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Elevated 5-hydroxymethycytosine and cell apoptosis induced by tetrachloro-1,4-benzoquinone in mouse embryonic stem cells

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Pentachlorophenol (PCP) has been extensively used as a fungicide, bactericide, herbicide, molluscicide, algaecide and insecticide worldwide (Ahlborg and Thunberg, 1980; Choudhury et al., 1986). Although PCP is primarily used as a preservative for wood and wood products, it has been also used in a variety of other products, including adhesives, construction materials, leather and paper. PCP is environmentally persistent with a half-life of up to 6–7 months in soil and water systems (Law et al., 2003). In 2015, the Stockholm Convention on Persistent Organic Pollutants added PCP to its list of persistent organic pollutants. Because of its extensive use and resistance to degradation, PCP has become a ubiquitous environmental contaminant that has been detected in surface water, drinking water, soil, and food (Zhu and Shan, 2009; Angelucci and Tomei, 2015). Consequently, the general public can be exposed to PCP as evidenced by its presence in human urine, serum, and adipose tissue samples from non-occupationally exposed people (Seiler, 1991; Morgan, 2015).

PCP was classified as a B2 carcinogen (possibly carcinogenic to human) by the International Agency for Research on Cancer (IARC, 1991). The incidence of liver tumors, pheochromocytomas, and hemangiosarcomas was significantly elevated in mice chronically exposed to PCP (McConnell et al., 1991; Chhabra et al., 1999). Epidemiological studies have shown that elevated risk of lymphatic and hematopoietic cancer is associated with the occupational exposure to PCP (Ramlow et al., 1996; Demers et al., 2006). Genotoxicity studies have been conducted to understand the effects of PCP exposure in humans and animals (Dahlhaus et al., 1996; Tisch et al., 2005). These studies have revealed that two reactive metabolites of PCP, tetrachlorohydroquinone (TCHQ) and tetrachloro-1,4-benzoquinone (TCBQ), play a key role in its genotoxicity. Conversion of PCP into its quinone metabolites takes place primarily in the liver (Renner and Mucke, 1986). The well-recognized pathway involves cytochrome P450-mediated dechlorination of PCP to generate TCHQ, which is further oxidized into TCBQ via its corresponding semiquinone, the tetrachlorosemiquinone radical (TCSQ·) (Zhu and Shan, 2009). Redox cycling associated with the oxidation of TCHQ and/or the reduction of TCBQ to semiquinones produces reactive oxygen species (ROS), which therefore lead to oxidative stress. Increased levels of hydroxyl radical-derived DNA lesion, such as 8-oxodeoxyguanosine (8-OHdG), was observed in V79 cells treated with TCHQ or TCBQ (Dahlhaus et al., 1996). Additionally, TCBQ can react with DNA to form DNA adducts (Lin et al., 2001).

Although the genotoxicity of TCHQ and TCBQ has been well studied, little is known about their impact on the DNA methylation. DNA methylation is an important epigenetic modification that is implicated in various biological functions, including regulation of gene expression, transposable element silencing, genomic imprinting, and X chromosome inactivation (Bird, 2002; Deaton and Bird, 2011; Wu and Zhang, 2011). In mammals, DNA methylation occurs predominantly at the C5

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position of cytosine in the context of Cytosine–phosphate–Guanine (CpG) dinucleotides, which leads to the formation of 5-methylcytosine (5-mC) through the action of DNA methyltransferases (Li and Zhang, 2014). Most CpG sites (~60%–80%) are methylated in mammals. 5mC can be oxidized to 5-hydroxymethycytosine (5hmC) by ten-eleven translocation (Tet) proteins (T1, T2 and T3) (Tahiliani et al., 2009; Ito et al., 2010; He et al., 2011). 5hmC can be further oxidized to removable 5-formylcytosine and 5-carboxylcytosine by Tet proteins, providing a pathway for DNA demethylation (Ito et al., 2011).

The oxidation activity of Tet proteins requires some cofactors, such as iron(II) and α-ketoglutarate (Tahiliani et al., 2009; He et al., 2011; Ito et al., 2011). Changing the levels of cofactors leads to an alternation of the catalytic activity of Tet proteins. Cellular levels of iron(II) and α-ketoglutarate have been reported to be affected by cellular redox status (Cairo et al., 1995; Kakhlon and Cabantchik, 2002; Deb et al., 2009). Because TCBQ and TCHQ are redox active, Zhao et al. hypothesized that TCBQ and TCHQ may alter the catalytic activity of Tet proteins by changing the cellular levels of iron(II) or α-ketoglutarate, and therefore impact DNA methylation process (Zhao et al., 2014). They tested whether TCBQ and TCHQ could alter the oxidation of 5mC to 5hmC because this oxidation is primarily driven by Tet proteins. An increase of 5hmC was observed when MRC-5 cells were treated with TCBQ or TCHQ and this increase was dose- and time- dependent. When other cell lines were examined, TCBQ/TCHQ treatment similarly resulted in an increase of 5hmC. To test whether the TCBQ/TCHQ-induced increase of 5hmC is mediated by Tet proteins, Tet1/Tet2 double-knockout mouse embryonic stem cells were used. 5hmC was barely detected in these embryonic stem cells treated with TCBQ/TCHQ, which suggested that the TCBQ/TCHQ-induced increase of 5hmC occurs via enhancing the activity of Tet proteins. To elucidate how TCBQ and TCHQ enhance the 5mC oxidation activity of Tet proteins, the cellular level of iron(II) was studied. Levels of cytoplasmic and nuclear ferritin light chain (FTL) were found to be up-regulated upon TCBQ treatment. FTL is a major iron-storage protein whose level is associated with the level of labile iron. The increase in FTL level indicates the elevation in the level of labile iron that generates iron(II) for the activity of the Tet proteins. Additionally, direct delivery of iron(II) to MRC-5 cells elevated 5hmC, whereas sequestering of cellular iron(II) by an iron-specific chelating agent reduced 5hmC. Therefore, TCBQ and TCHQ play a role in upregulation of 5hmC by increasing the cellular level of iron(II) which in turn enhances the 5mC oxidation activity of Tet proteins. TCBQ-induced increase of 5hmC is a genome-wide process, which suggests that TCBQ could regulate gene expression via increase of 5hmC. By using RNA-sequencing technology, Zhao et al. found that 3414 genes of 5751 5hmC-enriching genes showed a significant difference in expression (Zhao et al., 2014). These genes are associated with various cellular processes, including protein catabolic process, cell localization and transport, RNA processing, and apoptosis.

The same group further used mouse embryonic stem (ES) cells to study the impact of TCBQ on DNA methylation change and cell apoptosis (Li et al., 2016). Recently, stem cells are increasingly being used to replace animals in the field of environmental toxicology (Jennings, 2015; Faiola et al., 2015; Li et al., 2015; Yin et al., 2015) because animal tests are expensive, time-consuming, and labor intensive. More importantly, interspecies variations could decrease the predictability for relevant human toxicological outcomes. The use of primary human cells is generally impractical because derivation of primary human cells can be very invasive and it is often challenging to culture and expand the primary cells. ES cells have the ability to proliferate indefinitely in culture and differentiate into different types of cells of their organism of origin (Mori and Hara, 2013). Additionally, ES cells are often more sensitive than somatic cells and can be used for developmental toxicity and cell function assays. Therefore, the use of ES cells for TCBQ effects can produce data that are more relevant to human outcomes.

Specifically, the effect of TCBQ on 5hmC formation was first studied by treating mouse ES cells with varying concentrations of TCBQ. After incubation for 24 hr, genomic DNA was extracted and then digested into mononucleosides. UHPLC–MS/MS was used to quantify 5hmC and 5mC in digested DNA samples. 5hmC levels were positively correlated with TCBQ concentrations when cells were exposed to 10–40 μmol/L TCBQ. Therefore, TCBQ causes a concentration-dependent increase of genomic 5hmC in mouse ES cells, which is consistent with the observation of MRC-5 cells. To confirm whether this TCBQ-induced increase of 5hmC relies on Tet proteins, 5mC and 5hmC levels were detected in Tet1/Tet2/Tet3 triple-knockout (TKO) mouse ES cells. 5hmC was hardly detectable in these cells after TCBQ treatment, whereas the levels of 5mC were increased compared to that in wild-type (WT) stem cells. This is because Tet knockout removes the expression of Tet proteins and therefore minimizes the oxidation of 5mC to 5hmC. These results further suggest that TCBQ increases the 5hmC formation via enhancing oxidation activity of Tet proteins.

To test whether TCBQ-induced 5hmC formation is implicated in apoptosis, Li et al. (2016) compared WT and Tet1/Tet2/Tet3 TKO mouse ES cells for the TCBQ-induced apoptosis. An Annexin-V FITC/PI double staining assay was used to identify apoptotic cells. Apoptosis is a programmed cell death process that is characterized by specific morphologic features, including loss of membrane asymmetry and attachment, blebbing of the plasma membrane, condensation of the cytoplasm and nucleus, and chromosomal DNA fragmentation (Shi, 2001). Apoptosis can be classified into two stages, early stage apoptosis and late stage apoptosis. During early stage, the cell membrane loses its asymmetry, which allows membrane phosphatidylserine (PS) to be translocated from the inner side to the cell surface. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that binds to PS molecules on the cell surface with high affinity (Koopman et al., 1994). Annexin V labeled with a fluorescent dye such as FITC can be used to identify the early apoptotic cells. As apoptosis proceeds to the late stage, the membrane loses its integrity. A vital dye such as propidium iodide (PI) can enter cells and bind to nucleic acids, resulting in fluorescence increase. Therefore, late apoptotic cells are both Annexin V and PI positive.

When WT and TKO mouse ES cells were exposed to 0, 20, 30, 40, and 50 μmol/L TCBQ, TCBQ induced a dose-dependent increase in apoptotic cells; the increase of TCBQ resulted in a remarkable increase in the amount of apoptotic cells. When the concentration of TCBQ was 20 μmol/L, no significant increase in amount of apoptotic cells was observed in

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