



## ARAŞTIRMA / RESEARCH

# Investigation of the effects of nicotine and resveratrol on expression levels of Sox2 and Sox4 genes in human amniotic cell culture by real time PCR

İnsan amniotik hücre kültüründe nikotin ve resveratrolün Sox2 ve Sox4 genlerinin ekspresyon seviyeleri üzerindeki etkilerinin real time PCR ile araştırılması

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### Abstract

**Purpose:** The aim of this study was to show the effects of nicotine on expression levels of SOX2 and SOX4 genes, which are known for their master gene features, in human amniotic cell cultures. The study also aimed to show the effects of resveratrol, an antioxidant, on expression levels of these genes in human amniotic cell cultures, which are treated by nicotine.

**Materials and Methods:** Twenty patients were included in this study and for each patient; control, nicotine treated and nicotine + resveratrol treated cell culture groups are formed. The expression levels of SOX2 and SOX4 genes are examined in each group by using real time RT-PCR.

**Results:** Change in expression levels of SOX2 and SOX4 genes in nicotine treated group were found to be statistically significant. Also, when groups treated with nicotine and nicotine + resveratrol were compared the difference was found to be statistically significant.

**Conclusions:** Nicotine increased the expression levels of SOX2 and SOX4 genes by 60% in human amniotic cell cultures and resveratrol was found to be an important antioxidant that reduces the increased expression levels of SOX2 and SOX4 genes caused by nicotine treatment.

**Key words:** Amniotic cell culture, nicotine, resveratrol, SOX2, SOX4

### Öz

**Amaç:** Bu çalışmada, nikotinin master gen özelliklerine sahip genlerden olan SOX2 ve SOX4 genlerinin ekspresyon düzeylerine etkilerinin insan amniotik hücre kültüründe araştırılması amaçlanmıştır. Ek olarak, bir antioksidan olan resveratrolün nikotin ile muamele edilmiş hücrelerde bu genlerin ekspresyon düzeylerine etkileri insan amniotik hücre kültüründe incelenmiştir.

**Gereç ve Yöntem:** Çalışmaya 20 hasta dahil edilmiştir ve her hasta için kontrol, nikotin ve nikotin+resveratrol hücre kültürü grupları oluşturulmuştur. SOX2 ve SOX4 genlerinin ekspresyon düzeyleri her grup için real time RT-PCR yöntemi kullanılarak tespit edilmiştir.

**Bulgular:** SOX2 ve SOX4 genleri için nikotin ile muamele edilen grupta tespit edilen ekspresyon düzeylerindeki değişim istatistiksel olarak anlamlı bulunmuştur. Ayrıca, nikotin ve nikotin+resveratrol ile muamele edilmiş gruplar karşılaştırıldığında sonuç istatistiksel olarak anlamlı bulunmuştur.

**Sonuç:** Nikotin, insan amniotik hücrelerinde SOX2 ve SOX4 genlerinin ekspresyon düzeylerini %60 oranında artırmıştır ve resveratrolün bu artmış ekspresyon düzeylerini düşüren önemli bir antioksidan olduğu tespit edilmiştir.

**Anahtar kelimeler:** Amniotik hücre kültürü, nikotin, resveratrol, SOX2, SOX4

## INTRODUCTION

Smoking during pregnancy is considered to be quite dangerous for fetal development since it may cause different complications such as; miscarriage, perinatal deaths and low birth weight syndrome<sup>1</sup>.

Besides, a variety of toxic substances in cigarettes cause obstetrical and developmental complications<sup>2</sup>. One of the most important toxic components in cigarettes is nicotine<sup>1</sup>. Nicotine is a neuroteratogen, which affects the brain during critical stages of development and may cause cognitive, emotional, behavioral problems in children of mothers, who

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smoke during pregnancy<sup>3</sup>. Also, exposure to cigarette smoke during prenatal stage causes an increase in the rate of childhood cancers including brain tumors and leukemia<sup>4</sup>.

In this study expression levels of SOX2 (sex determining region homeobox2) and SOX4 (sex determining region homeobox4), which are involved in various signaling pathways, are examined to point out different aspects of damage caused by nicotine exposure during pregnancy. In addition, the effect of resveratrol, an antioxidant, on expression profiles SOX2 and SOX4 genes is examined. SOX2, an intronless gene, encodes a member of HMG-box (High mobility group-box) family, which is a transcription factor related with SRY (sex determining region Y) and is involved in the determination of molecular fate of the cell and regulation of the embryonic development<sup>5</sup>. SOX2 plays an important role in regeneration, proliferation and apoptosis in cells<sup>6</sup>. SOX4 gene, which encodes a very important transcription factor in vertebrates, is also essential for cell differentiation and proliferation for various tissues<sup>7</sup>. SOX4 gene is associated with development and tumorigenesis<sup>8</sup>.

Resveratrol is a polyphenol compound, which is found in grapes, strawberries and herbal medicines. It has been shown to exhibit a variety of biochemical activities such as regulation of cell cycle, stimulation of endothelial nitric oxide synthase and thrombocyte aggregation<sup>9</sup>. Resveratrol also reduces the O<sub>2</sub> (oxygen) products of mitochondria induced by the cigarette smoke extract, thus protects endothelial cells against DNA damage induced by cigarette smoke extract<sup>10</sup>.

This study aimed to determine the problems that may arise during development of infants due to the changes in expression levels of SOX2 and SOX4 genes, which play important roles in proliferation and tumor formation, by using human amniotic cell cultures treated with nicotine. Also, examination of the interaction between resveratrol and nicotine through those genes is aimed.

## MATERIALS AND METHODS

Working groups were composed of pregnant women (n=20), who were followed by Cukurova University Faculty of Medicine, Department of Obstetrics and Gynecology Clinic, required to have amniocentesis, non-smoker, aged between 25-30 and at their 16-17 weeks of pregnancy. People who

smoked, past gestational week 16-17 or were over 30 years old at the time of the study were excluded.

After the completion of required tests of the subjects, remaining amniotic fluids were used in this study. Study is approved by the ethical committee of Cukurova University Medical Faculty (5 April 2012, Meeting number: 7) and each subject was informed about the study and asked to fill in the signed informed consent form.

## Study design

Amniotic cells of the subjects with the desired parameters were inoculated to cell culture medium in cell culture flasks and incubated for 12-14 days in 37 °C incubator containing 0.5% CO<sub>2</sub> for proliferation. After 12-14 days of cell culture, each culture was passaged and divided into three flasks. Standard cell culture procedure was applied to the first flasks in each subject and was designated as the control. Nicotine (20 ng/ml) was added into the second flasks and they were designated as the nicotine group. Nicotine (20 ng/ml) and resveratrol (0.5 μM) were added into the third flasks and they were designated as the nicotine + resveratrol group. Flasks were incubated in a CO<sub>2</sub> incubator for 10 days to proliferate.

After the confirmation of proliferation by using invert microscope, the cells were collected. RNA isolation was performed with the collected cells by using RNA isolation kit (Vivantis Technologies). cDNAs were obtained from the RNA samples by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time RT-PCR reactions were carried out by using FAM tagged TaqMan Gene Expression Assays (Applied Biosystems), which were designed for SOX2 and SOX4 genes. Results of Real-time RT-PCR reactions were analyzed by using  $\Delta\Delta C_p$  method.

## Statistical analysis

In this study, the t-test was used to analyze the difference between the control group with constant value and  $2-\Delta\Delta C_t$ , which indicates the variation of the gene expression coefficient of the nicotine group with variable values. The  $2-\Delta\Delta C_t$  values of the nicotine group and the nicotine + resveratrol group, both with variable values, were compared with the Wilcoxin test and the differences between these groups were examined.

## RESULTS

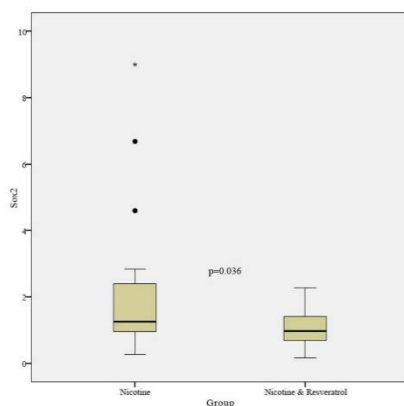
Nicotine was found to affect the expression levels of SOX2 and SOX4 genes ( $p=0.005$  for SOX2,  $p=0.000$  for SOX4). Expression level of SOX2 was found to increase in 60%, decrease in 30% and was found to remain unchanged in 10% of the nicotine treated group (Table 1). Expression level of SOX4 was found to increase in 60%, to decrease in 35% and was found to remain unchanged in 5% of the nicotine treated group (Table 1). Nicotine showed a

similar increasing effect on expression levels of both SOX2 and SOX4 genes. Effect of resveratrol on expression levels of SOX2 and SOX4 genes that were induced by nicotine was analyzed by using Wilcoxon test and results were found to be statistically significant ( $p=0.036$  for SOX2,  $p=0.026$  for SOX4) (Figure 1, Figure 2). Resveratrol was found to decrease the expression levels of SOX2 and SOX4 genes, whose expression levels were induced by nicotine.

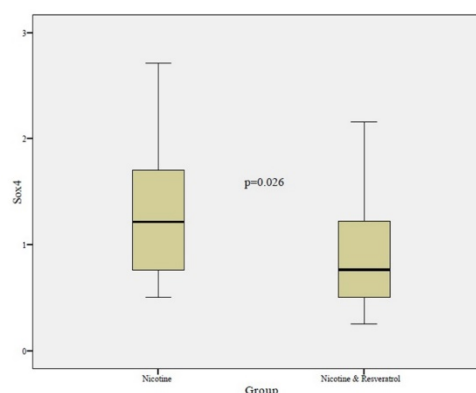
**Table 1. Expression levels of SOX2 and SOX4**

Subject #	SOX2		SOX4	
	Nicotine	Nicotine+Resveratrol	Nicotine	Nicotine+Resveratrol
1	0.259	0.165	1.248	1.602
2	2.8	0.28	2.5	0.297
3	0.94	0.94	0.5	0.6
4	0.97	0.8	1.22	1.73
5	1.390	1.96	0.750	0.66
6	1.240	1.17	0.970	1.19
7	1.000	1.141	1.341	1.11
8	1.376	1.007	2.144	1.464
9	1.266	0.42	1.206	1.24
10	4.595	2.266	1.548	0.33
11	0.586	0.865	0.727	0.346
12	1.007	1.257	1.173	0.611
13	0.993	0.674	1	1.206
14	1.682	1.705	0.774	0.865
15	6.681	1.558	2.713	2.158
16	0.483	0.646	0.7	0.63
17	2.84	1.14	1.77	0.98
18	9	1.90	2.1	0.60
19	0.8	0.86	0.63	0.41
20	1.98	0.72	1.63	0.25

\* Control flasks are accepted as "1"



**Figure 1. p value obtained by Wilcoxon test for SOX2 gene**



**Figure 2. p value obtained by Wilcoxon test for SOX4 gene**

## DISCUSSION

Despite evidence that cigarette smoking has harmful effects on fetal development and growth in pregnancy, one out of every three cigarettes smokes during early pregnancy<sup>11</sup>. Smoking during pregnancy increases the risk of developing health problems in babies and this risk continues to affect in adulthood<sup>12</sup>. Nicotine, which is one of the most important toxic compounds in cigarettes, is a dangerous chemical that developing fetus is exposed to<sup>1,13</sup>. Nicotine can be quickly absorbed into smokers' bloodstream through the oral cavity and lung, and quickly go through the placenta to reach the embryo and accumulate in fetal blood and amniotic fluid<sup>14</sup>. Nicotine easily crosses the placental barrier, and in humans it can be detected in the fetal circulation at levels exceeding maternal concentrations by 15%, while amniotic fluid concentrations of nicotine are 88% higher than maternal plasma<sup>15, 16</sup>. This transfer is rapid with peak concentrations in the fetus after 15-30 minutes<sup>4</sup>.

Epidemiological studies show that maternal smoking during pregnancy causes hypertension, obesity, dysglycemia and dyslipidemia in children<sup>13</sup>. Prenatal nicotine exposure affects the development of the structure and reactivation of blood vessels together with energy balance, the control of fat deposition and growth of pancreatic Langerhans islets<sup>4,13</sup>. These risks also include the pregnant women, who are passive smokers<sup>13</sup>. Since nicotine causes lipid peroxidation by inhibiting antioxidant enzymes, it also leads to formation of free radicals<sup>17,18</sup>. Antiapoptotic properties of nicotine are

shown on different studies<sup>19,20</sup>. Thus, Wright et al. state in their study, which was conducted to show the effects of nicotine on apoptosis, that nicotine inhibits the tumor necrosis factor and UV-stimulated apoptosis<sup>21</sup>. Heesch et al. determined that nicotine increases the number of cells depending on dose on endothelial cells of umbilical vein and coronary artery<sup>19</sup>. Although the effect of fetal and neonatal nicotine exposure on cancer development is not well studied, there is still a possibility that it may increase the cancer risk. Nicotine and its metabolites are known for their tumor growth inducing properties. Fetus may be defenseless against these effects due to its low detoxification capacity<sup>22</sup>.

This study showed the effects of nicotine on expression levels of SOX2 and SOX4, which were chosen due to their regulatory and master gene features. Generally speaking, approximately 60% of cell cultures are well suited to anticipate that the effect of nicotine increases the gene expression levels of the two genes involved. Because the Sox2 and Sox4 genes, which are involved in the HMG-box family and are also a strong transcription factor due to their DNA-binding properties, are among the most active genes in the early embryonic stage. Although amniotic cells are not early embryonic cells, they form a transition group with respect to differentiated and specialized cells. In fact, Sox2 and Sox4 genes have basal or naturally identifiable expression levels resulting from tissue and function. For this reason, we can say that the increase in expression of these genes detected in nicotine-treated amniotic cell cultures is due to nicotine. In

many studies done with different types of cells, SOX2 and SOX4 expression levels are associated with cancer and antiapoptotic effect<sup>6,23,24</sup>. SOX2 and SOX4 genes are determined as cancer markers in some of the adult cancers<sup>24,25</sup>. Bareiss et al. showed that SOX2 gene expression increases the expression levels of cancer stem cell markers in ovarian cancer cell lines. In addition, SOX2 expression has been reported to decrease the apoptosis, thus leading to resistance to chemotherapy<sup>26</sup>. In a study conducted by Ruizhe et al., SOX4 gene expression level was shown to increase in stomach cancer cell lines moreover silencing SOX4 gene by siRNA induced apoptosis in these cell lines<sup>27</sup>.

Since SOX2 and SOX4 genes play an active role in many pathways and have influence on carcinogenesis and apoptosis like nicotine, this study may suggest that nicotine performs its harmful effects by changing the expression levels of master genes. In light of this information, inhibition of apoptosis by the effects of nicotine may lead to the accumulation of damaged cells thereby increasing the risk of developing various diseases before birth or later in life. SOX gene family is one of the master regulatory gene families that is involved in the control of development<sup>28</sup>. Since SOX2 and SOX4 genes are master genes, changes in expression levels of these genes due to nicotine exposure may be associated with a variety of biological complications that may affect the fetus due to maternal smoking during gestation. This is noteworthy because it is one the most important outcomes of this study.

Expression levels of SOX2 and SOX4 genes in nicotine and nicotine + resveratrol groups were analyzed by using Wilcoxon test ( $p=0.036$  for SOX2,  $p=0.026$  for SOX4). Resveratrol treatment significantly decreased expression levels of both genes was. Resveratrol, which has a crucial role in neutralizing free radicals, has antagonist interaction with the action of nicotine on the cell because of this property. Likewise, resveratrol affecting the cells in the opposite direction of nicotine activity is consistent with the earlier studies<sup>29</sup>. Hongqiao et al., showed that resveratrol treatment prevents the loss of caspase activity caused by cigarette smoke extract and increases the apoptosis by increasing the caspase-3 and caspase-9 activity in human bronchial cell lines<sup>9</sup>. Ahmad et al. reported the dose-dependent apoptosis inducing effect of resveratrol in human epidermoid carcinoma A431 cells<sup>30</sup>. According to these data, this study may suggest that

resveratrol decreases the expression levels of SOX2 and SOX4 genes that are increased due to nicotine treatment and protects the cells against anti-apoptotic activity by acting like a nicotine inhibitor. Results of this study emphasize the importance of having a high resveratrol diet during pregnancy, which will help to reduce the detrimental effects of tobacco smoke.

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