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# Cigarette smoking during pregnancy regulates the expression of specific nicotinic acetylcholine receptor (nAChR) subunits in the human placenta

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ABSTRACT

Smoking during pregnancy is associated with low birth weight, premature delivery, and neonatal morbidity and mortality. Nicotine, a major pathogenic compound of cigarette smoke, binds to the nicotinic acetylcholine receptors (nAChRs). A total of 16 nAChR subunits have been identified in mammals (9 α, 4 β, and 1 δ, γ and ε subunits). The effect of cigarette smoking on the expression of these subunits in the placenta has not yet been determined, thus constituting the aim of this study. Using RT-qPCR and western blotting, this study investigated all 16 mammalian nAChR subunits in the normal healthy human placenta, and compared mRNA and protein expressions in the placentas from smokers (n = 8) to controls (n = 8). Our data show that all 16 subunit mRNAs are expressed in the normal, non-diseased human placenta and that the expression of α2, α3, α4, α9, β2 and β4 subunits is greater than the other subunits. For mRNA, cigarette smoke exposure was associated with increased expression of the α9 subunit, and decreased expression of the δ subunit. At the protein level, expression of both α9 and δ was increased. Thus, cigarette smoking in pregnancy is sufficient to regulate nAChR subunits in the placenta, specifically α9 and δ subunits, and could contribute to the adverse effects of vasoconstriction and decreased re-epithelialisation (α9), and increased calcification and apoptosis (δ), seen in the placentas of smoking women.


## Introduction

Despite major public health campaigns against cigarette (tobacco) smoking during pregnancy, it remains the most important preventable risk factor for a complicated pregnancy outcome in all developed, and an increasing number of developing countries (Jauniaux and Burton, 2007). Negative outcomes of smoking during pregnancy exist for both the mother and the foetus. For the mother, these include an increased risk of ectopic pregnancy, abruptio placentae, placenta praevia and preterm, and prelabour rupture of membranes (Odendaal et al., 2001), while for the foetus, prematurity and intrauterine growth restriction (IUGR) are two major adverse consequences which together, result in decreased birth weight of 170 to 250 g (Hardy and Mellits, 1972; Horta et al., 1997). Lower infant birth weight is strongly associated with increased infant morbidity and mortality, including an increased risk of heart, breathing and brain abnormalities (Hack et al., 1995), and increased risk of Sudden Infant Death Syndrome (SIDS) (Hoffman et al., 1988).

It is hypothesised that the above negative consequences result from the adverse effects smoking induces on the placenta including: calcification (Klesges et al., 1998), a reduction in the diameter of the chorionic villi, vasoconstriction, depression of active amino acid uptake (Sastry, 1991), increased apoptosis (Erel et al., 2001; Jauniaux and Burton, 2007), reduced placental weight and atrophic placental villi (Naeye, 1987), and significantly higher umbilical artery resistance, interpreted to be a surrogate measure for abnormal placental vascular formation, as well as reduced estimated foetal weights (Kho et al., 2009). While it is unclear which component(s) of cigarette smoke leads to these final downstream histological changes, nicotine is thought to be a major contributor.

Nicotine, a poisonous alkaloid, is the major psychoactive (Rollema et al., 2007) chemical identified out of the 4800 chemicals that exist in cigarette smoke (Green and Rodgman, 1996). Due to its low molecular weight and high lipid solubility, nicotine rapidly perfuses through the human placenta (Lambers and Clark, 1996) resulting in 15% higher

*Abbreviations:* ITGA3, integrin alpha 3; mRNA, messenger ribonucleic acid; nAChRs, nicotinic acetylcholine receptors; OD, optical density; RT-qPCR, reverse transcription quantitative polymerase chain reaction; YWAZH, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide.

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nicotine concentrations in the foetal circulation than in the maternal circulation (Eskenazi et al., 1995).

Nicotine induces its pharmacological effects after binding to its receptor(s) known as the nicotinic acetylcholine receptors (nAChRs). These nAChRs belong to the cys-loop family of ligand-gated ion channels and are formed as pentamers of subunits, arranged symmetrically around a central pore (Cooper et al., 1991). Genes encoding a total of seventeen subunits (α1–10, β1–4, δ, ε and γ) have been identified and are of mammalian origin, with the exception of α8 which is only found in avian species (Gerzanich et al., 1994). The predominant conformation of these subunits is heteromeric, although α7 and α9 homopentamers exist (Gotti and Clementi, 2004). The α1, β1, γ, δ, and ε subunits are classified as muscle type (Ke et al., 1998) while the remaining are classed as neuronal.

To date, there are only two studies concerning nAChR expression in the placenta (Kwon et al., 2006; Lips et al., 2005). The study by Lips et al. (2005) reported the mRNA and protein expressions of eight nAChR subunits (α2–7, α9 & α10) in normal human and rat placenta. The study by Kwon et al. (2006) found decreased expression of the α7 subunit mRNA and protein in pre-eclamptic compared to normal placentas. Thus, not only the relative expression of all nAChR subunits in the human placenta remains to be established, but also the effect of cigarette smoke on the expression of the nAChR subunits is still to be determined. We used real time reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot protein analyses, to identify which of the 16 mammalian nAChR subunits are expressed in the normal human placenta, and then the expression of the receptor subunits in the placenta of smoking women is compared to healthy controls.

## Materials and methods

*Tissue collection.* With the approval of the Sydney Local Health District Ethics Committee (Royal Prince Alfred Hospital (RPAH) zone) and after providing written informed consent, placental samples were obtained from women presented to both RPAH and Campbelltown hospital from April 2012 to July 2012. The gestational age range for the samples was between 37 and 40 weeks. Women were recruited into two study groups: 1 — those who had a healthy pregnancy and never smoked (controls) and 2 — those who had a healthy pregnancy and were active smokers during their pregnancy (smoking group). Within 30 min of placental delivery, a sample (2 × 2 cm) was dissected from the maternal interface, avoiding the amnion/chorion membranes, and immediately frozen with liquid nitrogen and stored at −80 °C for subsequent cDNA synthesis and reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analyses. As only 5 women were recruited in the smoking group during this time frame, 3 cases were included from a previous dataset collected from January to September 2011.

Venous blood (10 mL) was collected during birthing using a Vacuette system (Greiner Bio-One, Kremsmünster, Austria), allowed to clot at room temperature (RT) for 1.5 h, centrifuged for 10 min at 3600 rpm and 4 °C, and the serum extracted for the measurement of cotinine levels. The extent of cigarette smoke exposure is often determined by measuring cotinine levels rather than nicotine, given that cotinine is the major metabolite of nicotine, and has a much longer half-life (15–20 h) compared to nicotine (2 h) (Zevin et al., 1998).

The 3 cases of smokers from our previous dataset did not have serum collected and so cotinine measurements were made in serum extracted from the placental tissue itself. This involved allowing a 1 $cm^3$ placental sample to thaw at RT for 1 h, physical homogenisation using a blunt syringe tip, centrifuging for 10 min at 3600 rpm and 4 °C, and collecting the resulting serum for analysis. As testing cotinine from a direct tissue extract has not been reported in the literature, we validated the results by applying the same method of analysis to the placental samples collected in April–July 2012, and compared the values to those derived from the venous serum of the same women.

*Cotinine ELISA analysis.* To determine cotinine levels, a solid phase competitive ELISA (Calbiotech, Spring Valley, USA) was used according to the manufacturer's instructions. Briefly, 10 μL of standards and samples was pipetted into appropriate wells in duplicate. Enzyme conjugate was added to each well, mixed for 10–30 s, and incubated in the dark for 60 min at RT. At the end of the incubation period, wells were washed 6 times using an automated machine (Tecan Life Technologies, Australia), allowed to air dry and incubated in substrate reagent for 30 min at RT. Reaction was quenched by adding a stop solution, and the activity of the enzyme quantified using the PHERAstar microplate reader (BMG Labtech, USA) at 450 nm. Cotinine levels were calculated to ng/mL. Samples were run in duplicate within one assay. The intra-assay coefficient of variation was 12%.

*RNA isolation, quantity, purity and integrity.* Total RNA was isolated from placental samples using the RNeasy Mini Kit (Qiagen, Australia) following the provided kit protocol. The tissue was immersed in liquid nitrogen to minimise RNA degradation before being crushed into powder using a mortar and pestle. All steps were subsequently carried out at RT unless otherwise stated. Tissue (30 mg) was homogenised in 600 μL of lysis buffer and 6 μL of 2-mercaptoethanol in a sterile M tube (Miltenyi Biotec, Germany) using a gentleMACS Dissociator (Miltenyi Biotec) for 85 s and then centrifuged at 3750 rpm for 3 min. 70% ethanol was added and the lysate was transferred into the spin cartridge and centrifuged at 12,000 ×*g* for 15 s. The spin cartridge containing RNA was subsequently purified using wash buffers 1 and 2 according to the manufacturer's instructions. Finally, the RNA was eluted using 50 μL of RNase-free MilliQ water and centrifuged at 12,000 ×*g* for 1 min. The eluted RNA was immediately stored at −80 °C until further use.

The concentration and purity of each RNA sample were measured using the NanoDrop Spectrophotometer (Thermo Scientific, USA) and samples were ensured to be free from any protein or organic contamination by having a 260/280 UV absorbance measurement above 1.8 and 2.0 respectively. RNA integrity was measured using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA), which provided an RNA integrity number (RIN), and samples with RIN >3 were included in the study.

*Primer design.* Selected primers were purchased from Invitrogen and designed based on past studies (En-Nosse et al., 2009; Lam et al., 2007; Lips et al., 2005; Oh et al., 2011; Soeda et al., 2012). Created sequences were checked using the NCBI Primer BLAST and UCSC software to ensure primers were optimal for RT-qPCR. The following parameters were verified: forward and reverse primers were 17–28 nucleotides long, 50–60% GC content, melting temperature ($T_m$) between 55 and 80 °C, no more than 5 °C difference between the $T_m$ of primer pairs, 3′ end with a G or C (3′ clamp), no complimentary areas between primers (potential primer dimers), no genomic primer matches, and no/very weak secondary structures (such as hairpins). The sequences of the final primers for nAChRs used are presented in Table 1 with indication of any modifications made.

The reference (housekeeping) genes chosen were tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWAZH) (based on the findings of Murthi et al., 2008 that this was a suitable endogenous reference gene for placental tissue), and integrin alpha 3 (ITGA3) (Cotton EST Database, East Carolina).

*Real time reverse transcription quantitative polymerase chain reaction (real time RT-qPCR).* The transcriptor first strand cDNA synthesis kit (Roche Diagnostics GmbH) was used to convert total RNA into cDNA. Total RNA was treated with random hexamer primers and heated at 65 °C for 10 min to ensure denaturation of RNA secondary structures. The RT-qPCR parameters for cDNA synthesis were 25 °C for 10 min and followed by 55 °C for 30 min. The Transcriptor Reverse Transcriptase was then inactivated by heating at 85 °C for 5 min before stopping