Reactive Oxygen Species Mediate Terbufos-Induced Apoptosis in Mouse Testicular Cell Lines via the Modulation of Cell Cycle and Pro-Apoptotic Proteins

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Received 12 May 2015; revised 20 July 2015; accepted 26 July 2015

ABSTRACT: Terbufos (*S-t*-butylthiomethyl-*O,O*-diethyl phosphorodithioate) is a highly toxic organophosphate which is extensively used as an insecticide and nematicide. Chronic exposure to terbufos causes neuronal injury and predisposes to neurodegenerative diseases. Accumulating evidence has shown that the exposure to terbufos, as an occupational risk factor, may also cause reproductive disorders. However, the exact mechanisms of reproductive toxicity remain unclear. The present study aimed to investigate the toxic effect of terbufos on testicular cells and to explore the mechanism of toxicity on a cellular level. The cytotoxic effects of terbufos on mouse immortalized spermatogonia (GC-1), spermatocytes (GC-2), Leydig (TM3), and Sertoli (TM4) cell lines were assessed by MTT assays, caspase activation, flow cytometry,

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Conflict of Interest: All authors declared no conflict of interest.

Contract grant sponsor: Ministry of Science and Technology of Taiwan (MOST).

Contract grant number: 103-2314-B-024-002.

Published online 15 September 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/tox.22190

TUNEL assay, Western blot, and cell cycle analysis. The exposure to different concentrations of terbufos ranging from 50 to 800 μM for 6 h caused significant death in all the used testicular cell lines. Terbufos increased reactive oxygen species (ROS) production, reduced mitochondrial membrane potential, and initiated apoptosis, which was confirmed by a dose-dependent increase in the number of TUNEL-positive apoptotic cells. Blocking ROS production by *N*-acetyl cysteine (NAC) protected GC-1 cells from terbufos-induced cell death. The results demonstrated that terbufos induces ROS, apoptosis, and DNA damage in testicular cell lines and it should be considered potentially hazardous to testis. Together, this study provided potential molecular mechanisms of terbufos-induced toxicity in testicular cells and suggests a possible protective measure. Environ Toxicol 31: 1888–1898, 2016.
Keywords: terbufos; caspase-3; reactive oxygen species

INTRODUCTION

Terbufos is one of organophosphate pesticides used extensively worldwide as an insecticide and nematicide. The toxicity of terbufos is related initially to the inhibition of acetylcholinesterase in the central and peripheral nervous systems, causing headache, memory loss, confusion, nausea, depression, and chronic fatigue syndrome (Kim et al., 2005; Lorke et al., 2014).The oral LD_{50} of terbufos is from 1.3 to 1.57 mg/kg in female rats, and from 1.6 to 1.74 mg/kg in male rats (U.S. Environmental Protection Agency September 9, 1988). Although few studies have indicated that terbufos was non-mutagenic when assessed by bacterial reversion mutation test, chromosomal aberration test, dominant lethal study in rats, and DNA repair assay in rat liver, many others reported its ability to induce lung cancer, prostate cancer, leukemia, and non-Hodgkin's lymphoma (Bonner et al., 2010; Wu et al., 2011). In addition, terbufos also induced DNA damage, apoptosis, and the expression of tumor-related genes in hepatocellular carcinoma cells (Wu et al., 2011).

Infertility is a major reproductive health problem caused by many factors such as DNA damage, genetic, hypothalamic-pituitary, environmental and occupational (Mendiola et al., 2008; Dong et al., 2012). DNA damage can be induced by pesticides, smoking, reactive oxygen species (ROS), chemicals, fever or high testicular temperature with consequent reduced fertility in males (Wang et al., 1997; Safarinejad, 2008; Ranawat and Bansal, 2009). Pesticides may directly damage spermatozoa, alter Sertoli cell or Leydig cell function, or disrupt the endocrine function in any stage of hormonal regulation (Bjorge et al., 1995; Nakai et al., 2002). In addition, epidemiological studies had come to a conclusion that occupational exposure to pesticides increases the risks of sperm morphological abnormalities, decreased sperm count and decreased percentage of viable sperms (Tuc et al., 2007). Other studies indicated that pesticides can produce free radicals and change antioxidant capacity by inducing oxidative stress (Abdollahi et al., 2004; Karami-Mohajeri and Abdollahi, 2011). Oxidative stress has been established as one of the key factors determining the toxicity of chemical compounds such as pesticides. Excessive production of ROS has pathological effects on the male sperm, such as decreased viability, motility, and morphological changes in addition to DNA damage which possibly results in caspases activation and apoptotic cell death (Mahfouz et al., 2010; Mehany et al., 2013). The tumor suppressor p53, a major factor in the apoptosis process, is activated when mammalian cells are subjected to stress conditions such as hypoxia, radiation, chemotherapeutic drug, or DNA damage (Takimoto and El-Deiry, 2000; Horn and Vousden, 2007). The p53 tumor suppressor mediates apoptosis through bax transactivation, mitochondrial cytochrome c release, and caspase-9 activation, which is usually followed by the activation of caspase-3, -6, and -7 (Yoshida and Miki, 2010; Cheng et al., 2011). These processes limit the cellular proliferation by inducing cell cycle arrest and apoptosis in response to cellular stress (Arafa el et al., 2014).

Spermatogenesis is a complex continuous developmental process that involves cell division, differentiation and cellular interactions in the microenvironment of seminiferous tubules (Kao et al., 2008). ROS are required for maturation, capacitation, acrosomal reaction, binding to the zona pellucida, and oocyte fusion for spermatozoa (de Lamirande and Gagnon, 1993; Kao et al., 2008). However, spermatozoa are highly susceptible to oxidative damage because of the high content of polyunsaturated fatty acids in their plasma membranes (de Lamirande and Gagnon, 1995). Some studies reported that excessive ROS generation are associated with male infertility, sperm apoptosis, increased rates of early miscarriage, and impaired pre-implantation development of the embryo (Gandini et al., 2000; Baker and Aitken, 2005). Excess mitochondrial ROS generation induced by disruption of mitochondrial membrane potential caused sperm membrane lipid peroxidation and subsequent released of cytochrome c and enhanced a pro-oxidative/pro-apoptotic event followed by the activation of caspase-9 and caspase-3 (Barbonetti et al., 2013; Ferramosca et al., 2013). The reduction in mitochondrial functionality might be one of the reasons responsible for the decreased spermatozoa motility (Kao et al., 2008). Some studies also provided an evidence that ROS affect DNA quality of human sperm (Bennetts and Aitken, 2005). The degree of fragmentation of sperm DNA was inversely correlated with sperm quality and fertilization rates after *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (Loft et al., 2003). Altogether ROS quantity

could be a critical factor for seminal quality assessment before artificial reproduction (Gomez et al., 1996).

Here we report the ability of terbufos to induce ROS production and the consequent induction of apoptosis in mouse testicular cell lines. In addition, terbufos caused changes in the cell cycle and the morphology in GC-1 cells. Together, this study provided potential molecular mechanisms of terbufos-induced toxicity in testicular cells and suggests a possible protective measure.

MATERIALS AND METHODS

Cell Culture and Chemicals

Mouse testicular spermatogonia (GC-1), spermatocytes (GC-2), Leydig (TM-3), and Sertoli (TM-4) cell lines were obtained from ATC and maintained at 37°C in a 5% CO_2 incubator in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 g/mL streptomycin (Invitrogen, Ground Island, NY). Culture media were replaced every 2 days.

Chemicals and Materials

Antibodies against p53, bcl-2, cdc25, p-cdc25, and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX). Cyclin A1, cyclin B1, cyclin D1, cyclin D2, cyclin D3, cyclin E1, cyclin E2, cyclin H, anti-rabbit IgG-horseradish peroxidase (HRP) conjugates, and rabbit anti-mouse IgG-HRP conjugates antibodies were purchased from Cell Signaling (Beverly, MA). Terbufos, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and *N*-acetylcysteine were purchased from Sigma-Aldrich (St. Louis, MO). PE active caspase-3 apoptosis kit was purchased from BD Pharmingen (San Diego, CA). ECL Western blot detection system was purchased from Millipore (Billerica, MA).

Cell Viability Assay

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay in three replicates. GC-1, GC-2, TM-3, and TM-4 cells were seeded at 6×10^3 per well in 96-well flat-bottomed plates and incubated in 10% FBS-supplemented DMEM for 24 h as mentioned before (Omar et al., 2013). Cells were treated with terbufos at the indicated doses. Controls received vehicle (DMSO) at a concentration equal to that in drug-treated cells. The drug-containing media were replaced with 20 μL of 10% FBS-supplemented DMEM containing 0.5 mg/mL MTT, and cells were incubated in the CO_2 incubator at 37°C for 4 h. After removing the media, reduced MTT was solubilized in 100 μL per well of DMSO, and the absorbance of 100 μL aliquots of each well was measured at 570 nm.

Cell Cycle Analysis

To determine cell-cycle distribution, 5×10^5 cells in 6 cm dishes were treated with various concentrations of terbufos for 24 h as mentioned before (Weng et al., 2009). After incubation, the cells were fixed in 70% ethanol/PBS, pelleted, and resuspended in buffer containing RNase A and propidium iodide. Cell-cycle distribution was determined by flow cytometry analysis, and the percentages of cells were determined using the FlowJo software (FLOWJO, LCC).

Analysis of Caspase-3 Activity

Caspase-3 activity was determined using PE active caspase-3 apoptosis kit (BD Pharmingen) as mentioned before (Lee et al., 2014). Briefly, GC-1 (5×10^5) cells in 6 cm dishes were subjected to different drug treatments for 24 h and were resuspended cells in 0.5 mL Cytofix/Cytoperm solution for 20 min on ice and then incubated in 100 μL of Perm/Wash buffer containing 20 μL caspase-3 antibody for 30 min at room temperature. Each sample was then added with 400 μL Perm/Wash^{TM} (BD Pharmingen), and caspase-3 activity signals were analyzed by flow cytometry.

Western Blot Analysis

The cell lysates were collected with RIPA lysis buffer (50 mM Tris-Hcl pH 7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1mM PMSF, 1 mM EDTA; 5 μg/mL Aprotinin) containing protease inhibitors (1 mM PMSF, 1 mM orthovanadate, 1 mM EDTA, 10 μg/mL leupeptin) as described before (Arafa el et al., 2014). The protein concentrations were determined with the Micro BCA protein assay reagent kit (Pierce, Rockford, IL). The protein (20 μg per lane) was loaded and separated by SDS-PAGE and then transferred onto PVDF membranes (Millpore, Billerica, MA) using a semi-dry transfer cell (Bio-Rad Lab). Blotted membranes were washed twice with TBS containing 0.1% Tween 20 (TBST; 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20). After blocking with TBST containing 5% nonfat milk for 1 h, the membranes were probed with antibodies against: p53, Bcl-2, cyclin A1, cyclin B1, cyclin D1, cyclin D2, cyclin D3, cyclin E1, cyclin E2, cyclin H, cdc25, p-cdc25, and β-actin antibodies in 1% TBST nonfat milk at 4°C overnight. The membranes were then washed thrice with TBST for a total of 15 min. The secondary anti-mouse IgG-HRP conjugates or anti-rabbit IgG-HRP conjugates (1:2000 dilutions) were subsequently incubated with membranes for 1 h at room temperature and were washed extensively for 50 min with TBST. The blots were visualized with enhanced chemiluminescence reagent (GE, Pittsburgh, PA) according to the manufacturer's instructions. The blots were developed with the ECL-Western blot detection system according to the manufacturer's directions.

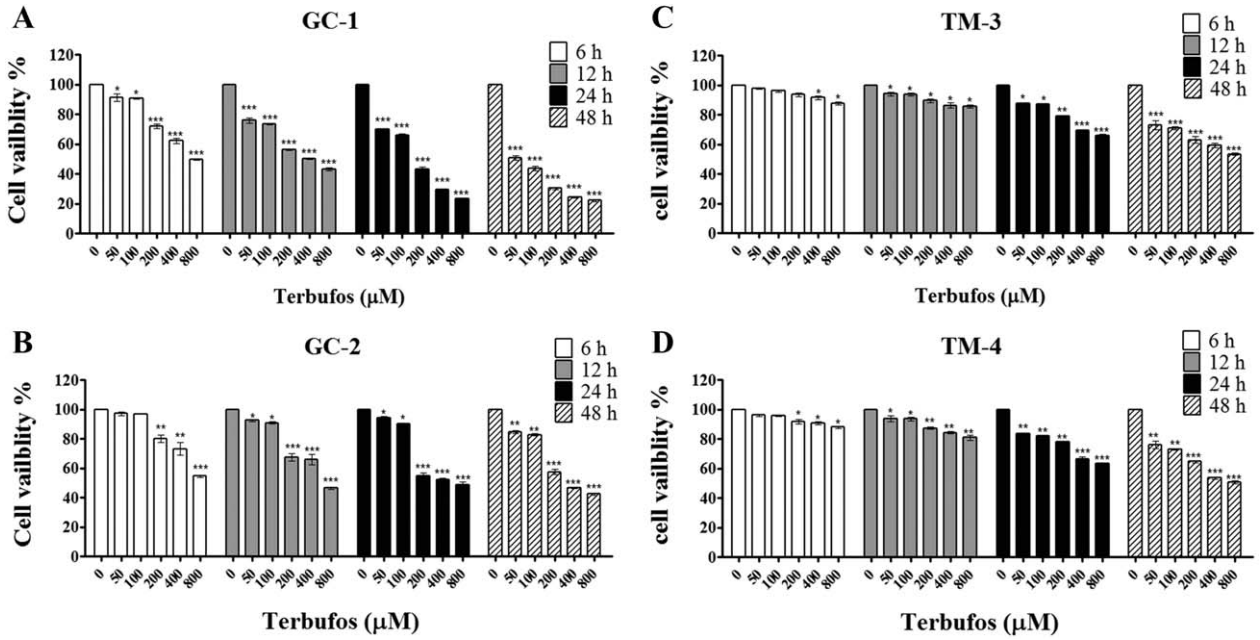

Fig. 1. Effects of terbufos on testicular cell lines viability (A–D). Cells were exposed to terbufos at the indicated doses and time intervals in 10% FBS-supplemented DMEM, and cell viability was assessed by MTT assay. Points, mean; bars, SD (n = 6). Data represent the mean ± SD. Significant differences (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) between the control and experimental groups are marked with asterisks.

Quantification of Apoptosis Using a Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay (TUNEL) Assay

DNA fragmentation was assessed in terbufos-treated cells by TUNEL staining using the APO–BRDUTM kit (BD-Pharmingen, San Diego, CA) following the manufacturer's instructions. Briefly, GC-1 cells were fixed in 1% paraformaldehyde (v/v) for 15 min on ice. GC-1 cells were then washed twice with PBS, pelleted and suspended in 70% ethanol. Cells were kept at −20°C in 70% ethanol overnight. The cells were washed twice with PBS, labeled with brominated deoxyribonucleotide triphosphates (Br-dUTP), and the enzyme terminal deoxynucleotidyl transferase (TdT) for 1 h at 37°C. After labeling, cells were washed and stained with FITC conjugated anti-BrdU mAb for 30 min. RNase-PI was used to stain the nuclei and the samples were incubated for an additional 30 min at room temperature. After staining, cells were observed using a fluorescence microscope.

Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential was assessed by the Rhodamine-123 (sigma, St. Louis, MO). Briefly, GC-1 cells were seeded at 1×10^5 per well in six-well flat-bottomed plates. GC-1 cells were exposed to terbufos with or without the NAC for 12 h. After treatment, the cells were stained with JC-1 for 30 min incubation at 37°C, cells were washed with PBS and collected. Mitochondrial membrane potential was analyzed by flow cytometry.

Statistical Analysis

Results were presented as the mean ± SD, and statistical comparisons were made using the Student's t-test. Statistically significant difference from control was defined at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, respectively.

RESULTS

Terbufos Induced Cell Death in GC-1, GC-2, TM-3, and TM-4 Cells

To investigate the inhibitory effect of terbufos on testicular cell growth, cell proliferation was examined by using MTT assay. Four mouse testicular cell lines GC-1, GC-2, TM-3, and TM-4 were treated with different concentrations of terbufos. As shown in Figure 1, terbufos reduced cell growth in GC-1, GC-2, TM-3, and TM-4 cells in a dose and time-dependent manner. Based on the IC_{50}, GC-1 and GC-2 cells were more sensitive than TM3 and TM4 cells to terbufos [Fig. 1(A–D)].

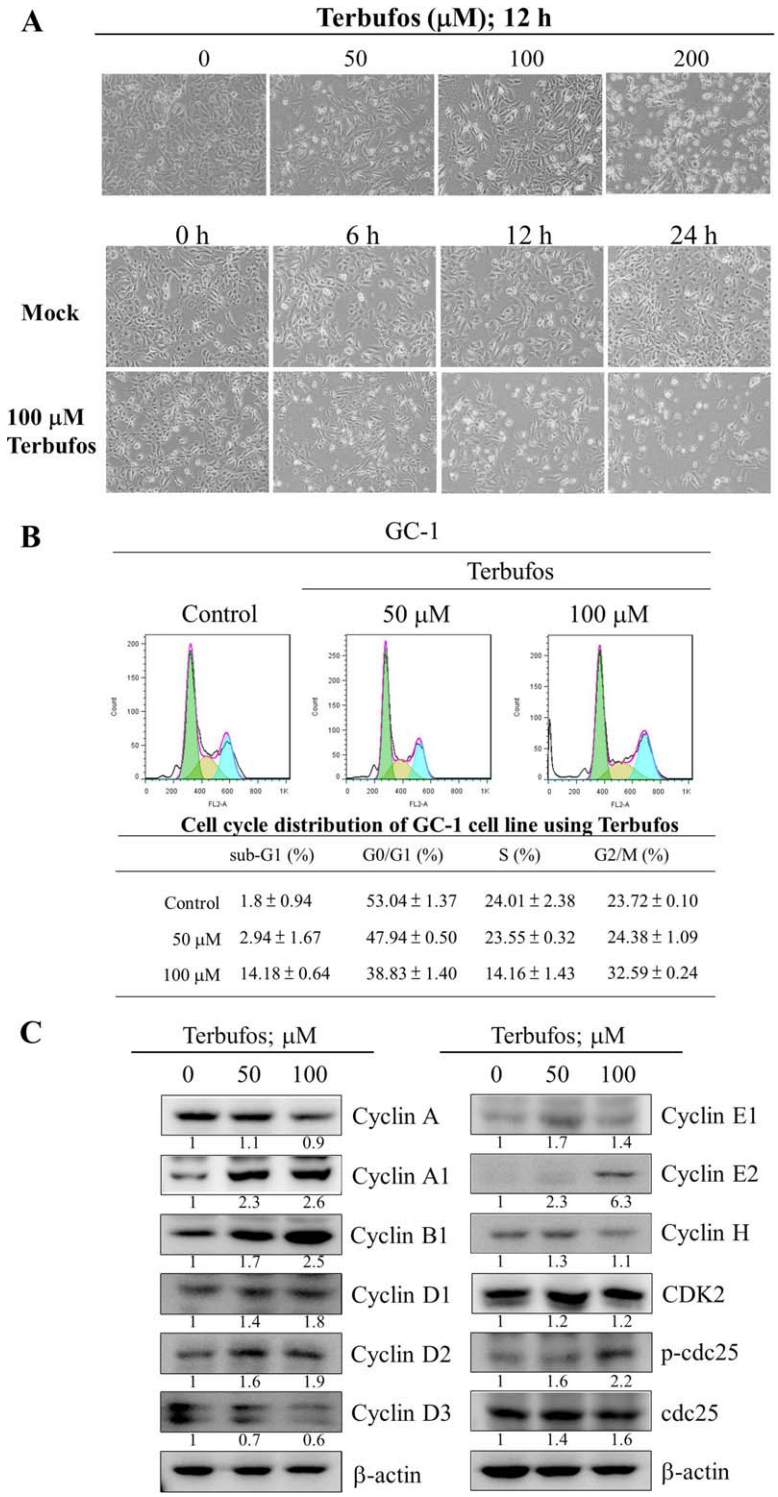

Fig. 2. Terbufos effects on the cell morphology and cell cycle in GC-1 cells. (A) The morphological changes after terbufos treatment at the indicated doses and time intervals. Cells were observed under phase-contrast magnification. (B) Flow cytometric analysis of cell cycle in GC-1 cells treated with terbufos for 12 h. Cells were analyzed by flow cytometry after staining with propidium iodide (PI). The percentages in the graphs represent the percent of cell cycle phases in the respective quadrants. Columns, mean; bars, SD. (C) Western blots of cell cycle-regulatory proteins, including cyclin A1, cyclin B1, cyclin D1, cyclin D2, cyclin D3, cyclin E1, cyclin E2, cyclin H, cdc25, p-cdc25. GC-1 cells were exposed to terbufos in 10% FBS–supplemented DMEM at the indicated doses for 24 h. [Color figure can be viewed at wileyonlinelibrary.com]

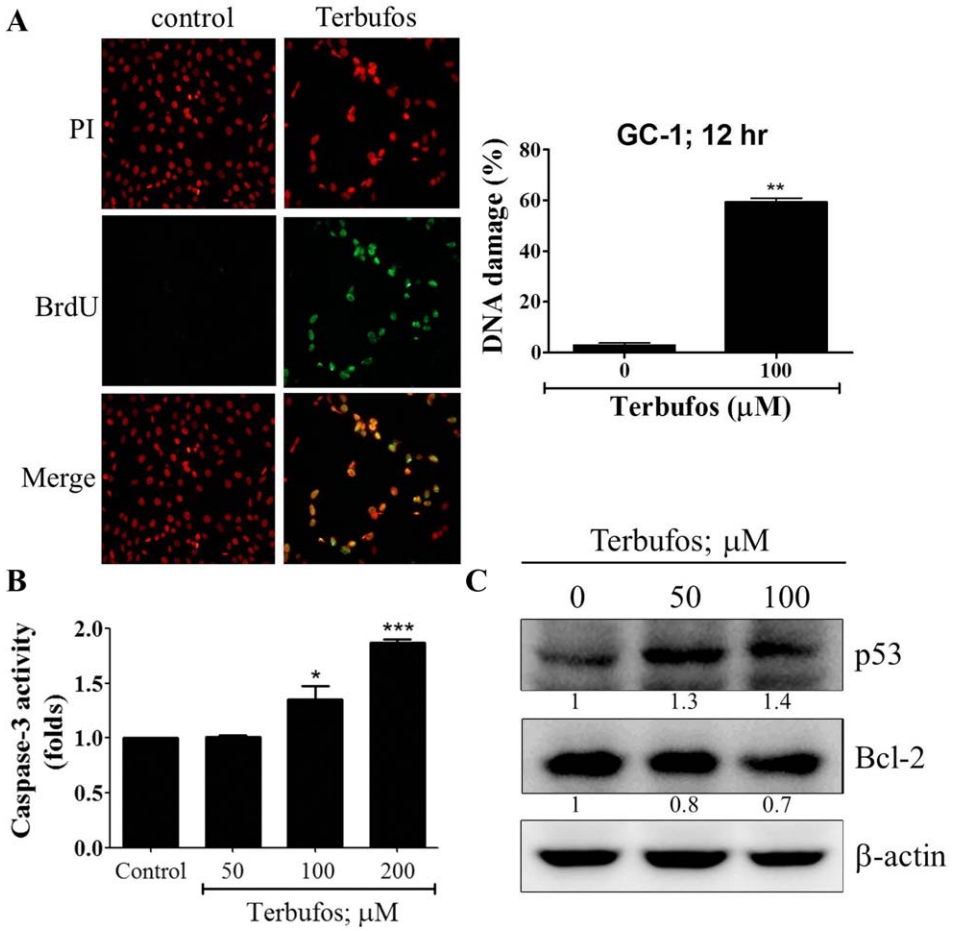

Fig. 3. Induction of GC-1 cells apoptosis in response to terbufos treatment. (A) GC-1 cells were treated with 100 μM terbufos for 12 h. Fluorescence microscopy ($\times$100) of cells subjected to propidium iodide/FITC double staining (left panel). Induction of cell apoptosis by terbufos was assessed by TUNEL assay with flow cytometric analysis. Cells were stained with propidium iodide and FITC, and TUNEL-positive (green fluorescent) cells were scored and normalized with controls (untreated cells) (right panel). Data shown are means $\pm$ SEM of three independent experiments. Significant differences ($^{**}P < 0.01$) between the control and experimental groups are marked with an asterisk. (B) Flow cytometric analysis of the dose-dependent effect of terbufos on caspase-3 activity in GC-1 cells. Columns, mean of three independent experiments; bars, SD. Significant differences ($^{*}P < 0.05$; $^{***}P < 0.001$) between the control and experimental groups are marked with asterisks. (C) Cells were treated with increasing doses of terbufos for various durations, and the expressions of p53, bcl-2, and β-actin were analyzed by Western blotting. [Color figure can be viewed at wileyonlinelibrary.com]

Terbufos Caused Cell Death and Cell Cycle Arrest in GC-1 Cells

GC-1 cells were used to assess the effect of Terbufos on cell survival and cell morphology. As shown in Figure 2(A), terbufos treatment resulted in a dose and time-dependent progressive changes from a flat to round morphology. For the determination of the effect of terbufos on cell-cycle distribution in GC-1 cells, the cells were incubated with terbufos at the indicated doses. Figure 2(B) shows the result of DNA flow cytometric analyses of GC-1 cells treated with different doses of terbufos for 12 h compared with control cells. Results indicated that GC-1 cells showed a dose-dependent accumulation in G1 phase from 1.8% (control) to 14.8% (100 μM terbufos) after 12 h of incubation. In addition, terbufos also caused accumulation in G2/M phase from 23.72% (control) to 32.59% (100 μM terbufos) after 12 h of incubation. To investigate the mechanism of terbufos-induced modulation of cell cycle progression, Western blot

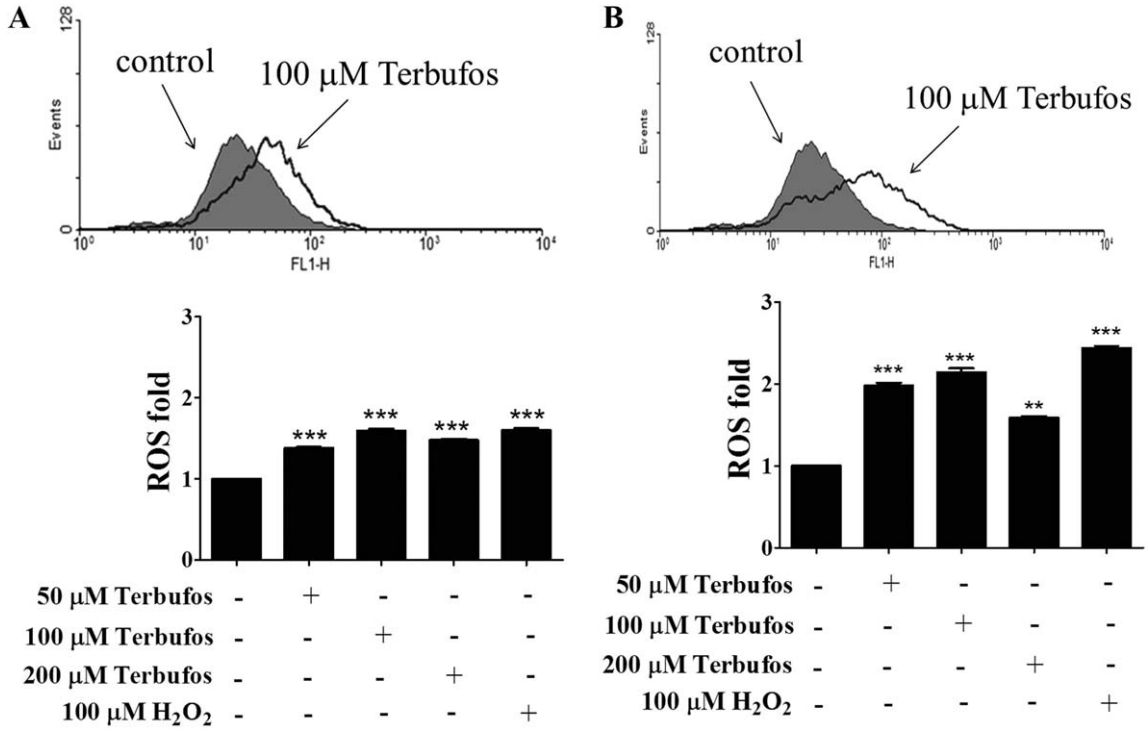

Fig. 4. Induction of ROS production in response to terbufos treatment. GC-1 cells were treated with terbufos or H_2O_2 at the indicated doses for (A) 1 and (B) 3 h. ROS levels in terbufos-treated cells were determined by flow cytometry. Columns, mean of three independent experiments; bars, SD. Significant differences (**$P < 0.01$; ***$P < 0.001$) between the control and experimental groups are marked with asterisks.

analysis was used to test the effect of terbufos on cyclin proteins expression. Results indicated the ability of terbufos to down-regulate cyclin D1 and cyclin H expression [Fig. 2(C)]. On the other hand, terbufos treatment increased expression of cyclin A1, cyclin B1, and phosphorylation of cdc25 (Ser 216) in GC-1 cells [Fig. 2(C)].

Terbufos Induced Cell Apoptosis, Capase-3 Activity, DNA Damage, and Alternation of p21 and p53 Protein Expression in GC-1 Cells

The effect of terbufos on DNA damage in GC-1 cells was examined using TUNEL assay, and the severity of DNA damage in GC-1 was determined by flow cytometric analysis [Fig. 3(A)]. The results indicated that terbufos induced approximately threefold increases in fluorescence intensity after 12 h of treatment with terbufos compared with the untreated counterparts. In addition, caspase-3 was suggested to play a critical role in pesticides-induced cell apoptosis. Therefore, we examined the effect of terbufos on modulating the activity of caspase-3 in GC-1 cells using flow cytometric analysis. As shown in Figure 3(B), exposure to terbufos led to a dose-dependent stimulation of caspase-3 activity. Furthermore, Western blot analysis revealed that terbufos treatment increased p53 expression and decreased Bcl-2 expression levels compared with control cells, which indicated their involvement in the regulation of apoptosis [Fig. 3(C)].

Terbufos Treatment Increased ROS Production in GC-1 Cells

To examine the mechanisms of cell death in response to terbufos exposure, the effect of terbufos on the production of intracellular of ROS was evaluated by DCFH-DA staining assay in GC-1 cells. As shown in Figure 4, treatment of terbufos enhanced ROS production in GC-1 cells. GC-1 cells treated with 100 μM terbufos caused approximately twofold increases in DCF fluorescence intensity after 3 h of treatment when compared with the untreated counterparts (Fig. 4). Hydrogen peroxide (H_2O_2) was used as a positive control.

ROS Production was Involved in Terbufos-Induced Cell Apoptosis

Growing evidence indicates that ROS plays an important role in the induction of cell apoptosis. To assess the effect of ROS production by terbufos on cell survival, cells were exposed to terbufos in the presence or absence of *N*-acetylcysteine (NAC), an antioxidant agent, and the effect of NAC on terbufos-induced cell death was investigated using MTT

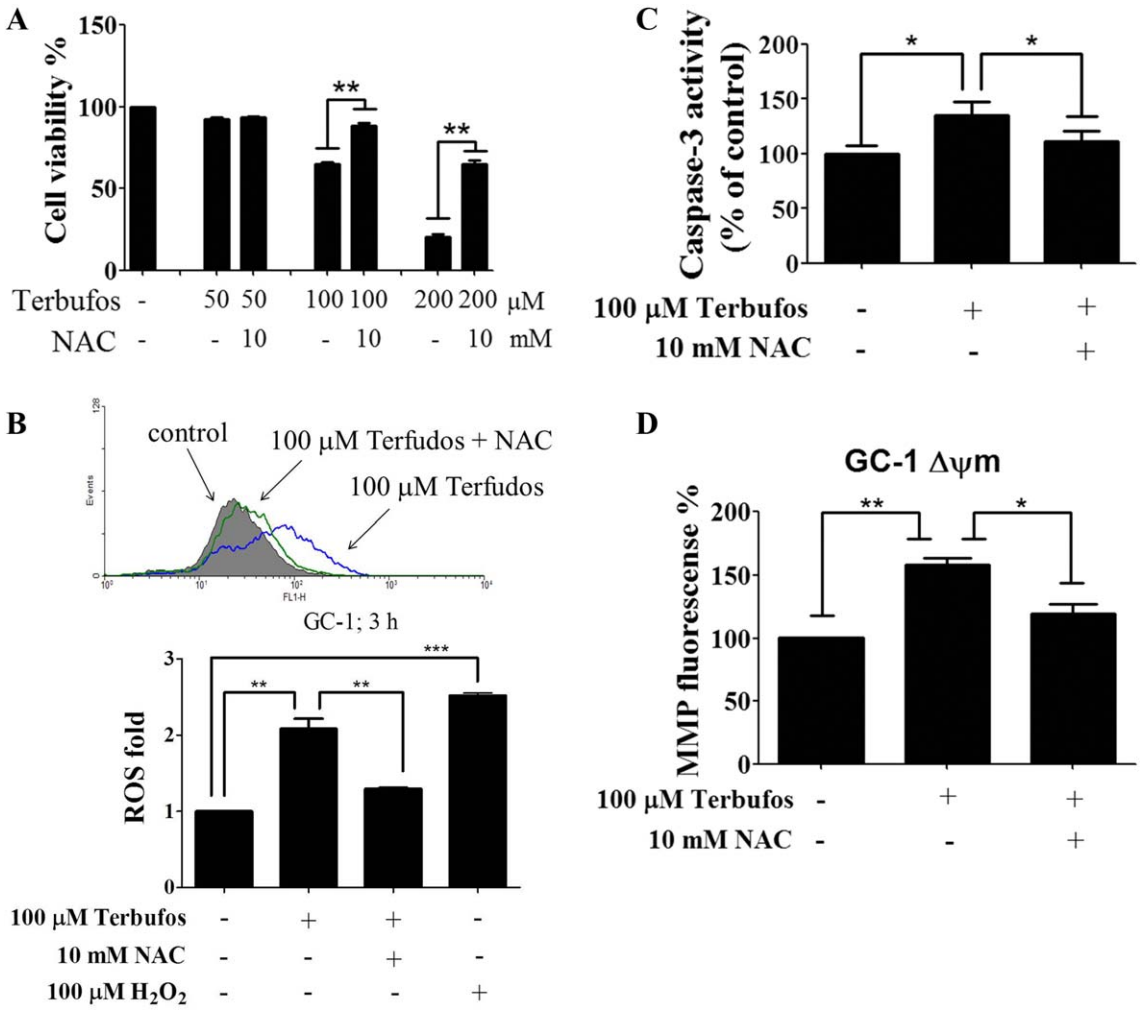

Fig. 5. ROS were involved in regulation of terbufos-induced cell apoptosis. (A) GC-1 cells were treated with terbufos in the presence of 10 mM NAC, and cell viability was assessed by MTT assays. Points, mean; bars, SD ($n = 6$). Data represent the mean ± SD. Significant differences (**$P < 0.01$) between the control and experimental groups are marked with asterisks. (B) NAC inhibited terbufos-activated ROS production in GC-1 cells. Cultures of GC-1 cells were treated with 100 μM terbufos in the presence of NAC for 3 h. ROS levels were determined by flow cytometry. Columns, mean of three independent experiments; bars, SD. Significant differences (**$P < 0.01$; ***$P < 0.001$) between the control and experimental groups are marked with asterisks. (C) Inhibition of ROS production by NCA significantly reduced caspase-3 activity during terbufos treatment in GC-1 cells. GC-1 cells were incubated with terbufos in the presence of 10 mM NAC for 24 h, and Columns, mean of three independent experiments; bars, SD. Significant differences (*$P < 0.05$) between the control and experimental groups are marked with asterisks. (D) GC-1 cells were exposed with 100 μM terbufos in the presence of 10 mM NAC for 24 h, and the mitochondrial membrane potential was evaluated by flow cytometry using JC-1 staining. The folds in the graphs represent the percent of cells in the respective quadrants. Columns, mean; bars, SD ($n = 3$). Significant differences (*$P < 0.05$; **$P < 0.01$) between the control and experimental groups are marked with asterisks. [Color figure can be viewed at wileyonlinelibrary.com]

assay. As shown in Figure 5(A), co-incubation with NAC protected GC-1 cells from terbufos-induced apoptosis. In addition, flow cytometric analysis indicated that ROS production by terbufos was inhibited with NAC treatment [Fig. 5(B)]. Furthermore, caspase-3 activity was also determined after GC-1 cells were co-incubated terbufos and NAC.

As shown in Figure 5(C), the combination of terbufos with NAC significantly reduced the caspase-3 activity.

Since the loss of the mitochondrial membrane potential (MMP) is often associated with early stages of apoptosis, the effect of ROS production on MMP ($\Delta\Psi_m$) during terbufos treatment was evaluated by flow cytometric analysis. The results revealed that NAC counteracted the effect of terbufos on MMP [Fig. 5(D)]. Therefore, these results suggest that ROS production was involved in the regulation of terbufos-induced cell apoptosis.

DISCUSSION

Terbufos is one of the organophosphates, which is extensively used as an insecticide and nematicide. Occupational or chronic exposure of terbufos causes neuronal injury and predisposes to neurodegenerative diseases. Accumulating evidence has shown that the exposure to terbufos may also cause many reproductive disorders such as sperm damage and infertility. However, the effect of terbufos on testicular cells is still unclear. The current study showed that the spermatogonia (GC-1) and spermatocytes (GC-2) cells were more sensitive than Leydig cell (TM3) and Sertoli cell (TM4) [Fig. 1(A–D)]. The toxic effect of terbufos on testicular cell lines was mediated by inducing ROS production and consequent modulation of Bcl-2 and p53 expression levels, reduction of mitochondrial membrane potential, and induction of DNA damage, leading to apoptosis. Antioxidants like NAC counteracted the toxic effect of terbufos on testicular cells and enhanced the cell survival through the inhibition of ROS production. Thus, the results of this study provided *in vitro* evidence that oxidative stress is involved in terbufos-induced testicular cell death.

In previous studies, oxidative stress has been established as one of the key factors determining the toxicity of several pesticides (Al-Gubory, 2014; Roustan et al., 2014; Sharma and Sangha, 2014). ROS are formed by incomplete reduction of molecular oxygen, and they include superoxide anion ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$). Mitochondria are the main source of sperm-produced ROS in the electron transport chain system or NADPH oxidase. ROS, if produced in excess, have many pathological effects on male sperm cells, such as decreasing the viability, motility, mitochondrial membrane permeabilization (MMP), and increasing DNA damage, morphology defects and lipid peroxidation, possibly resulting in poor semen quality and loss of motility (Kothari et al., 2010; Mahfouz et al., 2010; Aitken et al., 2012).

The toxic effects of terbufos on testicular cells were not reported and remain unclear. This study investigated the effects of terbufos on testicular cell lines. Results revealed that the exposure to terbufos induced reactive oxygen species (ROS) production and apoptosis in testicular cell lines. Terbufos down-regulated cyclin D1, cyclin E, and cyclin H expression [Fig. 5(A)] and increased the expression of cyclin A1, cyclin B1, and phosphorylation of cdc25 (Ser 216) in GC-1 cells [Fig. 2(C)]. Cyclin D1 and cyclin E are involved in regulating cell cycle progression and drives the G1/S phase transition (Dulic et al., 1992; Resnitzky and Reed, 1995). Cyclin H has an important role in regulating gene expression and cell fate in addition to being an important link between basal transcription controls and the cell cycle machinery (Liu et al., 2008; Patel and Simon, 2010). Terbufos down regulated cyclin D1 and cyclin H expression levels and resulted in cell arrest at G0/G1 phase and consequent cell apoptosis. Cyclin B1/Cdc2 complex is G2-M checkpoint regulator and it regulates many of the dramatic cellular rearrangements observed at mitosis (Kawamoto et al., 1997). Cyclin A/cdk2 is active during S and G2 phases of the cell cycle (Rosenblatt et al., 1992). The checkpoint kinases Chk1 and Chk2 phosphorylate cdc25C at Ser216 in response to DNA damage (Eymin et al., 2006). Our results showed that terbufos increased the expression of cyclin A1, cyclin B1 and phosphorylation of cdc25 (Ser 216) in response to DNA damage and cell cycle arrest caused by terbufos [Fig. 2(C)]. Antagonizing ROS production by *N*-acetyl cysteine (NAC) protected GC-1 cells from terbufos-induced cell death, alternation of mitochondrial membrane potential and caspase-dependent apoptosis. Taken together, the results of the current study demonstrated that terbufos induces ROS, apoptosis, DNA damage and should be considered potentially hazardous to testicular cells. In addition, it provided a molecular mechanism of terbufos-induced toxic effects in testicular cells, which can be employed for the selection of possible preventive measures.

In conclusion, our findings provided potential molecular mechanisms of terbufos-induced toxicity in testicular cell lines which were mediated through oxidative stress and its possible role in occupational pesticides-induced infertility. The ability of antioxidants to counteract the devastating effect of terbufos highlights its role as an effective protective measure should be employed in agricultural countries.

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