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Involvement of oxidative stress in hydroquinone-induced cytotoxicity in catalase-deficient *Escherichia coli* mutants

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Abstract

Hydroquinone is a benzene-derived metabolite. To clarify whether the reactive oxygen species (ROS) are involved in hydroquinone-induced cytotoxicity, we constructed transformants of *Escherichia coli* (*E. coli*) strains that express mammalian catalase gene derived from catalase mutant mice (Cs^b, Cs^c) and the wild-type (Cs^a) using a catalase-deficient *E. coli* UM255 as a recipient. Specific catalase activities of these tester strains were in order of $Cs^a > Cs^c > Cs^b >$ UM255, and their susceptibility to hydrogen peroxide (H_2O_2) showed UM255 $> Cs^b > Cs^c > Cs^a$. We found that hydroquinone exposure reduced the survival of catalase-deficient *E. coli* mutants in a dose-dependent manner significantly, especially in the strains with lower catalase activities. Hydroquinone toxicity was also confirmed using zone of inhibition test, in which UM255 was the most susceptible, showing the largest zone of growth inhibition, followed by Cs^b, Cs^c and Cs^a. Furthermore, we found that hydroquinone-induced cell damage was inhibited by the pretreatment of catalase, ascorbic acid, dimethyl sulfoxide (DMSO), and ethylenediaminetetraacetic acid (EDTA), and augmented by superoxide dismutase (both CuZnSOD and MnSOD). The present results suggest that H_2O_2 is probably involved in hydroquinone-induced cytotoxicity in catalase-deficient *E. coli* mutants and catalase plays an important role in protection of the cells against hydroquinone toxicity.

Keywords: *Hydroquinone, catalase-deficient E. coli* mutants, hydrogen peroxide, catalase

Introduction

Hydroquinone is one of the imperative metabolites from benzene. Long-term exposure to benzene has been reported to cause myelotoxicity, leukemia, lung cancer, etc [1]. However, the mechanism of its toxicity and its carcinogenicity still remains to be clarified. Snyder reported that a variety of metabolites from benzene instead of benzene itself are responsible for the cytotoxicity of benzene to biological systems [1]. Hakura et al. demonstrated that the hydroquinone was mutagenic and its mutagenicity was inhibited by SOD and / or catalase [2]. Several investigations also found that hydroquinone had an ability to induce DNA damage via generation of reactive oxygen species (ROS) [3–8], some suggesting the involvement of singlet oxygen, others blaming the contribution of hydrogen peroxide (H_2O_2), although a clear understanding of its mechanisms in cytotoxicity has not yet been reached.

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Catalase is an important component of the cellular defenses against ROS-induced damage [9,10]. In the sixties, Feinstein et al. [11] established catalase-deficient mouse strains, acatalasemic mouse (C_3-H/AnL/Cs^b) (hereinafter called Cs^b) and hypocatalasemic mouse (C_3H/AnL/Cs^c) (hereinafter called Cs^c). Recently, mutant catalase cDNAs from the Cs^c, Cs^b and their related wild-type (C_3H/AnL/Cs^a) (hereinafter called Cs^a) mice were cloned and expressed in bacteria [12]. A novel missense point mutation at amino acid position 439 (Asp to Ser substitution) of Cs^c catalase gene, at a different position with Cs^b catalase gene (Gln to His substitution at amino acid 11) [13], was identified and this mutation was shown to be responsible for the reduced activity of Cs^c catalase [12].

In the present study, we investigated the potential mechanism of hydroquinone cytotoxicity using a catalase-deficient *Escherichia coli* (*E. coli*) mutant strain (UM255) and its transformants expressing mammalian catalases derived from catalase-deficient mice (Cs^b, Cs^c) and the wild-type (Cs^a). We also examined the effect of some antioxidants on hydroquinone-induced cell damage in catalase-deficient *E. coli*. mutants.

Materials and methods

Chemicals and bacterial strain

Hydroquinone, hydrogen peroxide (30%), reduced glutathione (GSH), bovine erythrocyte superoxide dismutase (CuZnSOD and MnSOD), and dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bovine liver catalase was from Roche Diagnostics Co. (Indianapolis, USA). Ascorbic acid was from Tokyo Tanabe Co. (Tokyo, Japan). UM255 *E. coli* strain was kindly supplied by Dr P.C. Loewen, University of Manitoba (Canada).

Construction of catalase mutant E. coli *strains*

We employed a *katG katE* double mutant *E. coli* UM255 as a recipient since it is completely deficient in hydroperoxidase (both HP-I and HP-II) synthesis [14]. The mouse catalase mutant gene Cs^b and Cs^c and their related normal catalase gene Cs^a were introduced to UM255 using Z-Competent *E. coli* Transformation Kit (ZYMO Research, CA) according to the manufacturer's instructions.

Determination of catalase activity

Catalase activities of transformant bacterial pellet from each strain were determined spectrophotometrically by measuring the rate of H_2O_2 decomposition at 590 nm based on a method of Masuoka et al. [15] Protein determinations were carried out by the Biuret method [16] using bovine serum albumin as a standard. Specific activity of catalase was expressed as μ mol of H_2O_2 decomposed per second per gram protein at 25°C.

Hydrogen peroxide susceptibility assay

The susceptibility of catalase-deficient *E. coli* mutants to H_2O_2 was determined by the addition of H_2O_2 to each bacterial strain in LB broth to final concentrations from 2.5 mM to 150 mM at stationary phase. Aliquots of bacteria were removed after 1 h incubation at 37°C, diluted and plated on LB agar plates. Colonies were scored after overnight incubation at 37°C. Cell viability is expressed in percentages of control values.

Assessment of hydroquinone toxicity

Colony-forming efficiency test

To access the cytotoxicity of hydroquinone, the overnight cultures of catalase-deficient *E. coli* strains were diluted 50-fold in fresh LB medium and incubated at 37°C until the optical density reached 0.25–0.30 at 600 nm. Then, hydroquinone was added to bacteria in LB both to final concentrations from 2.5 to 10 mM. Aliquots of bacteria were removed after 2 h incubation with agitation at 37°C, diluted and plated on LB agar plates. Colonies were counted after overnight incubation at 37°C. Colony-forming efficiency is expressed as percent survival relative to control values.

Zones of inhibition test

The overnight cultures of tester strains were diluted 50-fold in fresh LB medium and incubated at 37°C. An aliquot (30 μl) of each culture with an optical density around 0.1 at 600 nm were added to molten top agar (3 ml) and poured on LB agar plates. Paper discs (6 mm in diameter) were placed on the solidified top agar plates, and 15 μl of hydroquinone solution (autoclaved distilled water was used as a solvent) were dropped on to the center of the discs and then all the treated plates were incubated at 37°C for 20 h. We recorded zones of inhibition by subtracting the diameter of the disc from the diameter (including the paper disc) of the zone.

Assay of H_2O_2 generated by hydroquinone

Hydroquinone (2.5, 5.0 and 10 mM) in 0.1 M potassium phosphate buffer (pH 7.0) was prepared. The solution was incubated at 37°C for 2 h. The mixtures were diluted with the phosphate buffer and concentration of hydroquinone was adjusted to 1 mM

since high concentration of hydroquinone inhibited determination reaction of H_2O_2. To the mixture, the same volume of a reagent solution [consisted of 10 volumes of 0.2 mM *meso*-tetrakis (4-methylpyridyl) porphinatoiron (III) pentachloride solution, 10 volumes of 41.2 mM *N,*N-dimethylaniline in 0.2 M hydrochloric acid, 10 volumes of 8.56 mM 3-methyl-2-benzothiazolinone hydrazone solution in 0.2 M hydrochloric acid and 1 volume of 20 mM EDTA solution] was added and incubated at 25°C for 1 h for the determination of H_2O_2. The absorbance at 590 nm was recorded.

Assessment of the effect of antioxidants and metal chelator

The experimental procedures for assessment of the effects of antioxidants (catalase, SOD, ascorbic acid, GSH) were the same with the above-mentioned zones of inhibition test except that LB agar lates were pretreated with 700 μl of antioxidant solutions before the molten top agar containing bacterial cultures were poured on LB agar plates. Reduced glutathione was in a neutralized condition when mixed with LB medium and the culture.

Effects of DMSO or EDTA on hydroquinone-induced cytotoxicity were tested by the above-mentioned Colony-forming Efficiency Test, in which 2% DMSO or 0.5 mM EDTA was applied to the culture before hydroquinone (5 mM) treatment.

Statistical analysis

Comparisons in catalase activity, effect of the chemical and the antioxidants among groups were performed using a one-way analysis of variance (one-way ANOVA) followed by Scheffe's or Bonferroni's multiple comparisons. Differences within and among groups in relation to cell survival were evaluated by two-way analysis of variance (two-way ANOVA) coupled with Bonferroni's multiple comparisons, using a Windows version SPSS 11.0 statistical program package (SPSS Inc., Illinois, USA). Differences with $P < 0.05$ were considered statistically significant.

Results

There was a significant difference in catalase activities among each catalase-deficient *E. coli* mutant (Table I). The level of catalase in Cs^a appeared higher than that in the others, the catalase level in Cs^c was 65.1% of Cs^a, Cs^b was 61.7%, and UM255 was 8.8% of Cs^a. Similar trends were observed in hemolysate and liver of the catalase mutant mice [17].

Susceptibility to H_2O_2 in catalase-deficient *E. coli* mutants showed a clear dose dependency by Spearman rank test with negative correlation to catalase activity levels (data not shown). The susceptibility order was UM255 > Cs^b > Cs^c > Cs^a (Table II). However, exposure to lower concentration of H_2O_2 (less than 1 mM) did not show a significant difference in the survival rates among the tester strains (data not shown).

Survival of catalase-deficient *E. coli* mutants was significantly reduced by hydroquinone treatment in a dose-dependent manner and the cell viabilities under hydroquinone exposure were also different significantly among each tester strain, showing 42.8% in Cs^a, 17.1% in Cs^c, 6.9% in Cs^b and 3.5% in UM255 at 10 mM hydroquinone exposure (Figure 1).

Hydroquinone-induced cytotoxicity was also confirmed by zone of inhibition test, in which UM255 was the most susceptible, showing the largest zone of growth inhibition surrounding the paper disc, followed by Cs^b, Cs^c and Cs^a (Figure 2).

As Table III demonstrated, no zone of inhibition was produced in solvent controls. The growth inhibition in catalase-deficient *E. coli* by hydroquinone exposure alone negatively correlated with intracellular catalase levels. Pretreatment with catalase or ascorbic acid completely blocked hydroquinone toxicity, leading to no difference in growth inhibition among the 4 catalase-variant tester strains, whereas, pretreatment with CuZnSOD or MnSOD increased the toxicity in a concentration-dependent relation, the later showed less effect on the growth of the tester strains. Glutathione pretreatment in lower concentration did not attenuate hydroquinone toxicity, however, glutathione pretreatment in higher concentration increased the susceptibility of bacterial cells to hydroquinone.

Table I Catalase specific activity in catalase-deficient *E. coli* mutants.

	Cs^a	Cs^c	Cs^b	UM255	*F* value (One-way ANOVA)	Bonferroni multiple comparison
Transformant bacterial pellets (μmol/s/g protein)	2.95 ± 0.82 (3)	1.92 ± 0.26 (6)	1.82 ± 0.29 (6)	0.26 ± 0.1 (6)	47.15**	$Cs^a > Cs^b$ > UM255**, $Cs^a > Cs_c$*, Cs_c > UM255**

Data are expressed as mean ± standard deviation (*n*). Each sample was run at least in duplicate for activity measurement. *$P < 0.05$; **$P < 0.01$.

Table II Effect of hydrogen peroxide on colony-forming efficiency in catalase-deficient *E.coli* mutants.

Treatment	Cs[a]	Cs[c]	Cs[b]	UM255	*F* value (Two-way ANOVA) F_1	F_2
H_2O_2					5.84‡	20.13¶
None	100	100	100	100		
2.5 mM†	126.20 ± 5.25	99.87 ± 43.54	107.12 ± 37.23	36.44 ± 11.63		
5 mM	104.69 ± 20.32	121.09 ± 12.29	111.33 ± 30.45	0.96 ± 0.31		
50 mM	75.76 ± 14.54	45.87 ± 13.07	33.01 ± 6.50	ND		
100 mM	45.47 ± 4.79	6.24 ± 2.90	4.03 ± 1.66	ND		
150 mM	0.65 ± 0.71	0.11 ± 0.10	0.05 ± 0.00	ND		

⋆Data are expressed as mean ± SD. The percentage of colony-forming cells with respect to the control values is based on three different experiments. †Concentrations of H_2O_2 were expressed as final concentrations in the reaction cultures. ‡F_1: comparison among groups of Cs[a], Cs[b], Cs[c] and UM255 ($P < 0.01$). ¶F_2: comparison among groups of hydrogen peroxide concentrations ($P < 0.001$).

Figures 3 and 4 showed that both the OH· scavenger DMSO and the metal chelator EDTA attenuated the cytotoxic effect of hydroquinone on catalase mutant *E. coli.*

Assay of H_2O_2 generation by hydroquinone showed that H_2O_2 was generated in phosphate buffer in the presence of 2.5, 5.0 and 10 mM hydroquinone. After incubation at 37°C for 2 h, concentrations of H_2O_2 in the buffer became 174 ± 45, 275 ± 49 and 374 ± 32 mM, respectively ($n = 6$). We also examined H_2O_2 generation by addition of 5 mM hydroquinone and 0.1 mM cupric chloride, the results showed that concentrations of H_2O_2 in the buffer were 214 ± 16 mM ($n = 3$) in the absence of cupric chloride, and 231 ± 16 mM ($n = 3$) in the presence of cupric chloride after incubation at 37°C for 2 h. The H_2O_2 generation by hydroquinone was slightly increased in the presence of cupric chloride.

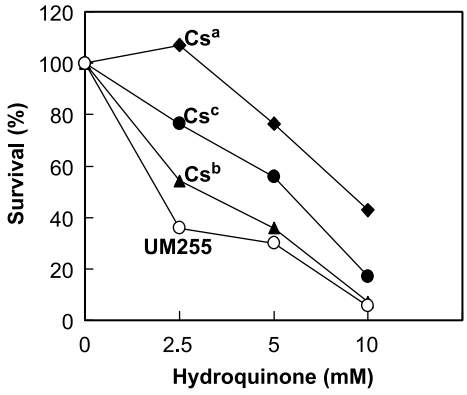

Figure 1. Effect of hydroquinone on the viability of catalase-deficient mutants of *E. coli*. Cell viability is expressed as percent survival relative to control values (in the absence of hydroquinone). The effect of hydroquinone on cell viability was dose-dependent significantly and the differences in viability among each catalase mutant strain were also significant ($P < 0.05$, two-way ANOVA). Concentrations of hydroquinone treatment are expressed as the final concentrations in the reaction cultures.

Discussion

In the present study, the evidence that the hydroquinone cytotoxicity occurred in a concentration-dependent manner and negatively correlated with intracellular catalase levels could be explained by assuming an increased production of H_2O_2 in the process of hydroquinone toxicity and a protective role of catalase in inhibiting the cell damage. This assumption was supported both by the observation that the pretreatment with catalase, an enzyme that detoxifies H_2O_2 generated by oxidative stress [18], completely blocked the toxic effect of hydroquinone on catalase-deficient *E. coli* mutants and by the direct observation of H_2O_2 generation in the presence of hydroquinone. Beckman et al. reported that *p*-benzoquinone was formed *in vitro* during the oxidation of hydroquinone, and he suggested that *p*-benzoquinone, instead of any free radical intermediate, is the main toxic agent produced during the peroxidase-catalyzed oxidation of hydroquinone [19]. However, Urios et al. [20] recently found that hydroquinone caused mutagenic and cytotoxic effects in *E. coli* tester strains deficient in the OxyR function but not in $OxyR^+$ strains, indicating its cytotoxicity might be mediated by H_2O_2.

It was known that hydroquinone can autoxidize readily to O_2^- and semiquinone radicals, the former can be dismutated afterwards to H_2O_2 by SOD. [4] Several studies found that hydroquinone could cause oxidative DNA damage in the presence of Cu(II). [21,22] We also observed Cu(II)-mediated damage in the experiment of hydroquinone exposure, in which addition of copper-containing enzyme, CuZnSOD, greatly exacerbated the hydroquinone toxicity, addition of MnSOD, however, showed a slight exacerbation of hydroquinone toxicity. In addition, EDTA treatment inhibited hydroquinone-induced damage to each tester strain. Based on the present results, it may be deduced that SOD functions as a weak oxidant. However, as it is known that SOD is

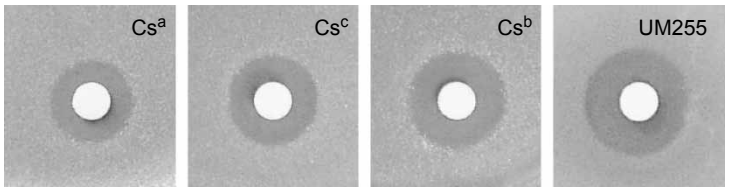

Figure 2. Hydroquinone-induced zone of growth inhibition in catalase-deficient mutants of *E. coli*. UM255 was the most susceptible to hydroquinone (750 nmol/paper disc), showing the largest zone of inhibition surrounding the paper disc, followed by Cs^b, Cs^c and Cs^a.

assumed to have protective effect on oxidative chemical-induced cytotoxicity even in the absence of catalase by suppressing metal reduction through eliminating superoxide. Since SOD we used was not metal-free, the present result that SOD augmented hydroquinone-induced cytotoxicity should be explained in caution.

Glutathione peroxidase (GPx) is also an important enzyme in regulation of intracellular H_2O_2 in biological systems. [23] However, *E. coli* lacks GPx, [24] therefore, catalase is a principal enzyme in degradation of H_2O_2 in *E. coli.* Some studies indicated that catalase is not cell permeable, [25,26] but H_2O_2 is capable of diffusing away from the sites of generation and can easily enter the cell where it can be detoxified by the intracellular catalase. If the amount of H_2O_2 produced exceeds that of the intracellular catalase could eliminate during the hydroquinone exposure, the excessive H_2O_2 may further react with trace metals to produce OH· by the Fenton reaction or the Haber–Weiss reaction [27,28]. Therefore, H_2O_2 or the highly reactive OH· seems playing a contributive effect on hydroquinone- induced cytotoxicity in the present investigation (Figure 5).

To further confirm the oxidative damage of the tester strains caused by hydroquinone, we also examined the effects of ascorbic acid, DMSO, and reduced GSH on hydroquinone-induced cytotoxicity. Ascorbic acid is known as a scavenger of O_2^- and OH [29,30], the addition of ascorbic acid attenuated the damage of hydroquinone in catalase mutant *E. coli*, revealing the possible involvement of O_2^- or OH produced from H_2O_2 in hydroquinone-induced cytotoxicity.

DMSO is reported as a scavenger of OH [30]. In this study, we demonstrated that the cell viability of each tester strain treated with both hydroquinone and DMSO was significantly higher than that treated with hydroquinone alone (Figure 3), indicating DMSO can reduce the deleterious effect of hydroquinone on catalase mutant *E. coli*, possibly by scavenging OH·.

Glutathione is a tripeptide thiol found in almost all cells and has been suggested to protect cells against oxidative damage. [31] In the earlier studies, however, Greenberg et al. found that GSH did not protect *E. coli* from lethal oxidative damage and proposed that the role of GSH in bacteria might differ from its function in mammalian cells [32]. In the present study, GSH pretreatment in a lower concentration did not play protective effect on oxidative damage of the bacterial cells, whereas, an enhanced damage was appeared in a higher concentration of GSH pretreatment. This phenomenon could be explained by that GSH is a

Table III Effect of antioxidants on hydroquinone-induced cytotoxicity.

	Zone of inhibition (mm)*				
	Cs^a	Cs^c	Cs^b	UM255	*F*-value (one way ANOVA)
Solvent control (H_2O)	ND	ND	ND	ND	—
Hydroquinone (750 nmol/paper disc)	4.5 ± 0.5	4.8 ± 0.4	6.0 ± 0.9	6.3 ± 0.5	12.32†
H + catalase (100 U/plate)	2.0 ± 0	3.0 ± 0	2.0 ± 0	2.0 ± 0	—
H + catalase (200 U/plate)	ND	ND	ND	ND	–
H + CuZnSOD (500 U/plate)	12.3 ± 0.6	11.3 ± 1.5	13.0 ± 1.0	12.7 ± 0.6	1.56
H + CuZnSOD (2000 U/plate)	14.3 ± 1.2	14.7 ± 0.6	14.7 ± 0.6	15.3 ± 0.6	0.62
H + MnSOD (500 U/plate)	5.7 ± 0.6	6.0 ± 1.0	5.3 ± 0.6	7.0 ± 0.0	3.73
H + MnZnSOD (2000U/plate)	7.3 ± 0.6	7.7 ± 0.6	8.0 ± 0.0	9.7 ± 0.6	12.89‡
H + Ascorbic acid (14 μmol/plate)	ND	ND	ND	ND	—
H + Reduced glutathione (14 nmol/plate)	4.7 ± 0.6	6.3 ± 0.6	5.7 ± 0.6	5.0 ± 0	3.17
H + Reduced glutathione (14 μmol/plate)	13.7 ± 1.2	17.3 ± 0.6	14.7 ± 1.5	16.3 ± 1.2	7.063¶

ND, no zone of inhibition was detected. H, hydroquinone (750 nmol/paper disc). *The diameter of the Zone of inhibition (in mm) was obtained by measuring the diameter (including the paper disc) of the zone and subtracting the diameter of the disc. All data are expressed as mean ± SD ($N \geqq 3$). † $Cs^a < Cs^b$ ($P < 0.01$), $Cs^a <$ UM255 ($P < 0.01$), $Cs^c < Cs^b$ ($P < 0.05$), $Cs^c <$ UM255 ($P < 0.01$); calculated by the Scheffe test. ‡ $Cs^a <$ UM255 ($P < 0.01$), $Cs^b <$ UM255 ($P < 0.01$), $Cs^c <$ UM255 ($P < 0.05$), calculated by the Scheffe test. ¶ $Cs^a < Cs^c$ ($P < 0.05$); calculated by the Scheffe test.

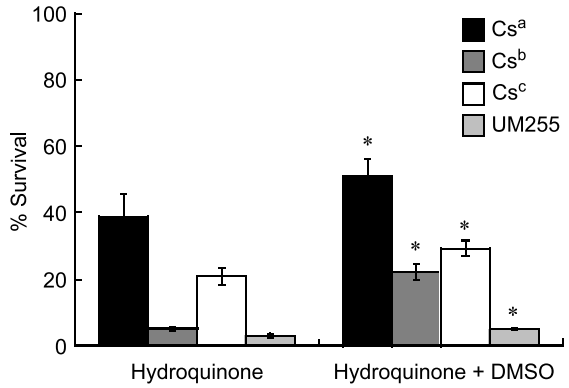

Figure 3. Effect of DMSO on hydroquinone-induced cytotoxicity to the catalase mutant *E. coli*. The cell viability of each catalase mutant strain treated with hydroquinone (5 mM) and DMSO (2%) was higher than that treated with hydroquinone alone (*$P \leqq 0.05$, Main-Whitney *U*-test). Values represent mean ± SD of more than three separate experiments.

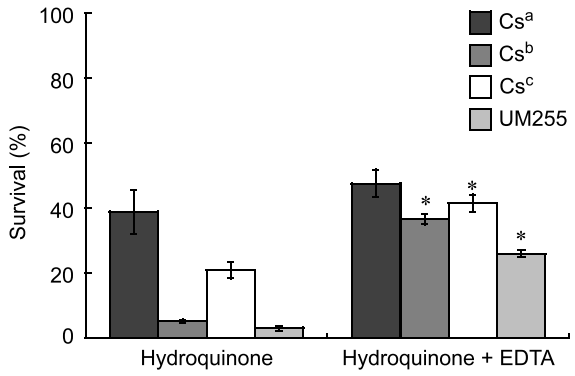

Figure 4. Effect of EDTA on hydroquinone-induced cytotoxicity to the catalase mutant *E. coli*. The cell viability of each catalase mutant strain treated with hydroquinone (5 mM) and EDTA (0.5 mM) was higher than that treated with hydroquinone alone (*$P \leq 0.05$, Mann-Whitney *U*-test). Values represent mean ± SD of more than three separate experiments.

co-substrate to GPx in regulation of cellular H_2O_2, since *E. coli* lacks GPx [24], GSH could not exert usual protective effects against the toxic actions of ROS. On the contrary, pretreatment of higher concentration of glutathione exacerbated cytotoxic effect of hydroquinone, a possible explanation might be that the presence of an excess of reduced GSH in extracellular environment was hard for catalase mutant *E. coli* to live.

Nowadays, more and more chemicals are synthesized for industrial and consumer use, it is impossible to finish long-term rodent bioassay for detection of carcinogens in all chemicals. Therefore, simple and efficient pre-screening alternatives to animal experimentation are greatly desirable. The successful construction of catalase mutant *E. coli* strains from catalase cDNAs of the Cs^a, Cs^b and Cs^c mice in our study raises a question whether these strains could be used for evaluation of toxicity and carcinogenicity resulting from oxidative stress of the chemicals. The present results suggest the possibility of these newly established strains for hazard identification of oxidative chemicals. More chemicals are needed to be tested by this system in order to evaluate the usefulness of the alternative approach to hazard assessment.

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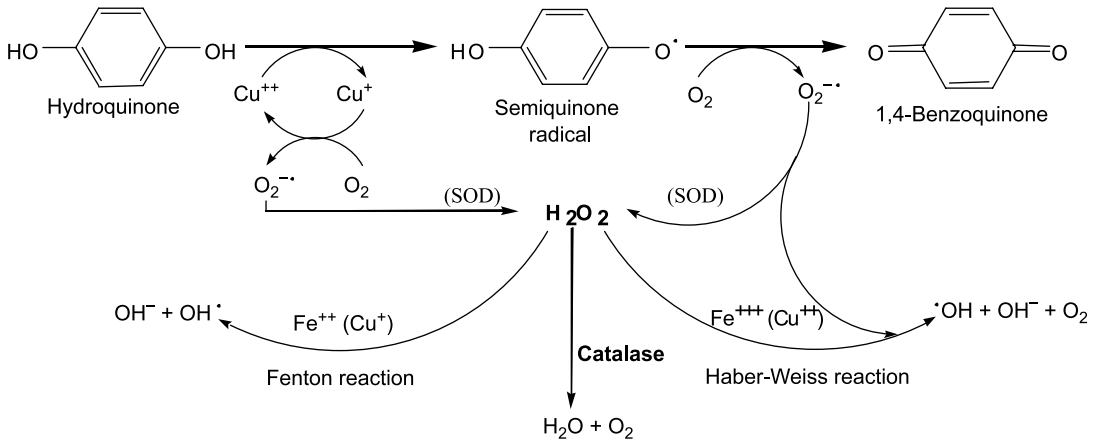

Figure 5. Potentially protective mechanisms of catalase against hydroquinone-induced cytotoxicity.

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