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1,2-Dichloroethane induced nephrotoxicity through ROS mediated apoptosis in vitro and in vivo

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Running title: **1,2-Dichloroethane generate ROS and induce nephrotoxicity**

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Abstract

1,2-dichloroethane (DCE) is a ubiquitous occupational environmental contaminant. Subacute exposure of DCE could cause severe toxic encephalopathy. However, the toxicity of DCE on kidney and the molecular mechanism still remain elusive. To address this issue, we established a DCE-exposed animal model by inhalation in SD rats and used human embryonic kidney 293 (HEK293) cells in *in vitro* tests. We showed that the kidney/weight ratios were obviously higher in DCE-exposed groups than that in control group. The renal distal tubules and distal convoluted tubule of rats treated with 577 ppm and 1000 ppm DCE obviously appeared abnormality. Moreover, apoptotic cells were found in the renal distal tubules from 1000 ppm DCE-exposed group. The antioxidant capacity was decreased and the levels of lipid per-oxidation were increased in kidney in exposure groups. In *in vitro* tests, we observed that there was no obvious toxicity in cells treated with DCE alone. However, over-expression of CYP2E1 or addition of S9 could remarkably increase the generation of ROS in HEK293 cells treated with DCE and decrease cell proliferation, even induce cells apoptosis. Antioxidant N-Acetyl-Lcysteine (NAC) pre-treatment could inhibit the generation of ROS and alleviate cell apoptosis induced by DCE in the presence of extra-metabolism system. Taken together, our findings provide direct evidence that excessive ROS generation may be the cause of the apoptosis effects induced by 1,2-Dichloroethane on kidney.

Key words: 1,2-dichloroethane, kidney lesions, oxidative stress, apoptosis

Introduction

1,2-Dichloroethane (DCE), a highly volatile organic liquid, is widely used as solvent, dry cleaning, and metal-degreasing agents in many industries. In china, it is also used as a kind of solvent thinner of adhesives. As an easily volatile solute, the main route of occupational exposure is inhalation through respiratory route. Following absorption, DCE could rapidly distribute and accumulate in the blood, lung, liver, brain, kidney, and abdominal fat [1, 2]. To date, DCE exposure was proved to have obvious toxic effects on brain, liver, and kidney. A line of evidence have proved that subacute exposure to DCE could obviously lead to severe toxic encephalopathy in both exposed workers [3] and mouse [4, 5]. Based on the data reported, DCE also cause the toxicity of kidney, several animals tests have showed that DCE resulted in an increasing in the absolute and relative kidney weights and abnormal serological indexes [2, 6, 7]. However, little is known about the mechanisms underlying DCE induces the dysfunction of kidney.

Cytochrome P450 mixed function oxidase enzymes are the main catalysts in exogenous components metabolism [8, 9]. CYP2E1 is one member of the CYP450 super-family and mainly metabolizes ethanol, carbon tetrachloride and so on [10]. Volatile organic compounds are specifically metabolized through CYP2E1 [11]. CYP2E1 was found highly expressed in the proximal tubule and distal tubule of the kidney besides liver tissue [12-15]. CYP2E1 assumes important roles in toxicity induced by exogenous components. Previous study have indicated that CYP2E1 could increase the generation of superoxide anion radical and hydrogen peroxide/hydroxyl radical, due to its enhanced NADPH oxidase activity and poorly coupled with NADPH-cytochrome P450 reductase [16-18]. Moreover, many studies have indicated that the generation of reactive oxygen species (ROS) and oxidative stress by CYP2E1 played a critical role in toxicity caused by exogenous components such as ethanol and CCl_4 [19-21]. ROS is proved to participate in a number of cellular processes including apoptosis, necrosis, cell proliferation, and carcinogenesis [22, 23]. In an organism, the biological consequences of oxidative stress exceeding background levels led to apoptosis contributing to the pathophysiology of a number of diseases [24]. One *in*

vitro study showed that CYP2E1 might involve in DCE-induced effects on cell division [25]. Another data from animal proved that DCE could increase the expression of CYP2E1 in liver [26]. However, the role of CYP2E1 and ROS in kidney toxicity induced by DCE remains unclear.

In this study, we examined the potential kidney toxicity of DCE *in vitro* and *in vivo*, and showed that DCE could obviously induce kidney cells apoptosis in both animal tests and human embryonic kidney 293 cells. The mechanism might be related with CYP2E1 and the generation of ROS in the process of metabolism of DCE. The addition of S9 and overexpression of CYP2E1 increased the toxicity induced by DCE. Moreover, antioxidant n-acetyl-L-cysteine (NAC) alleviates the cell apoptosis induced by DCE. Together, these fignding indicate that oxidative stress induced by CYP2E1 might contribute to exert kidney toxicity caused by DCE.

Materials and methods

Reagents

1,2-Dichloroethane (DCE) (purity greater than 99.8%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human liver S9 fraction was purchased from Moltox (Moltox CO., USA). Total antioxidant capacity (T-AOC), Superoxidase dismutase (SOD), Glutathione (GSH) and Malonaldehyde (MDA) assay kits were supplied by Beyotime Institute of Biotechnology (Shanghai, P.R. China). Antibodies against caspase-3 and caspase-9 were obtained from Cell Signaling Technology (Beverly, MA). The CYP2E1 antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Experimental animals and treatments

Male Spraque-Dawley rats (Guangdong Medical Laboratory Animal Center, China) around 10 weeks of age were used in this study. The experimental procedures were in compliance with The Guide for the Care and Use of Laboratory Animals that were approved by the China Animal Care and Use Committee. Maximal care was taken to minimize the number of the animals being sacrificed and their sufferings. The rats were housed under standard laboratory conditions, the temperature and the relative humidity during the study were maintained in the ranges of 22±2□ and 52±12%, respectively. Experiments were started after a one week quarantine period (body weight ranged from 180g to 210g). Rats were randomly divided into 4 groups including DCE exposure groups (333 ppm, 577 ppm and 1000 ppm) and a control group. Rats were exposed to DCE 6 h per day for 5 days successively in inhalation chambers. The DCE vapor concentration in the inhalation chambers was measured with gas chromatography (GC) using Agilent Technologies 6890 (Agilent Technologies, Santa Clara, CA, USA) every 15 min during the inhalation exposure period.

Detection of internal exposure

Blood collection was immediately followed by necropsy under ether anesthesia. 3 mL blood samples were collected into 20-mL headspace sampler (HS)-vials, and 2 mL of distilled water was added to each sample. The vials were immediately sealed

with an aluminum crimp cup. DCE concentrations in blood samples were analyzed by HS-GC/mass spectrometer (MS) using Agilent Technologies 7694 HS (oven temperature, 80□, loop temperature, 130□, 10 min vial equilibration time for blood samples) and Agilent Technologies 5989B GC/MS system (column, J&W DB-1 60 m×0.25 ×mm ID×0.25 μm; oven temperature, 100□; ion source temperature, 200□; carrier gas, helium at 1 mL/min; ionization, EI (electron ionization); fragment peak, 62 m/z).

Blood biochemical analysis

To determine the effect of 1,2-dichloroethane on kidney in rats, the blood of rats was collected from the tail of each rat treated with DCE for 5 days, the creatinine (Cr) and urea (UREA) were determined by auto-biochemical analyzer (Drew Trilogy, USA).

Pathological change

Organs were removed, weighed and examined for macroscopic lesions at the necropsy. Tissues for microscopic examinations were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections 5μm thick were prepared and stained with hematoxylin and eosin. The pathological changes and ultrastructures in renal tissue were examined by optical microscopy.

Terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL)

We detected apoptosis using the TUNEL technique (In Situ Cell Death Detection Kit; Roche Applied Science, Mannheim, Germany) according to manufacturer's instructions. Briefly, tissue sections were deparaffinized in xylene and re-hydrated in phosphate-buffered saline. After blocking the endogenous peroxidase activity, the sections were treated with proteinase K (20 mg/ml in Tris-HCl) for 10 min at room temperature, and incubated with TdT and fluorescein isothiocyanate-labeled dUTP. The immunolabel was developed with metal-enhanced diaminobenzidine (DAB) solution. For nuclear staining, we used 4′,6-diamidino-2-phenylindole (DAPI) (Dojindo Laboratories, Kumamoto, Japan).

Determination of T-AOC, SOD, GSH and MDA levels and activities

The levels of T-AOC, SOD, MDA, and GSH levels were examined according to

manufacturers' instructions. In brief, total antioxidant capacity (T-AOC) was measured by the reduction in the Fe^{3+}-TPTZ complex to the ferrous form Fe^{2+}-TPTZ. The optical density was measured at 593 nm. One unit (U) of T-AOC was defined as the amount of antioxidant capacity that increased absorbance by 0.01 at 37°C. Superoxide dismutase activity was determined using the xanthine/xanthine oxidase method. One unit of SOD activity was defined as the amount of protein that inhibited the rate of NBT reduction by 50%. Glutathione was determined by the reduction in glutathione disulphide (GSSG) by GR and NADPH and was measured according to absorbance at 340 nm. Malondialdehyde was measured by analyzing the reaction of MDA with thiobarbituric acid (TBA), which forms a MDA-TBA2 adduct that absorbs strongly at 535 nm. Results were expressed as perunit weight of tissue.

Cell culture

Human embryonic kidney 293 cells (HEK293) were purchased from the cell-bank of Chinese Academy of Sciences. Cells were maintained in Minimum Essential Medium α (MEMα) medium supplemented with 10% FBS.

Establish cell lines with over-expressed CYP2E1

In order to create pBabe-CYP2E1, we performed PCR using the sense primer ggcggcAGATCTatgtctgccctcggagtca and the antisense primer ggcggcGAATTCtcatgagcggggaatgacaca. The sequences of restriction enzymes used were underlined. The fragment was then subcloned into the retroviral vector pBabe-puro. To create stable HEK 293 cells, pBabe-CYP2E1 was introduced into HEK 293 cells by retroviral infection and selected with puromycin (2 μg/ml).

DCE treatment

Cells were plated in triplicate at a density of 5×10^4 cells in a six-well culture dish. HEK293 cells and HEK293-CYP2E1 cells were treated with DCE at a concentration of 125, 250, 500, 1000, 2000 ppm, vehicles (0.1% dimethyl sulfoxide) for dissolving chemicals were used as the controls. For S9 fraction supplement, HEK293 cells were treated with DCE in the presence of the addition of pooled human liver S9 fraction mix. The S9 fraction mix was freshly prepared according to protocols reported before and stored on ice during the experiment [27]. The concentration of treatment was

determined according to the cytotoxicity (LC50) measured from cells treated with DCE in the presence of S9 fraction mix. Cells were treated with DCE in the absence of S9 fraction mix (-S9) or presence of S9 fraction mix (+S9) for 24 h, 48 h, 72 h, respectively.

Measurement of cell viability

HEK293 cells and HEK293-CYP2E1 cells were seeded in 96-well plates with a density of 1×10^4 per well. After 24 h, these cells were treated with DCE (125, 250, 500, 1000, 2000 ppm) for 24 h, 48 h, and 72 h, respectively. Cytotoxicity was measured by trypan blue exclusion at indicated concentrations. The survival rate is calculated as the ratio of each dose of DCE to that observed in control cells treated with a vehicle.

Measurement of reactive oxygen species (ROS)

Briefly, HEK293 and HEK293-CYP2E1 cells were seeded in 6 cm plates with a density of 1.2×10^5 cells per plate. After 24 h, HEK cells were respectively treated with DCE (0, 250, 500, 1000, 2000 ppm) alone or co-treated with DCE and 10mM NAC (N-Acetyl Cysteine) in the presence or absence of S9 for 24 h, followed by an incubation with 50 μM 2',7'-Dichlorodihydro-fluorescein diacetate (H_2DCF-DA; Sigma-Aldrich) at 37℃ for 30 min. Cellular ROS levels were measured by fluorescence intensities determined by flow cytometry using a FACSCanto (BD biosciences).

Apoptosis analysis

Apoptosis analysis was performed by Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA). Briefly, harvested cells were re-suspended and rinsed in 1× binding buffer before 5 μl of Annexin V and 10 μl of propidium iodide (PI) were added. After incubated at room temperature in the dark for 15 min, the apoptosis status for these cells was determined by fluorescence intensities and measured by flow cytometry using a FACSVantage SE flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA)

Immunoblotting analysis

For the analysis of caspase-3 and caspase-9 protein levels, cells were lysed

directly on the plate using 2×SDS sample loading buffer (125 mM Tris-base, 138 mM SDS, 10% β-mercaptoethanol, 20% glycerol, bromophenol blue pH 6.8). Soluble proteins (50 μg) were subjected to 4%-12% gradient acrylamide gel for SDS-PAGE before immunoblotting.

Detection of CYP2E1 enzyme activity

The enzymatic activity of CYP2E1 was estimated by assaying P-nitrophenol hydroxylation in intact cells[28]. In brief, 5×10^5 cells were plated onto wells of six well plate. 0.4 mM P-nitrophenol was added to the wells and cells were incubated for 24 h at 37°C. Cells were scraped into the medium and 4% trichloroacetic acid was added. After centrifugation, 1 mM NaOH was added to the supernatant and the absorbance was measured at 510 nm.

Statistical analysis

The results are presented as mean±SD. for at least three replicate experiments. Differences between treatments groups were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered statistically significant at $P<0.05$.

Results

The effects of DCE on kidney of rats

To determine the effects of DCE on the kidney of rat, male SD rats were exposed to DCE vapor at a target concentration of 333, 577, 1000 ppm for 6 h every day for 5 days successively. At the end of the exposure, we found that the blood concentration of DCE in the exposure group was increased in a dose-dependent manner (Pearson r=0.951, $P<0.05$) (Fig. 1A). During the inhalation exposure period, body weigh of exposed rats drastically decreased at day 2, even more profound at day 5, compared with control group (F=29.250, $P<0.001$) (Fig. 1B). Moreover, the kidney/body weight ratios in DCE-exposed (557 and 1000 ppm) rats were significantly increased compared with control group ($P<0.05$) (Fig. 1C). We next examine the effect of DCE on renal function by performing blood biochemical analysis. As a result, we showed that the levels of UREA was significantly increased in rats treated with 1000 ppm DCE (F=29.504, $P<0.001$). Meanwhile, the serum levels of CR also decreased in a dose-dependent manner. However, there was no statistical difference compared with control (Fig. 1D, E).

Additionally, we also detected the kidney of pathological change upon exposure to DCE to assess the ultrastructure lesions. As shown in Fig. 2A, we found no abnormality in kidney glomerulus, proximal convoluted tubule, or distal kidney tubules in control rats, the structure were integrity without any intumesce or necrotic. In addition, the kidney glomerulus in three DCE-exposed groups was normal. However, in proximal convoluted tubule, there were slight intumesce at 333 ppm and mild atrophy at 557 ppm compared with control or 1000 ppm, the tubal wall were obviously collapsed, and internal structure derangement were noticed. About the distal kidney tubules, a great quantity of protein cast was observed at 1000 ppm and a few at 577 ppm (Fig. 2A). To assess whether DCE can induce the damage and apoptosis of kidney cells of rats treated with DCE, we examined the status of apoptosis in glomerulus renal, proximal convoluted tubules, and renal distal tubules through tunnel assay. As a result, apoptotic cells were detected in proximal convoluted tubules and renal distal tubules at 1000 ppm DCE-exposed groups

compared with the control group. In contrast, no obvious apoptosis was found in other groups (Fig. 2B). Thus, these results confirm that DCE exerts toxic effects on kidney of rat.

Effects of DCE on intracellular levels of T-AOC, SOD, GSH, and MDA in kidney of SD rats

Many physiological and environmental factors may contribute to upset the balance of pro-oxidative and anti-oxidative factors, resulting in oxidative stress, which in turn lead to oxidative damage [29, 30]. DCE has been reported to increase the ROS formation and induce cells proliferation inhibition [31, 32]. Thus, we examined the effect of DCE on the intracellular levels of T-AOC, SOD, MDA, and GSH in the kidney of SD rats treated with 333, 577, 1000 ppm DCE, respectively. The data in Table 1 revealed that the levels of malonaldehyde (MDA) increased in a dose-dependent manner upon exposure to DCE, especially there were significant differences at 577 and 1000 ppm compared with control group (P<0.001). In parallel, we observed a significant decrease in the levels of T-AOC, SOD, GSH at 577 ppm and 1000 ppm in kidney of male SD rats (Table 1). Taken together, these results indicate that DCE might contribute to the renal tubule cells damage and apoptosis through inducing cellular oxidative stress.

The cytotoxicity of HEK 293 cells induced by DCE

To further confirm the renal toxicity induced by DCE, we chose human embryonic kidney cell line HEK 293 and determined the cytotoxicity induced by DCE in *in vitro* tests. P450 CYP2E1 has been considered as the major metabolic enzyme for the activation of DCE [33, 34]. Consistently, we failed to observe the obvious cytotoxicity in HEK293 cells treated with different DCE concentrations for 24 h, 48 h, and 72 h, respectively (Fig. 3A). Thus, we determined chemical-induced cell viability in presence of two metabolic conditions in mediating including the addition of S9 fraction mix and over-expression of CYP2E1. To establish *in vitro* metabolic activation systems, we generated cells stably expressing CYP2E1 in HEK 293 cells.

As shown in Figure 3C-, we showed that introduction of an allele of CYP2E1 resulted in a 10-fold increase in protein expression (Fig. 3D) and a 18-fold increase in CYP2E1 specific enzyme activity compared to control cells (Fig. 3D). As shown in Figure 3E, the survival rate of HEK-CYP2E1 cells exposed to 500 ppm DCE decreased to 82% (48 h) and 91% (72 h) compared with control (Fig. 3E), indicating that CYP2E1 conferred cells to enhance cytotoxicity caused by DCE. Similar results were also obtained in HEK293 cells in presence of S9. We showed that the cell viability of HEK293 treated with DCE descended in a dose- and time- dependent manner. Moreover, the survival rate of cells treated with S9 and DCE at 500 ppm was decreased to 81% (48 h) and 62% (72 h), respectively compared with control cells (Fig. 3B). Taken together, these results indicate that DCE could exert cells toxicity upon metabolic activation condition.

DCE induced the apoptosis of HEK 293 cells in the presence of metabolic system

To explore the mechanism underlying cytotoxicity induced by DCE, the cells apoptosis rate was measured in cells exposed to DCE. HEK 293 cells were treated with different DCE concentrations (250-2000 ppm) for 48 h and followed by flow cytometric analysis. As shown in Fig. 4A, the percentage of apoptotic HEK293-CYP2E1 cells was increased by 17.1% (500 ppm), 23.0% (1000 ppm), and 29.8% (2000 ppm), respectively compared with control (9.43%). In contrast, when we treated cells with both S9 mixture and DCE, we found that the percentage of apoptosis of HEK293 cells was increased to 21.1% (250 ppm), 31.2% (500 ppm), 32.1% (1000 ppm), and 41.2% (2000 ppm), respectively (Fig. 4A). We next performed immunoblotting analysis to detect the effects of DCE on the activities of caspase-3 and caspase-9, which have been recognized as the best molecular hallmark for both early stages and late stages of apoptosis [35, 36]. As shown in Fig. 4B, the increasing levels of cleavered caspase-3 and caspase-9 were observed when we treated HEK293-CYP2E1 with 1000 ppm and 2000 ppm DCE for 48 h, which were consistent with the patterns of cells apoptosis detected by flow analysis (Fig. 4B). Thus, these observations demonstrate that DCE could induce apoptosis

Roles of Reactive oxygen species (ROS) in DCE -induced apoptosis

Excessive production of ROS generally damages the mitochondria and leads to cell apoptosis [37]. In the process of metabolism of xenobiotics, CYP2E1 could generated ROS and lead to cytotoxicity in various tissues [38]. To determine whether DCE metabolism via CYP2E1 could generate excessive ROS, we detected the levels of ROS in HEK 293 treated with DCE. As a result, the amounts of ROS increased remarkably in a dose-dependent manner in HEK293-CYP2E1 cells treated with DCE for12 h (Fig. 5A). Moreover, we showed that the level of ROS changed dynamically with a peak at 8 h and down to a basal level at24 h (Fig. 5B). These results indicated that DCE could generate excessive ROS in the presence of metabolism system. Accordingly, the mRNA levels of oxidative stress responsive genes including SOD1 (superoxide dismutase 1), SOD2 (superoxide dismutase 2), HO-1 (human heme oxygenase 1), and CAT (catalase) were significantly up-regulated (Fig. 5C), in line with previous study showing that oxidative stress responsive genes was induced by redundant ROS.

Given that ROS could lead to the disturbtion of cellular event, we speculated that excessive generation of ROS might be the important cause in apoptosis induced by DCE. To test this hypothesis, we pre-treated HEK293-CYP2E1 cells with 10 mM n-acetyl-L-cysteine (NAC), an antioxidant, and DCE for 48 h. As a result, we found that NAC treatment could obviously decrease the levels of ROS in HEK-CYP2E1 cells (Fig. 6A). Moreover, we observed that the percentage of apoptosis in HEK293-CYP2E1 cells was decreased by 50.1% (29.8±2.3% to 15.2±1.8%) in presence of NAC, suggesting that NAC did significantly alleviate cell apoptosis caused by DCE (Fig. 6B). Taken together, these results confirm the notion that redundant generation of ROS in the presence of CYP2E1 or S9 could play a critical role in the kidney cells apoptosis induced by DCE.

Discussion

As chlorinated solvents, DCE are known to have general effects on the central

nervous system [4, 39]. However, they also exert specific effects on multi-organ including liver, kidney, reproductive toxicity, and carcinogenicity [7, 39-41]. In this study, we found obvious kidney toxicity induced by DCE in *vivo*, and the decreasing anti-oxidative abilities might be the cause. We confirmed the speculate in vitro tests using human kidney cells with over-expressing CYP2E1 or adding S9 mix, and found that DCE metabolism through CYP2E1 obviously generated excessive ROS and could induce cells apoptosis, and this notion was confirmed by that antioxidant NAC could alleviate it. Thus, we conclude that DCE could induce the kidney toxicity through generating excess ROS in the process of metabolism via CYP2E1.

Previous studies have showed that DCE significantly led to an increase in the kidney weights and renal tubular cells regeneration [26]. Swelling of renal proximal convoluted tubules, proteinuria, and glucosuria are reported in mice given 4800 mg/kg by ip injection [26]. Consistently, we also found that subacute inhaled dosage regimen in male SD rats appeared to adversely affect the kidney of DCE. However, we noticed that there was obvious abnormality in proximal convoluted tubule, from slight intumesce, mild atrophy to internal structure derangement with the increasing concentration of DCE. We further showed that apoptotic cells were detected in proximal convoluted tubules and renal distal tubules at 1000 ppm DCE-exposed groups. Thus, we speculate that DCE exposure might damage the renal tubule through leading to cell apoptosis.

DCE is metabolized via two principal metabolic pathways, involving cytochrome P450-dependent oxidation and a glutathione S-transferase-mediated conjugation with glutathione have been proposed for DCE [42, 43]. The available animal data provide evidence that the majority of absorbed DCE is metabolized via oxidation mediated by CYP450s, with cytosolic glutathione conjugation representing a minor pathway [44]. And, DCE required metabolic bio-activation via CYP2E1 to exert effects on different types of cells [33]. Here, we showed that DCE treatment alone without any metabolic system did not cause any adverse effects on HEK293 cells. However, the addition of S9 or CYP2E1 over expression could obviously enhance the toxicity of DCE and induce cells apoptosis. Considering that CYP2E1 were also high expressed in the

proximal tubule and distal tubule of the kidney besides liver tissue [12-15], suggesting that DCE might be activated by CYP2E1 and cause cytotoxic effects in renal tubules.

It has been proved that CYP2E1 could generate reactive oxygen species (ROS) during catalytic cycles and play an important role in organic solvents-induced oxidant stress and cell toxicity, such as ethanol [45, 46] and trichloroethylene [39]. In our animal tests, we found that DCE exposure led to increasing malondialdehyde (MDA) levels and decreasing antioxidant status in the kidney tissues of rats. And, in *in vitro* tests, we used S9 or over-expression of CYP2E1 in HEK 293 as in vitro metabolic system, results showed that DCE in the presence of S9 or over-expression of CYP2E1 obviously increased the ROS levels. Reactive oxygen species (ROS) play a role in a number of cellular processes from apoptosis and necrosis to cell proliferation and carcinogenesis [47, 48]. The biological consequences of oxidative stress in an organism exceeding background levels can have effects on apoptosis and cell proliferation, contributing to the pathophysiology of a number of diseases, including cancer [24]. To test the proposal the ROS lead to cell apoptosis, The antioxidant n-acetyl-L-cysteine (NAC) was used to determine whether ROS play a causal role in DCE-induced cell apoptosis[49]. Our results showed that NAC obviously inhibited the apoptosis induced by DCE with CYP2E1 and S9. Therefore, we speculate that ROS played a causal role in DCE-induced cell apoptosis.

In summary, we showed that DCE could obviously induced kidney cells apoptosis in both animal tests and human embryonic kidney 293 cells. The generation of ROS in the metabolism of DCE through CYP2E1 plays a key role. And the antioxidant n-acetyl-L-cysteine (NAC) could decrease the adverse effects induced by DCE. These data indicate that anti-oxidative methods might be potential protective factors in kidney toxicity induced by DCE.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1 The damage in SD rat kidney induced by DCE.

Male Spraque-Dawley rats were exposed to DCE at indicated dose 6 h per day for 5 days successively in inhalation chambers. (A) The DCE vapor concentration in the inhalation chambers was measured by performing gas chromatography. The internal exposure of the rats was determined by detecting the blood concentration of DCE in exposure groups. (B)The body weight of the rats were measured. (C) The kidney /weight ratios of the rats were calculated. (D) The urea (UREA) and (E) the Creatinine (CR) of the rats were determined by auto-biochemical analyzer. *$P<0.05$, **$P<0.01$, compared to control group.

Fig. 2 The pathological changes and apoptosis of kidney the rats in DCE dose groups.

Rats were treated with DCE for 5 days successively, kidney tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. (A) Tissue sections were prepared and stained with hematoxylin and eosin. The pathology of kidney glomerulus, proximal convoluted tubule, and distal convoluted tubule were examined by optical microscopy (×100, ×200 magnification). (B) The epithelial cells of glomerulus, proximal convoluted tubule, and distal convoluted tubule were determined using an in situ apoptosis detection kit. Photographs were taken by optical microscopy (×200 magnification). Data are representative of at least three independent experiments.

Fig. 3 The cytotoxicity of HEK293 cells induced by DCE under two metabolic conditions.

(A) HEK-293 cells were treated with DCE at different concentration (0, 125 ppm, 250 ppm, 500 ppm, 1000 ppm, and 2000 ppm) for 24 h, 48 h, 72 h, respectively. Cytotoxicity was measured by trypan blue exclusion at indicated concentrations. The relative survival rate is calculated as the ratio of each dose of DCE to that observed in control cells treated with vehicle. Data were presented as mean ± SEM from three experiments. *$P<0.05$, **$P<0.01$, compared with control group. (B) Immunoblotting analysis of CYP2E1in HEK293 cells expressing CYP2E1. (C) The enzyme activity for CYP2E1 was determined in HEK293, HEK293-vector and HEK293-CYP2E1

cells. (D) HEK293-CYP2E1 cells were treated with DCE at indicated concentration and time. (E) HEK293 cells were treated with DCE at indicated concentration and time in the presence of S9 fraction mix (+S9). The survival rate of cells was measured by trypan blue exclusion.

Fig. 4 DCE induced apoptosis inHEK293 cells.

HEK293-CYP2E1 cells were treated with DCE at indicated concentrations for 48 h. (A) Flow cytometry analysis was performed to assess cells apoptosis. Data are shown as the percent for positive cells of necrosis, apoptosis, and live for three independent experiments. *P<0.05, **P<0.01, compared with control. (B) Immunoblotting analysis was performed with antibodies against Cleavered Caspase-9, Cleavered Caspase-3, and β-actin. Data are representative of at least three independent experiments.

Fig. 5 The effects of DCE in ROS generation and oxidative stress responsive genes expression

HEK293-CYP2E1 cells were treated with the indicated concentrations of DCE and indicated time. (A) The dynamic changes of ROS generation upon DCE treatment. (B) The relative mRNA levels of SOD1, SOD2, CAT, and HO-1 in HEK293-CYP2E1 cells exposed to 0, 250, 500, 1000, and 2000 ppm DCE. The data were shown as mean ± SD for three independent experiments. *, P<0.05, **P<0.01, compared with control cells.

Fig. 6 DCE induced cells apoptosis through excessive ROS generation.

HEK293-CYP2E1 cells were pretreated 10 mM N-Acetyl-L-cysteine (NAC) and followed by treatment with 2000 ppm DCE for 24 h. (A) The amount of ROS was measured by flow cytometry. (B) Flow cytometric analysis was used to detect apoptotic cells. The percent of necrotic, apoptotic, living cells were shown as indicated for three independent experiments.* P<0.05, **P<0.01, compared to the control groups without treatments.

Table 1 Effects of DCE on intracellular levels of T-AOC, SOD, GSH, and MDA in kidney

		T-AOC (mmol/g)	SOD (U/mg)	GSH (mmol/g)	MDA # (mmol/g)
Control		8.02±0.81	1.90±0.29	2.97±0.37	20.41±4.90
DCE (ppm)	333	7.60±0.66	1.71±0.32	2.88±0.38	22.42±2.51
	557	6.85±1.28*	1.26±0.42*	2.48±0.4*	31.86±3.09**
	1000	5.21±1.16*	1.13±0.43**	1.96±0.48*	51.27±5.53**

DCE, 1,2-dichloroethane; T-AOC, total antioxidant; SOD, superoxide dismutase; GSH, glutathione; MDA, malondialdehyde. *P<0.05, **P<0.01, compared to the control group. #: *Test for trend, P<0.05*.

Figure 1

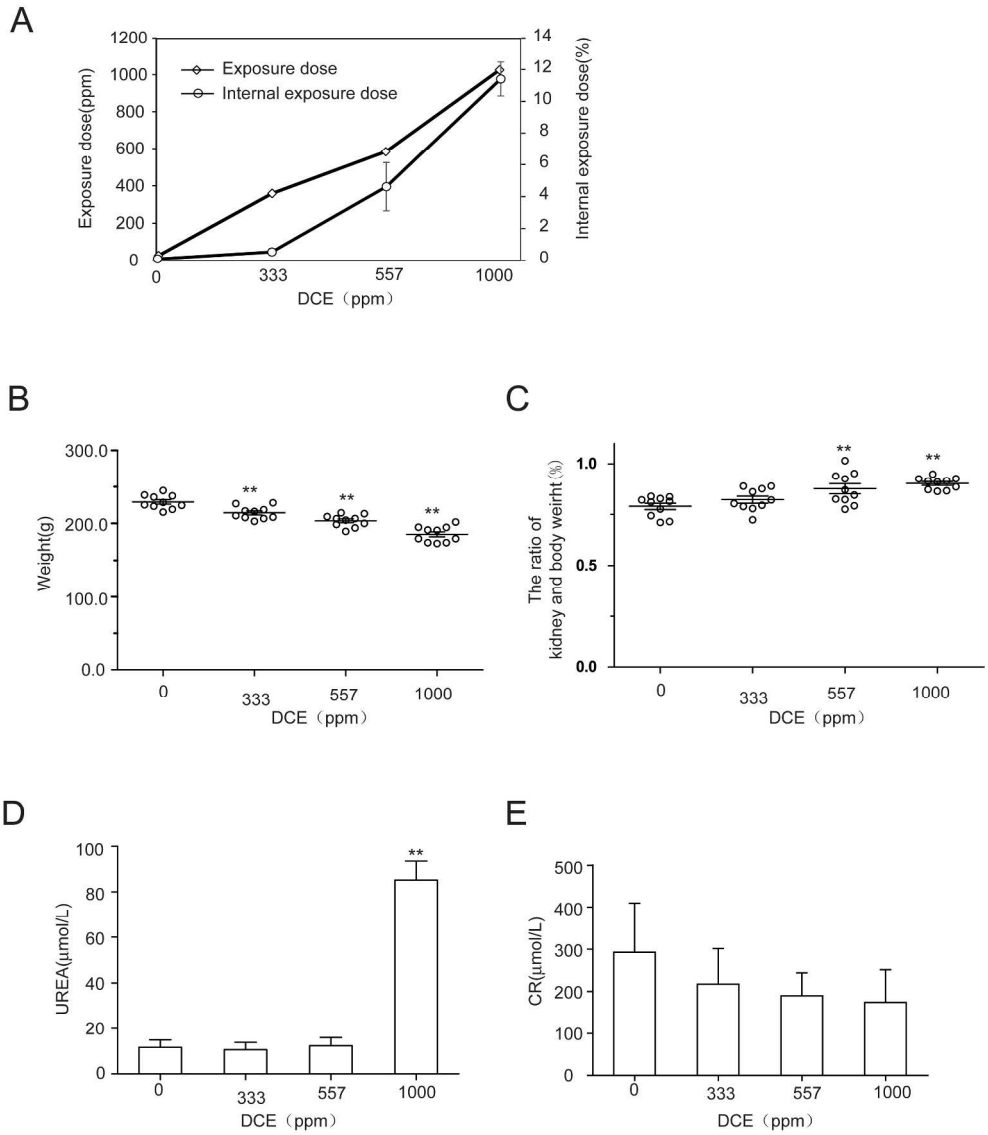

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Figure 2

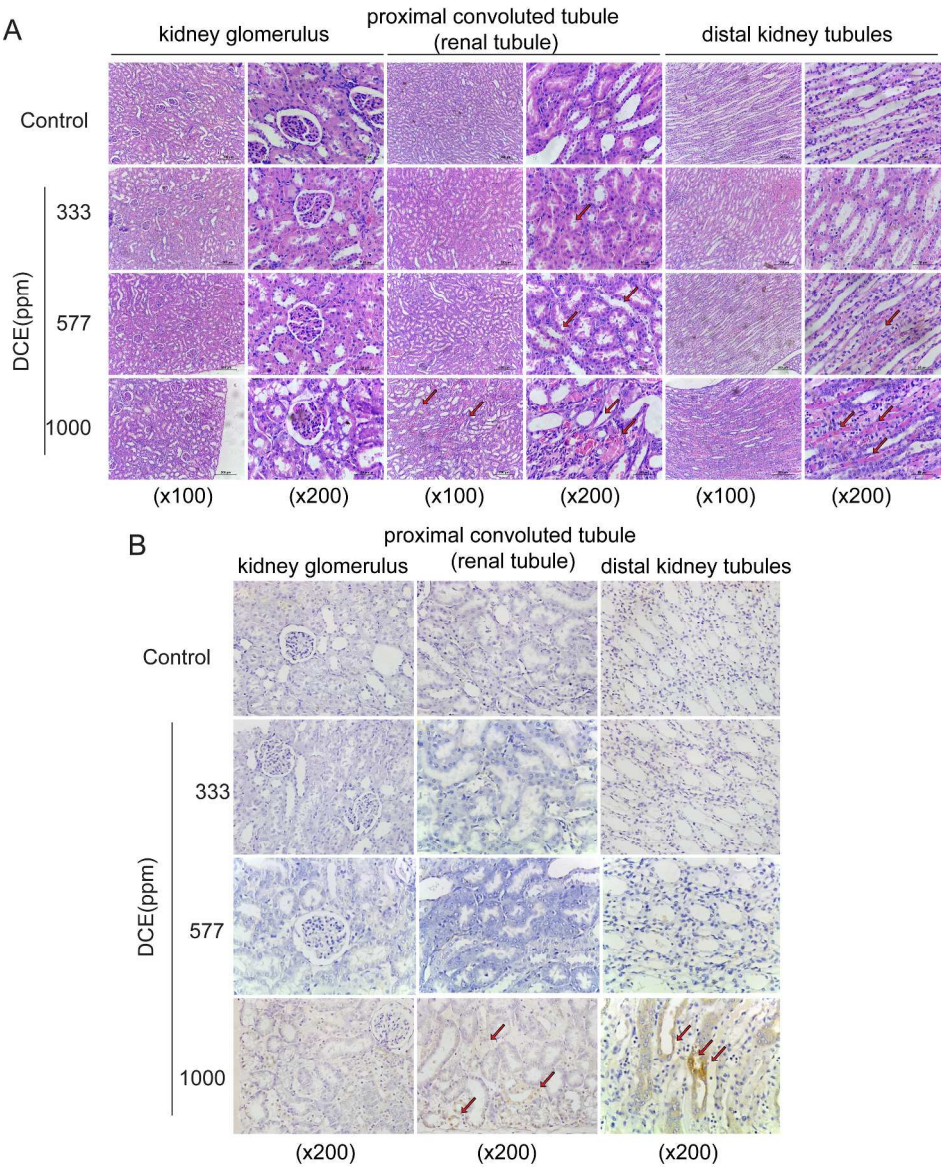

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Figure 3

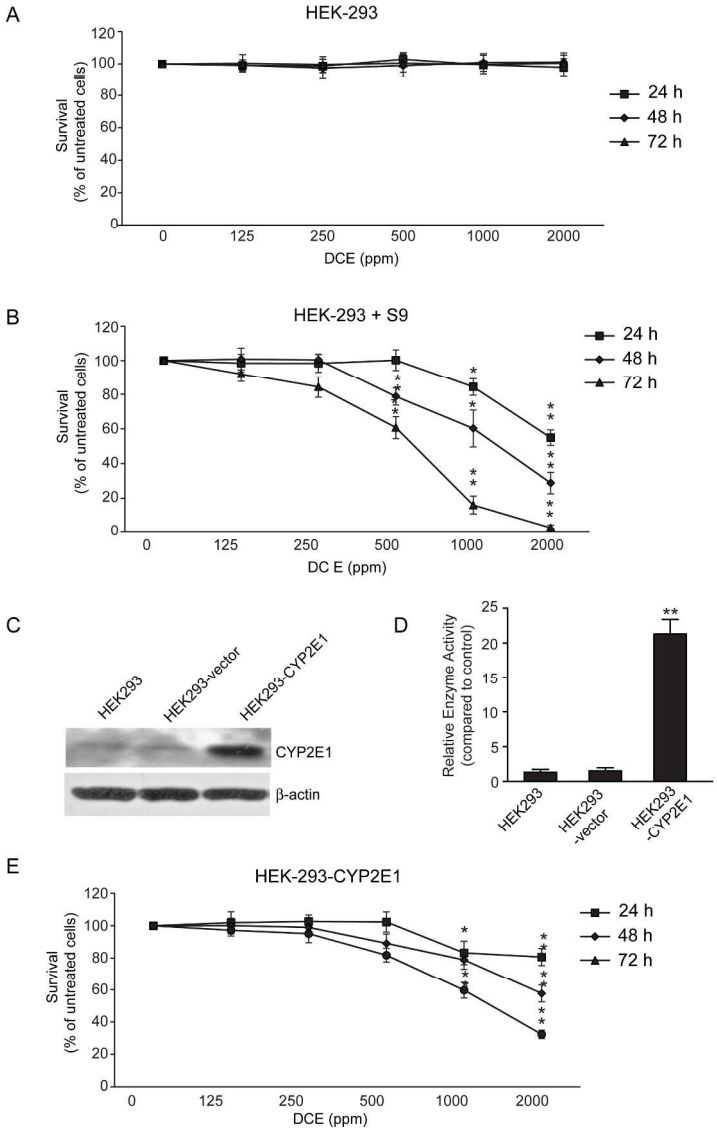

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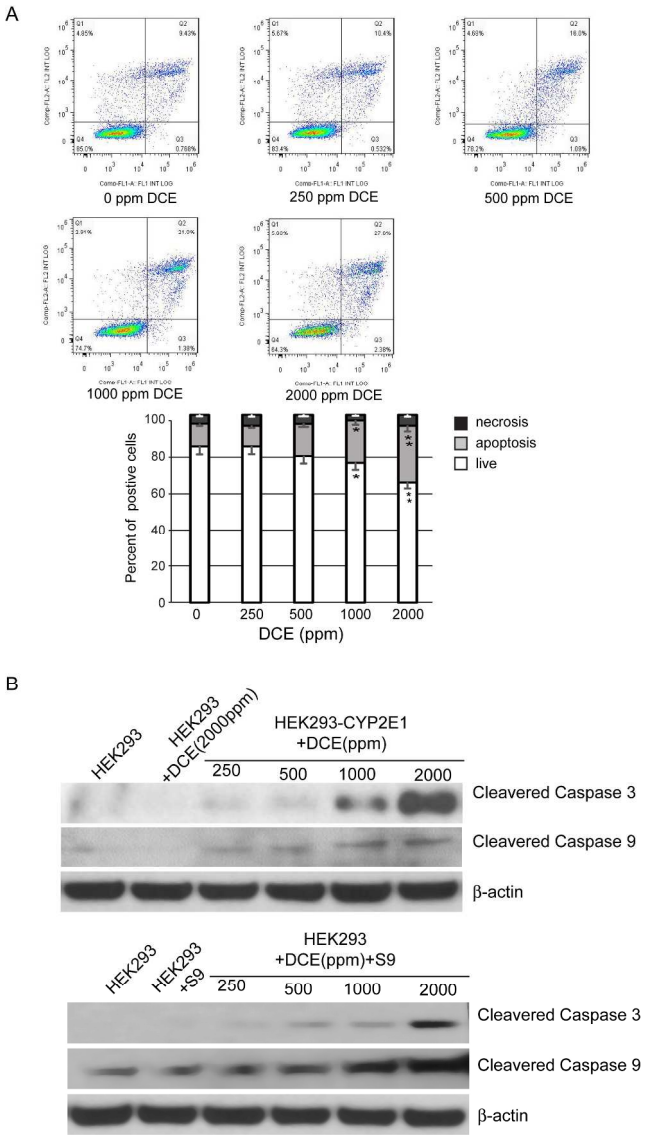
Figure 4



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158x298mm (300 x 300 DPI)

Figure 5

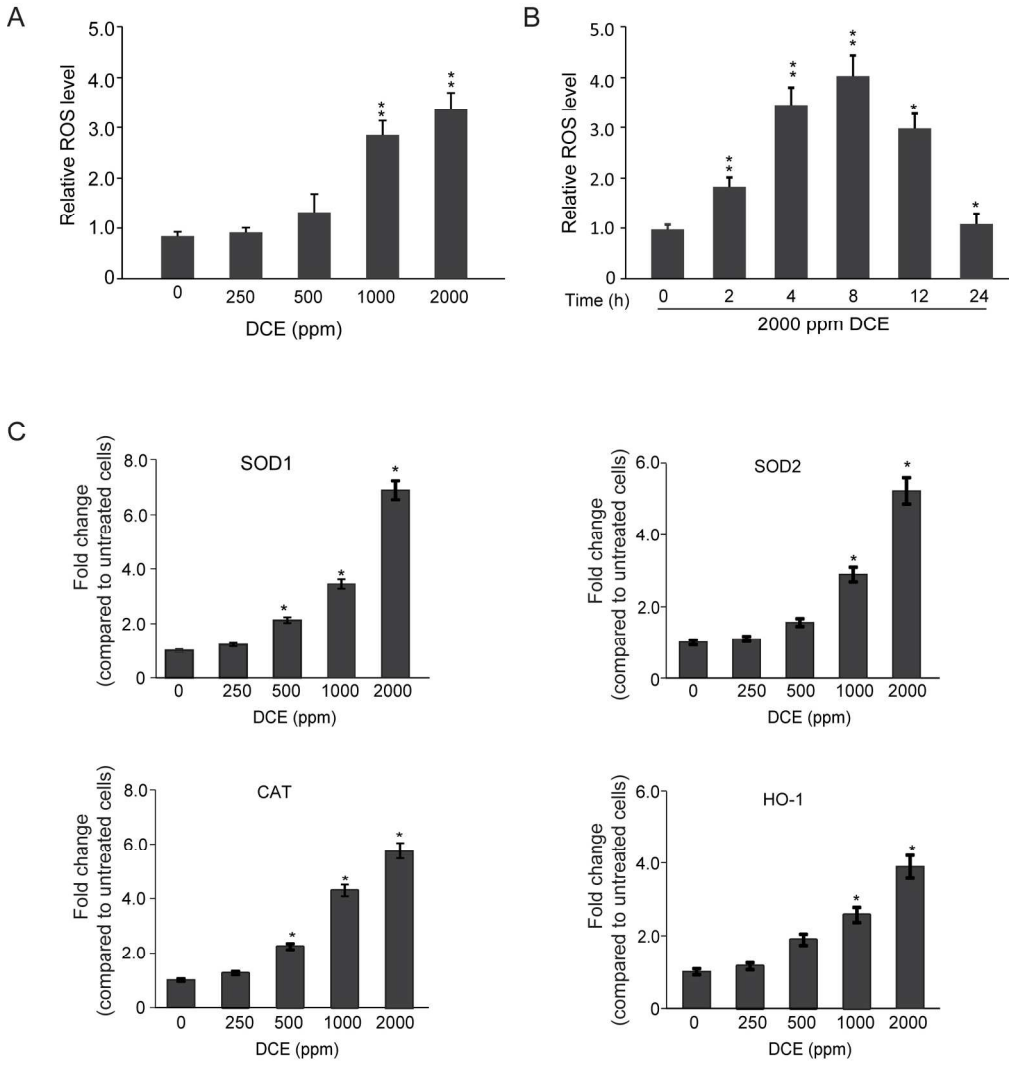

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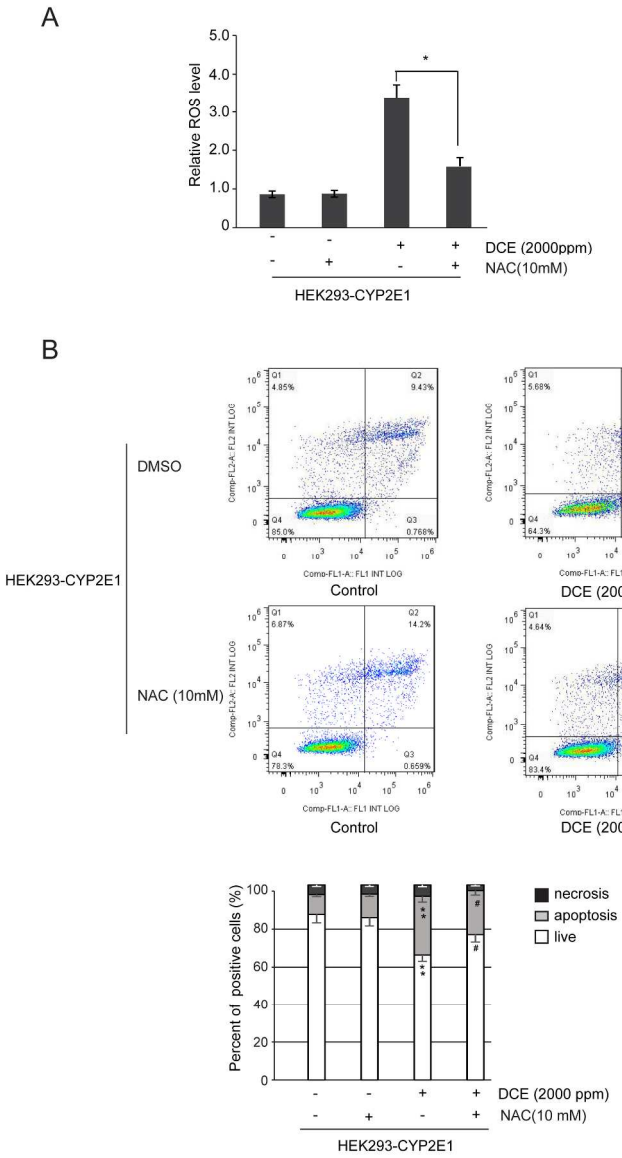
Figure 6



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