Chloro-benzoquinones cause oxidative DNA damage through iron-mediated ROS production in *Escherichia coli*

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HIGHLIGHTS

- CBQs have genotoxicity and cytotoxicity in *E. coli* cells.
- CBQs exposure to *E. coli* cells led to intracellular substantial ROS production.
- The addition of Fe²⁺ to the CBQs exposure system enhanced DNA oxidative damage.
- ROS scavengers and iron-chelators can partial protect from oxidative DNA damage.
- AhpCF, catalase E and catalase G protected *E. coli* from DNA damage corporately.

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ABSTRACT

Chloro-benzoquinones (CBQs) are a group of disinfection byproducts that are suspected to be potentially carcinogenic. Here, the mechanism of DNA damage caused by CBQs in the presence of ferrous ions was investigated in an *Escherichia coli* wild type M5 strain and a mutant L5 (*ahpCF katEG* mutant) strain that carried an enhanced green fluorescent protein reporter under the control of a SOS response gene (*recA*) promoter. All tested CBQs (including *para*-benzoquinone, 2-chloro-*para*-benzoquinone, and dichloro-*para*-benzoquinones with different substitutes) caused substantial oxidative DNA damage with *EC*₅₀ values in the micromolar range. Moreover, 2,5-dichloro-*para*-benzoquinone (2,5-DCBQ), a typical CBQ, caused substantial ROS production in *E. coli* mutant cells. And ROS scavengers provided partial protective effects on genotoxicity of 2,5-DCBQ to *E. coli* mutant cells. The addition of Fe²⁺ to the 2,5-DCBQ exposure system caused an increase in DNA oxidative damage; iron-chelating agents could partially prevent these cells from DNA damage. Finally, intracellular AhpCF, catalase E, and catalase G were all found to play an important role in the survival of *E. coli* cells exposed to CBQs, as indicated by an increased sensitivity of the *ahpCF katEG* mutant L5 strain to treatment compared with wild type M5 cells. Taken together, these results suggest that CBQs cause oxidative DNA damage in *E. coli* cells through the participation of iron-mediated ROS production.

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Abbreviations: CBQs, chloro-benzoquinones; HBQs, halogenated benzoquinones; DCBQ, dichloro-*para*-benzoquinones; DCMBQ, dichloromethylbenzoquinone; TriCBQ, trichloro-*para*-benzoquinone; DBBQ, dibromo-*para*-benzoquinone; OH-HBQs, OH-halobenzoquinones; AhpCF, alkyl hydroperoxide reductase; DMDBBQ, 2,3-dibromo-5,6-dimethyl-(1,4)benzoquinone; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; BQ, *para*-benzoquinone; 2-CBQ, 2-chloro-*para*-benzoquinone; 2,3-DCBQ, 2,3-dichloro-*para*-benzoquinone; 2,5-DCBQ, 2,5-dichloro-*para*-benzoquinone; 2,6-DCBQ, 2,6-dichloro-*para*-benzoquinone; DFO, deferoxamine; DIP, 2,2'-dipyridine; PHEN, *o*-phenanthroline; DHE, dihydroethidium; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; OD, optic density; *F*_i, induction factor; SFU, specific fluorescence unit; *t*-BuOOH, *tert*-Butylhydroperoxide; CuOOH, cumene hydroperoxide; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine.

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1. Introduction

Humans can be chronically exposed to halogenated benzoquinones (HBQs) created during the chlorine- and chloramine-disinfection process of phenol-like matrix chemicals (Heasley et al., 2004). HBQs can also be metabolized *in vivo* (Chignell et al., 2008) or dechlorinated chemically from polyhalogenated aromatic compounds, such as polychlorophenol, brominated flame retardants, hexachlorobenzene, and Agent Orange (Song et al., 2008; Meunier, 2002; Gupta et al., 2002; Sorokin et al., 1995). The production process of HBQs may result in the carcinogenesis of these polyhalogenated aromatic compounds (Zhu,

2009), however, no direct experimental data of HBQ-related carcinogenesis has yet been published. Nonetheless, a possible role of HBQ in bladder cancer has been proposed through quantitative structure–toxicity relationship analyses (Bull et al., 2006).

In fact, four different kinds of HBQs as chlorination disinfection byproducts in drinking water were initially discovered, including dichloro-*para*-benzoquinone (DCBQ), dichloromethylbenzoquinone (DCMBQ), trichloro-*para*-benzoquinone (TriCBQ), and dibromo-*para*-benzoquinone (DBBQ) (concentrations: 0.5–165 ng/L), among which DCBQ was the most easily formed chemical with the highest concentrations (Zhao et al., 2010). In subsequent investigations, as many as 12 kinds of HBQs in drinking water were found at ng/L levels (Huang et al., 2013). Similarly, UV irradiation use in water treatment plants can induce the transformation of HBQs to OH-halobenzoquinones (OH-HBQs), which can then be further modified into mono-halogenated benzoquinones (Qian et al., 2013). Moreover, specific conditions, such as elevated dissolved organic carbon, in addition to chlorine, higher temperatures, and personal care products can promote the formation of HBQs (TriCBQ, DMDBQ, and 2,6-DBBQ) in swimming pools originally absent in the input water; in fact, the concentration of 2,6-DCBQ has been found to be as high as 100 times that of the input water (Wang et al., 2013). Taken together, these findings indicate that chloro-benzoquinones (CBQs) are important HBQs that have the potential to impact humans via multiple pathways.

Previously, hydroxyl radicals were found to be formed through transition metal copper-mediated Fenton reactions *in vitro* and to cause oxidative DNA damage (Gunther et al., 1995; Lloyd and Phillips, 1999). In addition, copper ions could efficiently mediate any DNA damage caused by DCBQs in the presence of NADH, *in vitro* (Oikawa and Kawanishi, 1996). However, intracellular copper cannot cause oxidative DNA damage in *Escherichia coli* cells because of its low intracellular concentration and inactivation by its ligation to thiols (Imlay, 2003; Macomber et al., 2007). Another transition metal, iron, also participates in both the oxidative DNA damage process seen *in vitro* (Lloyd and Phillips, 1999; Henle et al., 1996) and in Fenton-like reactions in *E. coli* cells (Imlay, 2003; McCormick et al., 1998). It has not been proven if iron ions can participate in oxidative DNA damage caused by chloro-benzoquinones in *E. coli* cells.

An SOS protein, RecA, can be induced by oxidative stress in *E. coli* cells (Van Bogelen et al., 1987; Greenberg and Demple, 1989). Previously, we utilized the enhanced green fluorescent protein as a reporter under the control of the *recA* promoter to construct a pET16b-derived plasmid, pETPrecAegfp5 (Chen et al., 2012). The resultant *E. coli* L5 strain (*E. coli* LC106 strain with *ahpCF katEG* mutants carrying the pETPrecAegfp5 plasmid) is able to detect oxidatively DNA damaging agents with a sensitivity at least 10–20 times greater than that of other bacterial biosensors, demonstrating an incomparable superiority for investigating the mechanism of DNA damage caused by oxidative chemicals (Chen et al., 2011).

In this study, we utilized the *E. coli katEG ahpCF* strains to evaluate the oxidative DNA damage and intracellular reactive oxygen species (ROS) level caused by CBQs. Moreover, we also investigated the functions of iron ions, iron chelators, ROS scavengers, and antioxidative enzymes (including alkyl hydroperoxide reductase, catalase E, and catalase G) in the process of oxidative damage.

2. Material and methods

2.1. Chemicals

Para-benzoquinone (BQ), 2-chloro-*para*-benzoquinone (2-CBQ), 2,3-dichloro-*para*-benzoquinone (2,3-DCBQ), 2,5-dichloro-*para*-

benzoquinone (2,5-DCBQ), 2,6-dichloro-*para*-benzoquinone (2,6-DCBQ), deferoxamine (DFO), 2,2'-dipyridine (DIP), and *o*-phenanthroline (PHEN) were purchased from Sigma (USA) and used without further purification. Fluorescent probes including dihydroethidium (DHE) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), ROS scavengers including mannitol, benzoate, and ascorbic acid were purchased from Sigma (USA). Chemically pure H₂O₂ and FeSO₄ were purchased from Beijing Chemical Works, China.

2.2. Bacterial strains and cultivation conditions

The *E. coli* LC106 strain (*katEG ahpCF* in MG1655) was inoculated into fresh GCTM medium (0.2% (W/V) glucose, 0.2% (W/V) casein acidic hydrolysate, 0.2 mM tryptophan, 5 µg/mL thiamine, and M9 salts (Sambrook and Russell, 2001)) and modified slightly (Park et al., 2005). The *E. coli* L5 strain was inoculated in fresh GCTMA medium (GCTM medium added 60 µg/mL ampicillin). Anaerobic cultivation of the *E. coli* cells was performed at 37 °C, without shaking, in fresh anaerobic medium until log phase ($OD_{600} \sim 0.15$) in MGC AnaeroPack™ Series bag (Mitsubishi, Japan) in a YQX-II Anaerobic Chamber (Xinmiao Medical Instrument Co., Ltd., Shanghai, China). Both the LC106 strain and its derivative L5 strain were cultivated anaerobically until log phase. The wild type *E. coli* MG1655 strain and the M5 strain (the MG1655 strain carrying the pETPrecAegfp5 plasmid) were cultured in fresh GCTM medium and GCTMA medium aerobically, respectively.

2.3. Chemical induction

The L5 strain was anaerobically grown in the GCTMA medium at 37 °C overnight and inoculated into fresh GCTMA medium at a 1:50 ratio and grown to log phase ($OD_{600} \sim 0.15$). All aerobic exposure protocols were conducted in the same way as done previously (Chen et al., 2011). The treatment of a series of different CBQs concentrations (0, 0.1, 1, 10, 50 and 100 µM) was performed to log phase cells at 37 °C for 2 h at 130 rpm. Ferrous ion or ROS scavengers or iron chelators were mixed with 2,5-DCBQ before the treatment of L5 cells. Exposure with mixture was also performed at 37 °C for 2 h at 130 rpm. The treatment concentrations were as follows: 2,5-DCBQ (0, 1, 10, 20, 50, 75, 100 µM), ferrous ion (0, 5, 25, 50 µM), mannitol and benzoate (0, 1, 10, 50, 100 mM), ascorbic acid (0, 1, 5, 10, 20 mM), DFO and PHEN (2 mM), DIP (1 mM). The aerobic exposure was performed in a 96-well plate (Corning, USA) with 20 µL of chemicals in aqueous solution and 180 µL bacterial culture per well in MGC AnaeroPack™ Series bag (Mitsubishi, Japan) and shaken manually several times for uniformity. Induction was carried out at 37 °C in the time in the Anaerobic Chamber. Untreated L5 cells were added with 20 µL of sterile water as the control. The blank LC106 strain was anaerobically grown in GCTM medium to log phase; 20 µL of sterile water was added during the induction process to zero the auto-fluorescence emission of the *E. coli* cells. The M5 and MG1655 strains were aerobically grown to log phase; the induction protocols were the same as the L5 and LC106 strains.

2.4. Fluorescent microscopy analysis

Fluorescent microscopy analysis was performed as described previously (Chen et al., 2012). Fluorescence images of bacterial cells were obtained by confocal laser scanning microscope consisting of a Leica DMI 6000B inverted microscope (100× oil immersion objective, 1.40 numerical aperture NA) and a Leica TCS SP5

confocal scanning system. The fluorescence of *E. coli* cells were excited by the 488 nm line of an argon ion laser (4.5 mW), and emission was detected by a photomultiplier tube (PMT) at 500–560 nm; the PMT voltage was set at 890 V. Bright-field images were acquired with a PMT gain of 340 V. Each image was taken from a physical dimension of $155\ \mu\text{m} \times 155\ \mu\text{m}$.

2.5. Flow cytometry analysis

For a bivariate side scatter (SSC) vs. forward scatter (FSC) analysis, L5 strain cells under the exposure of 2,5-DCBQ performed as described in Section 2.3 with 10,000 counts were analyzed via flow cytometry (FACSAria™ III, BD Co., US). For intracellular ROS level detection, DHE (10 μM) and DCFH-DA (20 μM) were incubated with the log phase LC106 strain cells for 30 min at 37 °C. Then, serial concentrations of 2,5-DCBQ were added to the culture, which would be shaken at 130 rpm for 2 h at 37 °C. LC106 strain cells with 10,000 counts were analyzed via flow cytometry (FACSAria™ III, BD Co., US) with an excitation of 488 nm and an emission of 610 nm (for DHE) and 525 nm (for DCFH-DA). Data were analyzed through FACSDiva Version 6.1.3.

2.6. Fluorescence spectral scanning and optic density detection

Fluorescence scanning and optic density (OD) value detection of the aerobic induction were performed as previously reported (Chen et al., 2011). *E. coli* cells were excited at 480 nm, and the fluorescence was scanned from 500 to 600 nm in the 96-well plate (Corning, USA) with the spectral scanning multimode reader (Varioskan Flash, Thermo). The cell density (OD_{600}) was determined simultaneously using the same device. Data for these detections were recorded by SkanIt Software 2.4.3 RE for Varioskan® Flash.

2.7. Data analysis

The induction factor (F_i) and OD_x/OD_0 value were measured as previously reported (Chen et al., 2011). The specific fluorescence unit (SFU) value indicates the value of the detected fluorescence intensity divided by the optical density of *E. coli* cells at 600 nm (OD_{600}). The absolute SFU value indicates the SFU value of the treated sample minus that of the control strain. The induction factor was calculated by the absolute SFU value at 509 nm of the treated sample divided by the absolute SFU value of the untreated sample (Chen et al., 2011). The EC_{50} values were calculated as the concentrations of tested compounds causing a 50% decrease in the survival of the target organism; when the OD_x/OD_0 value of the tested compounds equaled 0.5, the exact concentration was deemed to be the EC_{50} value of this compound. The protection effect of the ROS scavengers (%) was equal to $[1 - (SFU_{CR}/SFU_C)] \times 100\%$, where: SFU_{CR} was the SFU value of the L5 strain cells exposed to CBQ-ROS scavengers, and SFU_C was the SFU value of the L5 strain cells exposed to CBQ. The DNA damage increment rate (%) was equal to $[(SFU_{CRF}/SFU_{CR}) - 1] \times 100\%$, where: SFU_{CRF} was the SFU value of the L5 strain cells exposed to CBQ-ROS scavengers- Fe^{2+} and SFU_{CR} was the SFU value of the L5 strain cells exposed to CBQ-ROS scavengers.

Experiments were performed at least three times, and the standard deviations between the experiments were determined. Statistical analyses were carried out using One-way ANOVA with Tukey's test with the aid of Origin 7.0 software.

3. Results

3.1. CBQs cause oxidative DNA damage to the L5 strain cells

CBQs (including BQ, 2-CBQ, 2,3-DCBQ, 2,5-DCBQ, and 2,6-DCBQ) caused oxidative DNA damage in a dose-dependent manner in the L5 strain cells (0.1–100 μM) (Fig. 1). When 25 μM H_2O_2 was added, the level of oxidative DNA damage increased slightly, as compared to L5 strain cells treated only with CBQs, except those exposed with two chlorine-substitution BQs at a concentration of 100 μM (Fig. 1). CBQs caused genotoxicity and cytotoxicity in the L5 strain cells; the EC_{50} values of the CBQs were in order of: $\text{BQ} < 2,3\text{-DCBQ} < 2,6\text{-DCBQ} < 2,5\text{-DCBQ} < 2\text{-CBQ}$ (Table 1). Moreover, as compared to both the CBQ system by itself and the $\text{CBQ} + \text{H}_2\text{O}_2$ system, the 2,3-DCBQ + H_2O_2 system exhibited the highest ΔEC_{50} value (Table 1).

The green fluorescence intensity of *E. coli* L5 cells indicated the level of oxidative DNA damage, while the cell density in bright field images indicated cell viability. As seen in Fig. 2, the *E. coli* L5 strain was highly sensitive to oxygen and clearly demonstrated a higher level of DNA damage than that of anaerobic condition (Fig. 2A and B). Whether under anaerobic or aerobic conditions, the L5 strain cells exposed to 2,5-DCBQ displayed substantial oxidative DNA damage and a large scale of cell death (Fig. 2E–H). Under aerobic conditions, the L5 strain cells exposed to 2,5-DCBQ displayed a significant increase in DNA damage as opposed to L5 strain cells without 2,5-DCBQ exposure (Fig. 2B and F). In addition, during the process of oxidative DNA damage caused by the exposure to 2,5-DCBQ, the *E. coli* L5 strain cells were larger and exhibited a damaged cell structure (Fig. 2I and J).

3.2. Intracellular ROS plays an important role

DCFH-DA was used as a fluorescent probe to detect the intracellular $\text{OH}^\cdot$ and H_2O_2 level while DHE was used to detect intracellular superoxide anions ($\text{O}_2^\cdot$). To avoid the interference of green fluorescence to DCF, a LC106 strain lacking EGFP expression was used as the target strain in the detection of intracellular ROS level. Results showed that the fluorescent intensity of DCF and ethidium (the oxidative product of DCFH-DA and DHE respectively) significantly increased with increasing concentrations of 2,5-DCBQ exposure concentration ($p < 0.01$) (Fig. 3A). We found a similar dose-ROS level effect when the LC106 strain was exposed to other CBQs (data not shown). When ROS scavengers, such as mannitol, benzoate, and ascorbic acid, were added into the L5 + 2,5-DCBQ (75 μM) exposure system, the oxidative DNA damage statistically decreased as compared with the L5 + 2,5-DCBQ exposure system (Fig. 3B). In addition, the ability of mannitol, benzoate, and ascorbic acid to prevent oxidative DNA damage was considered to be 31.4–37.3%, 27.3–42.5%, and 28.4–39.7%, respectively. These results demonstrate that the intracellular ROS yielded from 2,5-DCBQ exposure participates in the process of oxidative DNA damage in the mutant cells.

3.3. Iron ions participate in the process of oxidative DNA damage

3.3.1. The effect of iron ions on CBQ toxicity in the L5 strain cells

Whether 2,5-DCBQ was added or not, the level of oxidative DNA damage of the L5 strain cells significantly increased with rising concentrations of iron ions ($p < 0.01$) (Fig. 4A). When ferrous ions (50 μM) were added to the ROS scavenger + 2,5-DCBQ system, the oxidative DNA damage of the L5 strain cells was higher than that of the ROS scavenger + 2,5-DCBQ system (Fig. 4B).

Fig. 1. The response of L5 strain cells under the exposure of different types of CBQs with or without H_2O_2 . Data are expressed as the mean of the induction factors $\pm$ standard deviation.

Table 1
The EC_{50} values of CBQs in the L5 strain cells with or without H_2O_2 (μM).¹

CBQs	None addition	+ H_2O_2	ΔEC_{50}
BQ	9.56 $\pm$ 0.48	7.27 $\pm$ 0.36**	2.29
2-CBQ	41.98 $\pm$ 2.10	35.48 $\pm$ 1.77*	6.51
2,3-DCBQ	32.27 $\pm$ 1.61	15.80 $\pm$ 0.79**	16.47
2,5-DCBQ	37.48 $\pm$ 1.87	29.22 $\pm$ 1.46**	8.26
2,6-DCBQ	35.09 $\pm$ 1.75	28.07 $\pm$ 1.40**	7.02

The EC_{50} values of L5 strain cells exposed to CBQs with or without H_2O_2 (25 μM).

¹ Values in the same line are significantly different as analyzed by Tukey's test (** $p < 0.01$, * $p < 0.05$).

3.3.2. The effect of iron chelators on the CBQ toxicity

Three iron-chelators (DFO, DIP, and PHEN) were used to identify the function of iron in the process of oxidative DNA damage in the L5 strain cells. The addition of iron-chelators to the 2,5-DCBQ exposure system significantly decreased the levels of oxidative DNA damage in the L5 strain as compared to those with 2,5-DCBQ exposure only ($p < 0.01$). Furthermore, whether adding the Fe^{3+} -chelating agent DFO or the Fe^{2+} -chelating agents DIP and PHEN, the cell survival range of the L5 strain exposed to 2,5-DCBQ was statistically increased ($p < 0.01$) (Table 2).

3.4. Catalase E, catalase G, and alkyl hydroperoxide reductase defend together against oxidative DNA damage by CBQs

We found that the deletion of key antioxidant enzymes (alkyl hydroperoxide reductase (Ahp), catalase G, and catalase E) in the L5 strain allowed for micromolar levels of 2,5-DCBQ to cause substantial oxidative DNA damage. Concentrations as high as 75 μM 2,5-DCBQ failed to cause significant oxidative DNA damage in the wild type *E. coli* M5 strain cells (Fig. 5A). Simultaneously, the wild type M5 strain cells had a higher survival rate as compared to that of the L5 strain cells (Fig. 5B). This phenomenon indicated that Ahp,

catalase G, and catalase E provide a partial, but significant, protection against CBQ-induced oxidative DNA damage in *E. coli* cells.

4. Discussion

Bladder cancer has been associated with long-term exposure to disinfection by-products (DBPs) in drinking water (Cantor et al., 1998; King and Marrett, 1996; McGeehin et al., 1993; Villanueva et al., 2004). There was no direct proof, which demonstrated CBQs as a kind of DBPs were related bladder carcinogenicity in humans. *E. coli*, a model bacterial species, cannot degrade CBQs (McCarthy et al., 1997), was invoked as the target organism for the possible damage mechanism of CBQs to biological cells. And we confirmed that CBQs can cause oxidative DNA damage and cell death in a dose-dependent manner in *E. coli* cells. These results are consistent in mammalian cells (Du et al., 2013). The bacterial test system with CBQs exposure is sensitive, rapid and low-cost contrast with the expensive and long-term animal experiments. To our knowledge, CBQs have not been shown to be genotoxic in bacterial test systems except our mutant *E. coli* strains (Chen et al., 2011).

The cytotoxicity rank order of tested CBQs in *E. coli* L5 cells confirmed that BQ was more toxic to Gram-negative bacteria compared to chlorine-substituted *para*-benzoquinones (Beckman and Siedow, 1985). In addition, the higher ΔEC_{50} value of 2,3-DCBQ indicated that H_2O_2 , or the intracellular oxidative potential, likely has a more significant effect on the toxicity of 2,3-DCBQ than the other CBQs. Similarly, in a metal-free system, 2,3-DCBQ + H_2O_2 was found to have the largest effect on oxidizing calf thymus DNA to form 8-oxodG (Yin et al., 2013). Taken together, these data demonstrate the importance of 2,3-DCBQ + H_2O_2 and help to partially validate our results, *in vitro*.

Iron ions play an important role in the formation of oxidative DNA damage exposed to H_2O_2 in *E. coli* cells (Imlay, 2003; McCormick et al., 1998). However, the effect of iron ions on CBQ-induced toxicity of *E. coli* cells is unknown. Results also

Fig. 2. L5 strain cells exposed to 2,5-DCBQ at anaerobic and aerobic conditions. Laser confocal microscopic images (A, B, E, and F) and bright field images (C, D, G, and H) of L5 strain cells. SSC-FSC of L5 strain cells exposed to 2,5-DCBQ (I and J). Log phase L5 strain cells grew anaerobically (A and C) or were exposed to oxygen (B and D) for 2 h. The log phase of L5 strain cells were exposed to 20 μM 2,5-DCBQ, anaerobically (E and G) or aerobically (F and H). A bivariate SSC vs. FSC dot-plot showing the 'P1'-gate (the sample shown is the L5 strain cells with 0 μM 2,5-DCBQ exposure) (I). SSC vs. FSC of L5 strain cells exposed to 100 μM 2,5-DCBQ (J).

Fig. 3. Intracellular ROS levels and protective effect of ROS scavengers in mutant cells following the exposure of 2,5-DCBQ. (A) The fluorescent intensity of DCF (empty) and ethidium (black) in LC106 strain cells assayed via flow cytometry. (B) The response of the L5 strain cells following the exposure of 75 μM 2,5-DCBQ and ROS scavengers, mannitol, benzoate, and ascorbic acid. Data are expressed as the average fluorescent intensity (A) or mean value of the induction factor (B) $\pm$ standard deviation. Asterisks indicate statistical differences between treatments and control. * $p < 0.05$; ** $p < 0.01$.

revealed that the CBQ toxicity is a function of the presence of iron ions in the *E. coli* mutant cells. The addition of iron significantly enhanced the oxidative DNA damage potential of CBQs. The administration of iron chelators (DFO, DIP, and PHEN) resulted in a significant protection from oxidative DNA damage and bacterial death. ROS production also increased with increasing CBQ exposure, while the addition of ROS scavengers (the $\cdot\text{OH}$ scavengers, mannitol and benzoate, and the aqueous ROS scavenger, ascorbic acid) significantly protected the cells against oxidative DNA damage; the addition of extra iron in the CBQ + ROS scavenger system led

to enhancing oxidative DNA damage. Moreover, antioxidative enzymes (including Ahp, catalase G, and catalase E) protected the *E. coli* cells against oxidative DNA damage and cell death.

There are two kinds of catalase (HPI and HPII), both of which can catalyze H_2O_2 into H_2O and O_2 in the wild type *E. coli* genome. HPI catalase is encoded by *katG* under OxyR control while HPII catalase is encoded by *katE* under KatF control (Loewen et al., 1985a, 1985b). Furthermore, HPI catalase can reduce the concentration of H_2O_2 , while HPII catalase is not inducible by H_2O_2 , but rather is regulated through a growth phase-dependent manner

Fig. 4. The response of the L5 strain cells to iron ions in the presence of 2,5-DCBQ and ROS scavengers. (A) The anaerobic oxidative DNA damage response of the L5 strain exposed to 2,5-DCBQ and FeSO_4 . (B) The aerobic DNA damage increment rate in the L5 strain when 50 μM FeSO_4 was added to the exposure system (75 μM 2,5-DCBQ) with different concentrations of ROS scavengers. Data are expressed as the average induction factor (A) or DNA damage increment rate (B) $\pm$ standard deviation. $^{**}p < 0.01$ between treatments and control.

Table 2

The response of the L5 strain cells under the exposure of 2,5-DCBQ and iron-chelators.¹

2,5-DCBQ concentration (μM)	Fe ions chelators addition	Induction factor	OD_x/OD_0	2,5-DCBQ concentration (μM)	Fe ions chelators addition	Induction factor	OD_x/OD_0
0	–	1.00 ± 0.09^a	1.00 ± 0.01^a	100	–	4.55 ± 0.43^a	0.33 ± 0.03^a
0	DFO	1.74 ± 0.20^b	0.57 ± 0.01^b	100	DFO	2.67 ± 0.30^b	0.55 ± 0.02^b
0	DIP	1.40 ± 0.14^a	0.76 ± 0.03^c	100	DIP	3.27 ± 0.20^b	0.66 ± 0.04^c
0	PHEN	1.54 ± 0.21^a	0.53 ± 0.07^b	100	PHEN	1.97 ± 0.16^c	0.44 ± 0.02^d

The L5 strain cells were exposed to a range of 2,5-DCBQ concentrations with or without DFO (2 mM), DIP (1 mM), or PHEN (2 mM).

¹ Values in the same column with different letters are significantly different as analyzed by Tukey's test ($p < 0.01$).

Fig. 5. The response of the wildtype *E. coli* (M5) and the antioxidant mutants (L5) to 2,5-DCBQ. (A) The induction factors of the L5 and the M5 strains exposed to 2,5-DCBQ. (B) The OD_x/OD_0 (cell survival) of the L5 and the M5 strains exposed to 2,5-DCBQ. Data are expressed as the average induction factor (A) or OD_x/OD_0 (B) $\pm$ standard deviation. $^{**}p < 0.01$ or $^*p < 0.05$ between treatments and control.

(Farr and Kogoma, 1991; Schellhorn and Hassan, 1988). Another antioxidant enzyme found in the wild type *E. coli* genome is alkyl hydroperoxide reductase (Ahp), encoded by *ahpCF*. Ahp has been shown to provide additional defense to the cell by reducing the levels of either exogenous organic peroxides or peroxidized cellular components (Farr and Kogoma, 1991). Furthermore, *E. coli ahpCF katEG* cells under the exposure of oxygen produced submicromolar intracellular H_2O_2 , which was then found to play a key role in cellular Fenton-like reactions that produce hydroxyl radicals (Park et al., 2005). Similarly, we found that both endogenous ROS (due to the mutation of *ahpCF katEG*, Fig. 5) and exogenous ROS (the addition of H_2O_2 , Fig. 1) were able to enhance oxidative DNA damage. In addition, CBQs led to an increase of ROS, indicating that the toxicity of CBQs is facilitated by the participation of ROS in *E. coli* cells.

Previously, when the cytomembrane permeable Fe^{2+} chelator, PHEN (which has a similar chemical structure to DIP) was added to *E. coli* cultures prior to the addition of H_2O_2 , the cells were protected from death (Asad et al., 1994). PHEN can quickly react with Fe^{2+} to form Fe^{2+} -PHEN(I) and Fe^{2+} -PHEN(II) complexes, and the two products (mono and bis complexes) can react with H_2O_2 to produce hydroxyl radicals. The Fe^{2+} -PHEN(III) complex, which is the final and most stable product, is formed more slowly. Since DIP is a very similar compound to PHEN, we speculated that DIP would have the same reactions with Fe^{2+} and produce similar complexes. This protection would be due to the capture of Fe^{2+} ions leading the interruption of Fe^{2+} -mediated $\cdot\text{OH}$ production. Furthermore, another iron chelators, DFO, can permeate the cell membrane and form a non-reactive Fe^{3+} -DFO complex and has also been found to protect pUC19 plasmid DNA against damage caused

by the Fenton reaction, *in vitro* (Kim and Park, 2004) and inhibit H₂O₂-induced HL-60 leukemia cell death (Shim et al., 2003). DFO can effectively inhibit the formation of TCSQ[•], which was found to be produced following the exposure pure DNA (Zhu et al., 1998) and human fibroblasts (Witte et al., 2000) to tetrachlorohydroquinone (TCHQ), and to provide protection from DNA damage by chelating iron ions and directly interacting with TCHQ. As such, we speculate that the protective effect of DFO might not only be due to the chelation of Fe³⁺, but may also be due to direct interactions with CBQs.

Nonetheless, the addition of iron chelators and ROS scavengers was not in a position to completely protect the *E. coli* cells from DNA damage and cell death (Fig. 3B and Table 2), indicating that CBQs may also cause DNA damage through other pathways. This result piqued our interest, pushing us to further our biochemical understanding of the mechanisms involved in HBQ-induced toxicity, *in vitro*. It has been proven that CBQs (such as 2-CBQ, 2,5-DCBQ, and 2,6-DCBQ) directly react with dG and double-stranded DNA (calf thymus DNA) to form C8-dG O-adducts, *in vitro* (Dai et al., 2005). An additional study revealed that HBQs exhibit a capacity for binding to single- and double-stranded oligodeoxynucleotides to form 1:1 or 2:1 complexes through partial intercalation and H-bonding modes (Anichina et al., 2010). Organic hydroperoxides, such as *tert*-Butylhydroperoxide (*t*-BuOOH) and cumene hydroperoxide (CuOOH), were shown to nucleophilically attack CBQs in order to produce the respective organic alkoxyl radicals and *t*-BuO[•] or CuO[•] in a metal-independent manner, *in vitro* (Zhu et al., 2007). Moreover, subsequent research displayed that HBQs produce 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a DNA oxidative damage marker, more efficiently in double-stranded DNA (calf thymus DNA) than in dG under the participation of H₂O₂ via not only a metal-independent but also an intercalation-enhanced oxidation mechanism (Yin et al., 2013). Finally, H₂O₂ has been shown to attack the nucleophile of TCBCQ and form an unstable TriCBQ-OOH intermediate that decomposes homolytically through the cleavage of the O–O bond to produce a hydroxyl radical with the assistance of water molecules (Zhu et al., 2007; Li et al., 2013). All these findings provide the possibility that the CBQ-induced DNA damage revealed in this study does not depend on iron, thus resulting in the partial maintenance of DNA when CBQ was added in the presence of iron chelators or ROS scavengers.

We also assume that, besides the above-mentioned *in vitro* mechanism, the addition of increasing levels of CBQs and intracellular H₂O₂ can result in a concurrent increase in ROS levels. Submicromolar levels of H₂O₂ in host LC106 cells has been shown to oxidize the [4Fe–4S]²⁺ cluster exposed to the aqueous phase of dehydratase families, thus creating an inactive [3Fe–4S]⁺ cluster with the loss of one iron atom (Jang and Imlay, 2007). The released Fe³⁺ ion can react with cysteine or flavin within the cells to form ferrous ion (Woodmansee and Imlay, 2002; Park and Imlay, 2003). Reactions between ferrous ions and H₂O₂ produces the highly active hydroxyl free radical [•]OH, which in turn can cause oxidative DNA damage. We hypothesize that [•]OH attacks the Fe–S proteins in the L5 strain and thus releases high concentrations of Fe²⁺ that can then participate in the intracellular Fenton-like reaction. This process would produce much more intracellular ROS in the L5 strain cells treated with CBQs than in the untreated L5 cells, demonstrating the magnified effect of the oxidative DNA damage.

5. Conclusions

In conclusion, we found that CBQs (BQ, 2-CBQ, 2,3-DCBQ, 2,5-DCBQ and 2,6-DCBQ) caused oxidative DNA damage in *E. coli* cells. CBQs were also able to enhance intracellular ROS levels;

the addition of ROS scavengers partially protected the *E. coli* against DNA damage and cell death. Both the addition of endogenous and exogenous ROS led to oxidative DNA damage in *E. coli* cells exposed to CBQs; iron ions participated in this oxidative DNA damage process. Furthermore, iron chelators inhibited the production of oxidative DNA damage, while supplementing extra iron ions increased the level of oxidative DNA damage. Furthermore, antioxidative enzymes (Aph, catalase E, and catalase G) protected the cells from oxidative DNA damage and cell death. Taken together, these findings suggest that iron-mediated ROS production plays an important role in CBQ-induced toxicity in *E. coli* cells.

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