**Investigation of M3 Muscarinic Acetylcholine Receptor Expression in** **Placentas of Smoking Women**

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# **Abstract**

**Objective:** This study examined M3 muscarinic acetylcholine receptor expression in placentas of women who smoked during pregnancy and explored the affected signaling pathways via network and functional annotation analyses.

**Methods**: Placenta samples from 45 control subjects and 45 individuals who smoked during pregnancy were fixed in zinc-formalin and embedded in paraffin. Demographic data were recorded. Sections from the paraffin blocks were stained using Hematoxylin-Eosin and subjected to M3 muscarinic acetylcholine receptor immunostaining. Protein interaction networks were constructed with Cytoscape and analyzed with MCODE for module detection, while Enrichr was used for functional annotation.

**Results**: Histopathological analysis revealed significant degeneration in chorionic villi, increased fibrin deposition, a rise in syncytial knots, and vascular alterations, indicating that smoking adversely affects placental structure. In placental components, M3 muscarinic acetylcholine receptor immunoreactivity was mostly absent in the trophoblastic layer, syncytial knots, villous stroma, chorionic capillaries, and fibrin-rich areas. In the control group, moderate M3 muscarinic acetylcholine receptor expression was noted in connective tissue cells, whereas the trophoblastic layer and vascular structures displayed little reactivity. Conversely, placental sections from the smoking group showed a pronounced reduction in M3 muscarinic acetylcholine receptor expression, with a negative immunoreaction in key areas. Module and reactome pathway analysis indicated that Module 2, enriched in nicotinic acetylcholine receptor signaling, may mediate smoking-induced placental dysfunction. Additionally, Modules 1 and 3 were linked to GPCR and neurotransmitter pathways, respectively (p<0.05).

**Conclusion:** The diminished M3 muscarinic acetylcholine receptor expression appears to disrupt placental function via altered nicotinic receptor signaling, potentially affecting vascular tone and nutrient exchange.

**Keywords:** Pregnancy, smoking, muscarinic, placenta, nicotine

**Introduction**

Smoking during pregnancy is a major public health concern, negatively impacting maternal and fetal health. Cigarette smoke toxins, including nicotine and carbon monoxide, impair fetal development, leading to complications such as low birth weight, intrauterine growth restriction (IUGR), preterm birth, and placental abnormalities (1). A key mechanism underlying these effects is smoking’s direct impact on the placenta, which regulates oxygen and nutrient supply to the fetus. Cigarette smoke toxins disrupt placental blood flow, restricting fetal oxygen and nutrient availability, leading to growth restriction and health complications (2, 3).

Muscarinic acetylcholine receptors (mAChRs), G-protein-coupled receptors activated by acetylcholine, regulate autonomic functions such as heart rate, smooth muscle contraction, and glandular secretion. These receptors, classified into five subtypes (M1–M5), are expressed in various tissues, including the placenta, where they may regulate vascular tone and nutrient exchange(4, 5). Acetylcholine in the placenta modulates uterine function, prostaglandin production, and amino acid transport between mother and fetus(6) . Additionally, muscarinic receptors in ovarian tissue influence follicular development and ovulation, playing a vital role in fertility(7).

Studies suggest muscarinic receptors are essential for placental function. Pavia et al. (1997) identified mAChR subtypes in syncytiotrophoblast membranes, with M1 receptors in brush-border membranes and M2 receptors in basal membranes(8). Bhuiyan et al. (2006) reported that placental acetylcholine modulates nitric oxide (NO) production via muscarinic receptors, where carbachol (CCh) increases intracellular calcium levels, activating endothelial nitric oxide synthase (eNOS) and NO release(5). As NO regulates placental blood flow, muscarinic receptors likely contribute to vascular tone modulation.

Nicotine affects acetylcholine receptors, potentially disrupting muscarinic receptor function, leading to vascular dysregulation and impaired placental nutrient exchange(9). Altered M3 mAChR expression in smoking-exposed placentas may impair placental function(10). Additionally, muscarinic receptors influence hematopoietic system development, highlighting their broader impact on placental function (11).

This study investigates M3 mAChR expression in smoking-exposed placentas and assesses smoking’s effects on placental histology and in silico pathways.

**Material-Methods**

## **Study design**

Ethical approval was granted by Dicle University Faculty of Medicine Non-Interventional Studies Ethics Committee (Date: 20/11/2024, Issue: 21). Placental tissues were collected from 45 healthy non-smoking pregnant women and 45 women who smoked at least five cigarettes per day. Participants were recruited from the Gynecology and Obstetrics Clinic at Dicle University. Demographic data, ultrasonographic findings, pregnancy outcomes, and blood samples collected before delivery were documented. Inclusion criteria were maternal age between 18–40 years, absence of pregnancy complications, systemic conditions, or chronic diseases. Exclusion criteria included preeclampsia, severe anemia, diabetes, systemic lupus erythematosus (SLE), heart disease, and fetal anomalies. The control group consisted of healthy non-smokers with no pregnancy complications and fetal weight within the 10th to 90th percentile (12).

## **Histological tissue protocol**

Placentas were obtained from Dicle University Faculty of Medicine, Department of Gynecology and Obstetrics. Tissue samples were excised, fixed in zinc-formalin, and processed through tap water, alcohol series, and xylene stages before being embedded in paraffin. 5 µm sections were cut, stained with Hematoxylin-Eosin, and immunostained for M3 mAChR.(13, 14).

## **Immunohistochemical Staining**

Placental sections from paraffin blocks were placed onto polylysine-coated slides at 37°C using a double boiler. Excess paraffin was removed by incubating slides at 58-62°C for 6 hours. Sections were deparaffinized in xylene, rehydrated through decreasing alcohol concentrations, and rinsed in distilled water. Hydrogen peroxide was applied for 20 minutes, followed by PBS wash and treatment with Ultra V Block solution for 7 minutes. Sections were incubated overnight at +4°C with the primary antibody against M3 mAChR (Catalog no: ab-87199, Abcam, US). After washing with PBS, sections were exposed to a biotin-conjugated secondary antibody for 14 minutes, then incubated with streptavidin-peroxidase for 15 minutes. Diaminobenzidine (DAB) was used to visualize the antibody-antigen reaction, and sections were observed under a microscope. After three PBS rinses (15 minutes each), sections were counterstained with Harris hematoxylin. Coverslips were mounted, and slides were examined with a Zeiss Imager A2 photomicroscope(15).

## **Module and Pathway Analysis of M3 mAChR**

To investigate the signaling pathways potentially affected by the significant reduction in M3 mAChR expression observed in placenta samples of smoking women and to identify the proteins involved in these pathways, module and functional annotation analyses were performed. Initially, the protein interaction network of M3 mAChR was constructed using the Cytoscape software (v.3.10.3), including up to 100 additional interactors at a confidence level of 0.400. To identify clusters within the network, the MCODE plugin was utilized, which enabled the detection of densely interconnected regions indicative of potential functional modules. Subsequently, proteins identified within different modules were subjected to Reactome pathway analysis using the Enrichr platform [a]. The obtained pathways were ranked in ascending order based on their p-values, and the top five statistically significant pathways (p<0.05) were selected for further interpretation.(16, 17)

## **Statistical analysis**

Statistical analysis was performed using IBM SPSS 25.0 software (IBM, Armonk, New York, USA). Data were recorded as mean±standard deviation. Statistical distribution was evaluated with the Shapiro-Wilk test. Paired group comparisons were made with the independent t test. Significance was considered for p values ​​<0.05. The number of patients for each group was calculated by G Power analysis (version 3.1). Cohen criteria were defined according to the study of Alviggi et al(18).

# **Results**

## **Demographic findings**

Table 1 presents data on characteristics of pregnant smokers and non-smokers and their newborns. Measurements like weight, length, and head circumference highlight the effects of smoking. Newborns of smokers have significantly lower birth weight, length, and head circumference.

**Table 1.** Demographic parameters, measurements and pregnancy outcomes of the patients

|  |  |  |  |
| --- | --- | --- | --- |
| **Parameters** | **Control Group**  **(n=45)** | **Smoking group (n=45)** | **Significance**  **(p-value)** |
| **Maternal**  **age, years** | 25.15±4.62 | 24.89±5.6 | >0.05 |
| **GW 1** | 37.50±1.39 | 37.38±2.09 | >0.05 |
| **Birth weight, g** | 3340.20±511.37 | 2685.42±622.83 | <0.0001 |
| **Birth length, cm** | 49.90±2.44 | 46.69±2.31 | <0.0001 |
| **Head circumference cm** | 34.61±1.43 | 32.51±1.47 | <0.0001 |
| **1-min APGAR2 score** | 8.45±0.58 | 8.21±0.72 | >0.05 |
| **5-min APGAR score** | 9.79±0.48 | 9.56±0.53 | >0.05 |

GW: Gestational Week

2 APGAR: Appearance (skin color), Pulse (heart rate), Grimace (reflex irritability), Activity (muscle tone), and Respiration (breathing effort). Each criterion is scored from 0 to 2, with a maximum total score of 10

## **Histopathological findings**

The histological analysis of placental sections revealed distinct structural differences between the control group and smokers, highlighting the impact of smoking on placental morphology. Figure 1 presents the stained placental sections used for histological evaluation. In the placental sections of the control group consisting of healthy individuals, the chorionic villi were observed to have a regular, oval structure with well-defined borders. No signs of degeneration were observed in the structures of the chorionic villi. The syncytiotrophoblast cells were regularly arranged at the periphery of the villi, and the structural integrity of the chorionic vessels and villous connective tissue was maintained. Fibrin accumulation and the number of syncytial knots were minimal. The trophoblastic cells exhibited a homogeneous distribution within the placental section. No congestion, dilation, or any pathological changes were detected in the vascular structures (Figure 1A). The placental sections of the smokers are shown in Figure 1B. Histopathological findings in this group revealed degeneration and morphological alterations in the chorionic villi structures. A significant increase in fibrin accumulation and syncytial knot count was observed. Dilation of the vascular structures was prominent. Intense hemorrhage and the presence of inflammatory cells were noted in the intervillous space. Pyknotic nuclei were observed in the connective tissue cells.



**Figure 1.** Cross sections of placenta from control (A) and smoking groups (B). Arrow: syncytial knot, arrowhead: chorionic villus, \*: chorionic capillary, ct: villous connective tissue. Hematoxylin Eosin staining, Bar: 50 µm, magnification: 20X

The immunostaining analysis of M3 mAChR in placental sections revealed distinct differences between the control and smoker groups, highlighting a reduction in M3 mAChR expression in the placentas of smokers. Figure 2 illustrates the M3 mAChR immunostaining pattern observed in the placental sections of the control group. The M3 mAChR immunoreactivity was primarily observed at a moderate level in the cytoplasmic regions of the connective tissue cells of the villi. M3 mAChR expression was negative in the trophoblastic layer cells and the chorionic capillary endothelium. Negative M3 mAChR immunoreactivity was also observed in fibrin deposit areas and syncytial knots (Figure 2A). Placental sections of the smokers were stained using the M3 mAChR immunostaining method (Figure 4). A decrease in M3 mAChR expression was observed compared to the control group. In the components of the placenta, M3 mAChR immunoreactivity was generally negative in the trophoblastic layer, syncytial knots, villous stroma, chorionic capillaries, and fibrin deposit areas. Very slight M3 mAChR expression was observed in the connective tissue stromal areas (Figure 2B).



**Figure 2.** Placental section of the control group (A) and smoking group (B). Arrow: trophoblastic layer, \*: syncytial knot, star: villous connective tissue, f: fibrin, M3 mAChR immunostaining, Bar: 50 µm, magnification: 20X

**Identification of Functional Modules and Pathway Enrichment Analysis**

The M3 mAChR protein interaction network analysis identified three distinct modules using the MCODE plugin. Module 1, comprising 31 nodes and 379 edges, was the largest and most densely connected cluster and was significantly enriched in pathways related to G-protein coupled receptor (GPCR) signaling, including G Alpha (Q) signaling events, GPCR downstream signaling, and signaling by GPCR (p<0.05). Module 2, containing 13 nodes and 30 edges, was predominantly associated with nicotinic acetylcholine receptor signaling, highlighting pathways such as highly calcium permeable nicotinic acetylcholine receptors, presynaptic nicotinic acetylcholine receptors, and postsynaptic nicotinic acetylcholine receptors (p<0.05). Module 3, the smallest cluster with 8 nodes and 19 edges, was enriched in neurotransmitter receptor pathways, including neurotransmitter receptors and postsynaptic signal transmission, transmission across chemical synapses, and neuronal system (p<0.05) (Figure 3).



**Figure 3.**  M3 mAChR Protein Interaction Network and Functional Modules.The M3 mAChR network analysis identified three modules: Module 1 (31 nodes, 379 edges), Module 2 (13 nodes, 30 edges), and Module 3 (8 nodes, 19 edges). Statistically significant related Reactome pathways are shown for each module (p < 0.05).

**Discussion**

Smoking during pregnancy leads to numerous histological changes in the placenta (19). Toxic substances in cigarettes, such as nicotine, carbon monoxide, polycyclic aromatic hydrocarbons (PAHs), heavy metals (e.g., cadmium, lead), and reactive oxygen species (ROS), negatively affect placental structure and function, thereby threatening fetal development. Studies have demonstrated that nicotine in cigarettes induces morphological and functional alterations in the placenta. Due to its small molecular size and lipophilic nature, nicotine is rapidly absorbed (20), quickly reaching the brain and crossing the placenta, thereby exerting effects on the fetus as well. Nicotine impairs the differentiation and migration of trophoblast cells, adversely affecting their functions(3, 21). Additionally, nicotine may increase endoplasmic reticulum (ER) stress by affecting pathways such as PERK and JNK (22).

Smoking can trigger various mechanisms that impair placental angiogenesis and vascular integrity, leading to adverse effects on fetal development. Maternal smoking increases the risk of placental abruption and placenta previa, jeopardizing oxygen and nutrient supply to the fetus and potentially causing severe health complications (23). Several studies have reported reduced placental blood flow, edematous plasma, and increased trophoblast cell damage in pregnant smokers (24). Furthermore, smoking has been shown to cause abnormal placental vascular development and an increased tendency for hemorrhage, which contributes to reduced fetal oxygen and nutrient supply (25). Additionally, placental tissue from smoking mothers frequently exhibits signs of inflammation and cellular necrosis (19). Moreover, maternal smoking during pregnancy has been associated with intrauterine growth restriction and an increased risk of low birth weight (26). Our findings demonstrated that maternal smoking during pregnancy was associated with significantly lower birth weight, length, and head circumference in newborns compared to the control group. These results align with previous studies reporting that smoking impairs placental angiogenesis, disrupts vascular integrity, and reduces oxygen and nutrient supply to the fetus, contributing to intrauterine growth restriction and low birth weight. Histopathological alterations observed in the placentas of smoking mothers, such as increased fibrin deposition and inflammatory infiltration, likely underlie these adverse perinatal outcomes (2, 3, 27).

In this study, significant differences were observed in the histopathology and muscarinic acetylcholine receptor (M3 mAChR) immunoexpression of placental tissues from smoking mothers compared to healthy controls. Histopathological examination revealed that placental sections from the control group maintained the structural integrity of chorionic villi, exhibited minimal syncytial knot formation, and preserved vascular integrity. In contrast, placental sections from smoking mothers displayed pronounced degenerative changes, including structural abnormalities in chorionic villi, increased fibrin deposition, a significant rise in syncytial knot formation, and extensive hemorrhage and inflammatory cell infiltration in the intervillous space. Additionally, a notable dilation and congestion of vascular structures were observed. These findings support the detrimental effects of smoking on placental morphology and are consistent with previous studies.

Nicotine may disrupt the placental cholinergic system by increasing acetylcholine release and causing excessive stimulation of muscarinic receptors (M3 mAChRs) (28). The cholinergic system in the human placenta is characterized by the expression of acetylcholine and choline acetyltransferase (ChAT), the enzyme responsible for acetylcholine synthesis. This system regulates various cellular processes, including proliferation, differentiation, cytoskeletal organization, cell-cell interactions, motility, migration, cilia activity, and immune functions (29). Muscarinic receptors, which mediate the effects of acetylcholine through intracellular signaling, play a crucial role in modulating cellular responses. In the placenta, the activation of muscarinic receptors contributes to the regulation of cell growth and differentiation, thereby influencing placental functions. However, exposure to cigarette smoke has been shown to significantly disrupt these mechanisms.

Our study’s M3 mAChR immunostaining results further emphasize the impact of smoking on placental neurotransmitter regulatory mechanisms. In the control group, moderate M3 mAChR expression was observed in connective tissue cells, with minimal reactivity in the trophoblastic layer or vascular structures. In contrast, placental sections from smokers exhibited a marked reduction in M3 mAChR expression, with negative immunoreactivity observed in the trophoblastic layer, syncytial knots, chorionic capillaries, and fibrin accumulation sites. Literature suggests that alterations in muscarinic receptor expression within the placenta may lead to disruptions in acetylcholine-mediated signaling, which plays a critical role in fetal development (30). This decrease in expression may reflect the impact of smoking on acetylcholine-mediated signal transmission in placental cells and may also indicate alterations in placental stress responses.

To elucidate the molecular mechanisms underlying the reduced M3 mAChR expression and associated histopathological changes observed in the placental tissues of smoking women, pathway and protein interaction analyses were conducted, identifying three distinct modules(16). Module 1 included pathways related to GPCR signaling and G-protein-mediated signal transduction, which may be linked to smoking-induced vascular dysfunction through altered receptor modulation(17) . Module 3 encompassed neurotransmitter receptor and synaptic transmission pathways, suggesting potential disruptions in neurotransmission dynamics due to nicotine exposure(31). Notably, Module 2 was significantly enriched in nicotinic acetylcholine receptor (nAChR)-related pathways, including highly calcium-permeable nAChRs, presynaptic and postsynaptic nAChRs, and downstream acetylcholine binding events. Given that nicotine from cigarette smoke directly activates nAChRs, this finding suggests that reduced M3 mAChR expression may lead to a compensatory upregulation or increased activation of nAChR-mediated signaling (6). Such alterations could disrupt calcium homeostasis and neurotransmitter transmission, crucial for maintaining placental function and vascular integrity Studies have shown that calcium homeostasis is vital for fetal skeletal mineralization and neurotransmitter release, and its disruption is associated with placental dysfunction and pregnancy complications, including preeclampsia, where calcium transport is compromised by oxidative stress and ATP deficiency (32, 33). Additionally, it has been demonstrated that the placenta actively metabolizes and transports neurotransmitters like dopamine, norepinephrine, and serotonin, which are essential for fetal development and programming, further highlighting the potential impact of disrupted signaling pathways in smoking-related placental dysfunction (34). These results underscore the pivotal role of nAChRs in mediating the detrimental effects of smoking on placental function and fetal development. Thus, identifying the protein targets within Module 2 provides valuable insights into the specific signaling networks affected by reduced M3 mAChR expression, offering potential biomarkers and therapeutic targets to mitigate the adverse effects of smoking on placental health.

A reduction in muscarinic receptor expression may disrupt physiological processes such as placental vascular tone and blood flow, adversely affecting oxygen and nutrient transport to the fetus. Moreover, oxidative stress and inflammatory responses induced by nicotine and other cigarette components are proposed to exert a suppressive effect on the placental cholinergic system, contributing to these alterations (35). Particularly, the loss of immunoreactivity observed in the syncytiotrophoblast layer may weaken the placental barrier function, thereby compromising fetal circulatory homeostasis.

**Conclusion**

This study demonstrates that histological and immunohistochemical changes in the placental tissues of smoking mothers may impair placental function. The observed reduction in M3 mAChR expression suggests a disruption in placental acetylcholine signaling, potentially contributing to vascular abnormalities. In silico analyses highlight the crucial impact of reduced M3 mAChR expression on placental function through disrupted signaling networks, particularly those mediated by nicotinic acetylcholine receptors. Further experimental validation of these pathways could pave the way for novel therapeutic strategies to improve pregnancy outcomes in smoking women.

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