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Mainstream smoke and sidestream smoke affect the cardiac differentiation of mouse embryonic stem cells discriminately

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ABSTRACT

Epidemiology studies suggest that maternal smoking and passive smoking have strongly resulted in the occurrence of congenital heart defects (CHD) in offspring. Cigarette smoke (CS) can be divided into mainstream smoke (MS) and sidestream smoke (SS); CS chemistry study indicates that significant differences exist in the composition of MS and SS. Therefore, MS and SS were suspected to process toxicity dissimilarly. However, much less was known about the difference in the developmental effects induced by MS and SS. In the current study, heart development was mimicked by mouse embryonic stem cells (ESCs) differentiation. After MS and SS exposure, by tracing the bone morphogenetic protein (BMP)-Smad4 signalling pathway, interruption of downstream gene expression was observed, including Gata4, Mef2c and Nkx2.5, as well as myosin heavy chain and myosin light chain. Specifically, SS caused inhibition of Gata4 expression, even at non-cytotoxic concentration. Further, SS-induced hypoacetylation in promoter regions of Gata4 reflected the orchestration of CS-gene modulation-epigenetic regulation. Even though SS induced apoptosis in ESC-derived cardiomyocytes, the partial clearance in cells with down-regulated Gata4 caused these cells to survive and undergo further differentiation, which laid potential risk for abnormal heart development. These data uncovered the difference between MS and SS on heart development preliminarily.

1. Introduction

Congenital heart defects (CHD) have been reported to affect approximately 1% of newborns all over the world, however, apart from heredity, the full reasons remained unknown (van der Linde et al., 2011). Gene–environment interaction in heart disease raised public attention, as one of well-known environmental risk factors, exposure to cigarette smoke (CS) (Huang et al., 2008), was positively associated with increased cardiovascular morbidity and mortality (Lubin et al., 2016). Maternal smoking was identified to result in heart malformation in offspring, and smoking in the first trimester was more likely to result in infants born with CHD, with an odds ratio (OR) of 1.16 (95% confidence interval (CI) 1.08-1.24). Among various types of CHD, pulmonary valve anomalies, pulmonary artery anomalies and isolated atrial septal defects were strongly correlated to maternal smoking, with ORs of 1.48, 1.71 and 1.22 respectively (Sullivan et al., 2015). Also, secondhand exposure during pregnancy correlated to CHD, with an OR of 2.04 (95%CI 1.05, 3.98) for atrioventricular septal defects, and an OR of 2.48 (95%CI 1.04, 5.95) for left ventricular outflow tract obstructions (Deng et al., 2013; Patel et al., 2012). Conclusively, CHD induced by smoking and passive smoking suggested that CS produced toxicity anchoring the fetal heart. However, the mechanisms of CS-induced developmental cardiotoxicity were still poorly unveiled.

CS can be divided into two parts: mainstream smoke (MS) refers to the smoke inhaled by the smoker, and sidestream smoke (SS) refers to the smoke emanating from the cigarette between puffs. As major substances in cigarettes, nicotine, carbon monoxide, metabolites of some tobacco-specific nitrosamines, volatile organic compounds or polycyclic aromatic hydrocarbons (PAH) and heavy metals were carcinogenic and teratogenic; therefore, their combination in forms of MS or SS was considered to be strongly teratogenic (Carmines et al., 2003; Garcia-Canton et al., 2012). However, a great variety of compounds was produced when cigarettes combusted. CS chemistry was extensively studied and significant differences in the

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composition of MS and SS were found. The compounds detected in SS turned out to be more unsaturated and less oxygenated than those observed in MS (Schramm et al., 2011). The tar and hydrogen cyanide (HCN) in MS were nearly two-fold higher than in SS, while nitric oxide, ammonia, lead, cadmium and mercury in SS were less than half of that in MS (Moir et al., 2008). Consequently, MS and SS may process toxicity dissimilarly.

Even though some components, such as Benzo[a]pyrene and HCN, played a role in causing cardiovascular damage, as well as alterations in heart and blood vessels consisted of irreversible adverse effects in the formation of pathological lesions (Kerley-Hamilton et al., 2012; Tithof et al., 2001). At the time of the current study, however, much less has been clarified in terms of the different mechanisms between MS and SS. Previous studies elucidated more severe aromatic DNA adducts in SS than in MS, and yielding higher concentrations of PAH in SS (Thapliyal et al., 2004). In addition, CS caused platelet activation and increased the susceptibility of platelets to activation by shear stress, which was much more remarkable in SS, due to higher concentration of nicotine in SS (Rubenstein et al., 2004). These data were elucidated from adult heart, therefore it did less to promote the understanding of the differences in MS- and SS-induced developmental toxicity.

Under this circumstance, use of developmental model animals was applicable to investigate CS-induced developmental toxicity. In one study, cardiac developmental defects were observed in zebrafish following exposure to CS extracts (Palpant et al., 2015). Another popular model to study developmental biology was embryonic stem cells (ESC). This aroused attention due to its advantages of pluripotency, self-renewal ability and tissue-specific differentiation potential, which could further mimic the organogenesis *in vitro* and process accurate determination of chemical-induced adverse effects during embryo development (Seiler and Spielmann, 2011). By applying them, the ESC-induced cardiomyocytes provided a deeper insight into understanding the possible molecular mechanism during cardiac differentiation *in vitro* following xenobiotics exposure (Lin et al., 2010; Liszewski et al., 2012; Palpant et al., 2015), and this model was expected to study the difference between MS- and SS-induced interference in heart development.

Previously, three major differentiation pathways involved in cardiogenesis, Notch, canonical Wnt and transforming growth factor-β, had been identified to be affected by CS in ESCs, resulting in various disturbances in downstream gene expression (Liszewski et al., 2012; Palpant et al., 2015). As one of key signalling pathways, bone morphogenetic protein (BMP) signalling contributed to the normal development of heart (Harada et al., 2008; Uchimura et al., 2009). Biologically, BMP2 activated phosphorylation of Smad1 to form hetero-oligomeric complexes with Smad4, and modulated the transcription of the target genes after they were translocated into the nucleus. As a downstream effector of BMP2, Smad4 can physically interact with Gata4, one of the zinc finger-containing transcription factors, which further interacted with Tbx5 physically, and activated endocardial and myocardial gene expression cooperatively (Gittenberger-de Groot et al., 2014). Nkx2.5, a cardiac specific homebox gene, was one of the earliest markers for cardiac progenitor cells; combined expression of Gata4 and Nkx2.5 induced early cardiac gene expression (Chi et al., 2005; Stefanovic et al., 2014); Mef2c was required for cardiac muscle formation, interacting via its MADS-box with the T-box domain of Tbx5 and synergistically activating several downstream myogenic genes encoding myosin heavy chain and myosin light chain (Morimoto, 2008). This "GTMN" complex functioned together during cardiac morphogenesis and conduction system development precisely (Schlesinger et al., 2011). Up to date, whether BMP signalling could be affected by CS developmentally is unclear.

Therefore, to fill in gaps, the aim of our study was to investigate the potential difference in developmental adverse effects caused by MS and SS during heart development. Here, mouse ESCs were applied and were induced to differentiate into cardiomyocytes efficiently, which constructed an ideal platform to address the question about differences in the impact on cardiac differentiation between MS and SS. CS-induced adverse effect was investigated based on BMP signalling pathway, with special interest in effects observed at non-cytotoxic concentration.

2. Materials and methods

2.1. Culture and cardiac differentiation of ESCs

ESC line R1 was used in the current study between passages 25 and 30. Pluripotency of ESCs were maintained by cultivating cells on mitomycin C inactivated feeder layer of C57BL/6 mouse strain-derived mouse embryonic fibroblasts. ESC medium consisted of KnockOut Dulbecco's Modified Eagle's medium (KO DMEM, Gibco, USA), 15% fetal calf serum (Gibco, USA), 0.1 mM beta-mercaptoethanol (Gibco, USA), 2 mM L-glutamine (Gibco, USA), 0.1 mM non-essential amino acids (Gibco, USA) and 1000 U/ml leukaemia inhibitory factor (LIF, Millipore, USA). During cardiac differentiation, ESCs were digested into single cells with a 0.25% Trypsin/EDTA, the feeder was discarded and the LIF was removed. A total of 750 cells of R1 ESC were cultured in 20 μl hanging drops to generate embryoid bodies (EBs) in DMEM medium with 15% fetal calf serum (Gibco, USA), 30 EBs in one petri dish (Alpha, China) per concentration of CS extract for 3 days; then, EBs were cultivated in suspension as one petri dish per concentration of MS and SS extracts for 2 days. On day 5, EBs were plated into each well of a 96-well plate (Corning, USA). On day 10 of differentiation, contracting cardiomyocytes were determined microscopically, normalised to control group. Cardiac differentiation was considered positive if at least one contracting foci was observed. It is notable that the fetal calf serum should be pretested to assure its quality for differentiation culture, as described previously (Seiler and Spielmann, 2011).

Histone acetyltransferases inhibitor curcumin (CC) (CAS: 458-37-7, Sigma, USA) and histone deacetylases inhibitor valproic acid (VPA) (CAS: 99-66-1, Sigma, USA)were selected as controls in further experiments, with final concentrations of 10 μM and 5 μm respectively, according to previous study (Kawamura et al., 2005).

2.2. Cigarette smoke extracts preparation airflow rotary flowmeter

Cigarettes were purchased commercially, with specifications of 8 mg tar and 0.7 mg nicotine. Cigarettes were smoked, applying a self-made extraction system to execute the separation of MS and SS (Fig. 1). The smoking parameters and smoking specifications were set following the International Organization for Standardization Standard 3308 (ISO:3308). Briefly, in order to generate CS extract, 100 ml of DMEM medium was placed in two gas washing bottles separately; two vacuums were applied to pump MS and SS into the media through gas diffuses to generate media containing all contents of burned cigarettes respectively. The standard conditions employed a puff volume of 35 ml, a puff duration of 2 s and a puff interval of 60 s. The DMEM medium with MS and SS were made into a final concentration of 0.5 puff/ml, adjusted to pH 7.4 and then filtered with a 0.22-μm syringe filter.

According to previous study (Wan et al., 2012), detection of tobacco-specific nitrosamines (TSNA) in MS and SS by LC–MS–MS was performed to acquire the concentration of *N*-nitrosonornicotine (NNN), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosoanatabine (NAT) and *N*-nitrosoanabasine (NAB) in MS and SS, in purpose of characterizing the difference between components in MS and SS partially.