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Impact of commercial cigarette smoke condensate on brain tissue co-cultured with astrocytes and blood–brain barrier endothelial cells

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ABSTRACT

The purpose of the current study was to investigate the effect of two commercial cigarette smoke condensates (CCSC) on oxidative stress and cell cytotoxicity in human brain (T98G) or astrocytes (U-373 MG) in the presence of human brain microvascular endothelial cells (HBMEC). Cell viability of mono-culture of T98G or U-373 MG was markedly decreased in a concentration-dependent manner, and T98G was more susceptible than U-373 MG to CCSC exposure. Cytotoxicity was less prominent when T98G was co-cultured with HBMEC than when T98G was co-cultured with U-373 MG. Significant reduction in trans-epithelial electric resistance (TEER), a biomarker of cellular integrity was noted in HBMEC co-cultured with T98G (HBMEC-T98G co-culture) and U-373 MG co-cultured with T98G (U-373 MG-T98G co-culture) after 24 or 48 hr CCSC exposure, respectively. TEER value of U-373 MG co-cultured with T98G (79–84%) was higher than HBMEC co-cultured with T98G (62–63%) within 120-hr incubation with CCSC. Reactive oxygen species (ROS) generated by CCSC in mono-culture of T98G and U-373 MG reached highest levels at 4 and 16 mg/ml, respectively. ROS production by T98G fell when co-cultured with HBMEC or U-373MG. These findings suggest that adverse consequences of CCSC treatment on brain cells may be protected by blood–brain barrier or astrocytes, but with chronic exposure toxicity may be worsened due to destruction of cellular integrity.

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Introduction

Cigarette smoking corresponds to 434,000 casualties/year in the USA (US Department of Health and Human Services 2014) and produces not only cancers but also various other diseases including cerebrovascular, cardiovascular, and chronic obstructive pulmonary disease (COPD). (Bhalla et al. 2009; Ng et al. 2013; Plöttner et al. 2012; Pittilo 2000; Swan and Lessov-Schlaggar 2007).

The blood–brain barrier (BBB) is comprised of tight junctions of the brain microvascular endothelial cells as well as regulates passage of endogenous and exogenous compounds between blood and brain parenchyma cells (Kim et al. 2013). BBB forms an interface between circulating blood and brain as well as possesses various carrier-mediated transport systems for small molecules to support and protect central nervous system (CNS) functions (Ohtsuki and Terasaki 2007; Ronaldson and Davis 2012). Although it is considered to be impermeable to large molecules, several studies established that some large molecules or highly reactive substances may permeate the BBB, entering the brain and consequently affecting brain functions (Brines et al. 2000; Kreuter et al. 1995; Qian and Shen 2001).

Oxidative stress is associated with production of reactive oxygen species (ROS), and induces mitochondrial dysfunction as well as degenerative diseases in brain (Panov et al. 2007; Waris and Ahsan 2006). Mazzone et al. (2010) reported that cigarette smoke enhanced the risk of cerebrovascular and neurological disorders largely due to ROS generation. Previous investigators found that exposure to cigarette smoke extracts induced neuronal cytotoxicity as evidenced by oxidative stress in BBB endothelial cells in an *in vitro* HBMEC model (Naik et al. 2014; Prasad et al. 2015). Cellular ROS generation in BBB was significantly increased by exposure to cigarette smoke extract from 3R4F and ultra-low nicotine (ULN) cigarettes (Naik et al. 2015). In addition, cigarette

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smoke contains numerous harmful and reactive substances including polycyclic aromatic hydrocarbons (PAH), N-nitroso compounds, reactive aldehydes, and toxic heavy metals including cadmium which induce oxidative stress tissue damage (Piasek et al. 2016; Brajenović, Karačonji, and Bulog 2015; Abraham and Khandelwal 2013; Anbarasi et al. 2006; Luchese, Pinton, and Nogueira 2009; Vida et al. 2014; Bernhard, Rossmann, and Wick 2005).

To date, cigarette consumption and the total number of daily smokers have risen globally (Ng et al. 2014). Previously Kim et al. (2015) demonstrated that smoke extracts of 3R4F, standard cigarette produced cell cytotoxicity in brain cells. However, data on how commercial cigarette smoke extracts induce oxidative stress through an action on the BBB and other brain tissues including astrocytes, which physiologically support tight junction barrier, remain to be determined in particular in susceptible populations.

According to survey data from Brand stock, which brand value, Evaluation Company, ESSE, and This Plus are high ranked among Korean commercial cigarettes. Hence, the current study aimed to compare the effect of commercial cigarette smoke condensates (CCSC) on cell cytotoxicity, cellular membrane damage, and generation of ROS in brain cells by mono-culture and co-culture using HBMEC, to reflect the BBB, and astrocytes.

Experimental

Standards and chemical reagents

Dulbecco's modified Eagle's medium (DMEM) were obtained from Sigma-Aldrich (St. Louis, MO). Penicillin/streptomycin (Pen/Strep) and fetal bovine serum (FBS) were purchased by Gibco (Introgen Corporation, Grand Island, NY). Nu-Serum was purchased from BD Biosciences (San Jose, CA). L-Glutamine was obtained from Irvine Scientific (Santa Ana, CA). MEM non-essential amino acids, sodium pyruvate, and MEM vitamins were provided by Cellgro (Herndon, VA). Two commercial cigarettes, namely This Plus (commercial cigarette X) and ESSE Prime (commercial cigarette Y) were provided by the Korea Tomorrow and Global KT&G (Daejeon, Republic of Korea). Commercial cigarette X had 0.6 mg nicotine and 5.5 mg tar. Commercial cigarette Y contained 0.45 mg nicotine and 4.5 mg tar.

Preparation of commercial cigarette smoke condensate

CCSC obtained from two worldwide brands of commercial cigarette was obtained from Korea Institute of Toxicology (KIT, Jeongeup-si, Jeollabuk-do, Republic of Korea). All samples were conditioned and marked according to the International Organization for Standardization 3308 (ISO 3308), routine analytical cigarette smoking machine definitions, and standard conditions. Total particulate matter (TPM) was filtered and collected via a 44 mm Cambridge filter and then solubilized in dimethyl sulfoxide (DMSO) to 20 mg TPM/ml. This was filtered with a 0.45 micron PTFE filter and then kept at −80°C. Nicotine concentration was 819.87 and 833.59 mg/l for CCSC X and Y, respectively.

T98G/U-373 MG cell culture

The human glioma cell lines T98G and human glioblastoma astrocytoma U-373 MG were provided by the Korean Cell Line Bank (KCLB, Seoul, Republic of Korea). Cells were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified 5% CO_2 atmosphere, and subcultured when reaching 80% confluence. For T98G, the cell passage used for this study was between 290 and 295. For U-373 MG, the passage employed for this study was between 20 and 25.

Human brain microvascular endothelial cells culture

HBMEC cell line was provided by the Division of Pediatric Infectious Diseases, Johns Hopkins University School of Medicine. Cells were maintained in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, MEM vitamins, Pen/Strep, and MEM non-essential amino acids at 37°C in a humidified 5% CO_2 atmosphere, and subcultured when reaching 80% confluence. The cell passage utilized for this study was between 23 and 26.

HBMEC/T98G and U-373 MG/T98G co-culture

Co-culture of HBMEC and T98G (HBMEC-T98G co-culture) or U-373 MG and T98G (U-373 MG-T98G co-culture) were prepared separately. HBMEC

(1×10^5) or U-373 MG (1×10^5) was plated on collagen-coated tissue culture inserts with 12-mm diameter and 0.4-μm pore size (No. 3493, Corning, Corning, NY) for 3 days, while T98G (4×10^4) was plated in basolateral compartment of 12-well plate (No. 3513, Corning, Corning, NY) for 2 days before incubating in co-culture. The inserts were moved to the plate of T98G for incubation with CCSC.

Measurement of cell cytotoxicity

Cell cytotoxicity was determined on the mono-culture and co-culture incubated with CCSC of two commercial cigarettes using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Yang, Ko, and Shim 2014; Kim et al. 2016). Cell viability was expressed as percentage of control. T98G (4×10^3) or U-373 MG (1×10^4) were plated in each well of the 96-well culture plates. After 24 hr incubation, medium was removed and each well washed with PBS. Cells were treated with various concentrations of CCSC (0.05, 0.1, 0.2, 0.4, 0.8, 2 or 4 mg/ml) for T98G and CCSC (4, 8, 12 or 16 mg/ml) for U-373 MG, respectively, to calculate the concentration that inhibits cell growth by 50%. After 24 hr, incubation for 4 hr in the dark at 37°C after treatment, the medium was removed and 100 μl MTT solution was added to each well. The MTT solution was removed, and purple formazan precipitates in each well were sterilized in DMSO. Absorbance was measured at 570 nm wavelength using a microplate reader (Varioskan Flash, Thermo Scientific, San Jose, CA). This experiment was repeated three times, and average values were used in calculations as follows:

$$\text{Percent of cell viability}(\%) = \frac{[\text{Average of test}(\text{O.D.}) - \text{Average of blank}(\text{O.D.})]}{[\text{Average of control}(\text{O.D}) - \text{Average of blank}(\text{O.D})]} \times 100$$

Measurement of intracellular reactive oxygen species

As per the method described in previous studies (Yang, Ko, and Shim 2014; Lee et al. 2015), 2′,7′-dichlorodihydrofluorescein diacetate (2′7′-DCFH-DA) assay was performed to measure ROS generated by CCSC in T98G or U-373 MG. T98G (4×10^3) or U-373 MG (1×10^4) was plated into a 96-well of black microplates. After 24 hr incubation, medium was removed and each well washed with PBS. Various concentrations of CCSC (0.05, 0.1, 0.2, 0.4, 0.8, 2, or 4 mg/ml) for T98G or CCSC (4, 8, 12, or 16 mg/ml) for U-373 MG were incubated with each cells for 24 hr at 37°C. After treatment, the medium was removed and cells dislodged by trypsin EDTA and subsequently treated with 2'7'-DCFH-DA. Finally, fluorescence of cells was measured by using the microplate reader (Varioskan Flash, Thermo Scientific, San Jose, CA) at 488 nm for excitation and at 525 nm for emission. This experiment was repeated three times, and means used in calculations are as follows:

$$\text{Percent of ROS generation}(\%) = \left(\frac{\text{Average of treament}}{\text{Average of control}}\right) \times 100$$

Measurement of cellular integrity

Trans-epithelial electric resistance (TEER) as an indicator of tight junction permeability was used for evaluating cellular integrity according to previous study (Kim et al. 2015). 1×10^5 cells of HBMEC or U-373 MG were plated on collagen-coated inserts with 12-mm diameter and 0.4 μm pore size (Corning, Corning, NY) for 3 days, while T98G was plated in basolateral compartment of a 12-well plate for 2 days. Then, these two types of cells were cultured together for further experiments. HBMEC or U-373 MG were treated with CCSC when confluency was occurred. TEER of the monolayers was measured before starting the experiment to check confluency of HBMEC or U-373 MG. For using transport study with HBMEC or U-373 MG, TEER values need to be higher than 100 Ω via the Millicell ERS-2 system (Millipore Corp., New Bedford, MA). This experiment was repeated three times and calculated as follows:

$$\text{Percent of TEER value}(\%) = \left(\frac{\text{Average of treatment}\,(\Omega)}{\text{Average of control}\,(\Omega)}\right) \times 100$$

The medium was removed from each culture well, and cell layers were washed with PBS at 37°C before adding a test medium. For the test

medium, each treatment CCSC was mixed with DMEM and co-incubated with HBMEC-T98G co-culture or U-373 MG-T98G co-culture for 1 day at 37°C. Then, ROS and cytotoxicity of T98G were determined using MTT and 2'7'-DCFH-DA assays as previously described, respectively.

Statistical analysis

Values were reported as mean ± standard deviation (SD) from at least three different experiments. Analysis of variance (ANOVA) and Tukey's post-hoc test were carried out to determine significant differences among groups at the significance level of $\alpha = 0.05$ by using Graphpad Prism 3.0 software (Graphpad, San Diego, CA).

Results

Cell cytotoxicity induced by CCSC in mono and co-culture of brain cell or astrocytes

Cell viability was measured in mono-culture after incubating two types of CCSC (X and Y) for 24 hr in either T98G or U-373 MG. As illustrated in Figure 1A, cell viability of T98G decreased in a concentration-dependent manner and markedly affected (<10%) when CCSC ranged from 0.4 to 4 mg/ml added. The pattern of cell viability between two types of CCSC was similar. For U-373 MG, cell viability after incubation with CCSC ranging from 4 to 16 mg/ml fell from 81% to 25% and 103% to 52% for CCSC X and Y, respectively (Figure 1B). The inhibition of cell growth by 50% (IC_{50}) value incubation with CCSC of U-373 MG was

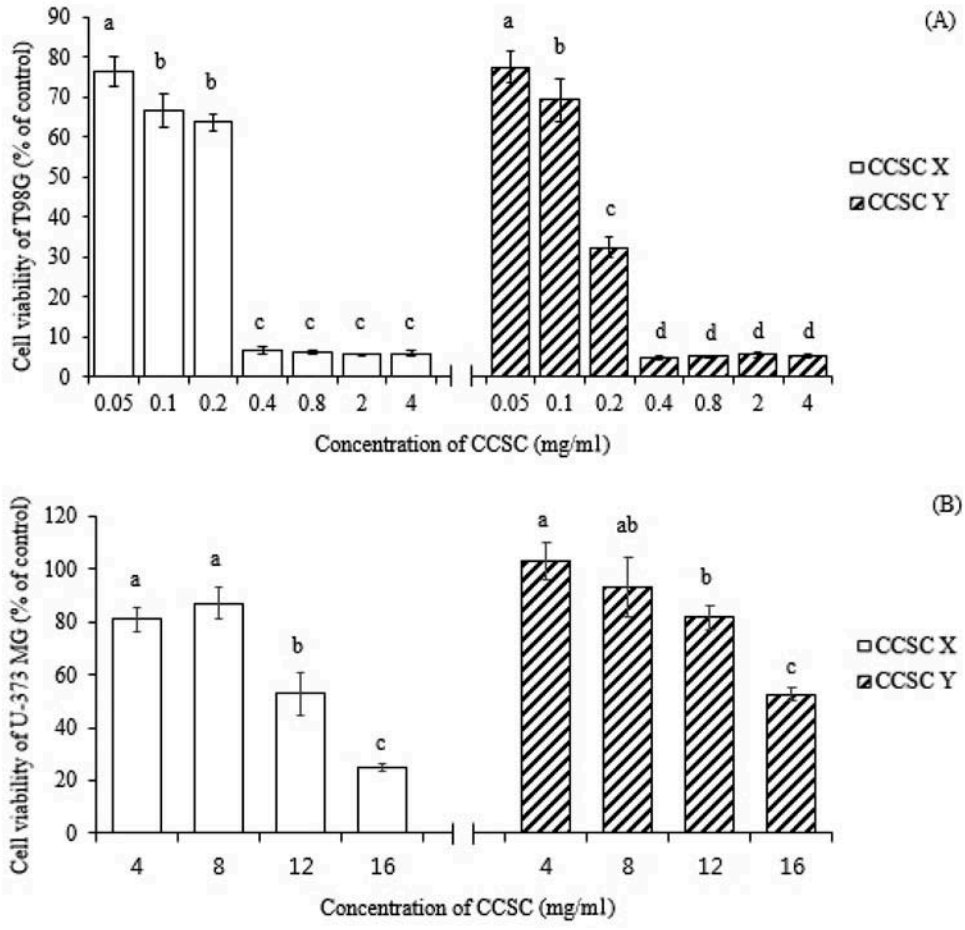

Figure 1. Cell viability measured in mono-culture of T98G (A) or U-373 MG (B) after CCSC exposure for 24-hr incubation. Control: T98G and U-373 MG were treated with DMEM without CCSC. Values are mean ± SD of triplicate samples. Each bar with different letters indicate significant difference among concentrations ($p < 0.05$).

determined to be between 12 and 16 mg/ml, which was higher than T98G (0.16–0.25 mg/ml). Data indicated that T98G appeared to be more susceptible than U-373 MG to CCSC exposure.

To evaluate protective ability of BBB and astrocytes, HBMEC and U-373 MG were co-cultured with T98G at IC_{50} value of T98G or U-373 MG, respectively. When T98G was co-cultured with HBMEC, cell viability of T98G ranged from 93% to 99% after 24 hr CCSC exposure (Figure 2A). Higher cell viability of T98G was observed in HBMEC-T98G co-culture than mono-cultured T98G. Cell viability of T98G co-cultured with U-373 MG was between 67% and 74% after incubating with CCSC, indicating that U-373 MG effectively protected T98G (Figure 2B). Our results indicate that HBMEC might protect brain cells from CCSC as a barrier when exposed to harmful materials.

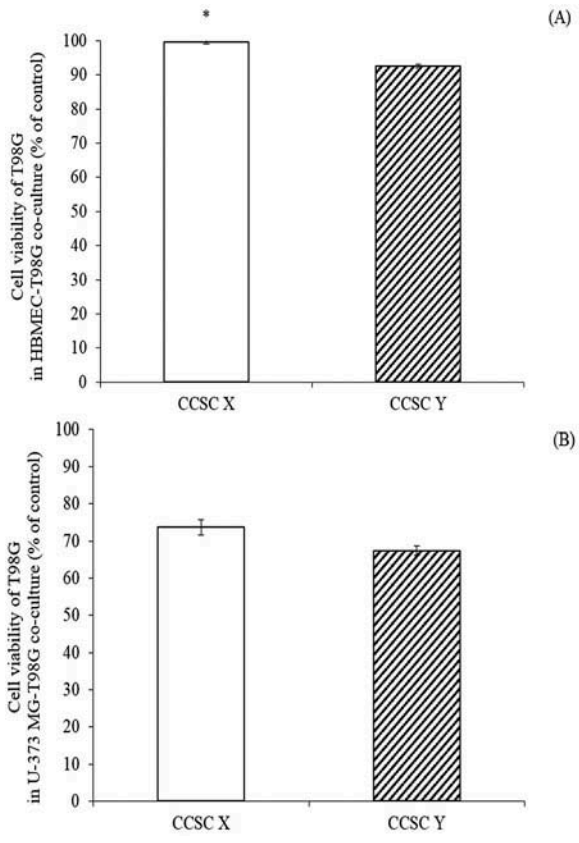

Figure 2. Cell viability of T98G measured in HBMEC-T98G co-culture (A) or U-373 MG-T98G co-culture (B) after 24 hr of CCSC exposure. Control: T98G incubated with DMEM without CCSC. Values are mean ± SD of triplicate samples. Asterisk indicates a significant difference between groups ($p < 0.05$).

Changes in cellular integrity by CCSC of BBB or astrocytes

Long-term exposure to CCSC may degrade either protective ability of BBB or function of astrocytes. In this study, 0.25 mg/ml CCSC X and 0.16 mg/ml CCSC Y were utilized in HBMEC-T98G co-culture and cellular integrity of HBMEC experiments, respectively. Twelve mg/ml CCSC X and 16 mg/ml CCSC Y were employed in U-373 MG-T98G co-culture and cellular integrity of U-373 MG experiments, respectively. For HBMEC co-cultured with T98G, TEER values were sustained and not significantly affected following 24-hr incubation of two types of CCSC (Figure 3A). However, a significant decline was noted between 24 and 48 hr incubation in both types of CCSC. TEER values declined to 62–63% for the two types of CCSC within 120 hr, respectively. For TEER of U-373 MG co-cultured with T98G, a steady reduced pattern was detected between 0 and 24 hr incubation in the two types of CCSC (Figure 3B). TEER fell to 79% for CCSC X and 84% for CCSC Y after 120 hr incubation, respectively. These results suggest that cellular integrity might be damaged when BBB or astrocytes are chronically exposed to CCSC.

ROS generation induced by CCSC in mono and co-culture of brain cell or astrocytes

ROS generation was measured in mono-culture of T98G and U-373 MG after incubating two types of CCSC for 24 hr. The levels of ROS generation in T98G continuously increased when it was incubated with CCSC ranging from 0.05 to 4 mg/ml (Figure 4A). Marked elevation of ROS generation was not found at concentrations ranging from 0.05 to 0.8 mg/ml both types of CCSC. However, a significant rise was found at higher than 0.8 mg/ml CCSC, ranging from 147% to 160% at 4 mg/ml for two types of CCSC. As presented in Figure 4B, a similar pattern in ROS generation was observed for U-373 MG. The levels of ROS generation were shown to be augmented at CCSC at concentrations ranging from 4 to 16 mg/ml CCSC which reached the highest levels of 115% and 108% at 16 mg/ml for the two types of CCSC. ROS generation induced by CCSC in co-culture model system was further

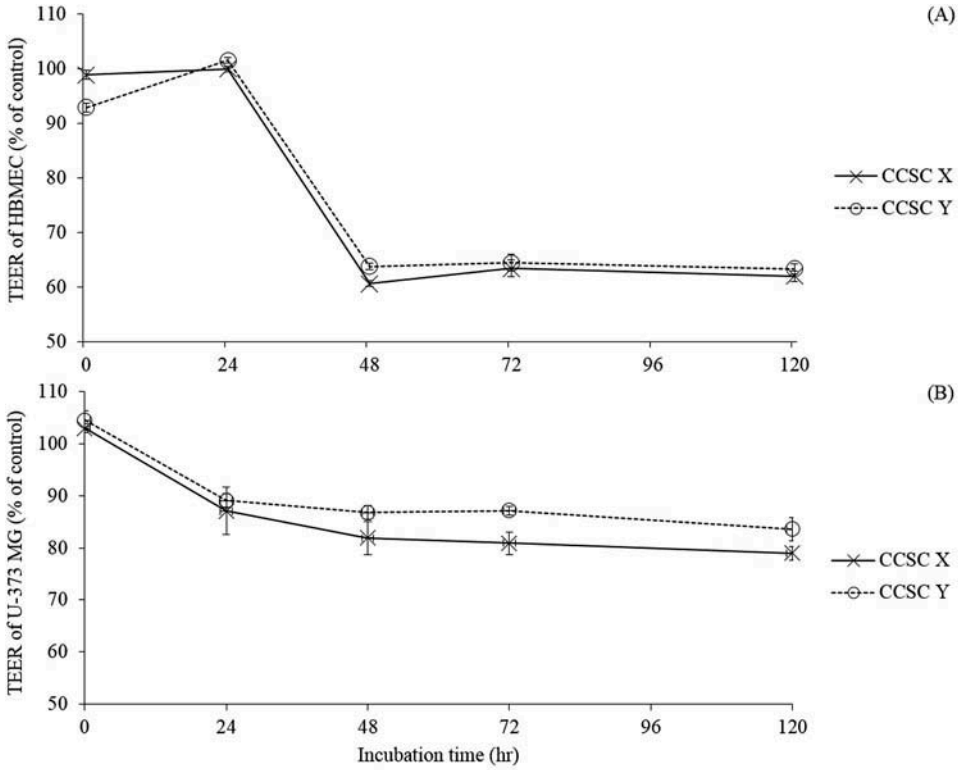

Figure 3. Cellular integrity of HBMEC (A) or U-373 MG (B) in co-culture after incubation with IC_{50} value of T98G and U-373 MG, respectively. Control: HBMEC and U-373 MG treated with DMEM without CCSC. Values are mean ± SD of triplicate samples.

investigated (Figure 5). ROS generation of T98G from HBMEC-T98G co-culture treated with IC_{50} amount of T98G produced from 101% to 107% levels for the two types of CCSC (Figure 5A). In case of T98G from U-373 MG-T98G co-culture, approximately 101% of ROS generation was found in both types of CCSC when it was treated with IC50 value of U-373 MG for 24 hr (Figure 5B). There was no significant difference in ROS generation compared to control.

Discussion

Exposure to standard cigarette (3R4F) smoke extracts produced oxidative stress involving alterations in brain functions accompanied by impairment of tight junction barrier (Comer, Elborn, and Ennis 2014; Kim et al. 2015), thereby suggesting the necessity of evaluating diverse brain tissues including astrocytes which physiologically support BBB. Findings from the current study demonstrated that CCSC exposure induced oxidative stress and cell cytotoxicity in human brain (T98G) or astrocytes (U-373 MG) coupled with HBMEC. T98G exhibited lower IC_{50} value following CCSC exposure than U-373 MG, indicating that T98G appeared to be more susceptible to CCSC exposure (Figure 1). These results support the observations that astrocytes play a crucial role in antioxidant defenses of the brain and possess a higher anti-oxidative potential (Pekny and Nilsson 2005; Schroeter., 1999). BBB protect the brain cell and CNS from harmful materials by building a protective and restrictive barrier between neuronal parenchyma and blood (Kuhlmann et al. 2009; Ballabh, Braun, and Nedergaard 2004). Astrocytes contribute to protection of neuronal damage on the brain by gliosis (Pekny and Nilsson 2005). When T98G was co-cultured with HBMEC or U-373 MG, it was protected from CCSC damage (Figure 2). Similar to our finding, Ballabh, Braun, and Nedergaard (2004) reported that astrocytes may defend brain cells by tightly attaching to the BBB when harmful materials cross the BBB. However, recent findings found that Cigarette Smoke Extract (CSE) exposure comparable to chronic smoker-produced BBB endothelial dysfunction and oxidative stress (Prasad et al. 2015). Thus, it is possible that long-term exposure to

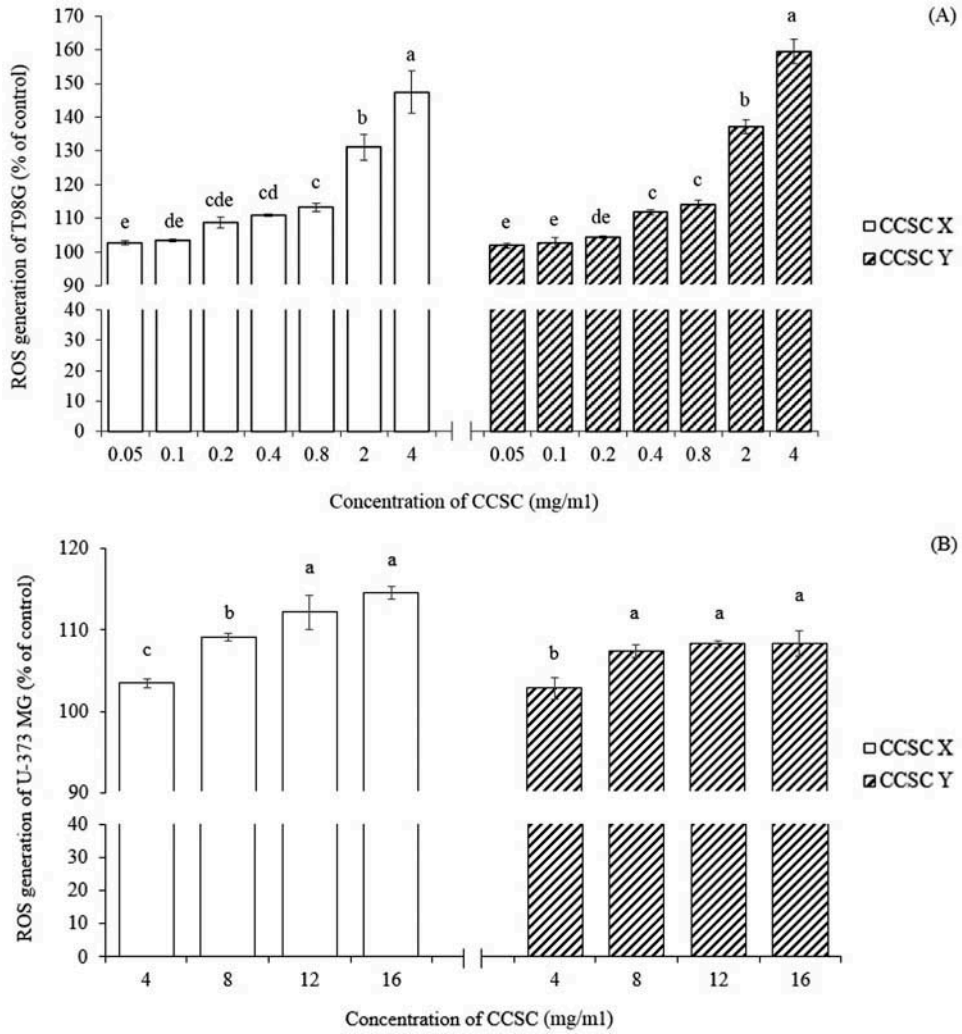

Figure 4. ROS generation induced by CCSC in mono-culture of T98G (A) or U-373 MG (B) for 24 hr of incubation. Control: T98G and U-373 MG incubated with DMEM without CCSC. Values are mean ± SD of triplicate samples. Each bar with different letters indicate significant difference among concentrations ($p < 0.05$).

CCSC might attenuate the function of BBB and astrocytes. Hence, it was worthwhile to examine the cellular integrity of HBMEC and U-373 MG when co-cultured with T98G. Data demonstrated that cellular integrity was damaged when BBB or astrocytes co-cultured with T98G were exposed to CCSC for a long period (Figure 3). The decrease in cellular integrity of BBB and astrocytes potentially modulated brain metabolism as well as increased susceptibility of risk to CNS alterations (Noseworthy and Bray 2000). Previous studies reported that ROS generation may lead to cell death (Ott et al. 2007) and ROS generation was associated with promoting brain aging and neurodegenerative disease including Alzheimer's disease and Parkinson's disease through neuronal cell death (Kwon et al. 2016; Castegna et al. 2002; Drechsel and Patel 2008). Our results showed that CCSC exposure to brain cells co-cultured with HBMEC or astrocytes elevated ROS levels within 24 hr but effects of long-term exposure to CCSC on ROS production remain to be determined. Since there is an ongoing awareness that oxidative stress plays an important role in brain-related diseases, it is conceivable that chronic exposure to CCSC may be detrimental to brain tissue and astrocytes.

In conclusion, data demonstrated that oxidative stress and cell cytotoxicity occurred in T98G and U-373 MG generated by CCSC in both mono and co-culture system. Compared to U-373 MG, T98G was more susceptible to cellular toxicity mediated by CCSC exposure attributed to cellular integrity damage produced by ROS. T98G in co-culture with either HBMEC or astrocytes showed less susceptibility to oxidative stress and cell cytotoxicity

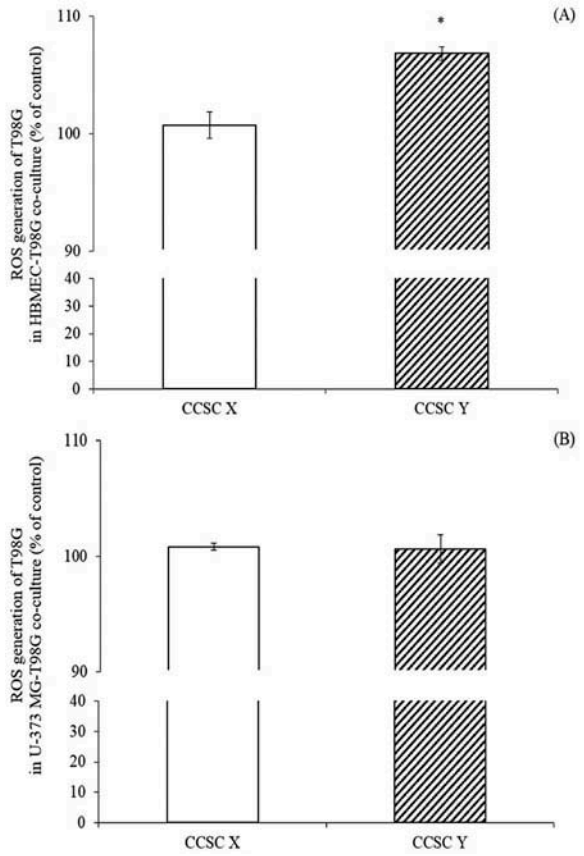

Figure 5. ROS generation induced by CCSC of T98G in HBMEC-T98G co-culture (A) or U-373 MG-T98G co-culture (B) after 24 hr of CCSC exposure. Control: T98G incubated with DMEM without CCSC. Values are mean ± SD of triplicate samples. Asterisk indicates a significant difference between groups ($p < 0.05$).

than those of mono-culture, indicating that BBB and astrocytes provide protective functions as a barrier for T98G from CCSC exposure. Taken together, it is possible to increase potential risk of brain damage following chronic exposure to CCSC by aggravating cellular integrity of BBB and astrocytes.

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