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Aqueous cigarette tar extracts disrupt corticogenesis from human embryonic stem cells in vitro

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ABSTRACT

Background: Cigarette butts are the most common form of litter in the world, and approximately 4.5 trillion smoked cigarettes are discarded every year worldwide. Cigarette butts contain over 4000 chemicals, many of which are known to have neurotoxic effects. Stem cell neuronal differentiation provides an excellent cellular model with which to examine the impact of aqueous cigarette tar extracts (ACTEs) on neurodevelopment.

Methods: We have developed a neurosphere-based stem cell neuronal differentiation protocol that can recapitulate corticogenesis and produce cell types that are similar to upper and lower layer cortical projection neurons found in the germinal zone of the developing human cortex. In this study, ACTEs were generated from smoked cigarette butts and then applied at different concentrations to neuronal progenitors and cortical neurons derived from human embryonic stem cells.

Results: ACTEs reduced the expression of the cortical neuronal progenitor markers pax6, tbr2, and neuroD and decreased the number of cortical layer neurons (tbr1, satb2, foxp2, and brn2) after exposure to as low as 1.87% of the extract from one smoked cigarette butt. Furthermore, our results showed that ACTEs increased reactive oxygen species (ROS) production in cortical neurons, which caused a substantial loss of the synaptic proteins PSD95, synaptophysin, vesicular glutamate transporter1 (vGlut1), and the extracellular matrix molecule reelin; all of those molecules are important for the maintenance of cortical neuron identity and activity.

Conclusion: ACTEs from smoked cigarettes have significant effects on cortical neuron development and neurodegeneration. The stem cell neuronal differentiation model holds great promise as a potentially powerful tool for the assessment of ACTEs on neurotoxicity.

1. Background

In the past decade, cigarette smoking in the U.S. has decreased by 28%, yet cigarette butts remain the most littered item in the U.S. and across the globe. Approximately 176,000,000 pounds of cigarette butts are discarded in the U.S. each year. Over 4.5 trillion cigarettes are littered worldwide each year (http://www.cigarettelitter.org). Cigarette butts contain over 4000 chemicals, including chemicals such as carbon monoxide, hydrogen cyanide, nitrogen oxides, polycyclic aromatic hydrocarbons, ammonia, acetaldehyde, formaldehyde, benzene, phenol, argon, pyridines and acetone, and many of them are known to be toxic to humans. A study showed that the introduction of a single cigarette to a liter of water resulted in the death of 50% of the fish in the water (Slaughter et al., 2011).

The environment plays a significant role in the development and health of an individual. Human brain development begins in the first few weeks after conception. Most of the structural features of the brain appear during the embryonic period (approximately the first 8 weeks after fertilization). These structures then continue to grow and develop during the fetal period (the remainder of gestation) (Stiles and Jernigan, 2010). Early brain development is the foundation of human adaptability and resilience. Various animal and human studies indicated that adverse prenatal and postnatal environmental conditions disrupt homeostasis and increase the risk of neurodevelopmental diseases or neurodegeneration (Abu-Taweel et al., 2012; Batstra et al., 2003; Modgil et al., 2014).

Neurodevelopmental or neurodegenerative diseases such as Fragile X syndrome (Irwin et al., 2005), Rett syndrome (Kerr et al., 2010), Alzheimer's disease (AD) (Chin-Chan et al., 2015), and Parkinson's disease (PD) (Hatcher et al., 2008) have been studied extensively, and various molecular mechanisms have been proposed for the onset of such diseases with regard to exposure to environmental toxins. Several

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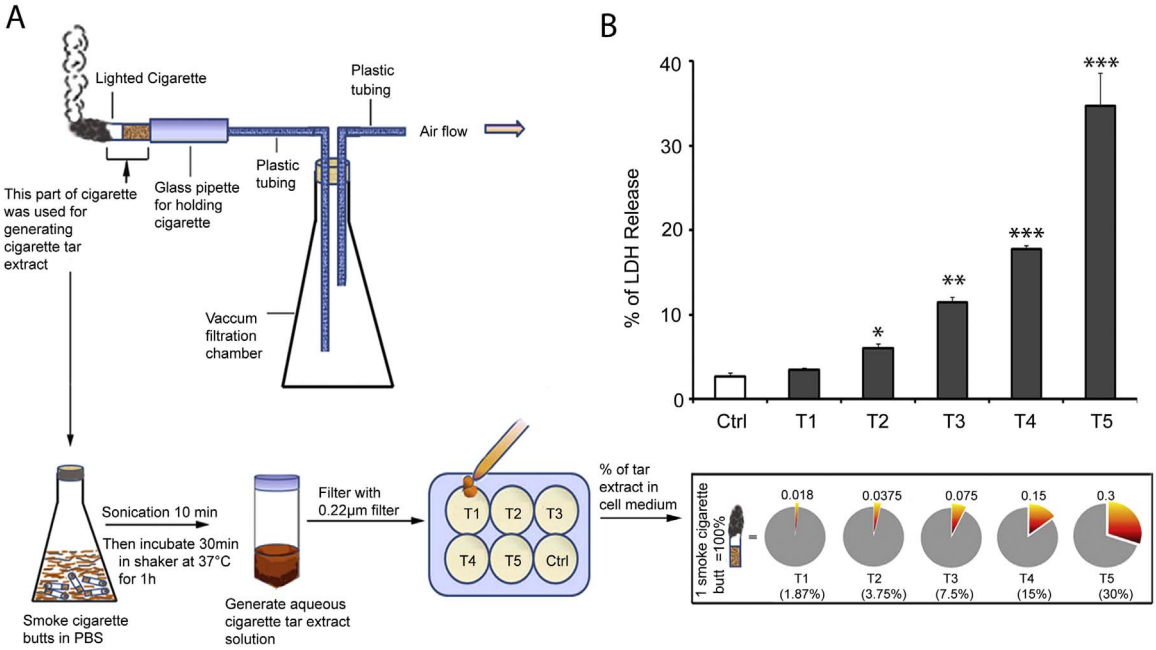

Fig. 1. Generation of aqueous cigarette tar extracts (ACTEs) and neurotoxicity dosing (**A**) ACTEs were generated from smoked cigarette butts. Five smoked cigarette butts were soaked in 5 ml of phosphate buffer saline (PBS) then sonicated in water for 10 min. The ACTE solutions were filtered with a 0.22-µm filter and then were diluted in the neuronal maintenance medium (NMM) at different concentrations. A total of 5 ml of ACTEs was obtained from 5 cigarette tars. The different dilutions of the ACTEs in NMM were referred to as T1, T2, T3, T4, T5, T6 which corresponded to 0%, 1.87%, 3.75%, 7.5%, 15% and 30% of cigarette tar, respectively, as shown in the experimental scheme. (**B**) LDH assay to determine the effect of ACTEs on neurotoxicity. The bar graph showed that ACTE increased the release of LA in a dose-dependent manner. The data are presented as the means ± SD, N = 3–4. *$p < 0.05$ represents a significant difference.

publications address the issue of cigarette tar and the generation of reactive species (ROS), its deleterious effect on blood microcirculation (Begum and Terao, 2002), and its involvement in the pathogenesis of cardiovascular diseases (Modgil et al., 2014), pulmonary hypertension (Li et al., 2015) and alterations in brain function (Lavezzi et al., 2012). However, there has been no systematic study regarding the neurodegenerative risk of aqueous cigarette tar extracts (ACTEs) on human neurons.

The cerebral cortex is the integrative and executive center of the mammalian CNS and constitutes over three quarters of the human brain (Mountcastle, 2003). Corticogenesis is a highly intricate process that requires multiple layers of regulation of cell behavior at the progenitor and post-mitotic cell stages. The cerebral cortex is divided into six different layers. Each layer is formed by radial glial cells located in the ventricular zone or subventricular zone, and it then migrates to its final destination (Meyer, 2007). Layer one is composed of Cajal-Retzius neurons and pyramidal cortex cells, which are also characterized by the expression of reelin, transcription factor T-box brain 1, and cortical migratory neuronal markers. The second, third, and fourth layers include pyramidal neurons, astrocytes, stellates, and radial glial cells. Pyramidal and stellate neurons express SATB2 and Brm2. The fifth and sixth layers include stellates, radial glia, and pyramidal neurons. During the production of these layers, the transcription factors Pax6, Tbr1, Tbr2, NeuroD, FoxP2, and Satb2 are expressed sequentially. Pax6 regulates diverse developmental mechanisms, including regional identity, neuronal fate, cell cycle kinetics, cell migration, cell adhesion, and axonal growth and guidance (Kroll and O'Leary, 2005). Tbr1 modulates the expression of RELN, which regulates the formation of the matrix for neuronal migration (Hevner et al., 2002). Tbr 2 plays important roles in neuron proliferation, fate specification, differentiation or migration (Eiraku et al., 2008). NeuroD is necessary for the proliferation of dentate gyrus neurons and for the postnatal differentiation of dentate gyrus neurons and cerebellar granule neurons (Liu et al., 2002). Diseases of the cerebral cortex are the major causes of morbidity and mortality in children and adults and include developmental conditions such as epilepsy and autism and neurodegenerative conditions that occur later in life, such as Alzheimer's disease (Geschwind and Miller, 2001). Many studies have been conducted on cerebral cortex development, function and disease in rodent models (Eiraku et al., 2008; Molnar et al., 2006; Wu et al., 2011). However, the primate cortex, and particularly the human cerebral cortex, differs in several respects from the rodent cortex. In this experiment, we used our recently developed neurosphere-based human cortical neuron (hCN) culture from human embryonic stem cells as a cellular model to investigate human cortical development and cortical degeneration in response to exposure to aqueous cigarette tar extract from smoked cigarette butts (Begum et al., 2015). The emerging stem cell neuronal differentiation model enabled us to examine the impact of cigarette tar extracts from smoked cigarette butts on cortical neuron development and the identities of cortical neurons and decipher the molecular mechanisms of neurodegeneration as a result of exposure to cigarette butt extract.

2. Materials and methods

2.1. Preparation of aqueous cigarette tar extract (ACTE) solution

ACTEs were obtained according to the method of Begum (Begum and Terao, 2002) with further modifications. Briefly, cigarettes (85 mm in length and 0.9 g in weight) were purchased commercially and smoked with an aspirator connected to airflow via tubing. The smoked tar was collected on glass fiber filter paper (diameter 1.5 cm, Thermofisher Scientific). The filters that contained tar from five cigarettes were soaked in 5 ml of 1x PBS, sonicated in a water bath for 10 min, and then the solutions were incubated with continuous shaking for 1 h at 37 °C. The tar extract solution was vortexed for 1 min and then filtered with a 0.22-µm stericup filter unit (Fisher Scientific) under sterile conditions. A total of 5 ml of the filter tar extract solutions was obtained such that 1 ml of extract corresponded to the tar from one cigarette. The resulting ACTE solutions were vortexed and immediately used for experiments. Five different concentrations of ACTEs were used in this experiment: control (only medium/without tar), and T1, T2, T3, T4 and T5, which represented 0%, 1.87%, 3.75%, 7.5%, 15% and 30% of one