The effect of nicotine on soluble fms-like tyrosine kinase-1 (sFlt-1) expression in trophoblasts and human umbilical vein endothelial cells (HUVECs)

Ja-Young Kwon

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Yong-Won Park

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Ja-Young Kwon

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This certifies that the
Doctoral Dissertation of Ja-Young Kwon
is approved.

Thesis Supervisor: Yong-Won Park

Young-Guen Kwon

Sang-Wook Bai

Se-Hoon Kim

Chul Hoon Kim

The Graduate School
Yonsei University

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I remember back in my second year of residency in Obstetrics and Gynecology reading Williams Obstetrics. I came across an interesting phrase..."Although smoking during pregnancy causes a variety of adverse pregnancy outcomes, ironically, smoking has consistently been associated with a reduced risk of hypertension during pregnancy" which made me always wonder why. My life-long pursuit for an answer to this question begins now and here with this naive and humble thesis.

My deepest and most sincere acknowledgement goes out to Professors Young-Guen Kwon, Sang-Wook Bai, Chul Hoon Kim, and Se-Hoon Kim for their dedicated guidance and support in this work. Above all, I bow with honor before Professor Yong-Won Park for being a great mentor and an inspiration whenever and wherever.

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My true affection and dear love will always remain with my family- Dr. Jae Yong Han (my husband, a soul mate and a warm-hearted supporter), Jung-Yeon (our daughter who never complains about mommy's absence), Dr. So-young (a sister to look up to), Dr. Wooil (a great surgeon), and last but not least, my mother who has always been and will always be my shelter and a guiding light.

Ja-Young Kwon

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Cigarette smoking is a unique factor known to reduce the risk of preeclampsia and serum soluble fms-like tyrosine kinase-1 (sFlt-1) level is known to be decreased in smokers. In light of this, present study was performed to evaluate the effect of nicotine on the sFlt-1 expression in trophoblasts and human umbilical vein endothelial cells (HUVECs) and to evaluate whether this effect is modulated by α7 nicotinic acetylcholine receptor (α7 nAChR).

The presence of α7 nAChR in placenta, commercially available term placental trophoblasts- 3A cells, and HUVECs extracted from umbilical cord of normal pregnancy was confirmed by immunostaining, RT-PCR, and western blotting.

Then 3A cells and HUVECs were each treated with nicotine. The gene expression levels of sFlt-1 evaluated by RT-PCR showed a significant decrease in 3A cells ($p<0.05$) but not in HUVECs. Concordantly, the levels of sFlt-1 protein secreted into the supernatant at 3, 6, and 12 h following nicotine treatment which was assayed by ELISA showed a significant reduction in 3A cells when compared with control group (19.0 ± 0.6 pg/mL vs. 29.0 ± 1.0

pg/mL, 23.7 ± 2.0 pg/mL vs. 41.5 ± 3.1 pg/mL, and 32.1 ± 1.9 pg/mL vs. 57.4 ± 5.6 pg/mL, respectively, $p<0.001$). However, this was not evident in HUVECs. When α-bungarotoxin (α-BGT), an antagonist for α7 nAChR was pretreated in 3A cells, aforementioned sFlt-1 suppressive effect of nicotine dissipated.

In conclusion, nicotine downregulated sFlt-1 production in trophoblasts via α7 nAChR but not in HUVECs. And this mechanism may be involved in preeclampsia risk reduction in smokers and thus may provide a potential therapeutic target for alleviating or treating preeclampsia.

Key words: trophoblasts, nicotine, soluble fms-like tyrosin kinase-1, α7 nicotinic acetylcholine receptor, preeclampsia

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I. INTRODUCTION

Preeclampsia is a multisystemic disease unique to pregnancy characterized by high blood pressure associated with proteinuria which develop beyond 20 weeks of gestation.[1] Exact etiology of the preeclampsia has not been elucidated yet, hence leaving us with no effective treatment option except for pregnancy termination. About 5 % of pregnancies are complicated with preeclampsia and it is the leading cause of maternal and fetal morbidity and mortality.[2]

As mentioned earlier, the root cause of preeclampsia remains unraveled. However, 2-stage process theory has gained spotlight over the years. The theory is suggesting that initially, shallow implantation of the placenta causing a hypoxic environment leads to placental elaboration of certain toxic factors

into maternal circulation (the first asymptomatic stage) followed by massive endothelial dysfunction leading to clinical symptoms pertaining to hypertension, proteinuria, edema, liver dysfunction, glomerular endotheliosis, coagulopathy, and etc (the second maternal stage).[1, 3-5]

The cause of insufficient integration of trophoblasts and maternal decidual vessels is not clear, however, studies suggested that abnormally overexpressed or underexpressed adhesion molecules involved in homing and vasculogenesis are involved.[6, 7] What is more important than the initial abnormal placentation early in pregnancy in clinical perspective is that following abnormal placentation, release of circulating factors from abnormally anchored placenta precipitates vascular damage.

Various candidate circulating factors in study are inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), reactive oxygen species (ROS), lipids, neurokinin B, syncytiotrophoblastic microvillous membranes, and angiogenic factors.[8-13]

Lately, an increasing body of evidence indicates that soluble fms-like tyrosine kinase-1 (sFlt-1) plays a crucial role in development of preeclampsia. Placental sFlt-1 expression and amniotic fluid sFlt-1 levels were found to be increased in preeclampsia compared to normal pregnancy.[14, 15] And in animal study by Maynard et al., [5] injection with adenovirus expressing sFlt-1 into pregnant rats increased blood pressure, induced endotheliosis in kidney, and excreted urine protein-characteristic signs in preeclampsia.

Although smoking has detrimental effects on pregnancy such as preterm delivery and fetal growth restriction, it is the only factor known to be related with reduced risk of preeclampsia.[16-18] In a large population study by Hammoud et al.,[16] incidence of preeclampsia was significantly lower in smoking pregnant women compared with nonsmokers and furthermore, the rate of preeclampsia correlated inversely to the number of cigarettes smoked

per day. As to whether smoking before pregnancy has any protective effect against pregnancy related hypertension, England et al. observed that women who smoked throughout pregnancy had the lowest incidence of preeclampsia followed by women who had quit smoking during pregnancy. The incidence was the highest in women who had never smoked as well as in women who had quit smoking before pregnancy.[17] However, exact mechanism via smoking reduces the risk of preeclampsia is still unknown.

In sFlt-1 perspective, Schmidt-Lucke et al. [19] reported that circulating sFlt-1 was decreased in healthy smokers when compared with non-smokers in the normal healthy population. Recently, Levine et al.[20] demonstrated a similar pattern of decrease of serum sFlt-1 level in pregnant women who had smoked during pregnancy compared with non-smokers. This insinuates a possible role of nicotine or another compound from cigarettes as a sFlt-1 suppressant. In this standpoint, smoking may play a beneficial effect in protecting pregnant women from preeclampsia by suppressing the placental production of sFlt-1.

Nicotine, a main component of cigarette, has been shown to exhibit its effect in part through activation of nicotinic acetylcholine receptors (nAChRs). In the past, nAChRs were known to exist only in neuronal tissue transmitting neuronal signals. But their presence in nonneuronal tissue such as endothelium,[21] macrophages,[21, 22] alveolar type II cells,[23] and smooth muscle cells[24] was only discovered to date. Recently, Lips et al. [25] immunolocalized the various type of nAChR in human placenta. However, the exact function of nAChRs found in nonneuronal tissue is still elusive.

Previously, we have shown a difference in placental α7 nAChR expression between normal and preeclamptic women and suggested that this receptor may in part play a role in the pathophysiology of the disease.[13] *In vitro* studies demonstrated that α7 nAChR exerts proangiogenic, anti-inflammatory, and antiapoptotic effects- possible protective effects of smoking against

preeclampsia.[21,26, 27] However, whether the receptor plays a role in suppressing sFlt-1 production in placenta, another possible mechanism by which smoking reduces preeclampsia, has never been studied. In light of this, we performed this study to evaluate nicotine's effect on trophoblasts and HUVECs as to sFlt-1 regulation and to evaluate the role of α7 nAChR in regulating sFlt-1 expression in trophoblasts and HUVECs.

II. MATERIALS & METHODS

1. Patient sample

Third trimester placenta (n=1) and umbilical cords (n=3) were obtained from elective cesarean section of normal pregnancies at the Department of Obstetrics and Gynecology, Yonsei University College of Medicine, Seoul, Korea. All patients consented to the use of tissue and present research was approved by the institutional review board.

2. Reagents and antibodies

Nicotine hydrogen tartrate and α-bungarotoxin (α-BGT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Immunofluorescent staining for α7 nAChR was performed using the monoclonal anti-nicotinic acetylcholine receptor, subunit α7, clone mAb 306 (Sigma, St. Louis, MO, USA) and the same antibody was used for α7 nAChR Western blot analysis. Western blot analysis for Flt-1 was done using monoclonal anti-Flt-1 antibody (Novus Biologicals, Littleton, CO, USA). Level of sFlt-1 in culture media was measured by commercial human sFlt-1 ELISA assay kit obtained from R&D systems (Minneapolis, MN, USA).

3. Cells and cell culture

The human cytotrophoblast cell line, 3A, which was transformed by SV40ts30 was purchased from American Type Culture Collection (Manassas, VA, USA). Cell line was cultured in minimum essential medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum

(FBS) (HyClone, Logan, UT, USA) and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 33°C in humidified 5% CO_2/95% air.

HUVECs were isolated from human umbilical cord veins as described previously by Jaffe EA et al..[28] Cells used in the experiments were in passages 4-5. Cells were cultured in M199 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 20% FBS and penicillin/streptomycin at 37°C in humidified 5% CO_2/95% air.

4. RT-PCR

Total RNA was extracted using an RNeasy extraction kit (Intron, Seoul, Korea). Total RNA was eluted from the columns into a final volume of 40 µl of RNase-free water and stored at 80°C or used immediately for cDNA synthesis. For cDNA conversion, total RNA was used in a reverse transcriptase (Invitrogen, Carlsbad, CA) reaction in a total volume of 20 µl with random hexamer primers and heated at 65°C for 5 min. The RT parameters for cDNA synthesis were were as follows: 50°C for 50 min and 85°C for 5 min and add 1 µl of RNase H 37°C for 20 min. The cDNA generated was subjected to PCR amplification in a GeneAmp PCR System 9700 (PerkinElmer, Waltham, MA, USA) amplification cycler. PCR reactions were carried out in a total volume of 20 µl containing 2 µg of cDNA, 0.4 mM dNTPs, each primer, and 0.2 units of GoTaq polymerase (Promega, Madison, WI, USA). The PCR conditions were 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C, followed by 72°C for 7 min. The PCR products were subjected to electrophoresis in 2% agarose gels. The sequences of the PCR primers for α7 nAChR, sFlt-1, Flt-1 and β-actin, which was used as an internal control, were as follows: α7 nAChR 5'-CCT GGC CAG TGT GGA G-3' (sense primer) and 5'-TAC GCA AAG TCT TTG GAC

AC-3' (antisense primer; 414-bp product); sFlt-1 5'-TTT GCA TAG CTT CCA ATA AAG TTG-3' (sense primer) and 5'-CAT GAC AGT CTA AAG TGG TGG AAC-3' (antisense primer; 450-bp product); Flt-1 5'-GCT CAC CAT GGT CAG CTA CTG-3' (sense primer) and 5'-CAG TGA TGT TAG GTG ACG TAA CC-3' (antisense primer; 505-bp product) and β-actin: 5'-AGG CCA ACC GCG AGA AGA TGA CC-3' (sense primer) and 5'-GAA GTC CAG GGC GAC GTA GCA C-3' (antisense primer; 313-bp product).

5. Western blot analysis

Proteins were extracted from the cells using lysis buffer (Cell Signaling Technology, Danvers, MA, USA). The proteins extracted were loaded onto a 10% mini-SDS-polyacrylamidegel (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane after electrophoresis. Blocking was carried out with 5% non-fat dry milk/PBS (MPBS) and 0.2% Tween-20 for 1.5 h at room temperature. The membrane was incubated with primary antibody overnight at 4°C with gentle shaking. After washing with PBS and 0.1% Tween-20, the membrane was exposed to anti-mouse or anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (Amersham Life Science, Buckinghamshire, UK) for 1 h at room temperature. The membrane was treated with ECL reagents (Amersham Life Science, Buckinghamshire, UK) and exposed to Hyperfilm ECL (Amersham Life Science, Buckinghamshire, UK). β-actin was used as an internal control to compare the data from different films.

6. Immunofluorescence

Placenta

Human term placenta (n=2) was obtained following elective cesarean section. None of the women smoked. Pregnancies were uncomplicated singletone pregnances. Tissue was washed in phosphate-buffered saline (PBS) to remove blood, fixed in cold 4% paraformaldehyde (PFA) for 12 h at 4°C Tissue was embedded in optimal cutting temperature (OCT) compound and frozen at -70°C until use. Cryosections (10 μm) were washed with PBS then incubated in 3% H_2O_2. After washing with PBS, sections were preincubated in blocking solution (Zymed, San Francisco, CA, USA) for 1 h at room temperature (RT) in a humid chamber. The primary antibody for α7 nAChR (1:100 in antibody diluent) was treated overnight at 4°C in humid chamber. After washing with PBS containing 0.05% Triton X-100, secondary FITC-conjugated anti-mouse IgG (1:200 in antibody diluent, Allexis Biochemicals, San Diego, CA, USA) was treated for 1 h at RT in humid chamber. After rinsing, DAPI was treated for 30 min. Sections were mounted, covered with coverslips, and evaluated by epifluorescence microscope.

HUVEC and trophoblasts

Cells were fixed in 2% PFA for 30 min then washed with cold PBS. Blocking solution containing 0.9g bovine serum albumin (BSA) in 30 mL PBS was treated for 1 h at RT. As a primary antibody, α7 nAChR (1:100 in PBS) was treated for 2 h at RT. After rinsing, cells were incubated with FITC-conjugated anti-mouse IgG (1:100 in PBS, Allexis Biochemicals, San Diego, CA, USA) for 1 h at RT. DAPI was applied for 20 min at RT, mounted, then examined by epifluorescence microscope.

7. Analysis of sFlt-1 protein level in culture media

Briefly, 2×10^4 cells were seeded onto 24-well plate and cultured overnight until 80-90% confluent. Cells were then treated with nicotine with or without α-BGT pretreatment. Culture supernatants were obtained according to schedule and assayed for sFlt-1 using commercial ELISA kits. Each experiment was repeated three times.

8. Triton X-100 extraction of α7 nAChR

Confluent monolayer of trophoblasts were overlaid with extraction buffer consisting of 0.5% Triton X-100, 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 300 mM sucrose, proteinase inhibitor mixture containing of phenylmethylsulfonyl floride, iodoacetamide, benzamidine (each 1 μM), aprotinin, leupeptin, pepstatin A and antipain (each 20 μg/ml) for 20 min at RT on a gently rocking platform. The soluble supernatant was collected and this fraction was defined as the membranous fraction of α7 nAChR.

9. Statistical analysis

ELISA result was expressed as means ± standard deviation (SD). Levels of significance were determined by a two-tailed Student's *t*-test, and a confidence level of greater than 95% was used to establish the statistical significance.

III. RESULTS

1. Confirmation of α7 nAChR expression in placenta, trophoblasts, and HUVECs

The presence of α7 nAChR in a normal human placenta was confirmed by RT-PCR and Western blot analysis. Furthermore, most intense immunoreactivity was found in syncytiotrophoblast, cytotrophoblast, endothelial cells of arterioles, and villous stromal cells (Fig. 1A-C).

3A cells, a human placenta-derived cytotrophoblasts, and HUVECs were evaluated for the expression of α7 nAChR by immunofluorescent staining. The staining on the cell cultures displayed a positive reactivity in 3A and HUVECs. The specificity of these findings were evaluated by RT-PCR and Western blot analysis in which the expression of α7 nAChR by both cell types were confirmed as demonstrated in the Figure 1 (Fig. 1D and E).

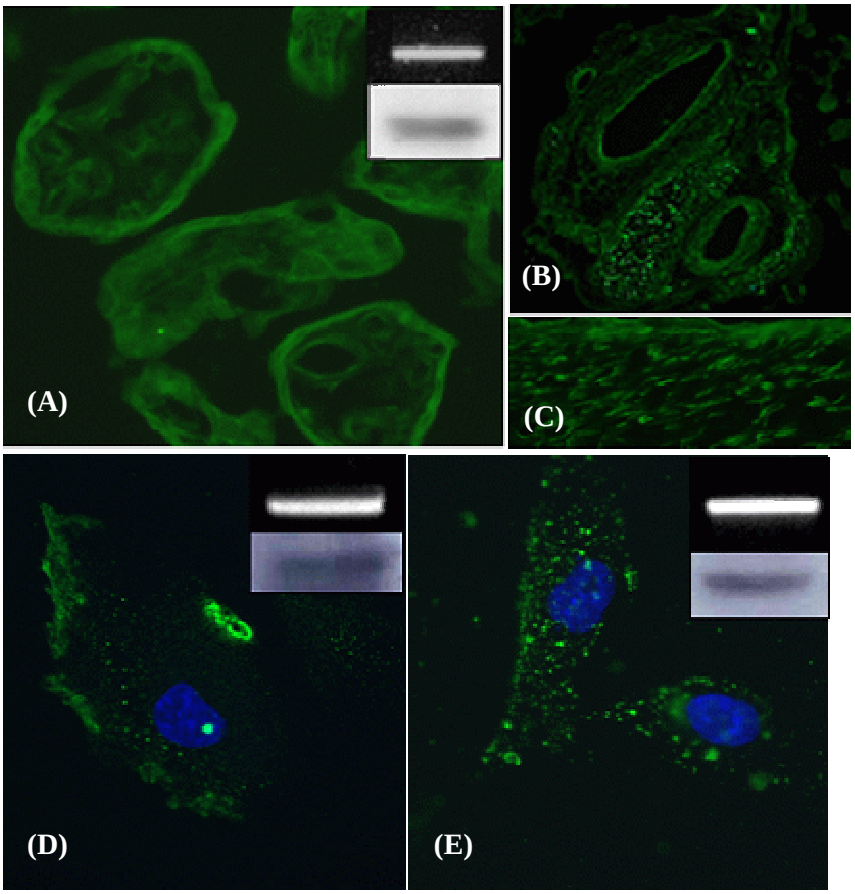

Fig 1. Expression of α7 nAChR in placenta (A-C), 3A cell (D), and HUVEC (E). The α7 nAChR was expressed in trophoblasts, fetal endothelium, and stromal cells. Likely, α7 nAChR was confirmed by RT-PCR, Western blotting, and immunofluorescent staining in 3A cells and HUVECs (A, x200; B, x100; C-E, x600).

2. Nicotine reduces sFlt-1 mRNA expression in 3A cells, but not in HUVECs

Messenger RNA expressions for sFlt-1 were examined by RT-PCR in 3A and HUVECs under different culture condition. As shown in Fig. 2A, sFlt-1 expressions in 3A cells and HUVECs during 12 h remained high in both cell types under normal condition without nicotine treatment.

In 3A cells, when nicotine was added to the culture condition, sFlt-1 gene expression showed a pattern of downregulation in cells treated with nicotine at a concentration of 10^{-6}M in a time-dependent manner when compared to the control cell group (Fig. 2B and 2D). To the contrary, in HUVECs sFlt-1 gene expression was not altered by nicotine treatment (Fig. 2C).

Furthermore, the effect of nicotine on 3A cells in suppressing sFlt-1 mRNA expression was reversed by pretreating the cells with α-BGT, an α7 nAChR antagonist reflecting this receptor's involvement in the aforementioned effect of nicotine (Fig. 2D).

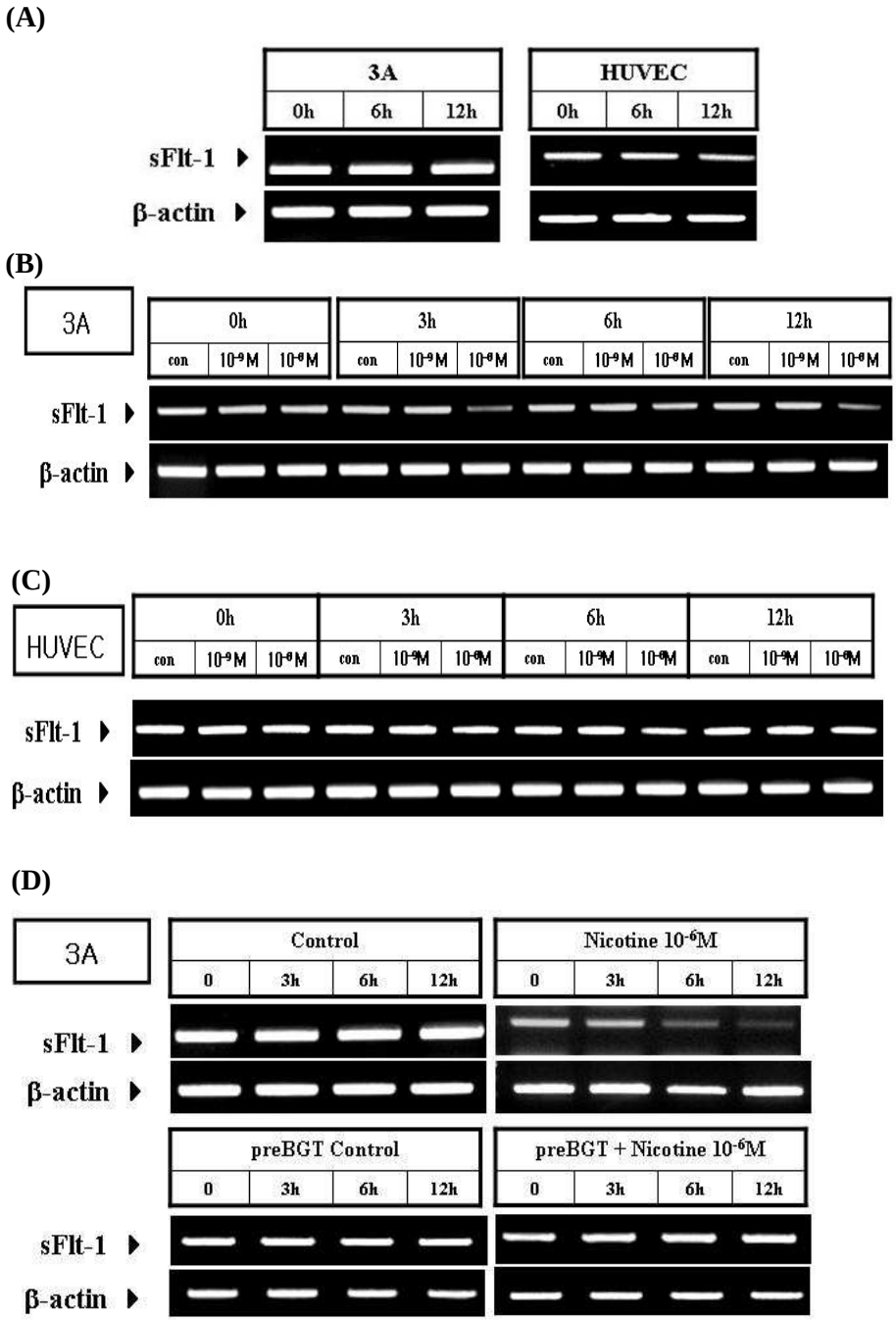

Fig. 2. Expression of sFlt-1 mRNA in 3A cells and HUVECs. In the control groups, sFlt-1 mRNA was persistently elevated at 3, 6, and 12h (A). But when

cells were treated with nicotine (10^{-9}M and 10^{-6}M), sFlt-1 mRNA expressions were downregulated at 10^{-6}M concentration in time-dependent manner in 3A cells but not in HUVECs (B and C). The nicotine's effect on 3A cells in reducing sFlt-1 expression was blocked by α-BGT pretreatment (D).

3. Nicotine reduces sFlt-1 protein production in 3A cells, but not in HUVECs

Levels of sFlt-1 in culture media following nicotine treatment were measured. In 3A cells, sFlt-1 protein level at 3, 6, and 12 h after 10^{-6}M nicotine treatment was 19.0 ± 0.6, 23.7 ± 2.0, and 32.1 ± 1.9 pg/mL, respectively whereas in the control cell group, it was 29.0 ± 1.0, 41.5 ± 3.1, and 57.4 ± 5.6 pg/mL, respectively ($p<0.05$). Nicotine markedly reduced sFlt-1 release from the 3A cells under 10^{-6}M nicotine exposure and this pattern of decrease was also observed when nicotine dose was adjusted to 10^{-7}M (Fig. 3A-C). Interestingly, when α7 nAChR was blocked by α-BGT, nicotine did not alter the sFlt-1 production in 3A cells.

The baseline sFlt-1 production at 3, 6, and 12 h in HUVECs was 68.3 ± 2.9, 230.6 ± 17.9, 457.9 ± 5.0 pg/mL, respectively, which was significantly higher than that of 3A cell ($p<0.05$). And the sFlt-1 level at 3, 6, and 12 h in nicotine-treated and nontreated HUVECs was 59.9 ± 4.3 vs. 68.3 ± 2.9 pg/mL, 204.0 ± 15.8 vs. 230.6 ± 17.9 pg/mL, and 427.8 ± 14.5 pg/mL vs. 457.9 ± 5.0 pg/mL, respectively, showing no significant difference ($p=0.23$, 0.08, and 0.94, respectively) (Fig.3D-F).

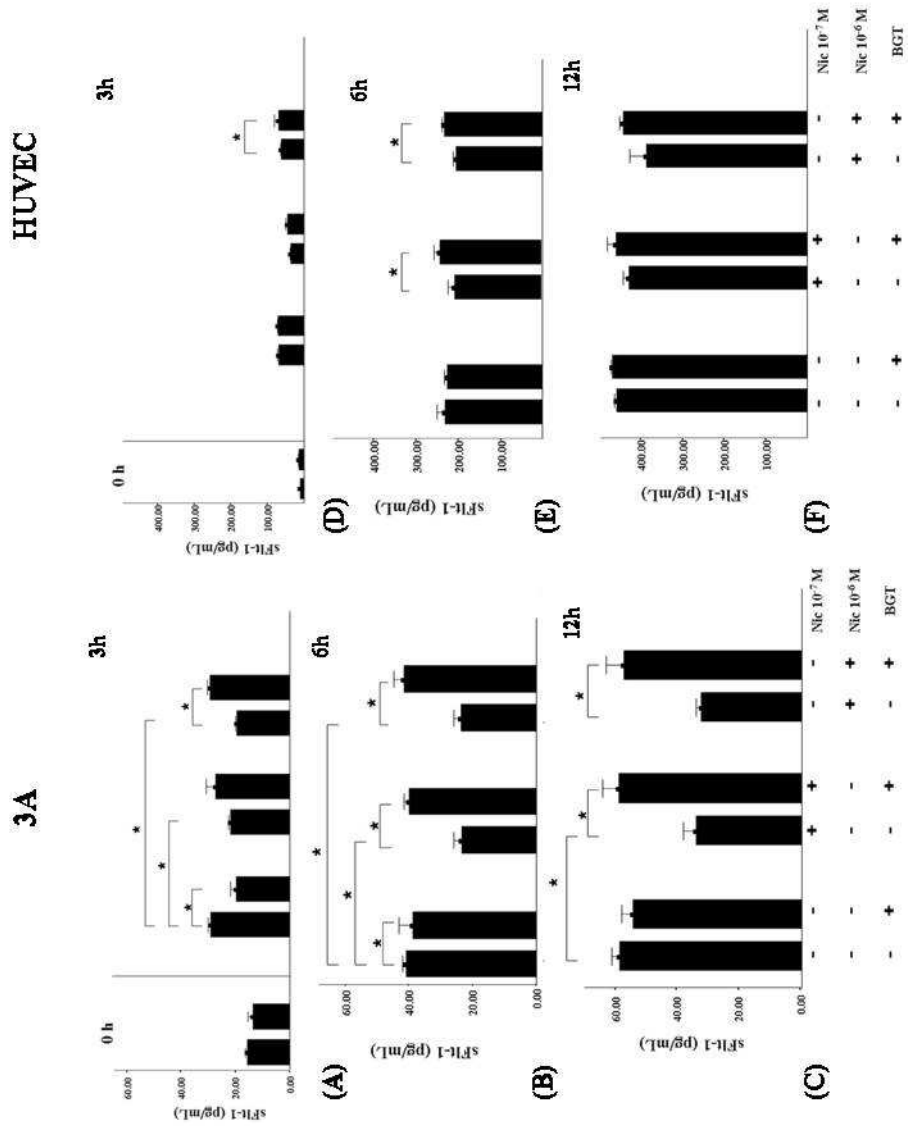

Fig. 3. The levels of sFlt-1 in supernatants of 3A cells (3A-C) and HUVECs (3D-F) according to nicotine (10^{-7} M and 10^{-6} M) and α-BGT treatments. When compared with the control cell group, nicotine treatment significantly decreased sFlt-1 secretion and the 2-h pretreatment of α-BGT

significantly reversed nicotine's effect in 3A cells (*$p<0.05$). On the other hand, nicotine did not decrease sFlt-1 secretion in HUVECs.

4. Flt-1 protein expression in 3A cells decreases with nicotine treatment in time-dependent manner.

As mentioned earlier, sFlt-1 is a byproduct of full-length Flt-1 transcription. So we evaluated the effect of nicotine on the full-length Flt-1 production to determine if sFlt-1 downregulation by nicotine is attributed to its full-length Flt-1 transcription suppression.

Flt-1 gene expression and protein level in 3A cells following nicotine and α-BGT treatments were evaluated. As a result shown in Fig. 4, no significant change was noted in Flt-1 gene and protein expressions at 6 and 12h in nicotine treated or α-BGT plus nicotine treated group when compared to the control cell group.

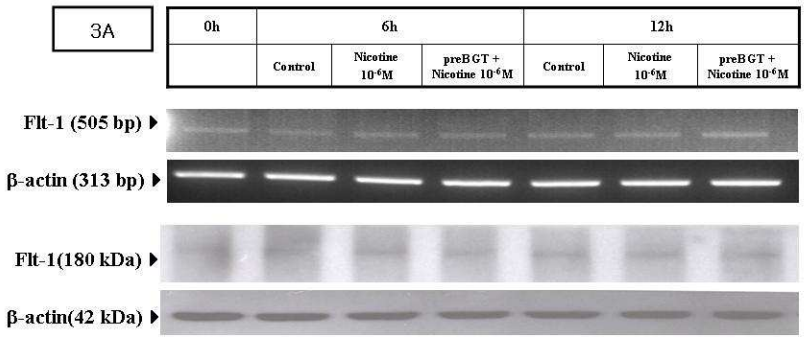

Fig. 4. Flt-1 gene and protein expressions in 3A cells. No significant rise or decline in Flt-1 expression was noted in the nicotine treated group when compared with the control group.

5. Membranous expression of α7 nAChR in 3A cells according to nicotine treatment in time-dependent manner

In the present study, we anticipated to evaluate the role of α7 nAChR in reducing sFlt-1 production by 3A cells after nicotine treatment. However, as α7 nAChR had been previously reported to be either downregulated or upregulated by its stimulation with its agonist, we evaluated the membranous expression of α7 nAChR after nicotine treatment according to exposure time to demonstrate that sFlt-1 reduction following nicotine treatment was not the resultant of membranous α7 nAChR downregulation. As shown in Fig. 5, membranous α7 nAChR expression was not attenuated by nicotine treatment during indicated time period. Thus, it can be concluded that the nicotine's effect on sFlt-1 reduction in 3A cells was not related to membranous α7 nAChR level change.

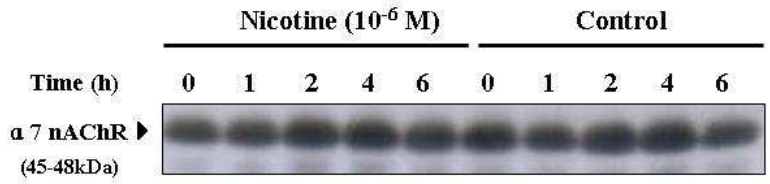

Fig. 5. Membranous expression of α7 nAChR in 3A cells after nicotine treatment. Confluent 3A cells were subjected to 10^{-6}M of nicotine. Triton X-100-soluble fraction representing the cell membrane protein was collected. Western blot analysis of the fraction for α7 nAChR demonstrated no difference in the level of expression between control and nicotine-treated cells.

Ⅳ. DISCUSSION

The sFlt-1 is known as a byproduct of VEGFR-1 which is generated by mRNA splicing of the Flt-1 gene. The uniqueness about the sFlt-1 when compared with Flt-1 lies in that it specifically lacks the cytosolic domain. Therefore, rather than remaining bounded to the cell membrane, it is excreted into the circulation acting as a potent antagonist of VEGF and placental growth factor. The mechanisms by which the sFlt-1 antagonizes the previously mentioned angiogenic factors are as follows: (a) decreasing the free active form of the angiogenic factors by directly binding or (b) blocking the downstream activation of Flt-1 by binding to the extracellular ligand binding region.[20, 29]

Preeclampsia, a syndrome of pregnancy is relatively common and remains a significant cause of maternal and fetal morbidity and even mortality. However, the etiology of the syndrome is comprised of theories. Thus the target for alleviation or treatment of preeclampsia for a healthy pregnancy and delivery is still not available to date. Generally acceptable potential causes are summarized as abnormal trophoblastic invasion of uterine vessels, immunological intolerance between maternal and fetoplacental tissues, maternal maladaptation to cardiovascular or inflammatory changes of normal pregnancy, dietary deficiencies, and genetic cause.[30-34] Nonetheless, the cascade of event leading to the preeclampsia syndrome is suggested as increased circulating sFlt-1 level that results in vascular endothelial damage with vasospasm, transudation of plasma, and ischemic and thrombotic sequelae.[5]

With respect to preeclampsia and sFlt-1, bodies of studies highlighted that the excessive amount of sFlt-1 most probably released from the placental tissues and eventually is involved in endothelial dysfunction playing a

contributory role in the pathogenesis of the maternal syndrome of preeclampsia.[4,5,35] This hypothesis seems plausible as sFlt-1 was demonstrated to inhibit tube formation by endothelial cells *in vitro*, as sFlt-1 expression was significantly increased in preeclamptic placenta when compared with normal placenta,[5,36] and as serum sFlt-1 levels were significantly increased in preeclampsia patients.[14,37] Moreover, the animal study conducted by Maynard et al. demonstrated that adenoviral gene transfer of sFlt-1 to normal rats resulted in characteristic clinical symptoms of preeclampsia such as hypertension, proteinuria, and glomerular endotheliosis.[5]

Conditions that are considered to be more susceptible to preeclampsia are nulliparous women, chronic hypertension, multifetal gestation, maternal age over 35 years, obesity, and African-American ethnicity. On the other hand, it is a long standing observation that cigarette smoking is associated with reduced risk of preeclampsia. Women who smoked cigarette throughout pregnancy are at a 33% reduced risk of developing preeclampsia.[18,38,39] Therefore, it certainly is worth pursuing for the mechanism by which smoking reduces this risk in hope to discover a novel therapeutic target. Unfortunately, the exact mechanism of this risk reduction is not yet understood.

North et al. hypothesized that smoking by compromising the immunity, decreases the exaggerated immune response seen in the preeclampsia thereby reducing the risk.[40] Others suggested that chronically induced endothelial events by cigarette smoking may eventually down-regulates endothelial sensitivity to triggering signals thus preventing preeclampsia development.[41] Bainbridge et al. proposed that carbon monoxide generated from the cigarette may act directly on the placenta to increase trophoblast invasion, placental flow, nitric oxide through hemoproteins activation, to decrease localized inflammatory response and syncytiotrophoblast apoptosis and to upregulate antioxidant system.[42]

In addition to this, we hypothesized that there may be a mechanism via cigarette smoking suppresses the sFlt-1 production thereby preventing preeclampsia. In a nested case-control study of pregnant women, Levine et al. evaluated the association between smoking and circulating sFlt-1 and found that those who smoked throughout the gestation had significantly lower levels of sFlt-1 when compared with those who had never smoked or had quit before the pregnancy.[20] This finding, along with other reports involving non-pregnant individuals strongly suggests that smoking may prevent pregnant women from preeclampsia through modulating angiogenic factor.[19,43] Furthermore, Mehendale et al. observed that cigarette smoke extract significantly reduced sFlt-1 secretion from the placental explants under hypoxia and normoxia in a dose-dependent manner recapitulating the nicotine effect in smokers.[29] However, because placental explants consist of vessels, stromal cells, and trophoblasts their study could not pinpoint as to which cells were mainly effected by the cigarette smoke and as to why.

Our findings demonstrated that sFlt-1 production from the trophoblast cell line was significantly reduced by nicotine treatment in a time-dependent manner. However, the mechanism of this decrease is unclear. In some ways, sFlt-1 is a byproduct of Flt-1. So at first, we hypothesized that decreased sFlt-1 production may be preceded by Flt-1 mRNA transcription suppression leading to a concurrent reduction of splicing variant generation. But to the contrary, no significant decrease in Flt-1 gene and protein expression was found with nicotine exposure. Thus we believe that sFlt-1 reduction by nicotine may involve modulation of post-transcriptional mechanism by which sFlt-1 is produced.

Nicotine binds to nicotinic acetylcholine receptors to elicit its effect. The distribution and expression of various types of nicotinic acetylcholine receptors in human placenta has been recently studied by Lips et al..[25] They

discovered that α-subunits present at the maternal-fetal interface were α3, 4, 5, 7, 9, and 10. However, α3, 4, and 5 subunits when bound with nicotine have been reported to function as a suppressor of active amino-acid uptake in the placenta,[44] and α9 and α10 subunits to inactivate nAChR rather than transmitting nicotine signal.[45] Therefore, we assumed that these subunits were least likely to be involved in mediating nicotine's effect on sFlt-1 suppression. This left us with α7 subunit. Also, the fact that selective antagonist was only available for α7 nAChR led us to favor this specific receptor.

Various effects of α7 nAChR in response to nicotine have been reported. According to Saeed et al, α7 nAChR stimulation by nicotine suppressed endothelial cell activation, inhibited TNF-induced adhesion molecule expression and chemokine production by endothelial cells, and blocked leukocyte migration during inflammation.[22] Moreover, α7 nAChR stimulation protected cells from hypoxia-induced apoptosis.[27, 46, 47] Since endothelial cell activation, inflammation, and apoptosis in placenta are being suggested as part of the process involved in the pathogenesis of preeclampsia, α7 nAChR mediating aforementioned effects may play a significant role in reducing the risk of preeclampsia in a cigarette smoker. This assumption gave us more reasons to favor α7 nAChR as a target of investigation.

As shown in this experiment, we demonstrated that nicotine downregulates sFlt-1 expression via α7 nAChR in trophoblasts by pretreating cells with α-BGT, an α7 nAChR antagonist. When α7 nAChR was blocked by its antagonist, nicotine's effect to suppress sFlt-1 production dissipated. This discovery of nicotine and α7 nAChR's involvement in decreasing sFlt-1 production from trophoblast may contribute to the development of potential pharmaceutical agent or target which could be used to either alleviate or treat preeclampsia while eluding nicotine's collateral toxicity.

Other reports demonstrated either an upregulation or a downregulation of the

membranous α7 nAChR expression in a time-dependent or a dose-dependent manner when exposed to nicotine.[21,48] And because this α7 nAChR desensitization phenomenon might have confounded our observation of suppressed sFlt-1 production in this study, we evaluated the membranous α7 nAChR expression levels according to nicotine-exposure time. As a result, we were able to discern the absence of α7 nAChR downregulation on the membrane of trophoblasts following nicotinic stimulation, thereby substantiating our initial hypothesis that nicotine directly suppresses sFlt-1 expression through α7 nAChR.

There are potential limitations to the present study. Firstly, the cell line which was used in this study was immortalized human trophoblasts by tsA mutants of simian virus 40 which cannot fully represent in vivo syncytiotrohoblasts. Thus, obtaining a consistent nicotinic effect from trophoblast cells that were not manipulated is crucial to substantiate our findings. Secondly, HUVECs is endothelial cells comprising the umbilical vein and not peripheral villous vessels. Therefore, *in vivo* response of placental vessels to nicotine as to sFlt-1 production cannot be extrapolated from the lack of response to nicotine in HUVECs demonstrated in this study.

Present study introduced the nicotine's potential proangiogenic role as a sFlt-1 suppressor in trophoblast cell line which may explain its protective effect from developing preeclampsia in smoking mothers. And we demonstrated that this effect was mediated through α7 nAChR. Nonetheless, precise downstream mechanism of α7 nAChR through which nicotine decreases endogenous excretion of sFlt-1 from trophoblasts was not elaborated in this study and still remains elusive. Therefore, further investigations must be deployed to clarify the downstreams of α7 nAChR.

Ⅴ. CONCLUSION

To evaluate the effect of nicotine on sFlt-1 production in 3A cells (a human trophoblast cell line) and HUVEC, sFlt-1 gene and protein levels were analyzed following nicotine treatment. Furthermore, the potential role of α7 nAChR in mediating aforementioned effect was evaluated.

1. Under normal culture condition, 3A cells and HUVECs expressed sFlt-1 gene and secreted sFlt-1 protein.

2. Nicotine treatment decreased both the sFlt-1 gene expression and protein secretion in 3A cells, however, these effects were not observed in HUVECs.

3. The sFlt-1 suppressive effect of nicotine on 3A cells dissipated when α7 nAChR was blocked by α-BGT.

In conclusion, nicotine downregulated sFlt-1 production in 3A cells via α7 nAChR but not in HUVEC. And this mechanism may be involved in preeclampsia risk reduction in smokers and thus may provide a potential therapeutic target for preventing or alleviating preeclampsia.

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Abstract (in Korean)

니코틴이 영양막세포와 제대정맥유래 혈관내피세포로부터의 soluble fms-like tyrosine kinase 분비에 미치는 영향

<지도교수 박용원>

연세대학교 대학원 의학과
권 자 영

전자간증은 독특하게 임신 중 발생하는 질환으로 산모와 태아의 이환률과 사망을 증가시키는 것으로 알려져 있다. 현재까지의 연구들을 보면 전자간증의 발생이 태반으로부터 다량으로 유리된 soluble fms-like tyrosine kinase (sFlt-1)로 인해 혈중 sFlt-1 의 농도가 증가되고 이 물질이 antiangiogenic 인자로서 작용하게 되면서 전자간증이 발생할 것이라고 한다. 그런데 흡연이 전자간증의 발생을 감소시킨다고 알려져 있으며 또한 정상인에 비해 흡연자에서 혈중 sFlt-1 농도가 유의하게 감소되어있다고 보고되고 있다. 이런 보고들을 바탕으로 본 연구를 통해 담배의 주성분인 니코틴이 태반을 이루고 있는 영양막세포와 혈관내피세포(HUVEC) 에서의 sFlt-1 분비감소 효과가 있는지와 이 과정에 니코틴에 대한 수용체 중 하나인 $\alpha 7$ nicotinic

acetylcholine receptor (α7 nAChR)가 관여하는 지 알아보고자 하였다.

인간 영양막세포주인 3A 와 HUVEC 에 니코틴을 처치하고 RT-PCR 을 통한 sFlt-1 유전자 발현을 본 결과 니코틴이 3A 세포에서 sFlt-1 유전자 발현을 유의하게 감소시키는 것을 확인하였으며 이러한 현상은 HUVEC 에서는 관찰되지 않았다. 니코틴 처치 후 배양액 내 유리된 sFlt-1 단백농도를 ELISA 분석방법을 이용해 측정한 결과 3A 세포주에서 3, 6, 12 시간 대 sFlt-1 단백농도가 니코틴을 처치하지 않은 군에서는 각각 29.0 ± 1.0, 41.5 ± 3.1, 57.4 ± 5.6 pg/mL 이였던 반면, 니코틴을 처치한 군에서 각각 19.0 ± 0.6, 23.7 ± 2.0, 32.1 ± 1.9 pg/mL 으로 니코틴에 의한 sFlt-1 분비감소효과를 확인할 수 있었다 ($p<0.001$). 그러나 HUVEC 에서는 이러한 현상이 관찰되지 않았다. 3A 세포주에서 관찰된 니코틴에 의한 sFlt-1 유전자 및 단백 분비억제 효과는 α7 nAChR 길항제인 α-bungarotoxin(α-BGT) 전처치로 인해 소실되었다.

위의 결과를 종합해 볼 때, 니코틴은 영양막세포에서 sFlt-1 발현 억제 효과가 있었으며 이 효과는 α7 nAChR 를 경유하였을 것으로 생각된다. 따라서 이 기전이 흡연에 의한 전자간증 발생률의 감소에 관여되었을 수 있으며 추후 전자간증 예방 및 치료제 개발에 적용 될 가능성이 있을 것으로 생각된다.

핵심되는 말: 영양막세포, 니코틴, soluble fms-like tyrosine kinase-1, α7 nicotinic acetylcholine receptor, 전자간증