What lessons have we learnt about the impact of maternal cigarette smoking from animal models?

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Abstract
Maternal first or second-hand tobacco smoking during pregnancy is still common albeit the known detrimental effects to the unborn child. Maternal tobacco cigarette smoking (SE) can affect multiple organ systems in the offspring, rendering them to increased risk of various conditions throughout life (eg. intrauterine underdevelopment, asthma, substance abuse, diabetes). However, this review will only focus on its impact on the brain and the related molecular changes in the offspring based on evidence from animal studies. Although epidemiological studies have identified the associations between maternal SE and brain disorders, animal models can help identify the underlying mechanisms and test interventions. Human studies have found that maternal SE is closely linked to small brain size and changes in brain structure and associated with a high risk of cognitive defects. Animal models suggest that this may be due to increased brain oxidative stress and inflammation during the neonatal period, leading to increased brain cell apoptosis in adulthood. There is a distinct gender bias of such impacts, where male offspring are more affected than female. Female offspring seem to have developed the adaptation by increasing endogenous antioxidant levels. Indeed, animal studies have shown that using antioxidant supplementation during pregnancy can improve neurological outcomes in male offspring, however the efficacy in humans is yet to be confirmed. Furthermore, some animal studies suggested nicotine as the key player in intrauterine underdevelopment due to maternal SE, whilst human clinical trials using nicotine replacement therapy do not support this mechanism. This review will discuss the possible reasons.

Keywords
Oxidative stress, inflammation, nicotine, maternal tobacco smoking, brain, offspring

Introduction

The concept of foetal programming of diseases is widely accepted. Maternal cigarette smoking disturbs the stability of the intrauterine environment, interrupting normal foetal development. Cigarette smoking cessation is difficult amongst all populations, and cessation rates during pregnancy are as low as 5%, forming an ongoing health risk to the next generation, including neurocognitive changes and brain injury [1]. Even after birth, the chemicals in the cigarette smoke can continue to exert direct adverse effects on the offspring's brain, transferred via breastmilk into the offsping's circulation, and then passing the blood-placental barrier to act locally on the developing brain.

Maternal smoking has been modelled in rodents either using direct cigarette smoke exposure (SE) or nicotine infusion to the mothers, to investigate the development of vaiours disorders in the offspring, including hypoxia-ischemia (HI) induced brain injury and cognitive changes [2,3]. Such studies aimed to understand the underlying mechanisms and investigate potential interventional strategies to mitigate the adverse impact of maternal cigarette smoking on the offspring.

While nicotine is addictive and quite often plays a key role in preventing successful smoking cessation, inflammation and oxidative stress are more likely to be the key players in neonatal neuronal HI injury and cognitive changes in adulthood [3]. Oxidative stress induced by certain toxic chemicals in the cigarette smoke inhaled by the mothers may be crucal in programming foetal susceptibility to certain diseases in their offspring. This theory is further supported by the evidence from rodent models showing that maternal antioxidant supplementation can prevent the adverse effect of maternal SE on the offspring [4]. This review will elaborate on the abovementioned theories.

1. Maternal tobacco cigarette smoking and brain development

Cigarette smoking is a significant risk factor for a number of chronic diseases. First hand and second-hand tobacco cigarette smoking affects a significant number of the world's population, including pregnant women [5,6]. The difficulties of quitting cigarette smoking during pregnancy, despite the widely available nicotine replacement therapy (NRT), leads to a high rate of cigarette smoking during pregnancy (20-45%) in some communities [5,6].

In developed countries, maternal cigarette smoking and second hard cigarette smoke exposure is a major contributor to intrauterine undernutrition and underdevelopment, and subsequently causes several birth complications and long term health issues in the offspring, such as sudden infant death syndrome, cerebral palsy, and attention deficit hyperactivity disorder (ADHD) [7,8]. This phenomenon is supported by the "foetal origin of adult disease" hypothesis by Barker [9], who proposed that intrauterine undernutrition can permanently change the physiological functions of the foetus which increase the risk of multiple organ dysfunctions later in life. Cigarette smoking is well known to reduce appetite and caloric intake in cigarette smokers, due to the appetite suppressive effect of nicotine [10]. However, smoking mothers had similar daily food intake to non-smokers, therefore intrauterine underdevelopment in smoker's offspring may be due to other factors [11]. One explanation is the dysfunctional endothelium of uterine small arteries caused by cigarette smoke, which can lead to vasoconstriction. This directly affects the blood flow and the delivery of nutrients and oxygen to the foetus causing foetal growth restriction [12].

The Barker hypothesis also suggests that the brain is among the vital organs to receive priority nutrition distribution at the cost of less vital organs, such as the liver. However, such advantages did

not prevent the small brain weight and size in smoker's offspring [7]. The third trimester seems to be the most critical period for determining brain development [8]. Human studies suggest that maternal cigarette smoking during pregnancy lead to smaller atrial width of lateral ventricle and smaller transcerebellar diameter in the growing foetuses across pregnancy, as well as smaller frontal lobes and cerebellar volumes [13]. This can directly lead to cognitive disorders in the smoker's offspring.

2. Maternal cigarette smoking and neurocognitive outcomes

Epidemiological studies have discovered the link between maternal cigarette smoking during pregnancy and the alteration in neurocognitive functions in the offspring (eg. schizophrenia) [13-16]. Size matters, which applies well with cognitive performance. In both human offspring from smoking mothers and rodent offspring from SE dams, reduced offspring brain size including reduced cerebral cortical gray matter and total parenchymal volume, as well as cerebellum and corpus callosum volume/thickness [13,17]. In humans, female offspring seem to be more affected in regional brain size, including lateral orbitofrontal and middle frontal cortices, and middle and posterior regions of the corpus callosum [13,17]. In animal models, males seems to be more prone to have smaller brain size than the female offspring [17,18].

Intelligent quotient positively correlates with total brain volume [19], while verbal ability positively correlates with cerebral volume [20]. Children from smoking mothers have persistentantly lower levels of intelligent quotient and problems with auditory functions [21]. Thinning of the orbital frontal cortex may also contribute to reduced competence and poorer social development due to maternal smoking [13]. Such offspring can also have increased risk of other neurocognitive defects, including ADHD, externalising disorders, internalising behaviours, conduct disorders, and a lack of coordination across brain regions during information and auditory processing [13,22-24].

The chemicals in cigarette smoke inhaled by the mothers can clearly affect the cognitive development of the offspring. There are alterations in nicotinic acetylcholine receptors (nAChRs) across various brain regions, which has been linked to the inhibition of DNA synthesis and increased apoptosis [13,25]. In offspring, dose-dependent responses to the number of cigarettes smoked by the mothers has been observed in humans. For example, increased risk of internalising behaviours (such as fear, anxiety and sudden change of mood) is prominent in 1.5 and 3 years old children if the mothers were heavy smokers (>20 cigarettes/day) during pregnancy [26]. The incidence of AHDH is also high in smokers' children, and the more the mother smoke the more severe the symptoms are [27]. However, the link between maternal smoking and ADHD was not found in all human studies, with some suggesting it due to a familial factor [22-24,28]. This discrepancy may be due to the study design, with some using self-reporting and others standard psychological diagnostic methods. Nevertheless, an imaging study suggested a reduced number of axons in the corpus callosum as the underlying reason for ADHD risk in the offspring from cigarette smokers [13].

Another factor contributing to foetal underdevelopment is the reduced oxygen supply due to vasoconstriction [29] directly affecting arobic glucose metabolism [30]. In addition, cigarette smoking can increase carboxyhaemoglobin levels in both foetal and maternal blood affecting the capacity of the red blood cells to carry oxygen [31]. This leads to hypoxia in the foetus, as shown by a study in the rhesus monkey [32]. Oxygen deprivation before and around the time of birth can result in HI brain damage within 12-36 hours of birth. The outcomes of HI are a diminished exchange of oxygen and

carbon dioxide, and severe lactic acidosis [33], increasing the risk of cerebral palsy after birth [30]. Studies in rodents using both maternal nicotine administration and SE showed an increase in the vulnerability of the neonatal brain to HI encephalopathy, leading to more severe neurological functional loss in the offspring from nicotine treated/SE dams than those from the control dams, including the risk of cerebral palsy [2,34]. Indeed, the risk of cerebral palsy is high in individuals whose mothers are heavy smokers (≥10 cigarettes/day) [35]. The cerebral cortex, hippocampus, and sub-ventricular regions are most vulnerable to HI damage [36]. HI encephalopathy increased infarct size in male pups from the SE dams compared with those from air-exposed control dams; however, this effect was not observed in the female pups [34]. This indicates a gender difference where male offspring are more severely affected by maternal cigarette smoking.

It needs to be noted that postnatal environmental factors in humans are far more complicated than occur in laboratory animal models and can confound intrauterine factors to exert negative health impacts. The prominent postnatal environmental factors are the socio-economic status of the parents, maternal mental health, alcohol consumption, maternal smoking during the postpartum period, paternal cigarette smoking, and genetic transmission of certain risks [26]. However, animal models can provide the opportunity to study a single or multiple risk factors in the offspring. Additionally, the investigation of the molecular mechanisms in the brain relies heavily on animal modelling. Although cigarette smoke contains thousands of different chemicals, the majority of animal studies only used nicotine instead of direct SE. Therefore, its relevance to human physiology is questionable.

3. Is nicotine the key player?

While it is well accepted that cigarette smoking during pregnancy is highly detrimental to the developing child, the field is still confused between the effects of cigarette smoke and nicotine. Although strong evidence arising from animal studies published by different groups showed detrimental effects of nicotine on foetal development and neurological outcomes, there is no evidence that nicotine delivery through NRT affects newborn birth weight in humans [37]. Neither is there any evidence that NRT during pregnancy chages neurological outcomes in the human offspring. In rhesus macaque, the brain size is similar between the offspring from nicotine and saline-treated mothers [38], similar to the effect of NRT during pregnancy. However, when the mothers are directly exposed to cigarette smoke in the animal model, the outcome of reduced brain size in the offspring is similar to humans [17]. This suggests that the other chemicals in cigarette smoke may play a critical role in foetal underdevelopment. Due to the complex nature of the chemical components in tobacco smoke, it is unlikely that one single component will cause all pathology.

Nicotine is, in fact, anti-inflammatory and may have neuroprotective properties [39]. However, it still does not rule out the possibility that nicotine from NRT may affect the fine-tuning of the connections between brain nuclei and neurological functions in the offspring at different ages. Animal studies using pure nicotine administration can't really answer this question. In the literature, nicotine dose ranges from 2-6 mg/kg/day [40,41]. For a human weighing 50kg, this dose is equivalent to smoking 84-250 cigarettes a day.

Furthermore, the dose of prescribed NRT to pregnant women is generally low, which may not be sufficient to exert significant influence on placental blood supply and nutrient delivery to the foetus [42,43]. As such, foetal growth itself is not changed. In a recent systematic review published in 2017 [44], the only serious adverse health effect due to NRT use during pregnancy was an increase in respiratory

congenital abnormalities. However, women included in this study had a high rate of chronic diseases, which can also independently affect foetal diseases [44].

The low NRT dose regimen used during pregnanacy may also contribute to lack of efficacy in quitting smoking during pregnancy, which is similar to placebo [45]. This is because that NRT does not effectively control the withdrawal symptoms in most users [42,43]. In addition, nicotine metabolism during pregnancy is much faster than during non-pregnant status. Therefore, the demand for nicotine or cigarettes during pregnancy may also be higher. However, there is no evidence to suggest that using a high dose of NRT is safe in humans. As a result, an effective cigarette smoking quitting method remains a challenge for both clinicians and smokers [42].

In recent years, e-cigarettes have gained significant popularity among the young generation, for its delivery of nicotine in an aerosol form with advertised lower toxicity than tobacco cigarettes [46]. Many women vape e-cigarette at the start of pregnancy aiming to reduce the number of tobacco cigarettes or stop using cigarette smoking [47]. Quite often pregnant women consider e-vaping is safe during pregnancy [47]. Now it is quite clear that certain flavoured e-fluids can be toxic and e-vaping harms not only the lung but also the other organs [48-50]. Growing evidence from animal studies including those from our own group has suggested that e-vaping during pregnancy may have detrimental impacts on multiple organ systems in the offspring at different postnatal ages, including the brain [51-55]. Against the traditional beliefs , the adverse impact of maternal e-cigarette vapour exposure on the offspring seems to be nicotine independent, suggesting noxious chemicals generated from the heated solvent may play a major role in the adverse impact on the health outcome in the offspring. For more detailed comparison between cigarette smoke and e-vapour exposure, please see the review by Li et al. [56]. Nevertheless, it can be concluded that e-cigarette should not be used to during pregnancy.

4. Potential cellular mechanisms

4.1 Inflammatory responses in the brain

The concept of detrimental inflammation has caught wide attention in different research fields. In the field of smoking research, it is not a new concept, as previous studies have profoundly investigated its impact on airway inflammation and the systemic immune response [57], which are the key to the development of common respiratory conditions in smokers, such as chronic obstructive pulmonary disease. Now, it is understood that such systemic inflammatory responses extend all the way to the brain.

It is believed that the free radicals (eg. reactive oxygen species (ROS)), as well as the toxic particle chemicals and gases, produced by combusting tobacco leaves, activate the *nuclear factor* kappa-light-chain-enhancer of activated B cells (NFκB) pathway [57]. They also activate resident macrophages, leading to the overproduction of various pro-inflammatory cytokines and heightened local oxidative stress response. This is most likely to be mediated via toll-like receptor (TLR)4 [58]. In the brain, SE can increase the levels of several pro-inflammatory cytokines, including TNFα, IL-1α, IL-1β, and IL-6 [59]. Interestingly, similar increases in the brain pro-inflammatory cytokines are found in the offspring from the SE dams. This suggests that the blood-placental barrier may not be sufficient to filter out the chemicals that can stimulate a pro-inflammatory response. Potential epigenetic modification can underlie persistent upregulation of pro-inflammatory cytokine expression in the adult offspring's brain [17]. However, in animal models, such effects are only apparent in male offspring [17,18], which is

similar to the gender difference in response to direct cigarette smoke extracts [60]. This can be explained by gender differences in the response of glial cells. Glial cells from the male brains are more pro-inflammatory especially during certain neurological insults, whereas those from the females seem to be more resistant and resilient to stressors [60,61].

Such changes in the offspring's brain may lead to brain dysfunction in the long term. Neuroinflammation has been found in various brain conditions, especially during the development of neurodegenerative disease [62,63]. Brain aging can also be accelerated by cigarette smoking [64], which may be replicated in smoker's offspring. The formation of β-amyloid, the hallmark of the development of Alzheimer's disease, can be increased if brain levels of TLR4, IL-1, and IL-6 are high [63]. Additionally, increased levels of IL-6 in the brain has also been linked to increased anxiety-like behaviour [65]. In the animal model, male offspring from the SE dams displayed an early decline in short term memory and significantly higher levels of anxiety even before puberty [2]. These behaviour may reflect poor school performance in smoker's offspring in human [66-68]. In addition, other neuropsychological conditions, such as schizophrenia or autism, tend to be more severe in the offspring of smoking mothers if a high level of inflammation is present [69,70], suggesting the key role of neural inflammation in maternal smoking-related neurocognitive behavioural disorders in the offspring.

4.2 Oxidative stress in the brain

Oxidative stress is always closely related to inflammatory responses, forming a positive loop between these two reactions. The implication of oxidative stress in disease pathophysiology has received heightened attention not just from the science community, but also the general public. As oxidative stress is found to be involved in nearly all disease processes, the claims of benefit from using antioxidants, especially from the commercial sector, makes oxidative stress a holy grail as the key to combat various conditions.

Cigarette smoke is well known to induce oxidative stress in the brain. Nicotine exerts its effects via nAChRs, which regulate cognitive, motor, and sensory functions [71]. Interestingly, the functions of nAChRs can be blocked by increased oxidative stress [72]. The high risk of neurological disorders in smokers may suggest that oxidative stress is more potent than nicotine to cause nAChR dysfunction. Indeed, changes in brain nAChRs have been observed in the offspring from SE mothers [25].

In homeostatic conditions the cellular antioxidant defense system can scavenge excess free radicals using enzymes copper-zinc superoxide dismutase, manganese superoxide dismutase (MnSOD), catalase, and glutathione peroxidase [73]. However, the overproduction of oxidative molecules can exhaust these enzymes leading to oxidative stress-induced damage. Brain tissue is especially susceptible to ROS damage since it is a major organ to metabolise oxygen (20% of the body consumption). The increase in ROS has been linked to mitochondrial calcium uptake which can increase the permeability of the mitochondrial membrane and eventually lead to cell death [74].

Maternal SE not only leads to an exhaustion of brain MnSOD in the mothers, but also in the adult offspring associated with significant cellular damage in the brain [17,18]. In this instance, perhaps only epigenetic modification can explain such prolonged impairment in brain antioxidant capacity long after the maternal source of oxidants are removed [75]. This may also explain why short-term

antioxidant supplement in the mothers during pregnancy and lactation can have such long-lasting effect to boost endogenous antioxidant capacity in the offspring [4,76-78]. Breastfeeding has been shown to protect the child from various metabolic conditions partially due to antioxidants in the breastmilk. However, its protection of smokers' offspring seems to be limited. Smokers do tend to have shorter breastfeeding period than the non-smokers [79] and chemicals in the tobacco smoke delivered via breastmilk can also negatively impact on postnatal brain development in smokers' offspring [80].

It needs to be noted that in animal models the increased oxidative stress and reduced antioxidants in the brain are only present in the male offspring from SE dams [17,18]. Thus, the levels of brain markers for oxidative stress injury, mitochondrial injury and cell death are increased in the male offspring, but not their female littermates [4,17,18]. It may not be a total surprise, as the male gender seems to be more susceptible to various medical conditions, including neurological disorders such as autism and ADHD [81]. Oestrogen is perhaps the most obvious explanation for such sex differences, as it is a potent antioxidant in addition to its classical role as a sex hormone [82]. This antioxidant function makes oestrogen capable of suppressing inflammation and oxidative stress, and protecting DNA, mitochondrial, and neurons [82-84]. However, we speculate that it may not provide complete protection to the female offspring from SE dams, but only delay the onset of certain disorders in later age when the male and female offspring are compared at different ages [4,17,18].

4.3 Change in mitochondrial function and repair machinery

Mitochondria are involved in a number of cellular processes, such as ATP production and metabolite synthesis. In the brain, there is a high density of mitochondria in the neurons due to the high metabolic and energy requirements. ROS is a major by-product of ATP synthesis. When ROS builds up, MnSOD is exhausted. ROS will firstly damage the mitochondria and DNA. Maternal SE is indeed linked to reduced mitochondrial density in multiple brain regions in the male offspring [2].

The production of ATP through oxidative phosphorylation (OXPHOS) takes place at the cristae of the mitochondrion and is facilitated by OXPHOS complexes I-V. As the brain consumes 50% of total glucose supply at rest, mitochondrial dysfunction is closely associated with neurological disorders [85]. The defects in different OXPHOS complexes are linked to different brain disorders. For example, Complex I is reduced in Parkinson's brains; Complex III is reduced in Alzheimer's brains [86]. Complex V is significantly reduced in the frontal cortex of patients with Down's syndrome [87].

Prolonged SE can cause brain energy shortages, as the activities of various ATPases in the brain can be reduced by smoking [88]. To compensate for the energy demand, all five OXPHOS complexes are increased in response to direct SE [17]. This effect seems to extend to the foetus if the mother is exposed to cigarette smoke during pregnancy, where brain OXPHOS Complexes I-V are increased in their offspring consistent with the changes in the mothers [17,18]. As mitochondrial DNA is inherited from the maternal lineage, one mechanism may be via epigenetic modification of the mitochondrial DNA passed from the mother to the offspring. Another mechanism would be the direct impact of the cigarette smoke inhaled by the mothers.

Mitochondrial integraity is maintained through a process called 'mitophagy', referring to mitochondrial self-eating, or self-renewal. The term derived from the word 'autophagy' means self-eating, which is a strategy for cells to remove or repair damaged components to maintain intercellular

homeostasis. Mitophagy is facilitated by fission and fusion. Fission is the separation of damaged mitochondria subcomponets from the healthy subcomponets; while fusion combines healthy mitochondrial fragments to form a new mitochondrion [89]. In adult male offspring, both fission and fusion markers are reduced in the brain by maternal SE, associated with reduced brain mitochondrial density and increased apoptosis [4]; whereas female offspring seem to be protected from mitochondrial damage. Mitophagy capacity seems to be increased in adult female offspring, associated with normal mitochondrial density and apoptotic activity in the brain [4]. This suggests that increased mitophagy activity is protective to the brain. A reduction in brain mitochondrial fusion was observed in Alzheimer's disease [90]. Although cigarette smoking is known to be closely linked to Alzheimer's disease and dementia [91], whether smoker's offspring have such risks is yet to be investigated by epidemiological studies.

5. The use of antioxidant supplement during pregnancy

Oxidative stress and inflammation are common pathways after HI injury. Even with mild HI injury, suckling pups had more H_2O_2 accumulation in the brain than the adult mice [92]. This is because the neonatal brain has a higher rate of oxygen consumptions and more redox-active iron molecules [92]. Antioxidants are also lower in neonatal brains, whose activity falls dramatically at 2 and 24 hours after HI injury [93]. The defects in brain antioxidant capacity occur in the offspring from SE dams [17]. This can be the reason for more severe neurological functional decline after HI injury in those offspring [2]. Therefore, increasing or restoring brain antioxidant capacity in such offspring may be a solution to protect them from future neurological dysfunction.

The use of antioxidant as a preventative or treatment strategy has long been debated in the literature. On one hand, animal studies showed convincing evidence of a variety of naturally occurred or synthetic antioxidants in different conditions, including some neurological disorders, such as stroke, traumatic brain injury, and dementia [94-96]. On the other hand, antioxidants have limited efficacy, if any, in clinical trials. One issue may be due to the choice of antioxidant used. Instead of using the best antioxidant, some studies used those easily available [97]. In addition, the concentrations of over-the-counter supplements are tightly controlled below therapeutic dosage by the regulatory bodies, such as the FDA, which can compromise the effect in humans. Most antioxidants (eg. Coenzyme Q10) has low bioaccessibility, therefore the cellular concentration is generally below the active level. Furthermore, the power in those human studies can also contribute to low clinical efficacy [94]. As a result, most clinicians are not in favour of recommending antioxidant suplementation.

In animal models, the therapeutic dose of antioxidants can be trialled. The naturally occurring L-carnitine plays a vital role in mitochondrial fatty acid oxidation [98]. L-carnitine also acts as a free radical scavenger that protects antioxidant enzymes, such as MnSOD, from oxidative damage [98]. In humans, L-carnitine has been shown to increase antioxidant enzymes activities, suggesting that it may be useful for improving mitochondrial function in certain diseases [98]. L-carnitine has also been shown to be neuroprotective, which may be applied in certain neurodegenerative disorders which are caused by oxidative stress-induced mitochondrial dysfunction [99].

Increased oxidative stress in the brain of the smokers and their offspring is very similar to neurodegenerative diseases. In the SE mothers, L-carnitine supplementation during gestation and lactation can increase brain levels of MnSOD and improve mitophagy markers in their offspring [4].

This did result in nearly normalised levels of apoptosis and DNA damage markers in the offspring's brain. This is not just confirming that oxidative stress plays a key role in maternal cigarette smoking-induced neurological disorders in the offspring, but also suggests a potential strategy to protect smoker's offspring from the risk of developing neurological disorders, and perhaps other diseases [78,100]. However, clinical trials using therapeutic doses of antioxidants are required to confirm the effects observed in animal models.

Conclusion

Maternal cigarette smoking during gestation and lactation is linked to abnormal neurological functions in the offspring at various ages. From the evidence of animal research, inflammatory response and oxidative stress may play a key role, especially in male offspring (outlined in Figure 1). Maternal supplementation of certain antioxidants may protect the offspring if smoking cessation is impossible to achieve.

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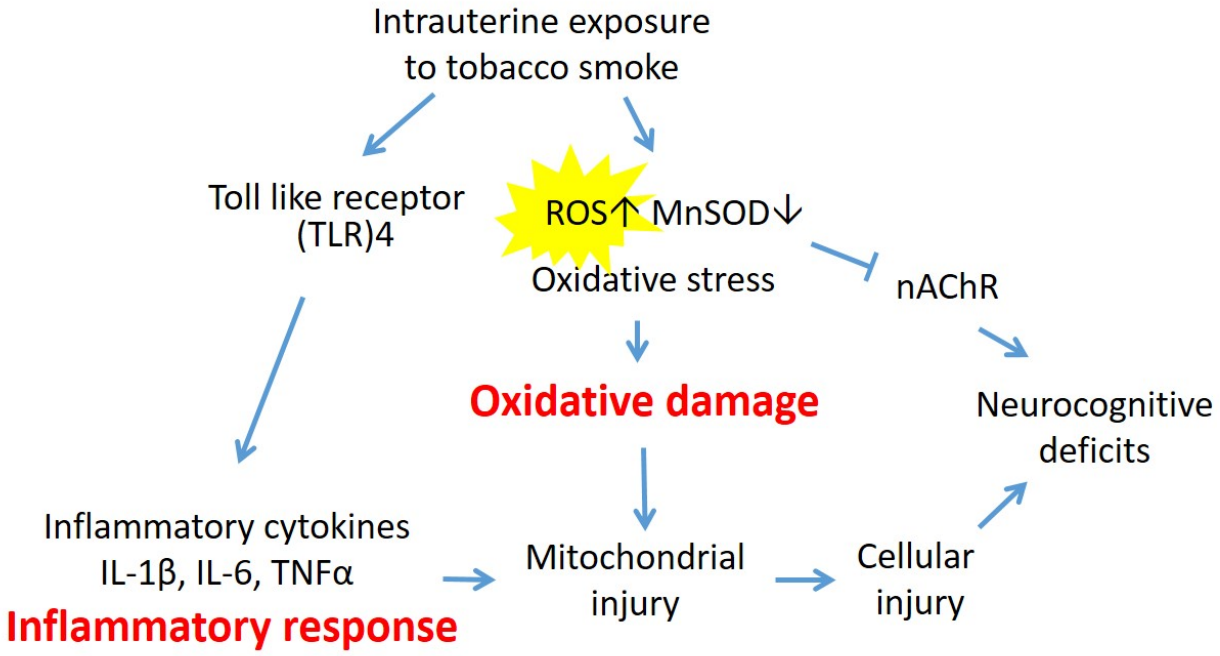

Figure 1: Schematic diagram of some of the mechanisms by which maternal smoking during pregnanacy can damage the developing offspring. IL: Interleukin; MnSOD: manganese-dependent superoxide dismutase; nAChR: nicotinic acetylcholine receptors; ROS: reactive oxygen species; TNF: tumor necrosis factor.