Induction of DNA Damage and \(G_2\) Cell Cycle Arrest by Diepoxybutane through the Activation of the Chk1-Dependent Pathway in Mouse Germ Cells

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Supporting Information

ABSTRACT: 1,2,3,4-Diepoxybutane (DEB) is a major carcinogenic metabolite of 1,3-butadiene (BD), which has been shown to cause DNA strand breaks in cells through its potential genotoxicity. The adverse effect of DEB on male reproductive cells in response to DNA damage has not been thoroughly studied, and the related mechanism is yet to be elucidated. Using mouse spermatocyte-derived GC-2 cells, we demonstrated in the present study that DEB caused the proliferation inhibition and marked cell cycle arrest at the \(G_2\) phase but not apoptosis. DEB also induced DNA damage as evidenced by \(\gamma\)-H2AX expression, the comet assay, and the cytokinesis-block micronucleus assay. Meanwhile, DEB triggered the Chk1/Cdc25c/Cdc2 signal pathway, which could be abated in the presence of UCN-01 or Chk1 siRNA. GC-2 cells exposed to DEB experienced ROS generation and pretreatment of N-acetyl-L-cysteine, partly attenuated DEB-induced DNA damage, and \(G_2\) arrest. Furthermore, measurement of testicular cells showed an increased proportion of tetraploid cells in mice administrated with DEB, alongside the enhanced expression of p-Chk1. Also, the defective reproductive phenotypes, including reduced sperm motility, increased sperm malformation, and histological abnormality of testes, were observed. In conclusion, these results suggest DEB induces DNA damage and \(G_2\) cell cycle arrest by activating the Chk1-dependent pathway, while oxidative stress may be associated with eliciting toxicity in male reproductive cells.

INTRODUCTION

1,3-Butadiene (BD; CAS No. 106-99-0) is an important industrial chemical that is widely used in the manufacture of rubber and thermoplastic resins.\(^1\) Epidemiology data showed that BD, a flammable gas, poses an occupational exposure risk to production workers, especially to those who are involved in its production and those exposed to industrial emissions during its synthesis of rubber. BD is also commonly found in automobile exhaust and cigarette smoke produced by the incomplete combustion of natural and manmade materials. A hazard analysis based on the results of 77 published studies identified BD as one of the nine priority air pollutants in residences in the United States and in countries with similar lifestyles.\(^2\) Thus, BD exposure has been extended from occupational workers to the general population. The potential health effects of BD have been extensively studied. Moreover, from the study, it has been proved that BD is carcinogenic in laboratory rodents, inducing tumors at multiple sites. More recently, the International Agency for Research on Cancer (IARC)\(^3\) raised the carcinogenic classification of BD from Group 2A (probable human carcinogen) to Group 1 (known human carcinogen), mainly based on the epidemiological evidence of BD workers, which supported the presence of a causal relationship between high cumulative exposure and high intensity of exposure to BD and leukemia.\(^4\) Because of the carcinogenicity, BD exposure and its potential adverse effects on various tissues and biosystems have become the topic of investigation.

Butadiene is a four-carbon compound with the molecular formula of \(\text{C}_4\text{H}_6\). The two double bonds in BD can be oxidized to epoxides, which are then rearranged to form reactive metabolites.\(^5\) A simplified scheme of the formation of reactive epoxides is illustrated in Figure S1 (Supporting Information). Studies have shown that the carcinogenicity of BD is attributed to its major epoxy metabolites, 1,2-epoxy-3-butene (EB), 1,2,3,4-diepoxybutane (DEB), and 1,2-dihydroxy-3,4-epoxybutane (EBD) rather than BD itself,\(^6,7\) with DEB being the most potent metabolite due to its capacity as a bifunctional electrophile.\(^8\) Previous reports indicate that the greater carcinogenicity of BD in mice over rats appears to be as a result of greater production of DEB in mice.\(^9\) There is also accumulating evidence that all three metabolites are genotoxic in vivo and in vitro, with the relative mutagenic potencies of DEB \(\gg\) EB \(>\) EBD.\(^10\) Experiments indicated that DEB induces not only gene mutations in different assay systems but also chromosome-level changes including aberrations, aneuploidy, and micronuclei (MN).\(^10\)
In addition to being carcinogenic in laboratory rodents and exposed workers, exposures to BD are associated with reproductive toxicity, and there was evidence indicating that male reproductive genotoxicity of BD may still depend on the production of potent metabolites, corresponding to the results from carcinogenicity studies.\(^{10,11}\) Previously in this study, we demonstrated the increased frequencies of MNi and nucleoplasmic bridges (NPBs) in BD-exposed workers.\(^{12}\) In line with the epidemiological study, experimental studies have shown DEB to be a potent inducer of chromosome aberrations likely that the mutagenic effect of DEB is mediated, to a large extent, by causing clastogenic damage of DNA. Generally, DNA damage activates a complex damage response pathway, namely, DNA damage response (DDR). This pathway coordinates cell cycle checkpoint arrest, DNA repair, or apoptosis to mediate the maintenance of the genomic integrity by activating various sensors and effectors, including ataxia-telangiectasia-mutated (ATM), ATM and Rad3-related (ATR), and their target proteins, which are often involved in response to DSB.\(^{13}\) However, DEB-induced DNA damage responses in testicular cells and candidate pathways are still undetermined.

As to the underlying mechanisms of genotoxic damage, it is well documented that DEB can react directly with DNA to produce covalent bonds at different sites in the molecule to form a variety of adducts, resulting in DNA damage.\(^{14,15}\) Alternatively, genotoxicity may be indirect. One manifestation of the indirect genotoxicity is the generation of oxidative DNA damage. Accumulated data pointed to an important role of reactive oxygen species (ROS) in DNA damage.\(^{16,17}\) When cellular production of ROS overwhelms its antioxidiant capacity, a multitude of oxidative modifications to DNA bases may ensue, leading to mispairing and DNA strand breaks, with the phosphorylation of variant histone H2AX (named γ-H2AX) representing DNA double-strand breaks and repair.\(^{18,19}\) DEB toxicity is also associated with oxidative stress since the epoxide structure of DEB implies redox-mediated catalysis in the rearrangement of oxygen bands.\(^{20}\) Meanwhile, initial studies by using sea urchins, a widely used assay for detecting developmental, reproductive, and cytogenetic toxicity of environmental toxicannts, indicated that DEB modulated catalase activity, led to substantial suppression of glutathione (GSH), and the formation of DNA oxidative damage by 8-hydroxy-2′-deoxyguanosine (8-OHdG).\(^{20,21}\) It was also documented that DEB-induced apoptosis was mediated by the generation of ROS in human TK6 lymphoblasts.\(^{22}\) Although oxidative stress has been implicated in DEB-induced cytotoxicity.\(^{23}\) The extent and biological impact of oxidative stress-mediated damage in testicular cells after DEB exposure requires further investigation.

The objective of this study is to understand the underlying mechanisms of action of DEB on male reproductive cell damage, with emphasis on the understanding of DNA damage response and oxygen-dependent toxicity. Both in vitro chemical exposed mouse spermocyte-derived GC-2 cells and in vivo mice were used as exposure models. The results confirmed that DEB could induce DNA double-strand breaks in germinal cells, and we first reported DEB-induced G2 phase arrest by activating the Chk1/Cdc25c/Cdc2 signal pathway. Direct exposure to DEB increased the level of ROS, and pretreatment of N-acetyl-L-cysteine (NAC), a potent antioxidant, could partly attenuate DEB-induced DNA damage and G2 arrest. These findings further demonstrated that oxidative stress might play roles in DEB-induced toxicity in the male reproductive system.

## MATERIALS AND METHODS

**Chemicals and Antibodies.** 1,2,3,4-Diepoxybutane (DEB, purity \(>99.5\%\)) was purchased from Chem Service, Inc. (West Chester, PA, USA) and subsequently diluted in phosphate-buffered saline (PBS). NAC was obtained from Beyotime Institute of Biotechnology (Beiyotme, China). Cytochalasin B was purchased from Sigma-Aldrich (St. Louis, MO, USA). UCN-01, which was used as a Chk1 inhibitor, was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). It was dissolved in dimethyl sulfoxide (DMSO). Chk1 siRNA and scrambled siRNA were from RIBOBIO (Guangzhou, China). Commercially available antibodies used were as follows: CyclinB1, Cdc25c, Chk1, and phospho-H3 (Ser 10) were purchased from Santa Cruz Biotechnology. Phospho-cdc2 (Y15) and phospho-Chk1 (Ser 345) were purchased from Cell Signaling Technology (Danvers, MA, USA). ATM, phospho-ATM (Ser1981), and cd2c were obtained from Abcam (Cambridge, MA, USA). Phospho-Cdc2S (Ser 216), horseradish peroxidase (HRP)-conjugated goat antibabt IgG (H + L), and goat antymouse IgG (H + L) secondary antibodies were from Faintbodyi (NY, USA), and Rhodamine-conjugated goat antiamouse IgG-R was from Santa Cruz Biotechnology.

**Cell Culture and Treatments.** The mouse premeiotic spermatoocyte-derived GC-2 cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). It was cultured in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS, GIBCO, Grand Island, NY, USA). The cells were incubated at 37 °C in a fully humidified atmosphere with 5% CO2. For DEB treatment, appropriate amounts of stock solutions of the chemical were added into the medium to achieve the indicated concentrations. Phosphate buffered saline solution was used as the control reagent.

For the siRNA-knockdown experiment, a double-stranded RNA duplex that targeted the mouse Chk1 gene was used (sense, 5′-GGUGGAUGUGACAGAGCUUUdTdTdT3′; antisense, 3′-dTdTGCA-CCAUACAGUCUCAGAA5′). siRNA was synthesized by RIBOBIO Co., Ltd. (Guangzhou, China). In vitro transfection was performed using riboFECTTM CP Transfection Kit (Guangzhou, China), according to the manufacturer’s protocols. Briefly, GC-2 cells were seeded into 6-well plates at a density of 1 × 105 cells/well in DMEM overnight. Cells were reversely transfected with siRNA at a concentration of 50 nM. After 36 h of incubation, cells were treated with 500 μM DEB for 24 h. Then, cells were harvested for the following experiments.

**Cell Viability and Proliferation Detection.** The effect of DEB on cell viability was assessed using a Cell Counting Kit-8 (CCK-8) assay. The cells were seeded in 96-well flat-bottomed plates (Corning, NY, USA) at a density of 5000 cells per well overnight, then treated with different concentrations of DEB for 24 and 48 h. Each well was filled with a 10 μl aliquot of CCK-8 solution and incubated for another 2 h. The optical density was measured at 450 nm wavelength using a microplate reader (Molecular Devices, Silicon Valley, USA). For cell proliferation determination, an EdU incorporation test was carried out using Cell-Light EdU Apollo488 In Vitro Imaging Kit (Guangzhou, China). The GC-2 cells were seeded into 6-well plates at a density of 1 × 105 cells/well in DMEM overnight.

**Mitotic Index Analysis.** GC-2 cells were treated with 50 μM and 100 μM EdU for 24 h. Then, the cells were rinsed with PBS three times and fixed with 4% paraformaldehyde for 15 min. Then, cells were labeled with Apollo488 azide and Hoechst 33342 dye, respectively. Fluorescence images were observed using Nikon Eclipse (Nikon, Japan), and the number of EdU-positive cells were counted.

**Mitotic Index Analysis.** GC-2 cells were treated with DEB for 24 h. Then, the cells were rinsed with PBS three times and fixed with 4% paraformaldehyde for 30 min. Next, the cells were stained with 5% (v/v) Giemsa for 30 min, subsequently flushed with tap water, and left to dry in the air. The mitotic cells were recognized as metaphase nuclei using a light microscope. At least 1,000 cells were randomly scored to determined the mitotic index (MI) of each culture.

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Figure 1. continued
were measured with a FC500 Cell Cycle Analysis Kit (Beyotime, China), cells were incubated in overnight. After centrifugation and resuspension in staining buffer, cells were harvested for cell cycle and apoptosis analysis. For cell cycle analysis, cells were stained with propidium iodide, and DNA contents of cells were measured with a FC500 flow cytometer. The percentage of cells in each phase of the cell cycle was analyzed using ModFit software. Values represent the mean ± SD of three independent experiments, **p < 0.01 and ***p < 0.001 versus the control. (C) After exposure to different concentrations of DEB for 24 h, the cells were stained with propidium iodide, and DNA contents of cells were measured with a FC500 flow cytometer. The percentage of cells in the each phase of the cell cycle was analyzed using ModFit software. Values represent the mean ± SD of three independent experiments, **p < 0.01 and ***p < 0.001 versus the control. (D) GC-2 cells were exposed to DEB for 24 and 48 h. Cells were collected, incubated with Annexin V-FITC and propidium iodide, and the level of p-H3 expression was detected by Western blot. (F) Mitotic index was analyzed by Giemsa staining after treatment with different concentrations of DEB for 24 h. Values represent the mean ± SD of three independent experiments, *p < 0.05 and **p < 0.01 versus the control.

Alkaline Comet Assays. The alkaline comet assay was carried out as described previously.23 GC-2 cells were mixed with 200 μL of low-melting-point agarose at 0.7% in PBS and added to microscope slides precoated with 0.8% normal-melting-point agarose. Then, the slides were carefully immersed in cold lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris-HCl, 1% Triton X-100, and 10% DMSO, pH 10) for 1 h at 4 °C. After lysis, DNA was allowed to unwind for 20 min in an alkaline solution (1 mM Na2EDTA and 300 mM NaOH, pH 13). Electrophoresis was carried out for 20 min and then stained with ethidium bromide (Sigma, USA). Cells were viewed with Nikon Eclipse (Nikon, Japan), and 500 randomly chosen cells were scanned per culture. The image analysis was performed with CASP software (CASP, Wroclaw, Poland).23 The tail length, percentage of DNA in the tail, and tail moment were recorded to evaluate DNA damage.

Cytokinesis-Block Micronucleus (CBMN) Assay. The CBMN assay was performed according to the protocol described by Fenech,26 with minor modifications. The GC-2 cells were treated with DEB for 24 h, and cytokalasin-B was added to each culture, reaching a final concentration of 3 μg/mL to prevent cytokinesis. Cells were harvested and fixed in methanol/acetic acid (3:1). Subsequently, a cell suspension was dropped onto clean slides. Slides were air-dried and xed in methanol for 10 min, then stained with Giemsa. For each culture, a total of 1,000 binucleated cells were analyzed for the presence of MNi, NPBs, and nuclear buds (NBUDs). The numbers of mono-, bi-, tri-, and tetra-nucleated cells in 500 GC-2 cells were also scored for the nuclear division (NDI) calculation.

Measurement of Reactive Oxygen Species (ROS). The level of intracellular ROS was measured with the nonfluorescent probe DCFH-DA (Beyotime, China). DCFH-DA can passively diffuse into cells and be oxidized to fluorescent DCF by the action of cellular oxidants. After DEB treatment, the cells were collected, incubated with DCFH-DA (10 μM) for 30 min at 37 °C, and washed twice with PBS. The fluorescence of DCF in cells was detected by a FC500 flow cytometer (Beckman, USA).

Cell Cycle and Apoptosis Analysis. After DEB treatment, the cells were harvested for cell cycle and apoptosis analysis. For cell cycle analysis, cells were fixed in 70% ice-cold ethanol and stored at 4 °C overnight. After centrifugation and resuspension in staining buffer of Cell Cycle Analysis Kit (Beyotime, China), cells were incubated in propidium iodide (PI) for 30 min at 37 °C. The DNA contents of cells were measured with a FC500 flow cytometer (Beckman, USA) or FACS Calibur flow cytometer (BD, USA). The percentage of cells in different phases was quantitated using ModFit 2.0 software. For cell apoptosis analysis, cells were processed according to the instructions of the manufacturer of Annexin V-FITC Apoptosis Detection Kit (Beyotime, China). The samples were immediately analyzed using an FC500 flow cytometer, and data were analyzed using CXP analysis software (Beckman, USA).

Immunofluorescence Staining (IF). Cells were grown on glass-bottomed cell culture dishes (NEST, China) in media. After washing with PBS, cells were fixed in 100% methanol followed by permeabilization with 0.2% Triton X-100/PBS. After blocking in 3% bovine serum albumin (BSA)/PBS, cells were incubated with anti-Cyclob1 antibody (1:50 dilution) at 4 °C overnight. Rhodamine-conjugated goat antirabbit IgG-R (1:100 dilution) was added for an additional 1 h of incubation. The cell nuclei were then labeled with Hoechst 33258 for 10 min. The cells were observed with a laser scanning microscope (LSM 700; Carl Zeiss, Oberkochen, Germany).

Western Blot Analysis. After DEB treatment, cells were lysed in cell lysis buffer (Beyotime, China). Protein concentration was determined by the BCA assay (Beyotime, China). Fifty micrograms of protein was separated by 8%–12% SDS–PAGE and transferred onto a polyvinylidene fluoride membrane. The membranes were soaked in 5% skim milk or 3% BSA at room temperature for 2 h and incubated with primary antibodies overnight at 4 °C. After washing with TBST (20 mM Tris-HCl, 1500 mM NaCl, and 0.1% Tween 20), the membranes were incubated with HRP-conjugated secondary antibodies for 1 h. The proteins were then detected using the ECL detection kit.

Animals and Treatment. Adult Kun-Ming mice (10–12 weeks old) weighing 28.1–32.3 g were purchased from the Animal Center of Third Military Medical University (TMMU), China. The animals were kept in plastic cages and fed with food and tap water. Individual mice were weighed and randomly assigned to either the control or the treatment group composed of 6 animals each under standard conditions of a 12 h light/dark cycle in 20 °C and 50% humidity. The mice received a single intraperitoneal injection of DEB on days 1, 3, and 5 injection intervals, dissolved in PBS at doses of 0, 17, 42.5, and 85 mg/kg. Dilutions were adjusted in order to inject the mice with 0.01 mL/g body weight. The animals were weighed and sacrificed 7 days after the last treatment. This study was approved by the Institutional Animal Care of TMMU.

Epididymal Sperm Collection and Assessment. Sperm were collected from the cauda epididymis of mice and placed into 0.2 mL of capacitation medium (HTF medium; CooperSurgical Company, Trumbull, CT, USA) at 37 °C. A 5 μL drop of sperm was wet-
Figure 2. Effect of DEB on DNA damage in the GC-2 cells. (A) GC-2 cells were exposed to DEB for 24 h, then the effect on chromosomal damage was assessed using the CBMN assay. The representative pictures of MN, NPBs, and NBUD are presented. (B) After treatment with DEB for 24 h in GC-2 cells, DNA damage was detected by an alkaline comet assay as described in Materials and Methods. The representative pictures of comets in each group are shown. (C) GC-2 cells were treated with DEB for 24 h. The level of γ-H2AX was detected using Western blot analysis.

DEB Inhibits Cell Proliferation and Induces G2 Phase Arrest but Not Apoptosis in GC-2 Cells. In this study, DEB has shown strong antiproliferation activity in GC-2 cells. The cytotoxic effect of DEB on GC-2 cells was evaluated by CCK-8 and EdU assays. As shown in Figure 1A, DEB induced significant reduction of cell viability compared to that of control in cells treated with DEB of 100, 300, and 500 μM for 24 and 48 h. Concomitant with the decrease in cell viability, DNA synthesis rates were significantly decreased in DEB-treated cells as evidenced by an EdU assay (P < 0.05) (Figure 1B). On the basis of these results, it was deduced that DEB did inhibit the proliferation of GC-2 cells. To clarify whether DEB-induced cell growth inhibition was ascribed to cell cycle arrest or apoptosis, we examined the cell cycle distribution and the staining of Annexin V-FITC by flow cytometry. As shown in Figure 1C, it was revealed that compared with the control, the cell proportion in the G2/M phase was accumulated significantly accompanied by a decrease in the G0/G1 phase after exposure to DEB with 100, 300, and 500 μM for 24 and 48 h. For DEB, previous studies have reported that DEB possesses an apoptosis-inducing ability in human lymphoblasts. On the basis of this, we assumed that DEB might play an important role in the inhibition of cell growth in vitro. In order to determine whether the growth decrease in DEB-treated GC-2 cells was due to apoptotic death, we tested the effect of DEB on apoptosis using flow cytometry. Result showed that there was no effect of DEB on apoptosis after treatment for 24 and 48 h (Figure 1D). The results indicated that DEB-induced G2/M arrest but not apoptosis.
DEB could cause G2 arrest and prevent GC-2 cells from damage in germline cells as well as in somatic cells. To clarify Cells. entering mitosis (M-phase).

The entry into mitosis is regulated by the activation of CyclinB-cdc2 complex (M-phase promoting factor, MPF), wherein Cdc2 is maintained in an active status via dephosphorylation modification at residues Tyr-15 by phosphatase Cdc25c.29 Consistent with the changes of the phosphorylated inactive state of Cdc25c after DEB treatment, the level of phospho-Cdc2 was upregulated (Figure 3B), suggesting the inactivation of Cdc2. Although DEB treatment upregulated the expression of CyclinB1 by Western blot analysis, the distribution of CyclinB1 in the nucleus was gradually decreased in a dose-dependent manner using immunofluorescence staining (Figure 3C). These results indicated that the DEB-induced Chk1 signaling pathway may relay the cascade that ultimately serves to inactivate the CyclinB1-cdc2 complex.

DEB Induces G2-Phase Arrest by Activating Chk1 Signaling. Since the phosphorylated expression of Chk1 was upregulated with DEB treatment, we next evaluated whether Chk1 is responsible for DNA damage signal transduction and G2-phase arrest induced by DEB. A potent Chk1 phosphorylation (Ser 345) inhibitor, UCN-01, was used as a tool compound to study Chk1-dependent pathway.30,31 In the study, GC-2 cells were treated with 300 nM UCN-01 for 4 h prior to DEB treatment for 24 h. Flow cytometry analysis showed the proportion of cells in G2 phase was 37.1% in UCN-01 + DEB group, while the DEB-treated group showed 47% of G2-phase cells (Figure 4A), indicating the inhibition of Chk1

Table 1. Chromosomal Damage Detected by the Cytokinesis-Block Micronucleus Assay

<table>
<thead>
<tr>
<th>DEB (μM)</th>
<th>NDI</th>
<th>MN (%)</th>
<th>NBUD (%)</th>
<th>NPB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.89 ± 0.03</td>
<td>1.3 ± 0.4</td>
<td>1.2 ± 0.5</td>
<td>7.8 ± 0.7</td>
</tr>
<tr>
<td>100</td>
<td>1.32 ± 0.04</td>
<td>13.8 ± 0.5</td>
<td>1.8 ± 0.5</td>
<td>28.5 ± 0.8</td>
</tr>
<tr>
<td>300</td>
<td>1.13 ± 0.01</td>
<td>33.2 ± 3.2</td>
<td>3.9 ± 0.3</td>
<td>38.95 ± 0.95</td>
</tr>
<tr>
<td>500</td>
<td>1.14 ± 0.01</td>
<td>43.5 ± 1.68</td>
<td>3.85 ± 0.45</td>
<td>42.2 ± 3.8</td>
</tr>
</tbody>
</table>

Data represent the mean ± SE of three independent experiments. a p < 0.05 versus the control. b Nunuclear division index. c Micronuclei. d Nuclear bud.

control, DEB-treated cells showed a dramatic increase of DNA strand breaks in all three concentrations. The phosphorylation of histone H2AX (Ser 139) is a sensitive molecular marker for DNA double-strand breaks.18,28 Western blot analysis further confirmed that DEB could produce DNA damage by the upregulated expression of γ-H2AX (Figure 2C). These results indicated the clastogenicity of DEB in GC-2 cells.

DEB-Induced DNA Damage Response Is Involved in Chk1 Signal Pathways. The G2/M DNA damage checkpoint serves to prevent the cells from entering mitosis in response to genomic DNA damage. We went a further step to clarify the molecular mechanisms of the DEB-induced G2/M DNA damage checkpoint. Accumulating data have reported that the ATM and ATR acted as sensory kinases to transduce DNA damage signals and mediate cell cycle checkpoint regulation.13 First, the level of total protein and phosphorylated protein expression of ATM and ATR was examined in cells treated with DEB for 24 h by Western blot. The results showed that when compared with the control, DEB treatment markedly increased the phosphorylation of ATM in a dose-dependent manner, while the total protein expression remained unaffected as shown in Figure 3A. In contrast, the expression level of ATR, including total and phosphorylated protein, showed no obvious variation (data not shown). To ascertain phosphorylated downstream substrates of ATM, phospho-Chk1 and phospho-Chk2 were detected. The phosphorylation level of Chk1 was upregulated in a dose-dependent manner (Figure 3A), whereas there was no obvious effect of DEB on phospho-Chk2 expression (data not shown). This result suggested that DEB could induce the activation of ATM and Chk1 and that the relayed signal cascade may be involved in the regulation of the DNA damage response after DEB exposure. Additionally, Chk1 and Chk2 have been proposed to share the common substrate of Cdc25c protein phosphatase, which is phosphorylated at serine-216 and then lost its activity of acting as the positive regulator in driving cell cycle progression.29 We found in the present study that DEB elevated the phosphorylation of Cdc25c using phospho-Cdc25c antibody (Ser 216), while the total protein level of Cdc25c did not change significantly (Figure 3A).

The entry into mitosis is regulated by the activation of CyclinB-cdc2 complex (M-phase promoting factor, MPF), wherein Cdc2 is maintained in an active status via dephosphorylation modification at residues Tyr-15 by phosphatase Cdc25c.29 Consistent with the changes of the phosphorylated inactive state of Cdc25c after DEB treatment, the level of phospho-Cdc2 was upregulated (Figure 3B), suggesting the inactivation of Cdc2. Although DEB treatment upregulated the expression of CyclinB1 by Western blot analysis, the distribution of CyclinB1 in the nucleus was gradually decreased in a dose-dependent manner using immunofluorescence staining (Figure 3C). These results indicated that the DEB-induced Chk1 signaling pathway may relay the cascade that ultimately serves to inactivate the CyclinB1-cdc2 complex.

DEB Induces G2-Phase Arrest by Activating Chk1

Signaling. Since the phosphorylated expression of Chk1 was upregulated with DEB treatment, we next evaluated whether Chk1 is responsible for DNA damage signal transduction and G2-phase arrest induced by DEB. A potent Chk1 phosphorylation (Ser 345) inhibitor, UCN-01, was used as a tool compound to study Chk1-dependent pathway.30,31 In the study, GC-2 cells were treated with 300 nM UCN-01 for 4 h prior to DEB treatment for 24 h. Flow cytometry analysis showed the proportion of cells in G2 phase was 37.1% in UCN-01 + DEB group, while the DEB-treated group showed 47% of G2-phase cells (Figure 4A), indicating the inhibition of Chk1

Table 2. Alkaline Comet Assay for DNA Damage after Treatment with DEB

<table>
<thead>
<tr>
<th>DEB (μM)</th>
<th>tail length</th>
<th>tail DNA %</th>
<th>tail moment</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>5.07 ± 0.54</td>
<td>0.37 ± 0.08</td>
<td>0.92 ± 0.32</td>
</tr>
<tr>
<td>100</td>
<td>44.39 ± 2.7b</td>
<td>8.61 ± 0.62b</td>
<td>7.13 ± 0.70b</td>
</tr>
<tr>
<td>300</td>
<td>54.99 ± 2.67b</td>
<td>8.45 ± 0.72b</td>
<td>6.06 ± 0.80b</td>
</tr>
<tr>
<td>500</td>
<td>52.67 ± 1.94b</td>
<td>7.73 ± 0.44b</td>
<td>5.41 ± 0.48b</td>
</tr>
</tbody>
</table>

Data represent the mean ± SE of three independent experiments. a p < 0.05 compared with the control group. b DNA fragmentation was measured by CASP software.
partly attenuated the accumulation of G2-phase cells induced by DEB. Furthermore, Western blotting analysis demonstrated that the expression of phospho-Chk1 (Ser 345) was significantly downregulated in the UCN-01 pretreatment group as shown in Figure 4B. Ser 216 on Cdc25c, a known substrate for Chk1, showed a decrease of phosphorylated level in UCN-01 + DEB group compared to that of DEB treatment alone (Figure 4B). Consistent with the data described above, UCN-01 pretreatment inhibited DEB-induced upregulation of phospho-Cdc2, a downstream effector of the Chk1 signal transduction cascade.

To further confirm the role of Chk1 on mediating DEB-induced G2-phase transition, we conducted RNA interference to knock down the expression of the Chk1 protein. As shown in Figure 4C, the expression of Chk1 protein was efficiently downregulated after cells were transfected with Chk1 siRNA. Meanwhile, knockdown of Chk1 partly abrogated the changes induced by DEB on phospho-Cdc25c and phospho-cdc2 (Figure 4C). When the results described above are combined, activation of Chk1 could account for, in part, the DEB-induced G2-phase arrest in GC-2 cells.

**DEB-Induced DNA Damage and G2 Arrest Is Associated with Oxidative Stress.** To determine the mechanisms contributing to the DNA damage induced by DEB, we examined the level of ROS using flow cytometry in GC-2 cells. Figure 5A showed that DEB markedly increased the mean DCF fluorescence, indicating that DEB could cause a significant increase in cellular ROS levels. Next, an antioxidant, NAC, was applied to further determine whether oxidative stress is responsible for DEB-induced DNA damage and G2 arrest. As shown in Figure 5B, pretreatment with 10 mM NAC for 1 h was followed by 500 μM DEB for 24 h in GC-2 cells, and the flow cytometry analysis showed NAC pretreatment significantly decreased the production of ROS in 500 μM DEB-treated GC-2 cells. As shown in Figure 5C, we also found that NAC effectively attenuated the expression of γ-H2AX (phospho S139), suggesting oxidative stress may be causative in DEB-induced DNA strand breaks. Next, we further investigated the effect of NAC on DEB-induced cell cycle arrest and phosphorylation level of G2-phase regulatory proteins. The results showed that pretreatment with NAC could partly attenuate the G2-phase arrest induced by DEB (Figure 5D). There was significant difference in the percentage of G2 cells between groups treated with DEB and DEB plus NAC (P < 0.05). Accordingly, the increased phosphorylation level of Chk1 and Cdc25c was relatively reversed with NAC pretreatment (Figure 5E). These data demonstrated that the induction of oxidative stress was involved in mediating DEB-induced DNA damage and G2 arrest.

**DEB Induces Reproductive Cell Cycle Arrest in the Male Mouse Model.** To further evaluate whether DEB induced reproductive cell cycle arrest in vivo, mouse testicular cells were collected, and the cell cycle distribution was analyzed by DNA content determination with flow cytometry. Typical DNA content distribution histograms were characterized by three main peaks representing haploid, diploid, and tetraploid peaks (Figure 6A), with S-phase located between the diploid and tetraploid peaks. Flow cytometry analysis showed that the percentage of tetraploid cells was significantly increased in DEB-treated animals compared with that of the control group (Figure 6A), indicating that DEB could induce the accumulation of testicular cells in the G2/M phase. We further analyzed the expression of p-H3 with immunohistochemistry and found that it was predominantly localized in the nuclei of spermatocytes and dramatically decreased with the increasing treatment of DEB (Figure 6B). The data confirmed the results...
from an in vitro study that DEB inhibited cells from entering mitosis and accumulated in cells in the G2 phase. We also observed the increase of p-Chk1 immunostaining in testicular cells with DEB administration (Figure 6B), which was consistent with the results of in vitro GC-2 cells.

**Evaluation of DEB-Mediated Reproductive Toxicity.** Sperm motility and morphology are important parameters for evaluating the normal spermatogenetic process. As shown in Figure 7A, the abnormal forms of sperm, which consisted of banana-shaped head, nonhook type, amorphism, big head, double tail, and folded-in tail, were noticed in mouse epididymis. The effect of DEB on epididymal sperm parameters is presented in Table 3. It showed the sperm malformation rate was enhanced, while the sperm motility was reduced markedly with the increase of DEB administration to mice. Further investigation on H&E stained sections from testes displayed remarkable histological changes in the seminiferous tubules, including decrease in the number of spermatogenic cells, disorder of spermatogenic cells, empty tubules, pyknotic nuclei, and intraepithelium vacuolation (Figure 7B). These results...
Figure 5. continued
Figure 5. DEB potentiated the generation of ROS, and NAC pretreatment partly attenuated DEB-induced DNA damage and cell cycle arrest. (A) ROS formation in GC-2 cells after treatment with DEB (100, 300, and 500 μM) for 24 h was measured by FC500 flow cytometry analysis. The mean fluorescence intensity was determined with a fluorescence probe DCFH-DA as described in Materials and Methods. Results are expressed as the percent of fluorescence intensity relative to controls; **p < 0.01 and ***p < 0.001 versus the control. (B) GC-2 cells were treated with 10 mM NAC for 1 h prior to the treatment of 500 μM DEB for 24 h. The DCF fluorescence intensity was shown; *p < 0.05 and ***p < 0.001. (C) DNA damage related protein γ-H2AX was analysis by Western blot in the presence or absence of 10 mM NAC for 1 h and 500 μM DEB for 24 h. (D) The percentage of cells in each phase of the cell cycle was determined in the presence or absence of 10 mM NAC for 1 h and 500 μM DEB for 24 h. Data are given as the mean ± SD of three independent experiments, * p < 0.05 versus control group. (E) GC-2 cells were pretreated with 10 mM NAC for 1 h, followed by treatment with 500 μM DEB for 24 h. G₂ phase-related proteins were detected by Western blot.

Figure 6. DEB-induced G₂ arrest in mouse testis. (A) Mice were administrated with different doses of DEB by intraperitoneal injection. Cell cycle distribution was detected by FC500 flow cytometry. The percentage of cells in each phase of the cell cycle was analyzed using FCS Express 4 software. Values are presented as the mean ± SD of six animals; **p < 0.01 and ***p < 0.001 versus the control. Red represents haploid, blue represents diploid, and green represents tetraploid in the graph. (B) G₂ phase related proteins in the testis were detected by immunohistochemistry staining. The expression of p-H3 and p-Chk1 proteins was observed in mouse testis (×20 magnification). The mean density of immunoreactivity was determined by Image Pro Plus6.0 software. Values are presented as the mean ± SD of three independent experiments. **p < 0.01 and ***p < 0.001 versus the control.
demonstrated that DEB induced male reproductive toxicity in mice.

**DISCUSSION**

An increasing number of studies have showed that exposures to BD are associated with reproductive toxicity. The adverse effects of BD on the mouse ovary have been identified as a particularly sensitive end point, with DEB shown to be the causative agent for ovary damage. Meanwhile, the testicular genotoxicity of BD and its metabolites has been assessed for a long time, and there was evidence of congenital malformations and dominant lethal mutations in male mice after BD exposure but not in rats. Clastogenic data of BD’s epoxy metabolites, especially that of DEB, were also available in germinal cells in both mice and rats from spermatid micronucleus tests and heritable translocation assays. These results indicated that male reproductive genotoxicity of BD may still depend on the production of potent metabolites. Our studies showed that DEB-induced long tails in the comet assay and the increase of MN frequencies in the CBMN assay, and the upregulation of γ-H2AX expression in Western blot in GC-2 cells further confirmed such damage. Previous studies in germ cells on the effects of DEB have been restricted to DNA damage. However, its effects on cell-cycle checkpoint signaling and the involved mechanisms are not clear. Hence, we focused on the mechanism of DEB-induced oxidative stress, which may cause DNA damage and G2 phase arrest in male reproductive cells in vivo and in vitro.

The cytotoxicity of DEB has been certified on germ cells. In the current study, the effect of DEB exposure on the proliferation inhibition of GC-2 cells in vitro was considered to be one of the cytotoxic effects of DEB. It was generally accepted that the cell growth inhibition may be the result of cell cycle arrest or apoptosis. Previous studies, it has been documented that DEB could induce apoptosis. However, we have not found apoptotic cell death to be a cause for the decreased cell proliferation as evidenced by the absence of any increase in apoptosis rates using flow cytometry. In contrast, our results demonstrated that DEB induced G2/M arrest in GC-2 cells. Thus, we assumed that the G2/M checkpoint provides an opportunity and capacity for cells to repair DNA damage, which contributes to the maintenance of genomic stability. Apoptosis is induced only when the extent of damage is too great to repair. Furthermore, mitotic index count and mitosis marker phospho-histone H3 expression in

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**Figure 7.** Reproductive toxicity data for male mice after exposure to DEB. (A) Sperm with banana-shaped head, nonhook type, amorphism, big head, double tail, folded-in tail was observed by light microscope (×100 magnification). (B) Histological changes in the tubule structure of mouse testes were observed (×20 magnification). The control group showed normal architecture of the testis in mice. In low-dose DEB treated mouse testis, pycnotic nuclei (small arrow) and intraepithelium vacuolation (asterisk) were observed. Medium-dose DEB treated groups showed that the seminiferous epithelium was disorganized. High-dose DEB treated groups showed empty tubules (large arrow), pycnotic nuclei (small arrow), intraepithelium vacuolation (asterisk), and a decrease in the number of spermatogenic cells.

**Table 3. Effect of DEB on Sperm Parameters**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sperm motility (%)</th>
<th>Sperm malformation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>86.15 ± 2.25</td>
<td>1.83 ± 0.79</td>
</tr>
<tr>
<td>17 mg/kg</td>
<td>74.20 ± 1.35</td>
<td>7.75 ± 2.34</td>
</tr>
<tr>
<td>42.5 mg/kg</td>
<td>65.50 ± 3.11</td>
<td>13.12 ± 2.72</td>
</tr>
<tr>
<td>85 mg/kg</td>
<td>52.13 ± 8.59</td>
<td>32.6 ± 5.10</td>
</tr>
</tbody>
</table>

Data represent the mean ± SD. *p < 0.05 compared with the control group. †Measured by a CASA system. ‡Analyzed by counting under a light microscope.
this study with GC-2 cells confirmed that DEB could cause G<sub>2</sub> arrest. Equally important is the activity of M-phase-promoting factor (MPF), a complex of CyclinB1 and Cdc2, which also plays an important role in the G<sub>2</sub> to M transition. The active CyclinB1-Cdc2 complex depends not only on the dephosphorylation of Cdc2 but also on the accumulation of cyclinB1 protein in the nucleus. Evidence has been obtained that nuclear export of CyclinB1 plays a key role in the DNA damage-induced G<sub>2</sub> checkpoint. In this study, we observed an obvious increase of CyclinB1 and Cdc2; although surprising, it is in agreement with a previous report. Furthermore, we also found the phosphorylated inactivation of Cdc2 and decreased expression of CyclinB1 in nucleus of cells exposed to DEB, indicating the reduced initiation of cell mitosis. Collectively, these results suggested that the proliferation inhibition by DEB in GC-2 cells in vitro is ascribed to G<sub>2</sub> arrest.

ATM and ATR are members of the phosphatidylinositol 3-kinase (PI3K) family, and they play important roles in the cellular response to DNA damage for the G<sub>2</sub> checkpoint. ATM and ATR were activated by DNA double-strand breaks (DSBs) and single-stranded DNA, respectively. In this study, we found that ATM was activated after treatment with DEB; however, there was no change in the level of ATR. Generally, ATM phosphorylates downstream effector Chk2, and ATR phosphorylates downstream effector Chk1. However, recent reports indicated a cross-talk between the ATM/Chk2 and ATR/Chk1 cascades. Guo et al. showed that ATM (pSer-1981) and Chk1 (pSer-345) activations were concomitant with cucurbitacin B induced G<sub>2</sub>/M arrest in human lung adenocarcinoma epithelial A549 cells, but they did not observe ATR (pSer-428) and Chk2 (pThr-68) activations. In our study, a significant increase of the phosphorylation of Chk1 on Ser-345 after DEB exposure was observed, whereas the phosphorylation of Chk2 on Thr-68 was not affected, which is consistent with the result of a previous study. We also found that the increased expression of phosphorylates Cdc25c and Cdc2, which are downstream factors of the Chk1 signal cascade, in response to DNA damage. To establish the role of Chk1 in DEB-mediated G<sub>2</sub> phase arrest in GC-2 cells, Chk1 siRNA was transfected to knock down Chk1 gene expression, which efficiently reversed downstream effectors such as p-Cdc25c (Ser 216) and p-Cdc2 (Tyr 15). This is also in agreement with the observations with UCN-01. Thus, all of the results demonstrated that DEB might induce DNA damage and the G<sub>2</sub> checkpoint in GC-2 cells through the Chk1 signaling pathway. Previous studies reported that DEB could cause accumulation of ROS and induce oxidative stress. A recent study has shown the abnormal cell cycle distribution of human lung cells with DEB treatment. These data indicated the possible abnormal cell cycle distribution of human lung epithelial A549 cells, but they did not observe ATM/Chk2 and Chk1 in DEB-mediated G<sub>2</sub> phase arrest in GC-2 cells. In the mouse model, DEB showed toxicity by histopathological evaluation in the testes. The increase of tetraploid cells indicated that DEB caused G<sub>2</sub> arrest in mice testicular cells. Collectively, these findings provide insights into the molecular mechanism of DEB-induced male reproductive cell damage.

ASSOCIATED CONTENT

Supporting Information
Simplified scheme of the formation of reactive epoxides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

ABBREVIATIONS
DEB, 1,2,3,4-diepoxybutane; BD, 1,3-butadiene; EB, 1,2-epoxy-3-butene; EBD, 1,2-dihydroxy-3,4-epoxybutane; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; ROS, reactive oxygen species; NAC, N-acetyl-L-cysteine; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; ATM, ataxia-telangiectasia-mutated; ATR, ATM and Rad3-related; MN, micronucleus; NBUD, nuclear bud; NPB, nucleoplasmic bridge

REFERENCES