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Role of B-Cell Lymphoma 2 Ovarian Killer (BOK) in Acute Toxicity of Human Lung Epithelial Cells Caused by Cadmium Chloride

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Background: B-cell lymphoma 2 (BCL-2) ovarian killer (BOK) is a Bcl-2 family member with sequence homology to pro-apoptotic BAX and BAK, but its physiological and pathological roles remain largely unclear. Exposure of cells to cadmium may cause DNA damage, decrease DNA repair capacity, and increase genomic instability.

Material/Methods: The present study investigated the effects of BOK on the toxicity of cadmium chloride ($CdCl_2$) to human bronchial epithelial (16HBE) cells. We constructed BOK over-expressing (16HBE-BOK) cells and BOK knockdown (16HBE-shBOK) cells using the BOK-ORF plasmid and BOK-siRNA. qRT-PCR for BOK mRNA expression. We used Trypan blue exclusion assay for cell growth, MTT colorimetric assays for cells inhibition rate, and Comet assays for detecting damaged DNA.

Results: $CdCl_2$, at various concentrations and exposure times, increased BOK mRNA. 16HBE-BOK cells (BOK over-expressing) proliferated more than 16HBE cells after 72 h; 16HBE-shBOK (BOK knockdown) cells proliferated less. In addition, BOK deficiency enhanced cell death induced by $CdCl_2$. Similarly, $CdCl_2$- and H_2O_2-induced DNA damage was greater in BOK-deficient cells.

Conclusions: These findings support a role for BOK in $CdCl_2$-induced DNA damage and cell death.

MeSH Keywords: bcl-Associated Death Protein • Cadmium Chloride • Comet Assay

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Background

Epidemiological studies show that, in industrialized countries, pollution of the natural environment by cadmium is a serious problem, creating a threat to public health [1,2]. Cadmium, an occupational and environmental toxicant, exists naturally in the earth's crust, and its chemical compounds are frequently used in paints, welding, pigments, electroplating, and nickel-cadmium batteries. Cadmium can be introduced into the air, soil, and water via its industrial uses. As a result, humans in various occupational settings can be exposed to cadmium compounds by inhalation, via consumption of contaminated food or water, or by smoking. The International Agency for Research on Cancer (IARC) has classified cadmium and cadmium-containing compounds as a class I human carcinogen [3]. The main organs affected by cadmium exposure are kidney, bone, the respiratory tract, liver, prostate, and the hematopoietic system [4]. The mechanisms of cadmium-induced damage have yet to be fully elucidated. Cadmium is not mutagenic and does not induce direct DNA damage; however, it increases production of reactive oxygen species (ROS), which in turn induce DNA damage and interfere with cell signaling [5–7]. Cadmium decreases anti-apoptotic proteins (Bcl-2/Bcl-xL); however, these proteins are higher in cadmium-transformed BEAS-2B cells than in non-transformed cells [8]. The abnormal expression of proteins caused by cadmium leads to an imbalance of cell growth and apoptosis, which promotes damage and tumor development [9,10].

BCL-2 gene family members, which are involved in the regulation of cell apoptosis, are frequently altered in cancers [11,12]. The BCL-2 ovarian killer (BOK) possesses 3 Bcl-2 homology domains, similar to BAX and BAK, and thus could act in a similar pro-apoptotic pathway. However, the role of BOK in the cell cycle and cell death is controversial. BOK is widely expressed in tissues of animals and human cells; it is frequently deleted in certain human cancers, such as colorectal cancers, liver cancers, and non-small-cell lung carcinomas [13–16]. BOK mRNA/protein is increased in cells exposed briefly to chemicals (diethylnitrosamine, paclitaxel, thapsigargin, or bortezomib [15,17]; bacteria (*Aeromonas hydrophila* or *Vibrio harveyi*); or viruses (white spot syndrome virus, Singapore grouper iridovirus, or *M. rosenbergii* norovirus) [18,19]. BOK links the cell cycle and cell death machinery upstream of mitochondrial and endoplasmic reticulum damage [15,17].

Cadmium is an inducer of endoplasmic reticulum stress [20,21]. However, the role of BOK in cadmium-induced damage has not been explored. The aim of the present study was to investigate the role of BOK in the toxicity of human bronchial epithelial (16HBE) cells exposed to cadmium chloride ($CdCl_2$).

Material and Methods

Chemicals and reagents

$CdCl_2$, RPMI 1640 medium, and L-glutamine were purchased from Sigma Chemical Co. (St Louis, MO). TRIzol reagent and penicillin/streptomycin were purchased from Life Technologies Corp. (Grand Island, NY). The antibody against to BOK was purchased from Abcam Co. (Abcam, ab233072), and monoclonal anti-actin was purchased from Oncogene Research Products (Cambridge, MA). The Bradford Protein Assay kit was obtained from BioRad Laboratories (Hercules, CA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Corp. (St. Louis, MO). Human airway epithelial cells (16HBE) were obtained from Shanghai Biotechnology Co. Enzyme Research (Shanghai, EK-Bioscience).

Cell culture

16HBE cells were cultured in RPMI 1640 medium containing L-glutamine and supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution (Life Technologies Corp.) at 37°C in a 5% CO_2 humidified atmosphere. Cells were passaged twice each week and maintained in the log phase of growth at 2×10^5~5×10^5 cells/ml.

Plasmids

The BOK-ORF plasmid was purchased from Origene (Origene, USA), and its sequence is shown in Supplementary Figure 1. ORF cDNA was excised from the pCMV6-Entry vector using F-xbal and R-ecoR1 restriction enzymes and subcloned into a pCDH expression vector. BOK downregulation was achieved using Invitrogen lentiviral plasmids (Life Technologies) expressing shRNA targeting human BOK (5'-*AAAAGAATTCCGGATGGACTGATGTCCT CAAGTGTGTCAAGAGCACACTTGAGGACATCAGTCCAT CCTTTTTGCGGCCGCAAAA*-3'). BOK-shRNA was also subcloned into the pCDH vector.

Transfection

16HBE cells (2×10^5) were plated onto 6-well plates and were transfected with BOK-ORF, BOK-siRNA, or a negative control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as a transfection reagent.

Isolation of total RNA and qRT-PCR

Cellular RNA was extracted by use of TRIzol reagent. From each sample, 10 ng of RNA was used for reverse transcription by RNA Reverse Transcription kits (Promega, A5001). Real-time RT-PCR was performed with 2X SYBR Green Mix kits (Biomiga, USA).

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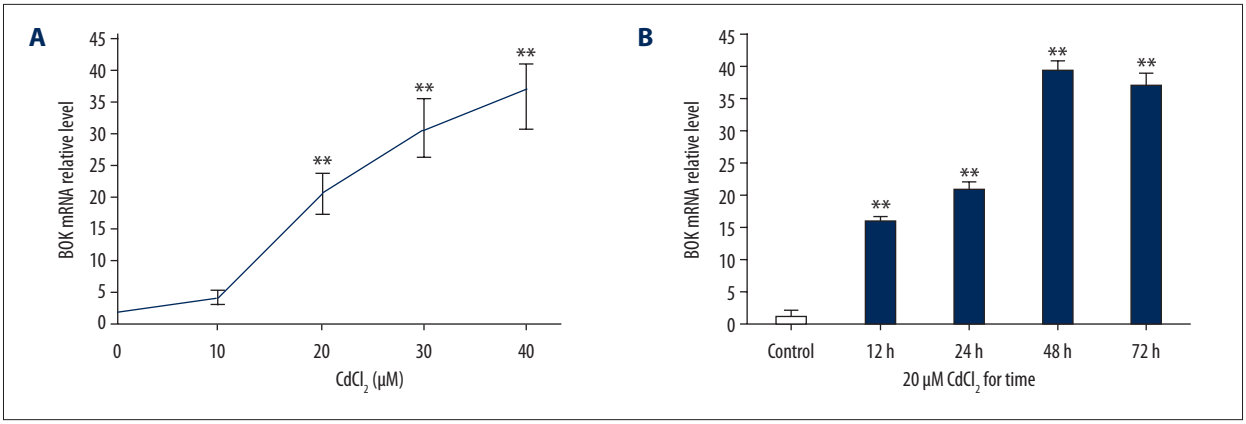

Figure 1. BOK mRNA levels in 16HBE cells exposed to $CdCl_2$. (**A**) 16HBE cells were exposed to 0, 10, 20, 30, or 40 μM $CdCl_2$ for 24 h, or (**B**) to 20 μM $CdCl_2$ for 0, 12, 24, 48, or 72 h. BOK mRNA expression is expressed relative to untreated control cells. All data are presented as means ±SEM. ** $P<0.01$.

Table 1. Primers for BOK and β-actin used for RT-PCR.

Gene	Primers
BOK	Forward: 5'-GCGATGAGCTGGAGATGATCC-3' Reverse: 5'-CTGCAGAGAAGATGTGGCCA-3'
β-actin	Forward: 5'-ACAGAGCCTCGCCTTTGCCGAT-3' Reverse: 5'-CTTGCACATGCCGGAGCCGTT-3'

The gene-specific primers are listed as Table 1. Changes in expression of genes were calculated by a comparative threshold cycle (Ct) method using the formula 2-(ΔΔCt) using ABI7900HT software (ABI 7900HT, USA). Values were normalized to an internal reference.

Western blotting

Total protein was extracted with Enhanced BCA Protein Assay kits (Beyotime, P0009), electrophoresed on NuPAGE 4–12% Bis-Tris gels, and transferred to nitrocellulose membranes according to the manufacturer's directions (Invitrogen, Carlsbad, CA). Immunoblotting was performed with rabbit antibodies against human BOK and β-actin, coupled with horseradish peroxidase-conjugated anti-rabbit secondary antibody according to the kit's instructions. Densitometric analysis of Western blotting blots was done using AzureSpot software.

Trypan blue exclusion assay for cell growth

Cells were seeded into 6-well plates at 3×10^5 cells per well. Fresh medium (2 ml) was added. The numbers of cells were counted at 12, 24, 48, and 72 h, and assays were performed in triplicate. The cells (50 μl) were mixed with the same volume of 0.4% trypan blue. The numbers of stained cells were counted in a hemocytometer under a light microscope [22].

MTT colorimetric assays

16HBE cells were seeded into 96-well cell culture plates at 4000 cells per well. Plates were incubated for 24 h at 37°C with 5% of CO_2 in a humidified incubator. The cells were exposed to 0, 10, 20, 30, or 40 μM $CdCl_2$ for 24 h, or to 20 μM $CdCl_2$ for 12, 24, 48, or 72 h. Following incubation, 20 μl of a 5 mg/ml solution of MTT in 0.01 M phosphate-buffered saline (PBS) was added to each well, and incubation was continued for an additional 4 h. Cells were then solubilized by the addition of 150 μl of DMSO to each well. Plates were read on a BioRad enzyme-linked immunosorbent assay (ELISA) plate reader using a 570-nm filter. It is calculated according to the following formula: Cells inhibition rate=[1–(exposure group-blank group)/(control group–blank group)]×100%.

Comet assays

Cells (1×10^5~2×10^5) were seeded in 6-well plates and treated with 0, 10, 20, 30, or 40 μM $CdCl_2$ for 24 h, or with 20 μM $CdCl_2$ for 12, 24, 48, or 72 h. Following incubation, cells were digested with 0.25% trypsin-EDTA and suspended in PBS, then the total numbers were counted, and portions (1×10^6 cells/tube) were placed in microcentrifuge tubes. The cell suspensions were mixed with low-melting point agarose (Genview-bio, 901203606, final concentration: 0.45% (w/v)) and spread on glass slides printed with water-repellent marks. The slides were immersed in cold lysis solution (100 mM Na_2EDTA, 2.5 M NaCl, and 10 mM tris (hydroxymethyl) aminomethane containing 1% (v/v) Triton X-100 and 10% (v/v) DMSO, pH 10.0) overnight at 4°C. Two slides per sample were incubated in alkaline solution (300 mM sodium hydroxide and 1 mM Na_2EDTA, pH >13) for 20 min at 4°C, followed by electrophoresis in the same buffer for 20 min at 0.7 V/cm with a constant voltage of 300 mA. After electrophoresis, the slides were immersed in neutralization buffer [0.4 M tris (hydroxymethyl) aminomethane, pH 7.5]

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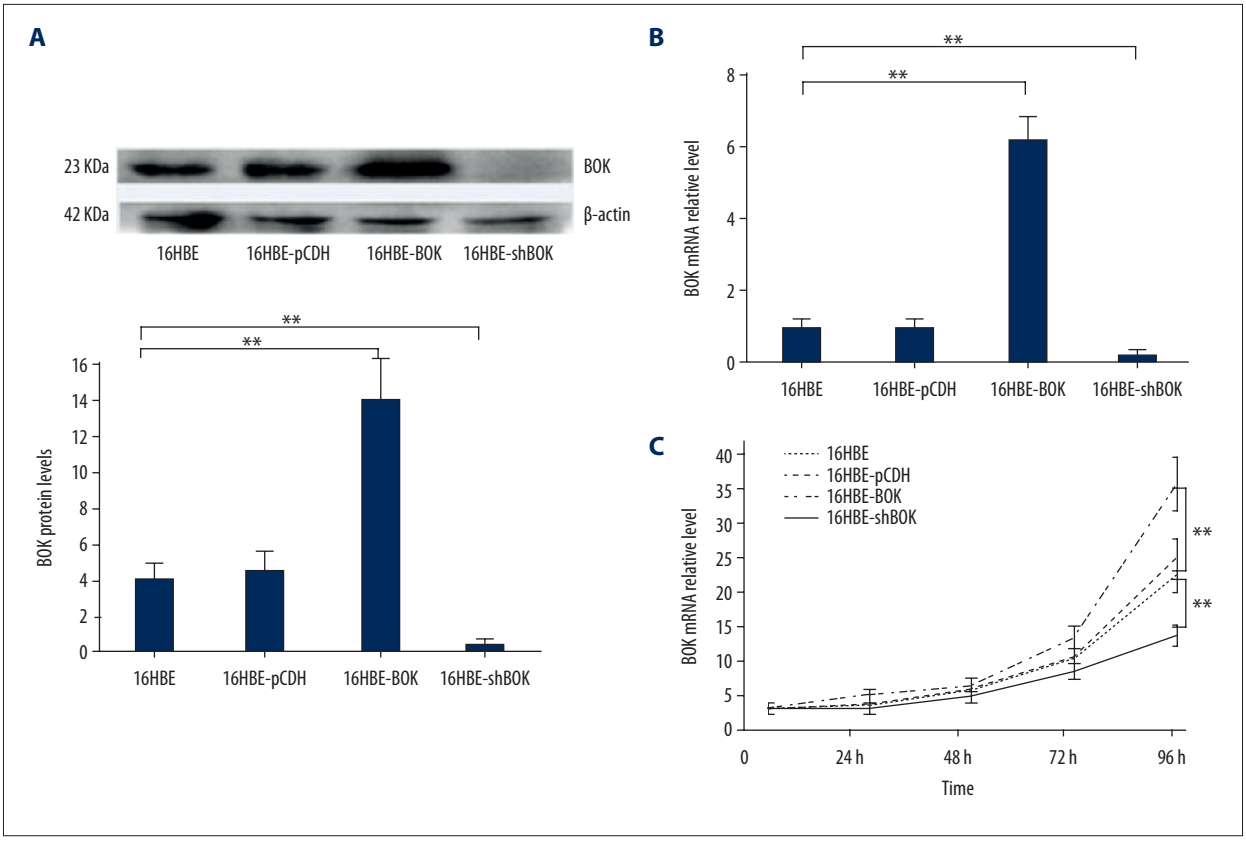

Figure 2. BOK affects cellular proliferation. Measurements of BOK protein (**A**) and mRNA (**B**) were used to confirm BOK expression in cells over-expressing BOK (16HBE-BOK) and in cells with BOK knockdown (16HBE-shBOK). (**C**) The trypan blue exclusion assay was used to assess cell growth. All data are presented as means ±SEM. ** $P<0.01$.

for 5 min. The slides were dehydrated by immersion in absolute ethanol for 5 min and air-dried at room temperature. DNA was stained with ethidium bromide, and cells were observed under a fluorescence microscope (Nikon Eclipse Ti-E microscope) and analyzed with Comet Score TM Version 1.5 software. Two replicate slides were prepared for each sample, and 50 randomly chosen cells on each slide were analyzed. The Olive tail moment (OTM), tail DNA%, tail length (TL), and tail moment (TM) of each sample were calculated [23,24].

Statistics

There were 3 parallel samples for each observation, and each experiment was repeated 3 times. SPSS 13.0 was used for Dunnett *t* tests. Data are presented as the means and standard errors of the mean (mean ±SEM). $P<0.05$ was considered as statistically significant.

Results

Upregulation of BOK in 16HBE cells exposed to cadmium

16HBE cells were exposed to 0, 10, 20, 30, or 40 μM $CdCl_2$ for 24 h, or to 20 μM $CdCl_2$ for 12, 24, 48, or 72 h. The expression of BOK mRNA, measured by qRT-PCR, was up-regulated at all concentrations and times of exposure to $CdCl_2$ as compared to control 16HBE cells (0 μM $CdCl_2$) (Figure 1). These data show that $CdCl_2$ exposure leads to an increase in expression of BOK mRNA.

BOK over-expression induced cell proliferation, and BOK deficiency limited cell proliferation

To determine whether BOK is involved in the toxicity of $CdCl_2$ to 16HBE cells, we constructed BOK over-expressing cells (16HEB-BOK) and BOK deficiency cells (16HBE-shBOK) by BOK-ORF and small hairpin RNA (shRNA). The results of Western blotting (Figure 2A) and qRT-PCR (Figure 2B) show that BOK was over-expressed in 16HBE-BOK cells and was knocked down (by approximately 85%) in 16HBE-shBOK cells. There were no clear phenotypic or apoptotic level changes between the

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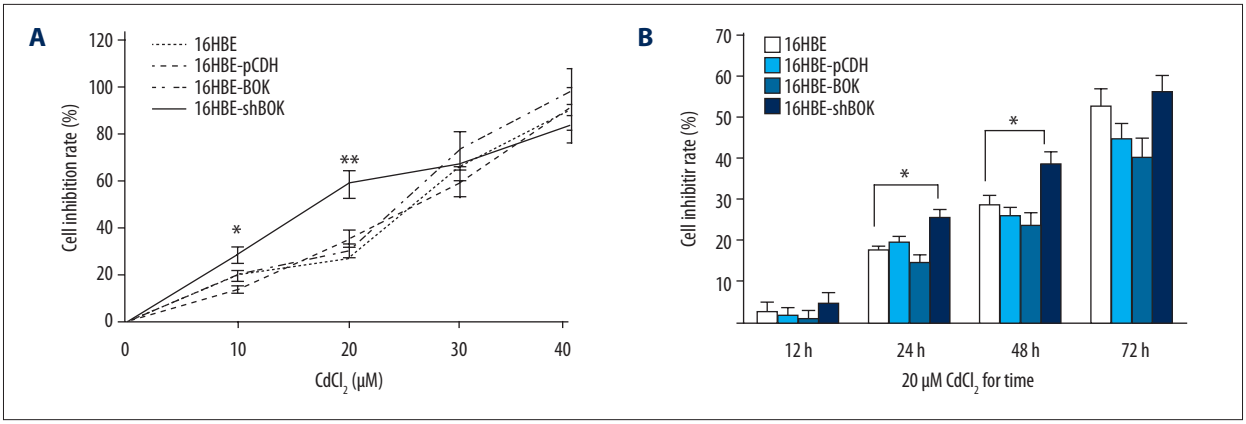

Figure 3. BOK affects cell growth inhibition by $CdCl_2$. Inhibition of cell growth by 24 h exposure to $CdCl_2$ (**A**) at concentrations of 10, 20, 30, or 40 μM and (**B**) inhibition of growth of cells exposed to 20 μM $CdCl_2$ for 12, 24, 48, or 72 h, as measured by the MTT assay. All data are presented as means ±SEM. * P<0.01, ** P<0.01.

constructed cells (16HBE-BOK and 16HBE-shBOK) and 16HBE cells (Supplementary Figures 2, 3). To determine if various expressions of BOK affected cell proliferation, trypan blue exclusion assays were used to assess cell growth. After 72 h, 16HBE-BOK cells had proliferated more than 16HBE cells, but 16HBE-shBOK cells had proliferated less (Figure 2C). These results indicated that high and low levels of BOK affect cell proliferation.

BOK deficiency enhanced cell death caused by$CdCl_2$

Data from the MTT assays of cells exposed to various concentrations of $CdCl_2$ for 24 h are shown in Figure 3A. For those exposed to 10 or 20 μM $CdCl_2$, the inhibition of 16HBE-shBOK cells was greater than that for 16HBE cells. Cells were also exposed to 20 μM $CdCl_2$ for 12, 24, 48, or 72 h. The growth of 16HBE-shBOK cells at 24 h and 48 h was less than that for the control cells (Figure 3B). These data indicate that BOK deficiency enhances $CdCl_2$-induced cell death.

BOK over-expression decreased H_2O_2-induced DNA damage; BOK deficiency had the opposite effect.

After exposure of cells to 125 μM H_2O_2 for 24 h, comet assays were used to detect damaged DNA (Figure 4A). For 16HBE-BOK cells, values for the tail DNA%, TM (tail moment), and OTM (Olive tail moment) were lower; these values for 16HBE-shBOK cells were higher compared with those for 16HBE (Figure 4B). Thus, low expression of BOK leads to more extensive oxidative damage to cell DNA, and high expression of BOK promotes the repair of such damage.

BOK deficiency increases $CdCl_2$-induced DNA damage

Cadmium does not directly cause DNA damage; however, it induces increases in production of ROS, which in turn cause DNA damage [5–7]. To gain insight into the role of BOK in DNA damage caused by $CdCl_2$, comet assays were performed with cells exposed to $CdCl_2$. There were no appreciable differences between BOK over-expressed/knockdown cells and control 16HBE cells in OTM, tail DNA%, TL, or TM for cells exposed to 0, 10, 20, 30, or 40 μM $CdCl_2$ for 24 h (Figure 5A, 5B). However, at 48 and 72 h, DNA damage in 16HBE-shBOK cells was greater than that in 16HBE cells exposed to 20 μM $CdCl_2$ (Figure 6A, 6B). These data indicate that, in 16HBE cells, elevated BOK lowers DNA damage caused by $CdCl_2$, whereas BOK deficiency increases DNA damage in cells exposed to $CdCl_2$.

Discussion

Cadmium is an industrial and environmental pollutant and a group I human carcinogen [3]. Epidemiologic studies have found that cadmium levels less than 1 μg in blood are a health risk for humans [1,2]. However, cadmium has a long biological half-life and accumulates in and remains in organs and tissues for long periods [25,26]. Acute or chronic exposure of cells to cadmium generates ROS, and these species are responsible for cadmium-induced DNA damage [5–7,27,28]. However, the mechanisms of cadmium-induced damage have yet to be fully elucidated.

Since BOK is a conserved and widely expressed BCL-2 family member with sequence homology to pro-apoptotic BAX and BAK, it has been proposed to act in a similar pro-apoptotic pathway [17]. However, it is controversial whether BOK is a BCL-2 family member and whether it induces apoptosis independently of BAX/BAK [17,29–36]. BOK mRNA/protein is increased after a brief exposure of cells to some chemicals [15,17], bacteria, and viruses [18,19]. Our findings show that the expression of BOK mRNA was up-regulated in 16HBE cells at all concentrations and times of exposure to $CdCl_2$. These data

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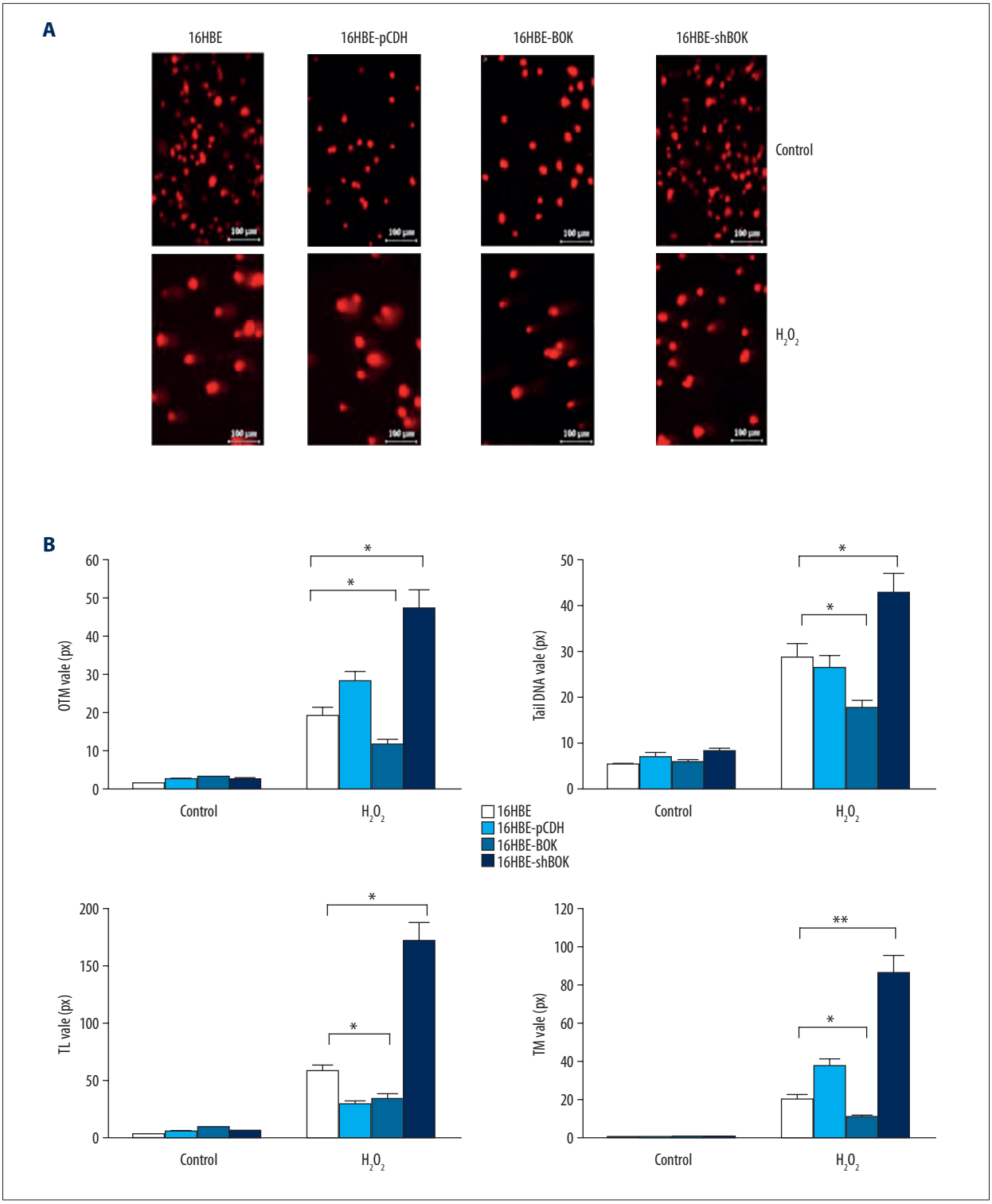

Figure 4. BOK affects H_2O_2-induced DNA damage. (**A**) Pictures from the comet assay of cells exposed to 125 μM H_2O_2. Two replicate slides were prepared for each type of cell, and 50 randomly chosen cells on each slide were analyzed. The Olive tail moment (OTM), tail DNA%, tail length (TL), and tail moment (TM) of each sample were calculated as the means of the median values for the slides (**B**) (asterisks show significant differences). All data are presented as means ±SEM. * $P<0.01$, ** $P<0.01$.

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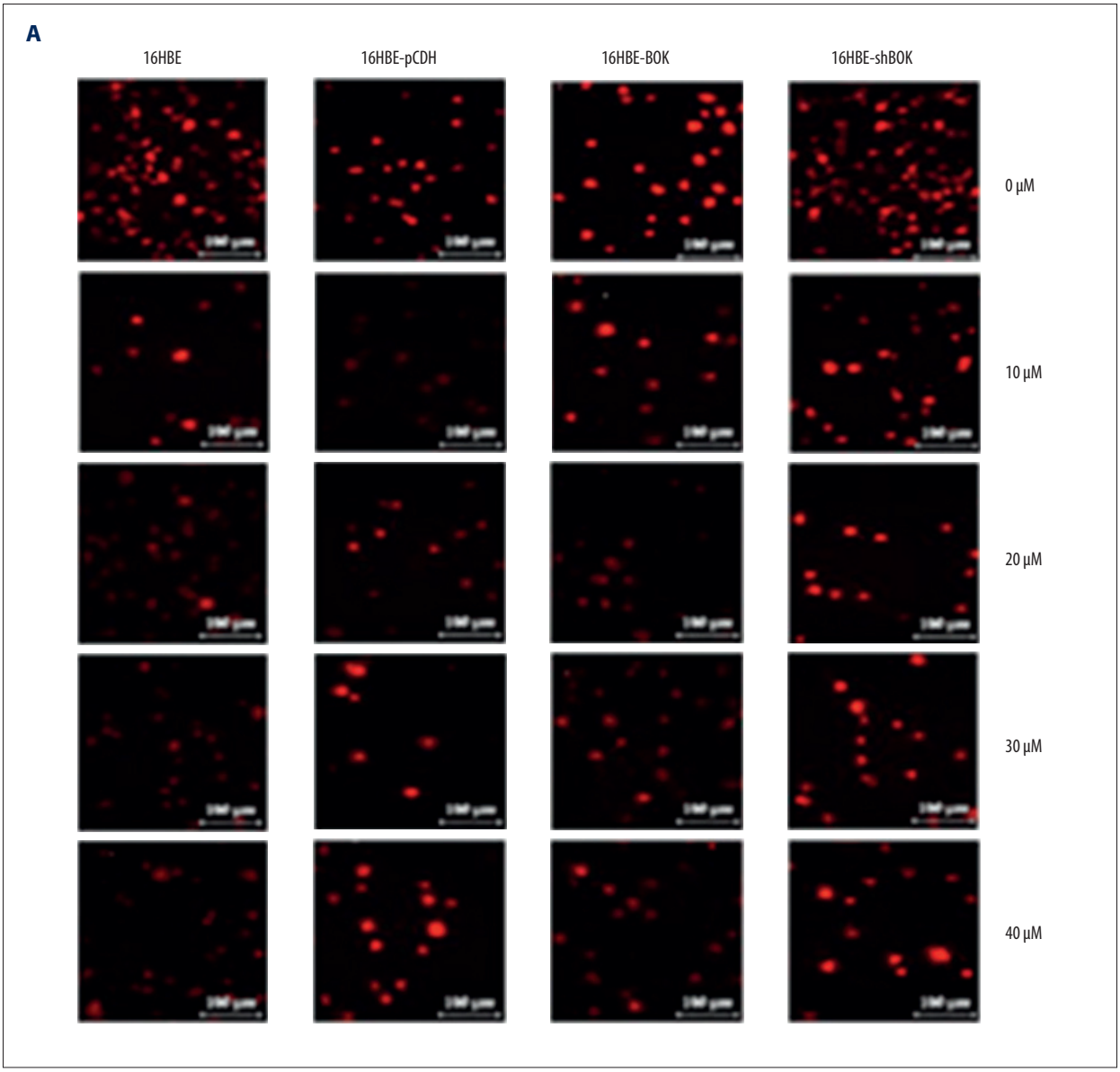
indicate that brief exposure to $CdCl_2$ can lead to increases in BOK mRNA expression.

Some BCL-2 family members are involved in cell proliferation and survival [37]. BOK links the cell cycle and cell death machineries upstream of mitochondrial and endoplasmic reticulum damage [15]. In $Bok^{-/-}$ animals, hepatocellular carcinomas (HCCs), BOK-deficient human HCC cell lines, and non-transformed cells, there is less proliferation than in BOK-proficient controls [17]. To investigate the role of BOK in cadmium-induced damage, we constructed BOK over-expressing (16HBE-BOK) cells and BOK knockdown (16HBE-shBOK) cells. There were no clear phenotypic or apoptosis changes between the constructed cells (16HBE-BOK and 16HBE-shBOK) and 16HBE cells. The data from cell growth experiments show that, after 72 h, 16HBE-BOK cells proliferated more than 16HBE cells and 16HBE-shBOK cells proliferated less. These results indicate that BOK over-expression induces cell proliferation and that BOK deficiency limits cell proliferation. Thus, BOK levels affect cell proliferation.

To gain further insight into the function of BOK in proliferation and survival of cells exposed to cadmium, the inhibition of 16HBE-BOK cells, 16HBE-shBOK cells, and 16HBE cells by $CdCl_2$ was assessed. The data from MTT assays showed that inhibition of the 16HBE-shBOK cells was greater than that for 16HBE cells exposed to $CdCl_2$. These findings show that BOK deficiency promotes cell death caused by cadmium.



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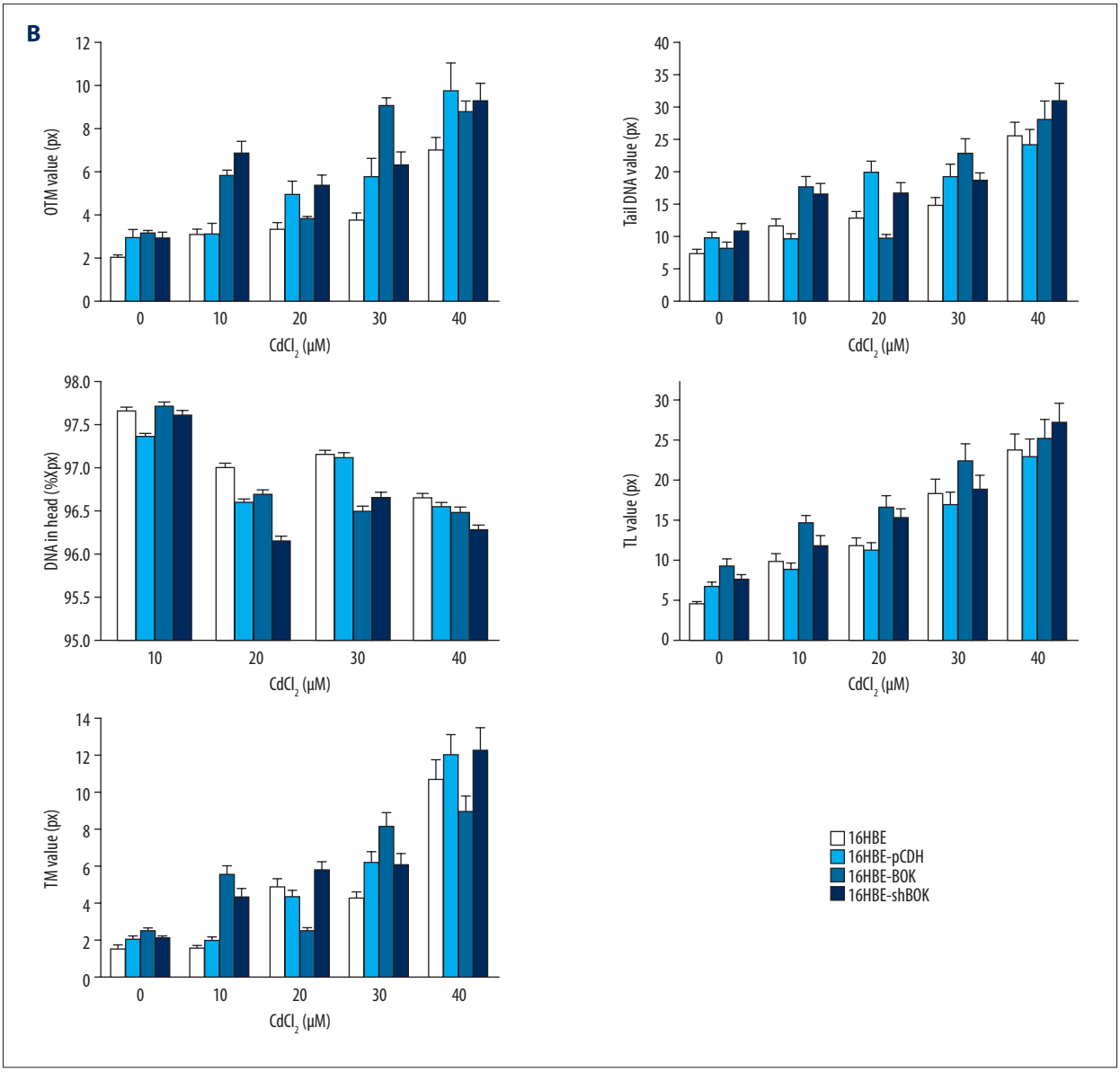

Figure 5. (**A**) Pictures for the comet assay of cells exposed to 0, 10, 20, 30, or 40 μM $CdCl_2$ for 24 h. (**B**) The Olive tail moment (OTM), Tail DNA%, Tail length (TL), DNA in head and tail moment (TM) of cells at different doses for 24 h were calculated as the means of the median values for the slides. (provide asterisks showing significant differences) All data are presented as means ±SEM. * *P*<0.01, ** *P*<0.01.Two replicate slides were prepared for each type of cell, and 50 randomly chosen cells on each slide were analyzed.

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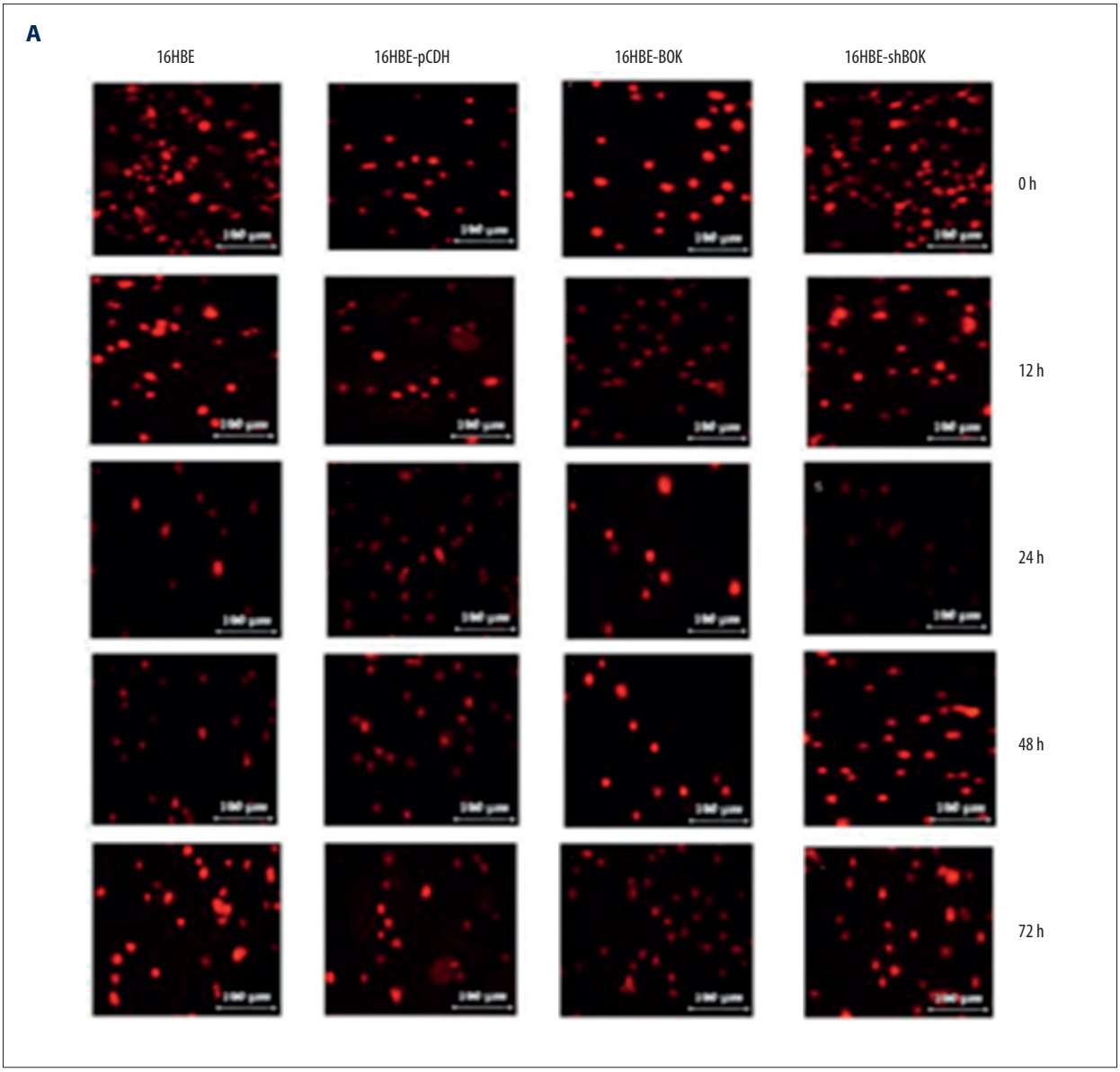

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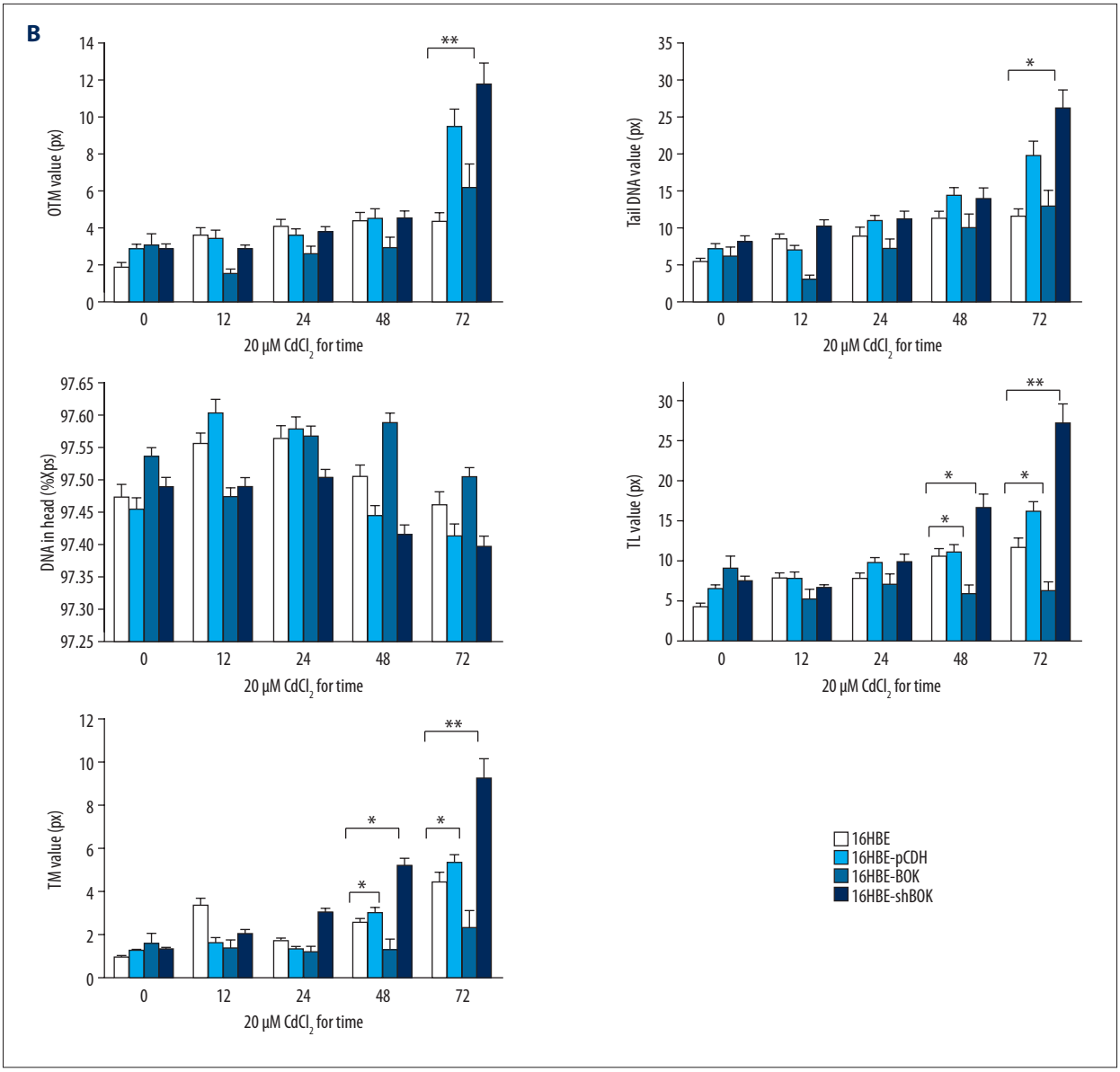

Figure 6. BOK affects DNA damage caused by $CdCl_2$ with different times. (**A**) Pictures for the comet assay of cells exposed to 0, 12, 24, 48, or 72 h with 20 μM $CdCl_2$. (**B**) The Olive tail moment (OTM), Tail DNA%, Tail length (TL), DNA in head and tail moment (TM) of cells at different doses for different hours were calculated as the means of the median values for the slides (provide asterisks showing significant differences). All data are presented as means ±SEM. * $P<0.01$, ** $P<0.01$. Two replicate slides were prepared for each type of cell, and 50 randomly chosen cells on each slide were analyzed.

Reactive oxygen species (ROS) are produced in aerobic organisms during normal physiological metabolism. However, excess ROS damages DNA, RNA, proteins, and other macromolecules. When cells are damaged in this manner, the process of apoptosis is initiated [38]. Whether intracellular or exogenous, H_2O_2 can cause DNA damage [39]. In the present study, data from comet assays of cells following exposure to H_2O_2 showed that BOK deficiency elevated oxidative damage to DNA and that over-expression of BOK promoted the repair of oxidative damage.

ROS, often implicated in cadmium-induced cytotoxicity, lead to mitochondrial dysfunction and inhibition of respiration. Cadmium causes oxidative damage, as demonstrated by DNA strand breaks and chromosomal aberrations [39,40]. In the present study, the DNA damage in cells exposed to $CdCl_2$ was greater in 16HEB-shBOK cells than in 16HBE cells. Thus, a BOK deficiency increases damage to DNA caused by cadmium.

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Conclusions

The present study investigated the role of BOK in acute damage to 16HBE cells exposed to $CdCl_2$. The results showed that short-term exposure to cadmium up-regulated BOK expression and that BOK deficiency decreased cell proliferation and enhanced the DNA damage and cell death caused by cadmium. Thus, in cells exposed to cadmium, BOK provides a protective effect by promoting cell proliferation and decreasing DNA damage.

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Conflicts of interest

None.

Supplementary Figures

The BOK-ORF sequence information:

ATGGAGGTGCTGCGGCGCTCCTCGGTCTTCGCCGCCGAGATCATGGACGCCTTTG
ACCGCTCGCCCACAGACAAGGAGCTGGTGGCCCAGGCCAAGGCGCTGGGCCGGGAGT
ACGTGCACGCGCGGCTGCTGCGCGCCGGCCTCTCCTGGAGCGCGCCCGAGCGTGCCG
CGCCGGTCCCGGGACGCCTGGCTGAGGTGTGCGCGGTGCTGCTGCGCCTGGGCGATG
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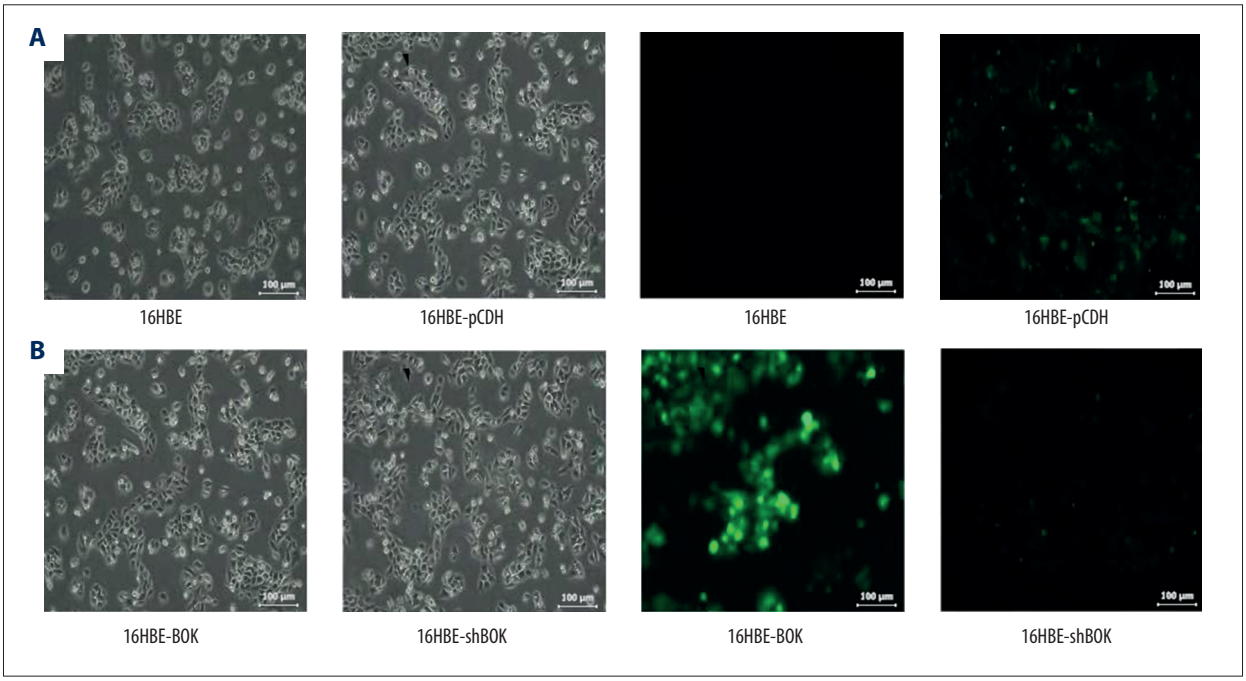
CCTGCAGTCTGAGCCTGTGGTGACCGATGCGTTCCTGGCCGTGGCTGGCCACATCTTC
TCTGCAGGCATCACGTGGGGCAAGGTGGTGTCCCTGTATGCGGTGGCCGCGGGGCTGG
CCGTGGACTGTGTGAGGCAGGCCCAGCCTGCCATGGTCCACGCCCTCGTGGACTGCCT
GGGGGAGTTCGTGCGCAAGACCCTGGCAACCTGGCTGCGGAGACGCGGCGGATGGAC
TGATGTCCTCAAGTGTGTGGTCAGCACAGACCCTGGCCTCCGCTCCCACTGGCTGGTG
GCTGCACTCTGCAGCTTCGGCCGCTTCCTGAAGGCTGCCTTCTTCGTGCTGCTGCCAG
AGAGA

Supplementary Figure 1. Information relevant to the BOK-ORF sequence. There are 636 bases within BOK-ORF.



Supplementary Figure 2. Pictures of cells. Four types of cell morphology as seen with an upright light microscope (20×) (**A**) and with a fluorescence microscope (20×) (**B**). Compared with the 16HBE cells, 16HBE-BOK and 16HBE-hBOK cells showed no obvious morphological differences. All the cells were transparent, polygonal, and well adhered. The over-expression or deficiency of BOK did not affect cell morphology.

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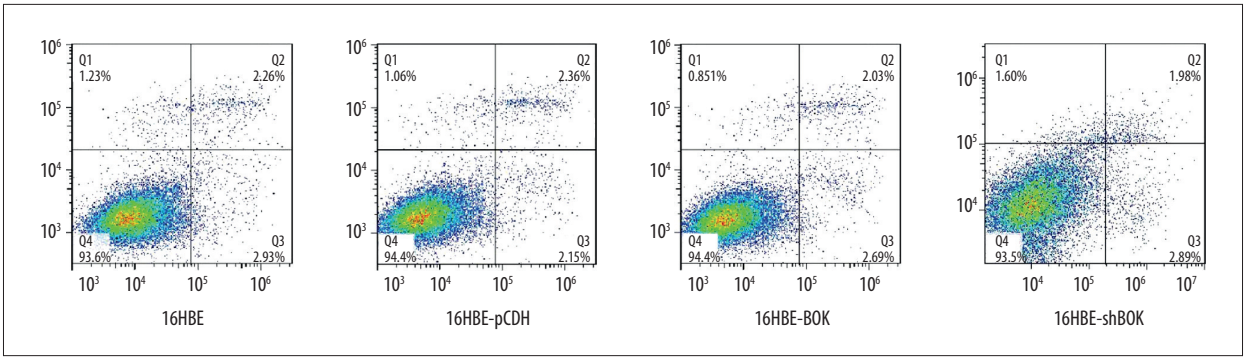

Supplementary Figure 3. 16HBE, 16HBE-pCDH, 16HBE-BOK, and 16HBE-shBOK cells were seeded into 10-cm dishes and cultured for 24 h. Annexin V-FITC/PI double staining kits were used to assess apoptosis. Among the 4 types of cells, there was no appreciable difference in apoptosis.

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