and pesticides on the reproductive system. To find the effect of nicotine on the endometrium, we investigated three markers of endometrium receptivity including fibroblast growth factor 2, vascular endothelial growth factors A, and C-X-C motif chemokine ligand 12 and also changes in methylation levels of CXCL-12 gene promoter. In addition, we evaluated the protective effect of CAPE against nicotine.

Methods

the appropriate treatment dose was selected based on the literature, the endometrial stromal cells were divided into 4 groups, including control, treated with nicotine, CAPE, and nicotine followed by CAPE. Finally, the quantitative polymerase chain reaction and Methylation-Specific PCR were carried out.

Results

The results showed that treatment of endometrial stromal cells with nicotine (10^{-6} μM) for 24 h significantly reduced expression of CXCL-12, FGF, and VEGFA genes. However, a decrease in CXCL-12 expression was not associated with increased methylation levels in the studied promoter region. In contrast, endometrial stromal cells treated with CAPE (4 μg/ml) for 24 h adverse significantly nicotine-induced reduction of CXCL-12, FGF, and VEGFA genes expression.

Conclusion

Exposure to nicotine has negative effects on uterine receptivity, implantation, and fertility, via reducing the expression of VEGFA, CXCL-12, and FGF2 genes. In contrast, CAPE has a protective effects and improves these genes expression.

Background:

Infertility is a disease that affects the reproductive system and is defined as a failure to achieve a clinical pregnancy after one year of unprotected intercourse [1]. Nearly, 186 million people around the world suffer from infertility for various reasons [2]. Several factors can contribute to infertility, including stress, occupational exposure to hazardous substances, alcohol consumption, and smoking [3]. Cigarette smoke contains various toxins and carcinogens which have many negative effects on the reproductive system. Smoking increases the risk of infertility about 1.6 times[4]. It also decreases the in vitro fertility (IVF)

success rate due to the reduction in uterine receptivity [5, 6]. Moreover, smoking reduces the expression of CXCL-12 and FGF2 genes [6]. CXCL-12 and FGF2 play important roles in endometrium receptivity, implantation, and pregnancy maintenance [7, 8]. CXCL-12 is extensively expressed in placenta and plays important roles in several reproductive processes including placentation, implantation, etc. [9–12]. Several studies conducted on rodents, pigs and rabbits have shown that FGF2 is involved in implantation, trophoblastic cell migration, and embryogenesis [13–15]. FGF2 provokes adhesion of primary human endometrial epithelial cells to extracellular matrix components by stimulating ERK1/2 phosphorylation, thus communicates with the blastocyst's trophectoderm [16, 17]. Similarly, VEGF-A is another uterine receptivity marker that is found in uterine fluid and regulates blastocyst implantation [18]; it also increases the mitotic activity of endometrium cells [19],elevates blastocyst development, and adhesion of uterine epithelial cells[18]. So, FGF2, VEGF-A, and CXCL-12 are three important maternal–embryonic junction axis [10, 18, 20, 21] and uterine receptivity markers [7, 8]. Maternal exposure to cigarette smoke increases VEGF-A expression in their placenta and cytotrophoblasts [22]. In addition, nicotine, as a toxin, can pass through placenta and result in placenta dysfunction [23, 24]. Totonchi et al. found that estrogen receptor (ER) and progesterone receptor (PR) mRNA expression, have significantly decreased in primary human endometrial cells, treated with nicotine. Also, VEGF mRNA expression has been increased in these cells [25]. Endometrial angiogenesis is regulated by the progesterone type B receptor (PR-B) [26, 27]. On the other hand, (CAPE), as a potent antioxidant, has protective effects against the oxygen free radical molecules, methotrexate and pesticides on reproductive system [28]. It is a nuclear factor (NFKB) inhibitor, anti-cancer, anti-inflammatory, immune modulator and blood pressure reducer [29, 30]. Furthermore, CAPE has been introduced as a DNA methyltransferase inhibitor [31]. Despite the increasing number of published scientific papers on the harmful effects of smoking and nicotine exposure on the reproductive system, women continue to smoke which can be due to nicotine dependence. In addition, maternal smoking reduces the success rate of IVF. Therefore, smoking can even disrupt therapeutic methods for treating infertility. Therefore, more knowledge about neutralizing compounds of cigarret smokingcan be effective in the treatment of infertility. Therefore, our purpose is to investigate the deleterious effects of nicotine on the CXCL-12, VEGF-A, and FGF2 genes expression and evaluation of protective effects of CAPE against nicotine.

Methods:

Chemicals:

Pen- strep, trypsin-EDTA, DMEM F12 and FBS (Fetal bovine serum) were purchased from Gibco-BRL (Paisley, UK), cDNA synthesis kit was supplied from YEKTA TAJHIZ AZMA (IRAN), caffeic acid phenylethyl ester was obtained from Cayman Company, Nicotine was purchased from Sigma Company (Poole, Dorset, UK), RealQ Plus 2x Master Mix Green, high Rox was supplied from AMPLIQON Company, RNA extraction kit was bought from RNX- plus solution for total RNA isolation- Sinaclon (Iran). Human Methylated & Non-methylated DNA set was purchased from Zymo Research Company. Hydroquinone and sodium bisulfite were obtained from Sigma-Aldrich Company.

Cell culture and cell treatment:

primary human endometrial stromal cells were obtained from (Muhammad Rasoololah Research Tower, Shiraz University of Medical Sciences). All experimental and surgical procedures were approved by the committee of investigations involving human participants of Fasa University of Medical Sciences. After 24 h of rest, cells were cultured in DMEMF12 media enriched with 10% heat-inactivated fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37°C and 5% CO2. Then, they were divided into 4 groups of a control group (untreated), a second group treated with nicotine (10^{-6} μM) for 24 h, a third group treated with CAPE (4μg/ml) for 24 h, a fourth group treated with 10^{-6} μM nicotine for 24 h. Next, the media was collected and discarded and then treated with CAPE (4μg/ml) for 24 h. All experiments were carried out using cells at 80% confluency, in passage numbers 3–5.

Cell viability assay (MTT test):

We selected the appropriate treatment dose for nicotine (10^{-6} μM) [25] and CAPE(4μg/ml) based on the literature[32].

Quantitative polymerase chain reaction (QPCR):

Briefly, total RNA was obtained by an RNA extraction kit pursuant to the manufacturer's protocol sheet (RNX- plus solution, Sinaclon, Iran). cDNA was synthesised by 400 ng of total RNA using Transcriptor First Strand cDNA Synthesis Kit (YEKTA TAJHIZ AZMA, IRAN). The RT-PCR was performed using the RealQ Plus 2x Master Mix Green, high Rox (AMPLIQON Company). Polymerase chain reaction for FGF2 and CXCL-12 was applied for 40 cycles at 95°C for 20 seconds, 60°C for 30 seconds, and 72°C for 25 seconds, and gene expression levels were computed by the $2^{-\Delta\Delta Cq}$ method [33]. All tests were carried out at least in triplicate. In this study, The FGF2, CXCL-12 and b-actin primers were used with the sequences shown in Table 1. Eventually, all gene expressions were normalized to B-actin expression levels.

Table 1: The sequence of the primers used for FGF2, CXCL-12, VEGF-A and B-actin

Name	Sequence (5' → 3')	References
FGF2	forward:5' CGACCCTCACATCAAGCTACAA 3' reverse:5' CCAGGTAACGGTTAGCACACACT 3'	[34]
CXCL-12	forward 5'-GTCAAGCATCTCAAAATTCTCAACAC-3' reverse 5'-CACTTTAGCTTCGGGTCAATGC-3'	[6]
VEGF-A	forward 5'- CTGGAGTGTGTGCCCACTGA-3' reverse: 5'-TCCTATGTGCTGGCCTTGGT-3'	[35]
B-actin	forward:5'-GCCTTTGCCGATCCGC-3' reverse:5'-GCCGTAGCCGTTGTCG-3'	[25]

DNA extraction and sodium bisulfate modification:

The genomic DNA was obtained from primary human endometrial stromal cells using a DNA extraction kit (Pars Tous Company, Iran) according to manufacturer instructions and was then treated by sodium bisulfate, as formerly explained, with minor changes[36-38]. In summary, 2 mcg of extracted DNA was placed at 42°C for 30 min, in a total volume of 50 µl containing 3.5 µl of 3M NaOH. Next, 30 µl of 0.1 M freshly provided hydroquinone (Sigma, USA, and 520 µl of 3 M sodium bisulfate (Sigma, USA) at pH 5.0 were added and incubated at 55°C for 16 h. Then, the solution containing treated DNA were transferred into the filtered column (Pars Tous Company, Iran) and centrifuged for 30 s at 12000 rpm (DNA adheres to the filter and other fluids are removed from the DNA). Afterward, centrifugation was repeated three times with 1 ml of 70% alcohol, for 30 s at 12000 rpm. Then, 50 µl of desulphurizing solution (50 µl of NaOH + 900µL of absolute alcohol + 93 µl of DEPC water) was poured into the column and incubated at room temperature for 5 min. After that, it was centrifuged twice with 1ml of 90% alcohol for 30 s at 12000 rpm. Finally, 30µl of DEPC water was added to the column and incubated at room temperature for 2 min and then was centrifuged at 12000 rpm for 2 m. The DNA was collected in a micro tube beneath the filtered column, which was immediately transferred to a container with 2 °C temperature to be used in MS-PCR.

Methylation-Specific PCR (MS-PCR):

MS-PCR was applied using 2 µl of bisulfite-treated DNA in a volume of 50 µl consisting of 5 µl of 10x buffer (Yekta Tajhiz Company), 1 µl of 4x10 mM dNTP, 0.6 µl of 10 mM of each primer, 3 µl of mgcl2, 0.5 µl of Taq DNA polymerase and the rest was DEPC water. The primers for MS-PCR were designed for the promoter region of the CXCL-12 gene) 493 to +168). The primers for the methylated CXCL-12 were 5’ -GGA GTT TGA GAA GGT TAA AGG TC-3’ (forward) and 5’ -TTA ACG AAA AAT AAA AAT AGA CGA T-3’ (reverse). The primers for the unmethylated CXCL-12 were 5’ -GAG TTT GAG AAG GTT AAAGGT TGG-3’ (forward) and 5’ -TAA CAA AAA ATA AAA ATA CAA CAA T-3’ (reverse) [39, 40]. The PCR conditions were: 95 ˚C for 10 min, followed by 45 cycles of denaturing at 95 ˚C for 45 s, annealing for 45 s at 61.1 ˚C, an extension for 45 s at 72 ˚C, and then a final extension for 10 min at 72 ˚C. Human Methylated & Non-methylated DNA Set (Zymo Research Company) was used as a positive and negative control for the methylated and unmethylated gene. The PCR products were analyzed on 2.5% agarose gels.

Statistical analysis:

The data were analyzed by Statistical Package for the Social Sciences (SPSS) version 16 software using the One-way-ANOVA method; $P<0.05$ was considered significant. Data were expressed as the mean ± (SD) of at least three independent experiments.

Results:

In this study, we checked out VEGF-A, FGF2 and CXCL-12 gene expressions in primary human endometrial stromal cells, before and after treatment with nicotine, nicotine along with CAPE, and CAPE (Figs 1-3).

As seen in Figs 1-3, our results showed that the treatment of endometrial stromal cells with nicotine (10^{-6} μM) for 24 h has significantly reduced the expression of CXCL-12, FGF, and VEGFA genes. However, the decrease in CXCL-12 expression was not associated with the methylation status in the studied promoter region. In contrast, the results from endometrial stromal cells after treating with CAPE (4μg/ml) for 24h, showed that, gene expression of CXCL-12, FGF, and VEGFA significantly increased

Methylation-Specific PCR (MSP) results:

We evaluated the methylation status of CXCL-12 in the primary human endometrial stromal cells in groups: untreated control group (C), treated with nicotine (10^{-6} μM) for 24 h (N), treated with CAPE (4μg/ml) for 24 h followed by treating with nicotine (10^{-6} μM): (CAPE+N), treated with CAPE for 24 h (CAPE), negative (NC) and positive control (PC), Fig 4.

Discussion:

Smoking has been introduced as a risk factor for some diseases including lung cancer [41], pediatric asthma [42], oral cancers [43], cardiovascular disease, etc. [44]. Moreover, maternal smoking has damaging consequences on most processes that contribute to fertility, including ovulation, ovarian-produced steroids, gametogenesis and implantation [45]. Cigarette smoke can disturb placental development and damagingly affects placental anchorage [46], invasion, and trophoblastic migration [47]. Smoking during pregnancy elevates the danger of stillbirth [48] and abortion [49].

Studies have shown that maternal smoking is associated with decreases in IVF success rate and implantation [50–52]. Nicotine exists in cigarette smoke and can pass through placenta and result in placenta dysfunction [23, 24].

Besides, smoking reduces the endometrium receptivity [6], which plays a decisive role in implantation and placentation [6, 53]. Several studies have shown that CXCL-12 and FGF2 play important roles in endometrium receptivity, implantation, and maintaining of pregnancy [7, 8]. CXCL-12 stimulates trophoblastic invasion and tissue angiogenesis after binding to its receptor (CXCR4) [20]. Likewise, several studies carried out on rodents, pigs and rabbits, have shown that FGF2 is involved in implantation, trophoblast cell migration, and embryogenesis. [13–15]. FGF2 provokes adhesion of primary human endometrial epithelial cells to extracellular matrix components by stimulating ERK1/2 phosphorylation; thus it communicates with the blastocyst's trophectoderm [16, 17]. Similarly, VEGF-A is another uterine receptivity marker found in uterine fluid and regulates blastocyst implantation [18]; it also increases the mitotic activity of endometrium cells [19]. VEGF-A elevates blastocyst development and adhesion of uterine epithelial cells [18]; therefore, VGF-A, FGF2, and CXCL-12 are three important maternal–embryonic junction axis [10, 18, 20, 21].

Maternal smoking changes the methylation levels of different genes of the reproductive system [54]. DNA methylation, histone modification, and diverse RNA-mediated processes are important epigenetic processes that regulate gene expression [55].

In this study, the gene promoter of the CXCL-12 was unmethylated during treatment with nicotine in primary human endometrial stromal cells. The decreased expression of the CXCL-12 gene may have been due to the increased expression of microRNAs that regulated the CXCL-12 gene expression and suppressed it. microRNAs can defect transcription factors of the promoter and regulating the CXCL-12 gene expression or histone modification.

Moreover, in some cancers including astrocytoma [56], gastric cancer [40], breast carcinomas [38], and papillary thyroid carcinoma, promoter hypermethylation results in a decrease in expression levels of CXCL-12 gene [57]. Studies showed that the expression of microRNA-135b increases hypoxic conditions, which leads to suppression of CXCL12 expression and trophoblastic invasion. [58]. microRNA-135b and microRNA-130a-3p target the 3'UTR sequence of CXCL-12 and suppress its expression. microRNA-130a-3p has an overexpression in nasopharyngeal carcinoma [58, 59]. In addition, Pillai et al. showed that miR-886-3p reduces the expression of CXCL-12 in human marrow stromal cells [60]. Moreover, microRNA-140 targets the 3'UTR sequence of CXCL-12 in mesenchymal stem cells. In general, the increases the expression of this miRNA lead to decreases the expression of CXCL-12 and vice versa [61]. Markovic et al. have shown that transcription factors including C/EBPβ, C/EBPα, STAT3, p53, FOXO3a, and HMG I/Y are capable of binding to the promoter and regulating the CXCL-12 gene in rat pancreatic insulinoma cell line [62]. Also, histone hypoacetylation can decrease the expression of CXCL-12 gene in colon cancer[63].

Our study showed a reduction in the expression of VEGF-A in human primary endometrial cells treated with nicotine. Sahin et al. indicated that smoking has decreased the expression of CXCL-12 and FGF2 [6]; our study confirmed their results and showed that nicotine exposure can decrease the expression of CXCL-12 and FGF2 genes. Furthermore, another study demonstrated that treatment of endometrial stromal cells with nicotine at concentrations of 10^{-11} μM, 10^{-8} μM, and 10^{-6} μM for 24 h results in decreased expression of DNA methyl-transferases (DNMTs) and decreases global genomic DNA methylation levels, which confirms our results [64].

On the other hand, Polyphenols are complex antioxidants with modification catastristics and are considered because of their beneficial effects in disease [65–67]. Propolis is one of the bee products which is rich in famous polyphenols such as CAPE. This compound, as a potent antioxidant, is protective against the harmful oxygen free radical molecules, methotrexate and pesticides on the reproductive system [28]. It is a potent inhibitor of nuclear factor (NFKB), an anti-cancer, anti-inflammatory, immune modulator and reducer of blood pressure [29, 30]. Moreover, Assumpcao et al. introduced propolis as a new source of DNA methyltransferase inhibitor. In addition, they found that CAPE has the highest contact with DNA methyltransferase-1 [31].

Our results showed that CAPE significantly increases, the nicotine-induced reduction of VGF-A, CXCL-12, and FGF2 gene expression, in human primary endometrial cells. Moreover, we found that, the methylation status in the promoter of CXCL-12 gene, is not associated with the expression level of its mRNA in the cells. In any case, CAPE, can be used as a powerful antioxidant to modified the harmful effects of cigarettes on the uterus and embryo implantation.

Conclusion:

Nicotine treatment decreases the expression of VEGF-A, CXCL-12, and FGF2 in human primary endometrial cells. Since VEGF-A, CXCL-12, and FGF2 are involved in blastocyst implantation, they are recognized as markers of endometrial receptivity. Maternal exposure to nicotine is associated to decrease the endometrium receptivity and fertility; but more investigation is needed to clarify this issue. In contrast, CAPE can improve fertility in maternal smoking, may be because of its capability in increasing the VEGF-A, CXCL-12 and FGF-2 gene expression.

Abbreviations

caffeic acid phenylethyl ester
CAPE
C-X-C motif chemokine ligand 12
CXCL-12
fibroblast growth factor 2
FGF2
vascular endothelial growth factors A
VEGFA
estrogen receptor
ER
progesterone receptor
PR
progesterone type B receptor
PR-B

Declarations

Ethics approval and consent to participate:

Prior to data collection, all respondents were informed about the aims of the study and data confidentiality, and gave written informed consent. All participants took part in the survey voluntarily.

Consent for publication:

not applicable.

Availability of data and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests:

The authors have declared that no conflict of interest exists (financial, non-financial) and they have consent to the publication.

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Authors' contributions:

Conceptualization: BM, methodology: AN, FM, data curation and analysis: BM, AN, FK, SK, writing—original draft preparation: AN, writing—review and editing: BM, the authors read and approved the final manuscript.

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Figures

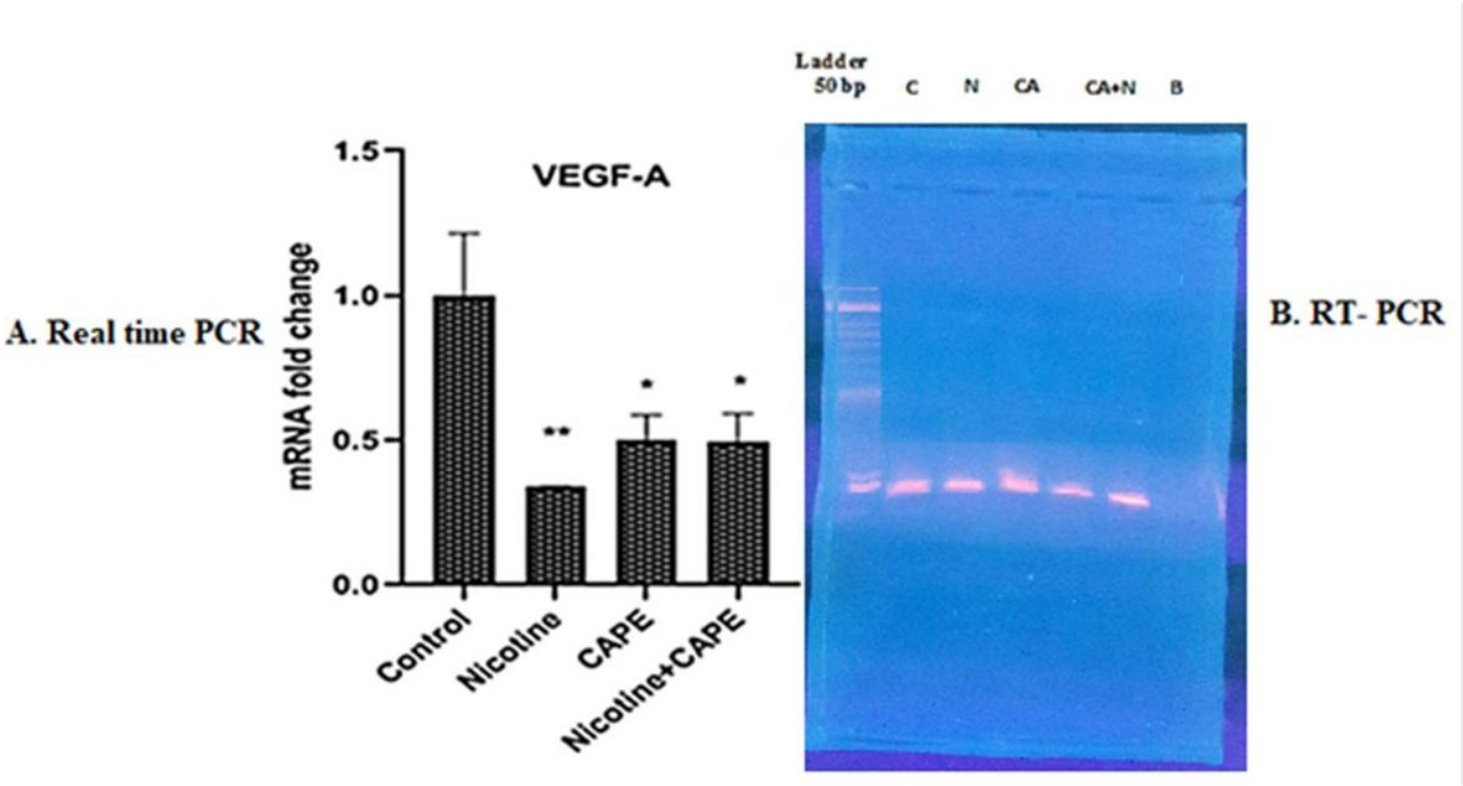

Figure 1

Real time PCR, and changes in the VEGF-A mRNA expression (A), in primary human endometrial stromal cells by nicotine, caffeic acid phenethyl ester (CAPE), and nicotine along with caffeic acid phenethyl ester and RT-PCR analysis (B). primary human endometrial stromal cells were treated with nicotine at a concentration of 10-6 μM for 24 h, CAPE at a concentration of 4μg /ml for 24 h and nicotine along with

caffeic acid phenyl ester . PCR products were analyzed by 1.5% agarose gel electrophoresis. The predicted sizes of RT-PCR products for VEGF-A and B-actin are 81, and 90 bp, respectively. Molecular weight markers (50-bp DNA ladder). Data shown are the 〖Mean〗_-^+ SEM of three independent tests (n =3). Treatment groups were significantly different from the control group

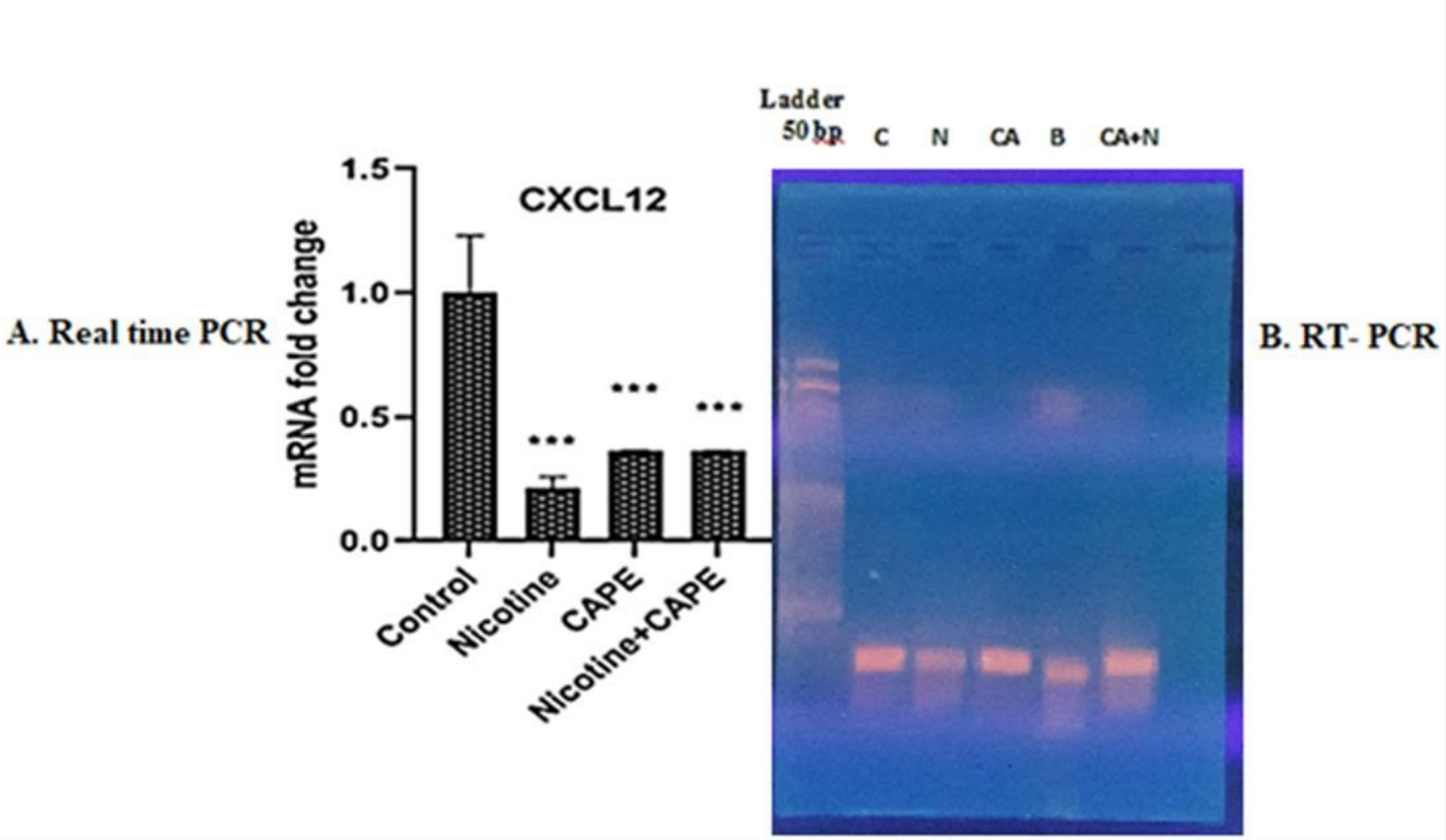

Figure 2

Real time PCR, and changes in the CXCL12 mRNA expression (A) in primary human endometrial stromal cells by nicotine, caffeic acid phenethyl ester (CAPE), nicotine along with caffeic acid phenethyl ester and RT-PCR analysis (B). primary human endometrial stromal cells were treated with nicotine at a concentration of 10-6 μM for 24 h, CAPE at a concentration of 4μg /ml for 24 h and nicotine along with caffeic acid phenyl ester. PCR products were analyzed by 1.5% agarose gel electrophoresis. The predicted sizes of RT-PCR products for CXCL12 and B-actin are 104, and 90 bp, respectively. molecular weight markers (50-bp DNA ladder). Data shown are the 〖Mean〗_-^+SEM of three independent tests (n =3). Treatment groups were significantly different from the control group (***P <.0001) by analysis of one way nova.

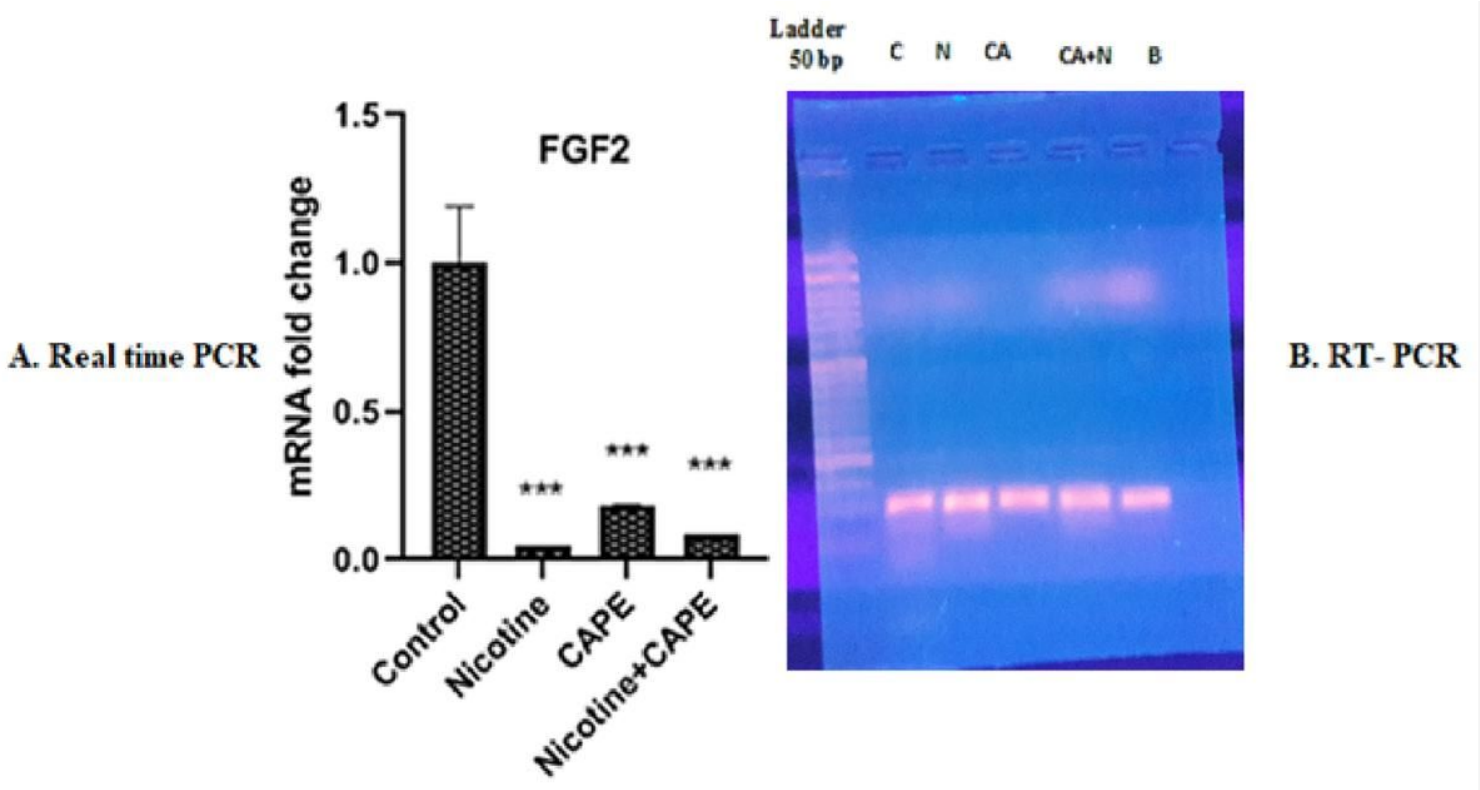

Figure 3

Real time PCR, and changes in the FGF2 mRNA expression (A) in primary human endometrial stromal cells by nicotine, caffeic acid phenethyl ester (CAPE), nicotine along with caffeic acid phenethyl ester and RT-PCR analysis (B). primary human endometrial stromal cells were treated with nicotine at a concentration of 10-6 μM for 24 h, CAPE at a concentration of 4μg /ml for 24 h, and nicotine along with caffeic acid phenyl ester. PCR products were analyzed by 1.5% agarose gel electrophoresis. The predicted sizes of RT-PCR products for FGF2 and B-actin are 83 and 90 bp, respectively. Molecular weight markers (50-bp DNA ladder). Data shown are the 〖Mean〗_-^+ SEM of three independent tests (n =3). Treatment groups were significantly different from the control group (***P <.0001) by analysis of one way nova.

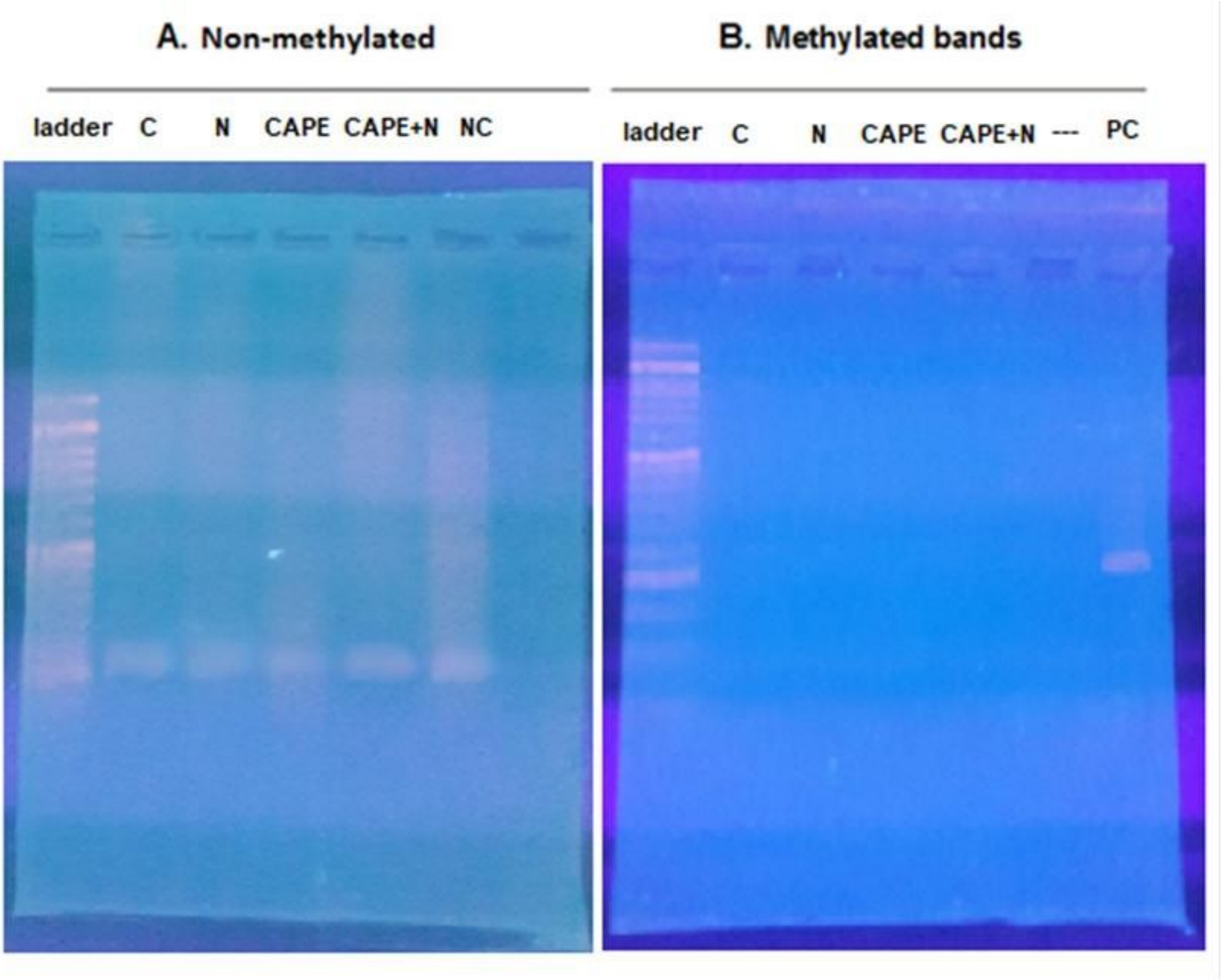

Figure 4

Specific PCR (MS-PCR): CXCL-12 unmethylated and methylated bands in primary human endometrial stromal cells. CXCL-12 was not methylated before and after the treatment with CAPE, showed that nicotin cannot change the methylation status of this gene. A. All groups: control (C), nicotine (N), CAPE + nicotine (CA+N), CAPE (CA) and negative control (NC) groups have shown non methylation status (non methylated primer) B. All of the groups with the methylated primer and positive control (PC).

Supplementary Files

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