

カタラーゼ変異遺伝子大腸菌を用いた Dithiothreitol の 細胞毒性に対する酵素系及び非酵素系抗酸化物質の効果

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要旨： Dithiothreitol (DTT) は、チオール性抗酸化物質として広く使用されているが、一方では、活性酸素種を産生し酸化促進剤として作用することも報告されている。本研究では、マウスのカタラーゼ遺伝子導入大腸菌 (Csa: 正常カタラーゼ活性菌、Csb: 低カタラーゼ活性菌) を用いた CAT Assay により DTT の細胞毒性及びそれに対する抗酸化物質の影響について検討した。その結果、Csa、Csb とも DTT 濃度依存性の細胞毒性が見られ、両者の間には有意な差異が認められた。カタラーゼの添加により、DTT の細胞毒性を完全に抑制され、アスコルビン酸、カテキン、及びレスベラトロールも DTT 毒性の予防に有効性を示した。以上の結果から、DTT の細胞毒性には O₂⁻ 及び H₂O₂ が関与されているというこれまでの報告に一致している。さらに、非酵素系抗酸化物質が DTT の毒性に対して有用であることを新たに示した。

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—キーワード—

Dithiothreitol, Cytotoxicity, Antioxidant, Catalase, *Escherichia coli*

1. Introduction

Since its introduction in 1964 by Professor Cleland, dithiothreitol (DTT) has gained widespread acceptance as the reagent of choice for the protection of sulfhydryl groups³⁾. Nowadays, DTT or commonly named as Cleland's reagent is widely used in biochemical studies.

At low concentration, DTT stabilizes enzymes and other proteins which possess free sulfhydryl groups and has been shown to restore activity lost by oxidation of these groups *in vitro*³⁾. Many studies have utilized DTT to investigate disulfide linkages in protein and immunoglobulin^{11,16)}.

Despite these beneficial effects, it has been reported that DTT induced cell death in attached cell lines (V79 cells)⁵⁾ and increased the toxicity of arsenic trioxide⁷⁾.

There are reports that the toxicity of DTT was mediated particularly through the production of H₂O₂ via

copper-catalyzed thiol oxidation and [•]OH via the Fenton reaction^{5, 8)}. Its toxicity on V79 cells has several characteristics. Some of the characteristics are the dependency on the medium used during exposure of cells to DTT and the toxicity is decreased or prevented by addition of catalase exogenously, but no effect by addition of superoxide dismutase⁵⁾.

Another report from the same author has shown that mechanism involved in production of [•]OH from DTT occurs in two stages. The first stage is when Cu²⁺ and DTT make a complex that catalyzes oxidation of free DTT. The second stage begins after oxidation of free DTT is complete and involves production of O₂⁻ and H₂O₂, followed by [•]OH via Fenton reaction⁸⁾.

Since previous study has shown that superoxide dismutase (SOD) has no effect against DTT-induced toxicity⁵⁾, it is needed to confirm the protection ability of

two types of SOD: Copper Zinc SOD (CuZnSOD) and Mangan SOD (MnSOD), beside confirm the effect of catalase.

In this study we confirmed the involvement of H₂O₂ in DTT cytotoxicity, the mutagenic effect of DTT and the efficacy of enzymatic (catalase, CuZnSOD and MnSOD) and non enzymatic antioxidants (ascorbic acid, catechin and resveratrol) against DTT-induced cytotoxicity, since it has not been fully resolved and investigated yet.

We used newly established strains of bacteria: catalase normal and deficient mutant *Escherichia coli* from catalase cDNAs of the Cs^a and Cs^b mice¹³ which are considered sensitive to detect the generation of H₂O₂ in chemical cytotoxicity.

2. Material and methods

2.1. Media and chemicals

Nutrient broths were Luria-Bertani (LB) broth and nutrient broth; solid medium were LB agar and minimal glucose agar. S9 fraction that prepared from livers of Sprague-Dawley rats pre-treated with phenobarbital and 5,6-benzoflavone, was purchased from Kikkoman Co. (Chiba, Japan).

Dithiothreitol, Cu/Zn type superoxide dismutase (CuZnSOD), ascorbic acid, catechin and resveratrol were purchased from Wako Pure Chemicals (Osaka, Japan). Bovine liver catalase and Mangan type SOD (MnSOD) were purchased from Roche Diagnostic Co. (Indianapolis, USA). All other reagents were of the best commercially available grade.

2.2. Bacterial strains source and construction of catalase mutant *E.coli* strains

A completely deficient in hydroperoxidase (both HP-I and HP-II) synthesis kat G kat E double mutant *E. coli* UM255 was kindly donated by Dr P.C. Loewen, University of Manitoba (Canada). The mouse catalase mutant gene Cs^b and their related normal catalase gene Cs^a were introduced to UM255 using Z-Competent *E.coli* Transformation Kit (ZYMO Research, CA)⁶.

2.3. CAT Assay

Growth inhibition test:

Growth inhibition experiments were evaluated by means of the disc-diffusion method. The cultures harvested from stationary phase were diluted 50-fold and incubated until the optical density reached around 0.1 at 600 nm. Then, 30 µl of cultures were added to 3 ml of molten top agar and

poured on LB agar plates. Paper discs (6 mm in diameter) were placed on the solidified top agar plates and 10 µl of DTT solution were dropped on the center of the paper disc and then all the treated plates were incubated at 37°C for 18 hours. The diameter of the zone of inhibition (in mm) was obtained by subtracting the diameter of the disc from the diameter of the zone (including the paper disc).

2.4. Investigation the effect of enzymatic and non enzymatic antioxidants

The investigation of the effect of catalase, SOD, ascorbic acid, catechin and resveratrol was the same with the above mentioned growth of inhibition test. LB agar plates were pretreated with 700 µl of catalase, CuZnSOD, MnSOD, ascorbic acid, catechin or resveratrol before poured by the molten top agar containing bacterial cultures.

2.5. Ames/Salmonella mutagenicity assay

The mutagenic effect of DTT was tested by Ames mutagenicity assay with *Salmonella typhimurium* using the pre-incubation method¹⁵. DTT was diluted in water and tested on TA 98, TA 100 and TA 102 in the absence and presence of exogenous metabolic activation by rat liver S9. The experiment was carried out by adding the following components in order: 100 µl test compound, 500 µl S9 mix or 0.1 M of NaPO₄ buffer, 100 µl of overnight incubation culture and 2 ml of molten top agar. Then, all treated plates were incubated at 37°C for 48 hours. Positive controls (2-nitrofluorene and 4-nitroquinoline 1-oxide) were included in all assays. The mutagenic index (MI) is expressed as the mean of revertant colonies divided into the mean of revertant colonies in the control of minimum three plates per dose level. It was considered mutagenic when MI was 2 or higher for at least one of the tested concentrations².

2.7. Statistical analysis

Values were given as mean ± SEM (n≥3) and analyzed using a Window version SPSS 11.0 statistical program package (SPSS Inc., Illinois, USA). Data was analyzed by one way analysis of variance (ANOVA) and confirmed by Bonferroni test. Differences within and among groups of strains and concentrations were evaluated by two-way ANOVA. The limit of statistical significance was set at $p < 0.05$.

3. Result

3.1. CAT assay: Effect of DTT on catalase mutant *E.coli*

Exposure of DTT 0.2 to 1.6 $\mu\text{mol}/\text{paper disc}$ led to a dose-dependent growth inhibition significantly. Cs^b was more susceptible to DTT than Cs^a in clear dose dependency as shown in Figure 1.

3.2. CAT assay: Protective effect of antioxidants against DTT on catalase mutant *E.coli*

The capacity of antioxidants to prevent DTT-induced cytotoxicity was evaluated. We examined the effect of enzymatic and non enzymatic antioxidants against 0.8 $\mu\text{mol}/\text{paper disc}$ DTT by growth inhibition test.

Fifty units of exogenous catalase enzyme was completely blocked the toxic effect of DTT and higher concentration of CuZnSOD (200 units) almost blocked the zone, respectively,

whereas MnSOD did not significantly protect Cs^b cells (Table 1).

Ascorbic acid effectively protected the bacterial cell from toxic effects of DTT, whereas the presence of catechin and resveratrol rendered Cs^a and Cs^b less susceptible to DTT as shown in Table 1.

3.3. Ames/Salmonella mutagenicity assay

The mutagenic activity data obtained in Ames/Salmonella mutagenicity assay are shown in Table 2. In this test, the series concentrations of DTT from 0.01 to 100 mM were not mutagenic to TA98, TA 100 and TA102 in the absence and presence of metabolic activation.

TABLE 1

Effects of dithiothreitol on the growth of normal (Cs^a) and catalase-deficient (Cs^b) *E.coli*

	Concentration	Zone of inhibition (mm) [†]	
		Cs^a	Cs^b
Solvent control (H_2O)		ND	ND
DTT	0.8 μmol (123.40 μg)/paper disc	9.2 \pm 0.87	13.2 \pm 0.83
DTT + Catalase	50 U/plate	0.0 \pm 0.00***	0.0 \pm 0.00***
DTT + Catalase	200 U/plate	0.0 \pm 0.00***	0.0 \pm 0.00***
DTT + CuZnSOD	50 U/plate	7.0 \pm 0.00	8.0 \pm 0.00**
DTT + CuZnSOD	200 U/plate	0.0 \pm 0.00***, Δ^{**}	1.3 \pm 1.33***, Δ^{**}
DTT + MnSOD	50 U/plate	0.0 \pm 0.00***	10.3 \pm 0.67
DTT + MnSOD	200 U/plate	0.0 \pm 0.00***	10.0 \pm 0.00
DTT + Ascorbic acid	7 $\mu\text{mol}/\text{plate}$	0.0 \pm 0.00***	2.0 \pm 1.15***
DTT + Ascorbic acid	14 $\mu\text{mol}/\text{plate}$	0.0 \pm 0.00***	0.0 \pm 0.00***
DTT + Catechin	0.3 $\mu\text{mol}/\text{plate}$	1.0 \pm 0.00***	8.7 \pm 0.33**
DTT + Catechin	1.2 $\mu\text{mol}/\text{plate}$	2.0 \pm 0.00***	6.0 \pm 0.00***
DTT + Resveratrol	68.1 nmol/plate	2.0 \pm 0.00***	7.0 \pm 0.00**
DTT + Resveratrol	1.02 $\mu\text{mol}/\text{plate}$	1.0 \pm 0.00***	7.7 \pm 0.33**

Zones of inhibition after DTT exposure were presented as mean \pm SEM ($n\geq 3$) and calculated by one-way ANOVA,

followed by Bonferroni test. ND: no zone of inhibition was detected. DTT: Dithiothreitol

[†]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 (6 mm).

Significant difference as compared to DTT alone.

^ΔSignificant difference between two concentrations.

* $p<0.05$, ** $p<0.01$, *** $p<0.001$

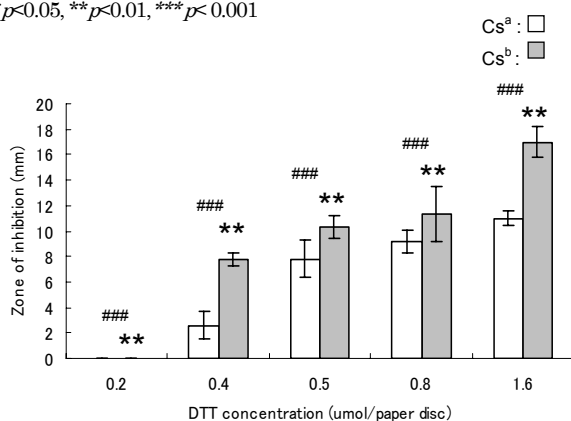


FIGURE 1. Zones of inhibition after dithiothreitol (DTT) exposure were presented as mean \pm SEM ($n\geq 3$) and calculated by two-way-ANOVA.

###: significant differences ($p<0.001$) between five (5) concentrations of DTT

** : significant differences ($p<0.01$) between the groups of Cs^a and Cs^b

TABLE 2.

Ames mutagenicity assay of various doses of dithiothreitol in TA98, TA100 and TA102, with or without metabolic activation

Strain	Dose (mM)	+S9		-S9	
		<i>His</i> ⁺ ^a	M.I. ^b	<i>His</i> ⁺	M.I.
TA98	0	26±4.2	-	24±1.4	-
	0.01	16±7.0	0.6	34±12.0	1.4
	0.1	20±6.7	0.8	21±7.5	0.9
	1	21±5.1	0.9	27±11.0	1.1
	10	15±2.1	0.6	8±4.0	0.3
	100	23±4.2	0.9	0	0
	2-NF ^c	697±54.0	26.8	-	-
	2-NF ^d	-	-	602±47.0	25.1
TA100	0	64±9.2	-	79±17.0	-
	0.01	67±2.5	1.0	82±7.5	1.0
	0.1	74±8.1	1.2	98±19.6	1.2
	1	81±1.2	1.3	112±17.1	1.4
	10	77±12.0	1.2	96±9.9	1.2
	100	68±11.0	1.1	109±7.8	1.4
	2-NF ^c	1893±123.0	29.6	-	-
	2-NF ^d	-	-	1860±134.0	23.5
TA102	0	73±8.5	-	64±9.9	-
	0.01	76±10.7	1.0	51±3.5	0.8
	0.1	83±11.1	1.1	69±5.1	1.1
	1	78±4.2	1.1	64±15.3	1.0
	10	73±1.0	1.0	60±5.0	0.9
	100	64±7.2	0.9	64±7.0	1.0
	4-NQO ^e	266±2.8	3.6	5140±149.0	80.3

^a*His*⁺: mean and standard deviation of the number of histidine revertants by triplicate plates from 3 independent experiments.

^bM.I.: Mutagenic index: mean of the revertant colonies induced by samples divided into the mean of spontaneous revertant colonies in the negative control.

^c2-Nitrofluorene, 21.12 µg/plate (positive control for TA 98 and TA100 with metabolic activation)

^d2-Nitrofluorene, 10.56 µg/plate (positive control for TA98 and TA100 without metabolic activation)

^e4-Nitroquinoline oxide 4.75 µg/plate (positive control for TA102 with and without metabolic activation)

4. Discussion

At relative high drug concentrations, DTT can poses as an effective antioxidant that decreases cell injury from various oxidative stresses. It could react with ROS (Reactive Oxygen Species: H₂O₂, ·OH, etc.), modulate the cellular redox potential by increasing intracellular level of glutathione and/or by acting as thiol reducing agents.

In contrast to that protective effect, we and others have shown that, under certain circumstances, DTT also can be a pro-oxidant producing ROS *in vitro*. Although we found DTT was toxic to mutant *E.coli* cells, it had no mutagenic effects in Ames/Salmonella test using TA98, TA100 and TA102, since the mutagenic index (MI) of all conditions were lower than 2 (Table 2).

There are reports indicating that the toxicity of DTT particularly through the production of H₂O₂ via copper-catalyzed thiol oxidation and ·OH via the Fenton reaction^{4,8}.

DTT causes biphasic toxicity pattern on V79 cells whereby the cytotoxicity occurred at intermediate drug concentration (0.2-1 mM), but is not seen at lower or higher drug concentrations⁴. These phenomena are probably due to that low concentration of DTT could not generate H₂O₂ at a sufficient rate right away and higher concentration of DTT could possibly have sufficient thiols to consume excessive H₂O₂ before damage occurs⁷.

The pattern seems to be more difficult to explain on *E.coli* mutant cells. Low concentration (0.2 µmol/paper disc) did not show toxic effect, whereas 0.4 to 1.6 µmol/paper disc showed toxic effects in dose dependent manner. We have not tested more concentrated DTT, since 1.6 µmol/paper disc is high enough (Fig.1).

To confirm previous study on the capability of enzymatic antioxidants in protecting cells from DTT, including the finding that showed SOD has no effect⁵, we tested CuZnSOD, MnSOD and catalase. Our findings showed that exogenous CuZnSOD and MnSOD have protective effects at relative high concentration (200 units per plate). We found that CuZnSOD was more effective than MnSOD. This is in line with the hypothesis that at second stage mechanism, DTT produces O₂⁻ and H₂O₂⁸.

We further investigated the protection ability of non enzymatic antioxidants against DTT. In this study, ascorbic acid, a hydrophilic nature and low molecular weight antioxidant, has shown protective effect definitely (Table 1), suggesting the involvement of ROS in DTT-induced cytotoxicity, since ascorbic acid has a potent O₂⁻ and OH· scavenger activity.

Catechin and resveratrol eliminated the diameter of the zone in dose dependent manner (Table 1).

Catechin, which is widely present in tea, and also many other foods, such as apples, grapes and their processed beverages, is known binding to metal ions, preventing them from participating in Fenton or Harber-Weiss reactions and also has the potentiality to scavenge reactive oxygen and nitrogen species¹⁴. It is also reported that

catechin pre-treatment showed restoration in the activities of glutathione metabolizing enzymes and other antioxidant enzymes, including catalase and superoxide dismutase¹⁰.

Resveratrol (3,4',5-trihydroxystilbene), a polyphenolic compound found in mulberries, grapes and its products, has been demonstrated to be capable of protecting against oxidative stress. Notably, induction of catalase was remarkable in resveratrol-inducible antioxidants on cultured aortic smooth muscle cells (ASMCs) in a concentration- and/or time-dependent fashion⁹.

The restoration and induction of enzymatic antioxidants might be one of the plausible reasons of the protective activity of catechin and resveratrol against DTT and in line with the exogenous catalase and SOD protective activity in this study.

5. Conclusion

DTT was not mutagenic in Ames test using TA98, TA100 and TA102, but it showed toxic effects on catalase mutant *E.coli*, probably by the involvement of O₂⁻ and H₂O₂. Enzymatic antioxidants (catalase, CuZnSOD and MnSOD) and catalase and SOD-restorable and inducible non enzymatic antioxidants showed protective activity against DTT-induced cytotoxicity.

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Protective ability of enzymatic and non enzymatic antioxidants against dithiothreitol-induced cytotoxicity on catalase mutant *Escherichia coli*

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Dithiothreitol (DTT) has gained widespread acceptance as one of the thiol antioxidants. In contrast to that protective effect, some studies have shown that, under certain circumstances, it also can be a pro-oxidant producing Reactive Oxygen Species (ROS) *in vitro*. This study was conducted to confirm the DTT cytotoxicity and mutagenicity, and to investigate the protective ability of enzymatic and non enzymatic antioxidants against DTT-induced cytotoxicity. We carried out CAT assay (a zone of inhibition test of bacterial growth of transformant *Escherichia coli* (*E.coli*) strains that express mammalian catalase gene derived from normal catalase mice (Cs^a) and catalase mutant mice (Cs^b)) and Ames mutagenicity assay. DTT was not mutagenic in Ames assay using TA98, TA100 and TA102. In the series of five concentrations of DTT by CAT assay, the results show that it was toxic to the catalase mutant *E.coli* cells in dose dependent manner and significantly different between Cs^a and Cs^b. Unlike previous study on V79 cells, not only catalase, other enzymatic antioxidants (CuZnSOD and MnSOD) also showed protective ability on catalase mutant *E.coli* cells. Fifty units of catalase and seven μ mol ascorbic acid abolished the zone respectively. Catechin and resveratrol, the catalase and SOD-restorable and inducible non enzymatic antioxidants rendered to decrease the zone. These results are in line with previous study that O₂⁻ and H₂O₂ involved in DTT-induced cytotoxicity. In additional, the present results suggest the beneficial effect of non enzymatic antioxidants against DTT toxicity.

Key words: Dithiothreitol, Cytotoxicity, Antioxidant, Catalase, *Escherichia coli*