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Reproductive toxicity of 4-octylphenol induced mitochondria-mediated apoptosis in male mouse-specific niche cells

Mingtian Zhang[1,†], Hyun Jung Park[1,†], Kwonho Hong[1], Chankyu Park[1], Hyuk Song [1,*]

[1] Department of Stem Cell and Regenerative Biology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 05029, Republic of Korea

† These authors contributed equally to this work

*Correspondence: Prof. Hyuk Song, Department of Stem Cell and Regenerative Technology, Konkuk University, 1 Hwayang-dong, Gwangjin-gu, Seoul 05029, Republic of Korea; Tel: +82-2-450-0562; e-mail: songh@konkuk.ac.kr

Abstract

The toxic effects of 4-octylphenol (4-OP) have been studied in species such as mouse and fish; however, the toxic effects of 4-OP in male specific niche cells has not been researched. In this study, we investigated the molecular mechanism of toxicity of 4-OP in mouse TM4 Sertoli cells. TM4 cells were treated with four concentrations (0, 10, 30, and 50 μM/mL) of 4-OP at time points 24, 48, and 72 h. Cell viability and apoptosis assay was conducted following exposure. 4-OP significantly decreased cell viability in a concentration- and time-dependent manner, and increased apoptosis. Quantitative PCR analysis showed that Bad, Bax, and Bak mRNA expression levels were higher in exposed cells than in the control, but Bcl-2 expression was decreased. Western blotting revealed that 4-OP induced activities of caspase-3 and phosphorylation of Bad in a concentration- and time-dependent manner. Additionally, cytochrome C protein did not colocalize with mitochondria marker dye by 24 h. Cytochrome c protein expression increased in a time-dependent manner with 50 μM/mL. These results suggest that 4-OP induces mitochondria-mediated apoptosis by regulation of Bcl-2 family proteins and caspase-3 activation in male Sertoli cells.

Keywords: 4-octylphenol, Male Sertoli cells, Reproductive toxicity, Apoptosis, Mitochondria

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1. Introduction

4-Octylphenol (4-OP), one of many long-chain alkylphenols (APs), has been reported to cause environmental contamination through its use by industries worldwide to optimize the manufacturing of common products such as lubricants, plastics, cosmetics, and detergents [1]. APs are accumulated in the human body via ingestion, inhalation, and dermal absorption, and have even been found in maternal blood plasma, amniotic fluid [2], and breast milk [3].

Several studies have reported that 4-OP is a typical endocrine disruptor with estrogenic action, and environmental exposure to endocrine disrupting chemicals (EDCs) has adverse effects on the human reproductive system [4]. It has been reported that long-term 4-tert-octylphenol (OP) exposure in bank voles resulted from disturbed androgen and estrogen synthesis and action (20850518). In addition, a study of mice leydig cells exposed to 4-OP showed a decrease in the secretion of dehydropiandrosterone, androstenedione, and testosterone, and reactive oxygen species overproduction [5]. Similarly, juvenile mouse exposure to OP inhibited steroidogenesis by decreasing the expression of steroidogenic enzymes in the testes [6]. Subcutaneous injections of 80 mg OP in an oil vehicle 3 times weekly decreased sperm production in adult male rats [7], and the number of mitotic germ cells and pre-spermatogonia was reduced in human fetal gonads during a 3-week culture period with 4-OP treatment [8]. In females, the proliferation of uterine luminal, glandular, and stromal cells, and vaginal epithelial cells were increased in adult ovariectomized rats following subcutaneous injection of OP [9].

Fetal exposure to the weak estrogenicity of OP enhanced the induction of mammary carcinomas in rats [10]. Early neonatal exposure to 4-OP (50 mg/kg) by oral gavage caused delayed sexual maturation and deceased ventral prostate weight [11].

Apoptosis is a common form of programmed cell death that causes morphological changes including cell shrinkage, nuclear fragmentation, and chromatin condensation [12]. Cellular apoptosis is regulated by two typical activation mechanisms: the intrinsic pathway and the extrinsic pathway [13]. Apoptosis via the intrinsic pathway can be induced by the release of cytochrome c from mitochondria and a change in the level of pro-apoptotic Bcl-2 family proteins such as Bax [14]. Among APs, 4-nonylphenol induced thymocyte apoptosis via the intrinsic pathway, including caspase-3 activation and mitochondrial depolarization [15]. It is well-known that Sertoli cells are necessary for the progression of germ cells into sperm in male testes. The damage of Sertoli cells caused by environmental toxicants can negatively affect spermatogenesis.

Although many studies have reported the toxic effects of OP in reproductive organs and various cells, the molecular mechanism of 4-OP in the niche of male germ cells has not been studied in detail. Therefore, the present study examined the molecular mechanism underlying 4-OP mediated toxic effects in TM4 sertoli cells.

2. Results

2.1. Effect of 4-OP on viability of TM4 cells

Figure 1A illustrates the chemical structure of 4-OP. We determined the cytotoxic effects of 4-OP on the cell proliferation of TM4 sertoli cells. MTT assay was done at 24, 48, and 72 h. TM4 cells were treated with the concentrations of 4-OP (10, 30, 50, and 100 μM/mL). As shown in Figure 1B, cell viability significantly decreased when cells were exposed to the lowest concentration of 4-OP (10 μM/mL) for 72 h, although cell viability was only slightly affected when the cells were exposed to the same amount for 24 and 48 h. Notably, marked decrease in cell viability was observed following incubation with 4-OP at concentrations of 30 and 50 μM/mL at 24, 48, and 72 h (Fig. 1B). The morphological change observed 72 h after treatment was dose dependent. 4-OP induced dose-dependent apoptotic cell death on TM4 cells. Cell shrinkage and cytoplasm condensation appeared in 30 and 50 μM/mL of 4-OP treatment (Fig. 1C).

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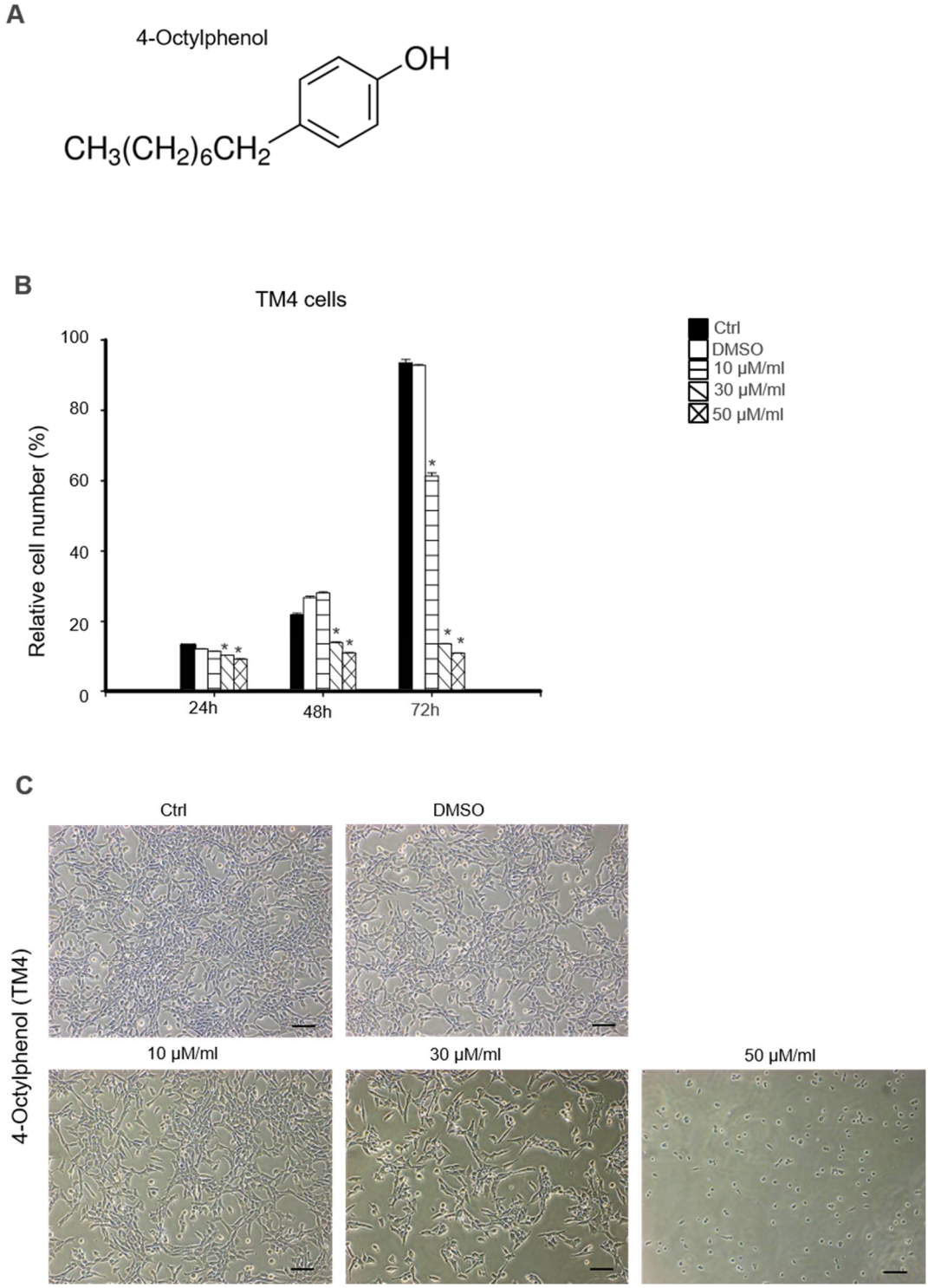

Figure 1. Cytotoxic effects of 4-OP on mouse TM4 Sertoli cells. (A) Chemical structure of 4-OP after treated with increasing doses of 4-OP (0, 10, 30, and 50 μM/mL) at different time points (24, 48, and 72 h), (B) TM4 cell viability was measured by MTT assay. Results are represented as mean ± SD from 3 determinations per condition repeated 4 times. P<0.05, compared with the control. (C) Morphological changes of TM4 cells were photographed under an inverted microscope after treatment with indicated concentration of 4-OP (0, 10, 30, and 50 μM/mL) for 72 h. Scale bars = 100 μm.

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2.2. Effects of 4- OP on apoptosis of TM4 cells

Cell apoptosis was examined using a TUNEL assay to quantify cellular death. As shown in Figure 3, 4-OP significantly increased the number of TUNEL-positive cells in a dose-dependent manner (0, 30, and 50 μM/mL). In particular, 30 and 50 μM/mL of 4-OP markedly increased TUNEL-positive TM4 cells (Fig. 2A). The data indicated that TUNEL-positive rates of TM4 and control were 13.95±0.14 and 53.29±1.13, respectively (Fig. 2B).

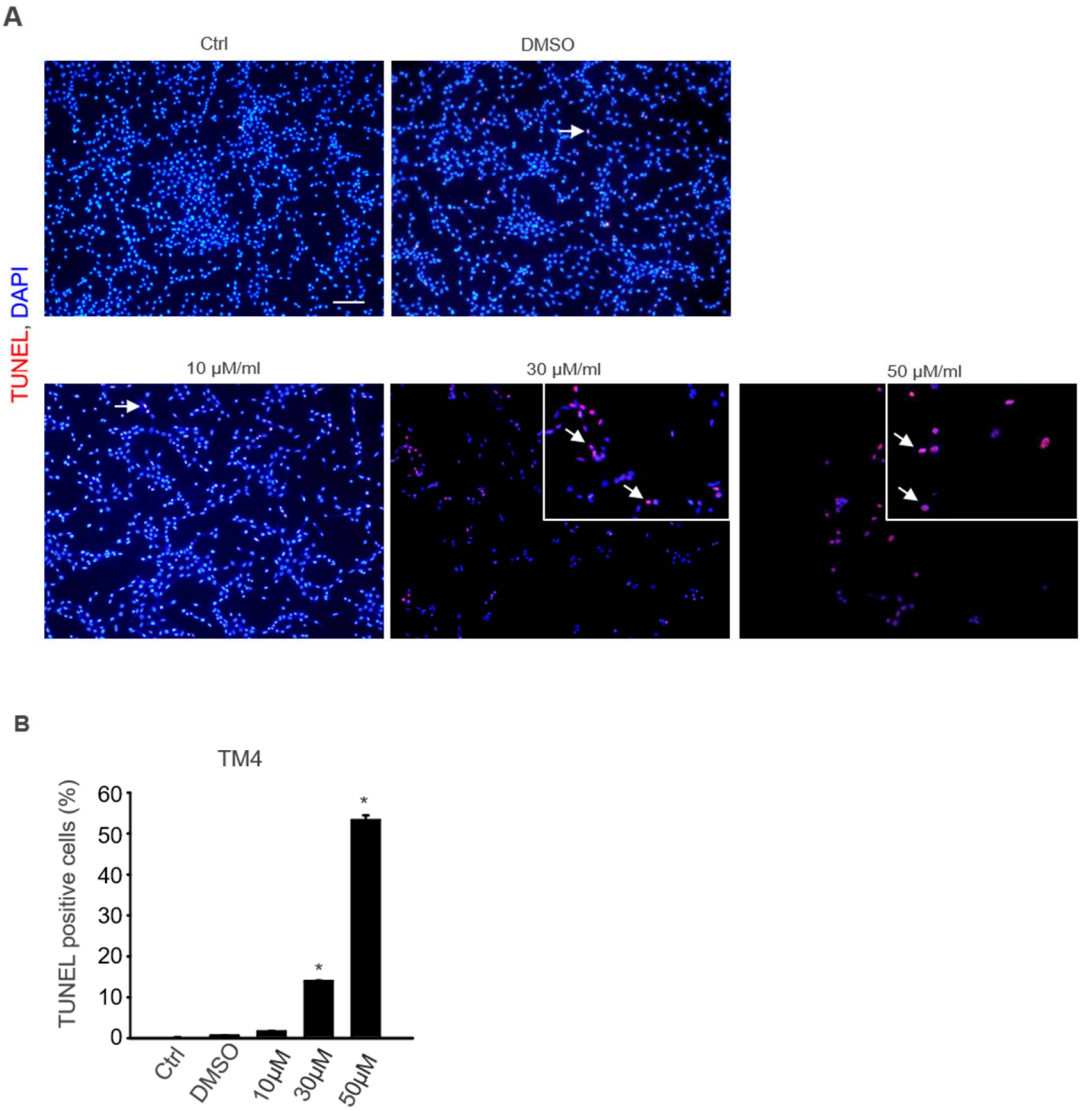

Figure 2. 4-OP-induced cell death in the TM4 cells by TUNEL assay. (A) Detection of in situ DNA breaks by TUNEL assay. Cells were exposed to 0, 10, 30, and 50 μM/mL 4-OP for 24 h, the TUNEL positive cells (arrow) increased dose-dependently in the 4-OP-treated TM4 cells. Bar represents 100μm. (B) Graph representing average number of TUNEL-positive cells in each group. The percentage of TUNEL positive cells in each case were counted and the cumulative data from 3 independent experiments in shown here as mean ±SD (n =3, *p < 0.05 significantly different from control).

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2.3. 4-OP induces the expression of pro-apoptotic gene and protein TM4 cells

To understand the apoptotic effect of 4-OP signaling in detail, we further analyzed several apoptosis-associated molecules. The expression levels of Bax, Bad, Bcl-2, and Bak, mRNAs that play essential roles in modulating apoptosis, were analyzed. Bax, Bad, and Bak mRNA levels in TM4 ells treated with 4-OP increased in dose-dependent manner (Fig. 3). Bad and Bax mRNA levels in TM4 cells treated with 4-OP increased in a dose-dependent manner (Fig. 3A and B). In addition, the expression level of Bak significantly increased in TM4 cells treated with 50 μM/mL of 4-OP, when compared with the control, and decreased Bcl-2 levels were observed in TM4 cells treated with 30 and 50 μM/mL of 4-OP (Fig. 3C and D). Since caspase-3 activation is considered a hallmark of the apoptotic process, activation of caspase-3 by 4-OP was confirmed by western blot analysis to confirm whether pro-apoptotic protein is involved in this apoptosis induction (Fig. 4). Caspase-3 activity was significantly increased in 4-OP exposed TM4 cells in a dose-dependent manner (Fig. 4A). Consistently, 50 μM of 4-OP treatment increased the level of cleaved caspase-3, the active form of caspase-3, in TM4 cells 24 h after treatment, but total caspase-3 was not elevated at all time points when compared with the 0 h control (Fig. 4B). Based on our QPCR result, we examined the effect of 4-OP on the phosphorylation of Bad and found remarkable detection of Bad phosphorylation in TM4 cells after exposure to 4-OP in a dose-dependent manner (Fig. 4C). We found that 50 μM/mL 4-OP also induced Bad phosphorylation of TM4 cells at 24 and 48 h, when compared with the control (Fig. 4D). For normalization, total Bad protein levels were determined with Bad antibody, which reacted with both the phosphorylated and non-phosphorylated Bad.

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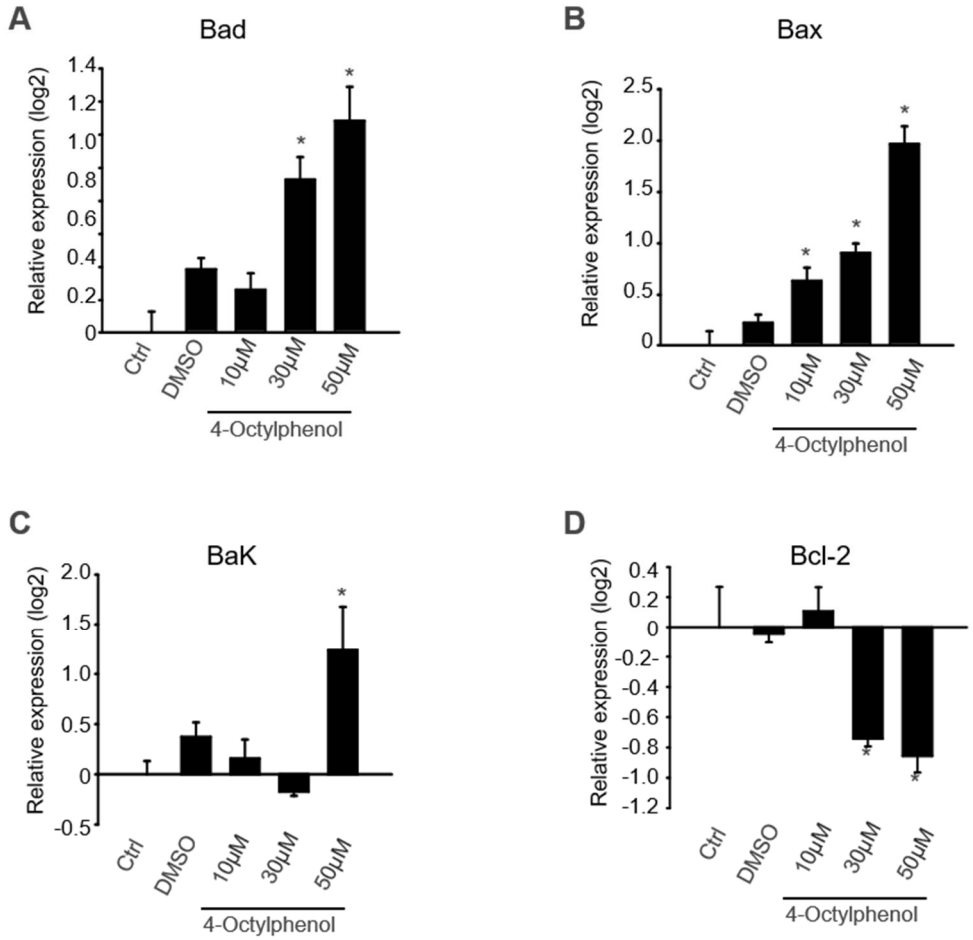

Figure 3. mRNA expression of anti-and pro-apoptotic genes in 4-OP-exposed TM4 cells. The mRNA levels of Bad (A), Bax (B), Bak (C), and Bcl-2 (D) were examined by QPCR on 4-OP exposed TM4 cells in a dose dependent manner (0, 10, 30, and 50 μM/mL 4-OP). Data shows significant difference of 4-OP exposed cells compared to the control group. Results are represented as mean ± SD from 3 determinations per condition repeated 4 times. (n=4, *$p < 0.05$).

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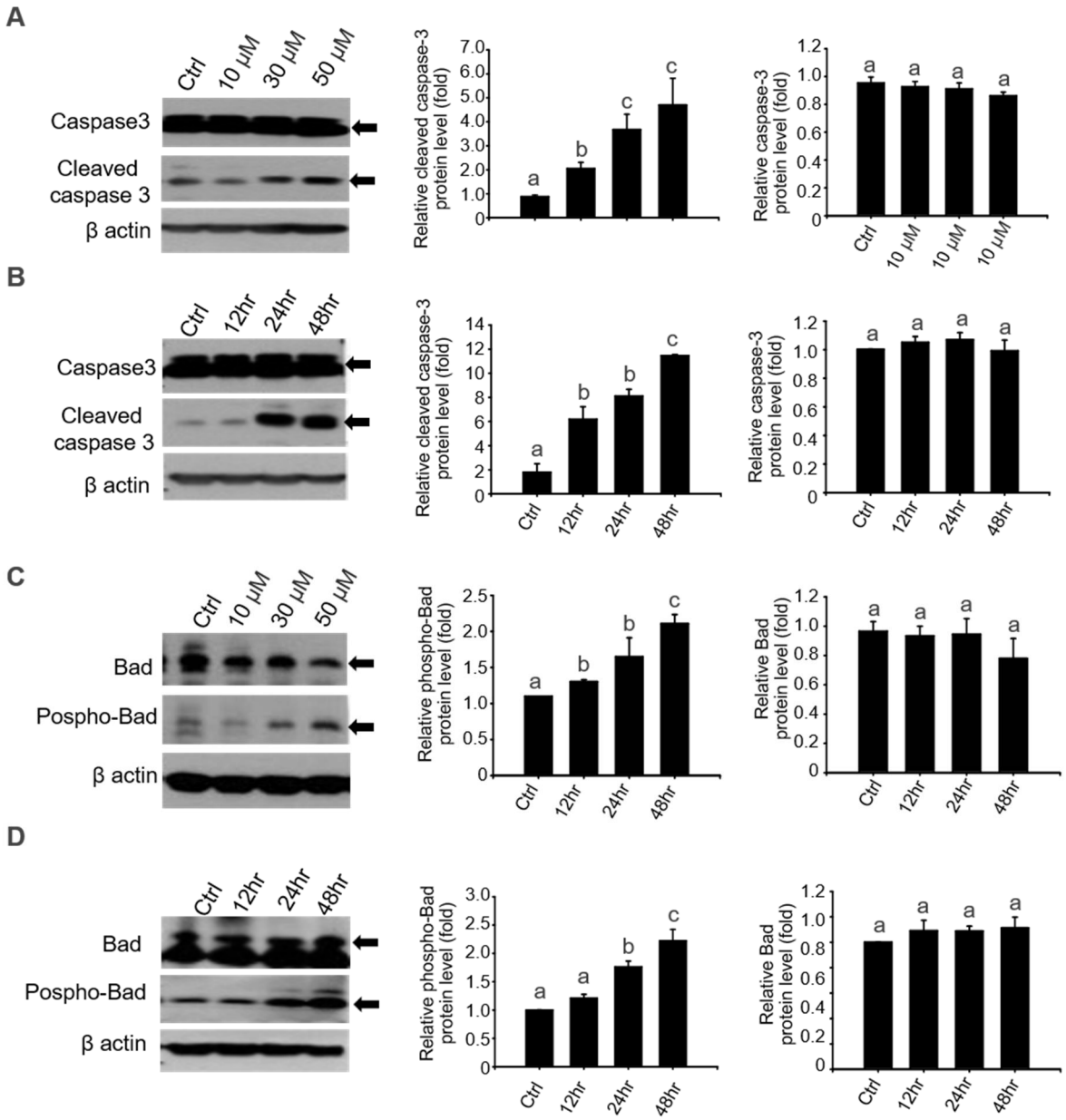

Figure 4. The expression of pro-apoptotic protein in 4-OP-exposed TM4 cells. 4-OP increased the expression of cleaved caspase-3 in 4-OP-exposed TM4 cells. (A) After treatment with different concentrations of 4-OP (0, 10, 30, and 50 μM/mL) for 24 h. (B) TM4 cells were treated with 50 μM/mL 4-OP, respectively, at different time points (0, 24, and 48 h). The expression levels of cleaved caspase-3, caspase-3 and β-actin were examined by western blot. (C) After treatment with different concentrations (0, 10, 30, and 50 μM/mL) for 24 h and (D) different time points (0, 24, and 48 h) of 4-OP. The expression level of phospho-Bad, Bad, and β-actin were examined by western blot. Bar graphs represent the relative density of each band normalized to β-actin or the no active form of each protein. Values represent the mean ± SD of 3 independent experiments (n=3, *$p < 0.05$ compared with the controls).

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2.4. 4- OP induces cytochrome c release from mitochondria in TM4 cells

We determined whether the mitochondria pathway is involved in TM4 apoptosis induced by 4-OP. Generally, cytocrome c release from mitochondria has been proposed to be a critical event that initiates apoptosis in mammals [16]. We examine whether 4-OP induced the release of cytochrome c from mitochondria to the cytosol. The cellular localization of cytochrome c protein was examined by confocal immunofluoresence microscopy in TM4 cells. Labeling was done with MitoTracker, a red fluorescent dye that targets mitochondria. The results showed an apparent difference in the levels of cytochrome c between the treatment and control groups at 24 h post treatment. The pattern of cytochrome c immunofluorescence and mitochondrial dye showed complete colocalization in the untreated controls as overlapping red and green pixels seen as yellow in the TM4 cells (Fig. 5A). In contrast, the pattern of staining observed after treatment with 4-OP revealed that mitochondria were stained with red MitoTracker dye but were no longer colocalized with cytochrome c in the TM4 cells (Fig. 5B). In addition, western blot analysis indicated that 4-OP significantly increased the expression level of cytochrome c (Fig. 5C).

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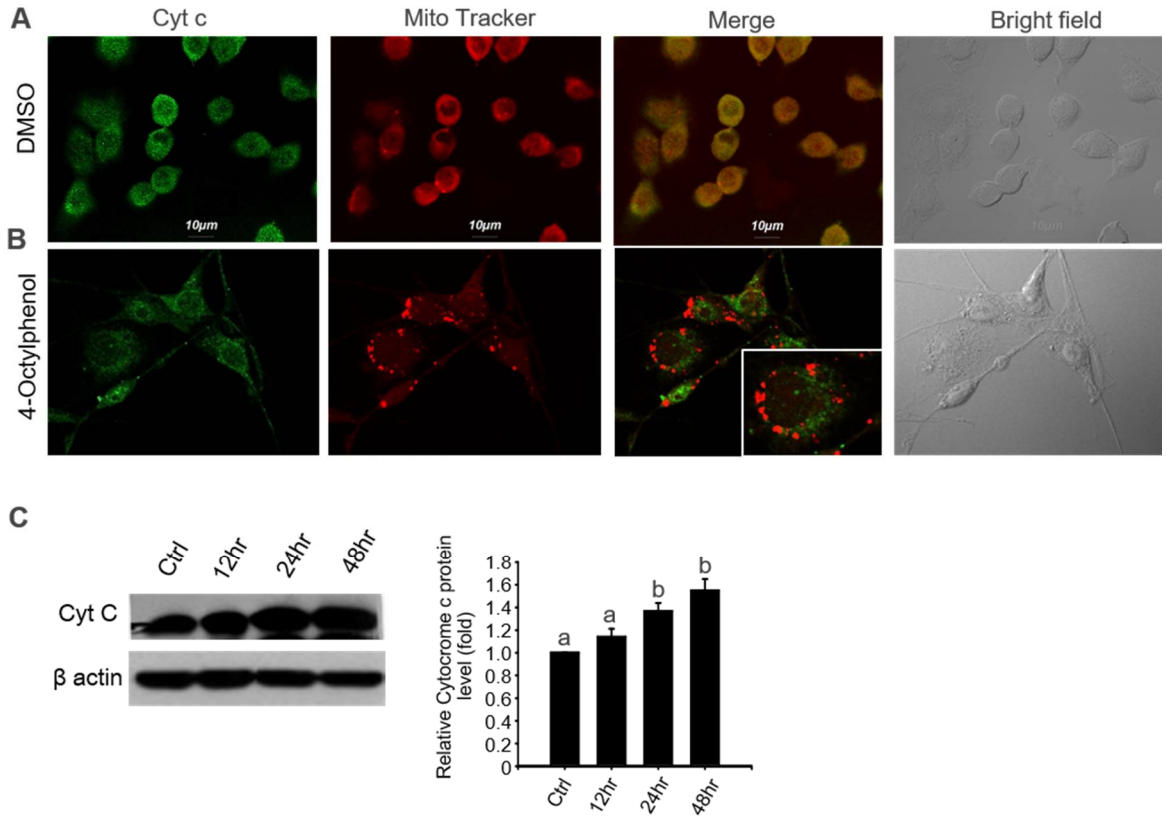

Figure 5. 4-OP induces cytochrome c release in TM4 cells in a time-dependent manner. (A) Effect of 4-OP on cytochrome c expression in TM4 cells. After treatment with 50 μM/mL 4-OP for 24 h, cells were stained with Mito Tracker red (Mito Red) dye followed by immunostaining for cytochrome c and observed under a confocal microscope. Scale bars = 10 μm. (B) Cytochrome c was examined by western blot on 50 μM/mL 4-OP-exposed TM4 cells in time-dependent manner (0, 10, 30, and 50 μM/mL). Bar graphs represent the relative density of each band normalized to β-actin. Values represent the mean ± SD of 3 independent experiments (n=3, *$p < 0.05$ compared with the controls).

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3. Discussion

Our results suggested that 4-OP was toxic to TM4 cells. In addition, we found that 4-OP could induce TM4 cell apoptosis, which was verified by TUNEL analysis. Qian et al similarly reported that OP decreased viability and increased apoptosis in concentration- and time-dependent manner in cultured Sertoli cells derived from Sprague-Dawley rats; however, 30 μM/mL OP did not affect the proliferation of Sertoli cells, in contrast to our TM4 cell viability results [17]. Another study reported that OP had a toxic effect on spermatogenic cells or Sertoli cells in rats [18].

4-OP induces apoptosis through various molecular mechanisms, such as the induction of apoptotic proteins and gene expression. There is much research showing that the Bcl-2 family plays an important role in the regulation of both pro- and anti-apoptotic signals in healthy and stressed cells. Anti-apoptosis proteins in the Bcl-2 family include Bcl-X_L, BCL-$_W$, and Bcl-2, and these proteins appear to directly or indirectly preserve the integrity of the outer mitochondrial membrane, thereby preventing cytochrome c release and cell death initiation. In contrast, Bax, Bak, and Bad are pro-apoptotic proteins that mediate mitochondrial outer membrane permeabilization (MOMP) during apoptosis [19, 20].

In this study, Bad, Bax, and Bak gene expression was markedly higher in 4-OP-exposed TM4 cells than in the control group. Results also showed that 4-OP induced activities of caspase-3 and phosphorylation of Bad in TM4 cells. These findings not only help us understand the anti-proliferation and apoptosis effect of 4-OP, but also improve our understanding of apoptotic signaling pathways. According to previous research, caspases are crucial mediators of apoptosis. In particular, caspase-3 has been extensively studied as a protease that activates cell death, catalyzing the specific cleavage of many proteins, and is dependent on mitochondria cytochrome c release function [20]. Caspase-3 is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types investigated. In addition, a previous study showed Bcl-2, Bax, and caspase-3 activation were involved in the regulation of the PO-induced apoptotic process in cultured rat Sertoli cells, supporting our results [16].

Generally, mitochondria play a key role in mediating apoptosis induction by diverse stimuli. The release of cytochrome c from mitochondria and downstream caspase activation are important in regulating apoptosis [21, 22]. Bcl-2 family proteins regulate MOMP and it has been reported that Bax and Bak are also essential for MOMP and both Bax and Bak deficient cells are resistant to cytochrome c release and apoptosis [23]. We found that Bax and Bak expression was increased in TM4 cells with 4-OP treatment; thus, we analyzed the cytochrome c in these cells and found that cytochrome c protein does not colocalize with mitochondria marker dye in exposed cells 24 h after treatment. In addition, cytochrome c protein increased in a time-dependent manner in 50 μM/mL 4-OP-exposed cells.

4-OP is one of many alkylphenol compounds and is a known an environmental pollutant. Moreover, it is a known EDC with estrogenic effects [24]. Multiple studies have shown the impact of 4-OP in the reproductive systems of different species such as frog [25], swine [26], fish [27, 28], and rodent [5, 29].

Even though Gregory et al. demonstrated that OP treatment of adult rats does not appear to have a major effect on the male reproductive system at a relevant environmental exposure dose [30], many studies have reported the negative effects of 4-OP in testes or testicular cells. In rodent testes, OP appears to inhibit cAMP formation and steroidogenesis in mLTC-1 leydig tumor cells [31]. In addition, 4-OP induced reproductive abnormalities including small testes weight and decreased daily sperm production [32]. Another study reported that administration of 80 mg/kg OP to adult male rats caused shrinkage of the testes and accessory sex organs, and disrupted spermatogenesis. In humans, 4-OP significantly reduced the mitotic index and the number of pre-spermatogonia in cultured fetal gonads [8].

The seminiferous tubules in testes are composed of two major cell types, spermatogenic cells and Sertoli cells. It is well-known that Sertoli cells normally control germ cell apoptosis and spermatogenesis, and facilitate the progression of germ cells to spermatozoa through direct contact in seminiferous tubules [33]. For example, Mono-(2-ethylhexyl) phthalate (MEHP), the active metabolite

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of di-(2-ethylhexyl) phthalate, targets Sertoli cells and makes them dysfunctional, resulting in the rapid induction of testicular germ cell apoptosis [34]. In addition, several models of Sertoli cell injury showed that spermatogenesis is vulnerable to disruption and that targeting critical Sertoli cell functions can lead to rapid and massive germ cell death [35]. Therefore, the apoptosis of Sertoli cells may result from the abnormal effect of 4-OP. Maternal exposure to OP suppressed gonadotropin secretion with decrease in testis size and Sertoli cell number during the fetal life of lamb but there was no effect to development of the reproductive tract in male or female rats, although body weights were significantly decreased [10]. Toxicity of OP has been extensively investigated in other organs as well. For example, OP induced splenocyte apoptosis in rats and mice through Ca2+-dependence [36] and OP also had toxic effects on liver in male rats [37]. Therefore, toxicity evaluation of 4-OP in TM4 Sertoli cells is needed to ensure normal sperm production in humans and animals.

In summary, our data suggest that 4-OP-induced TM4 cell apoptosis occurs in a time- and dose-dependent manner directly through the mitochondrial apoptotic pathway. Of particular importance is that high dose of 4-OP induced TM4 apoptosis via the down-regulation of Bcl-2; in contrast, Bak, Bad, Bax expression was upregulated with subsequent activation of the caspase-3 pathway. These findings can contribute to understanding the mechanism of 4-OP on the male reproductive system through its action on the TM4 Sertoli cells.

4. Materials and Methods

4.1. Cell culture and treatment

Mouse TM4 Sertoli (TM4) cells were purchased from the Korean cell line bank (KCLB 21715, South Korea). The cells were cultured in Dulbecco's Modified Eagle's medium, 10% fetal bovine serum (FBS), and 1% penicillin streptomycin solution, in a humidified atmosphere of 5% CO_2 at 37 ℃. 4-OP was purchased from Sigma Aldrich (Sigma-aldrich, St. Louis, MO, USA), and dissolved in dimethyl sulfoxide (DMSO) to make a stock solution. The stock solution was diluted into the cell culture media prior to treatment to prepare the desired concentration.

4.2. Cell viability and morphologic analysis

Cell viability was determined using the EZ-Cytox Viability assay kit (Daeil Lab Services Co, Seoul, Korea, #EZ1000) following the manufacturer's instructions. TM4 cells were seeded in 96-well plates at a density of 5×10^3 per well in culture medium and incubated for 24 h at 37 ℃. After 24 h, medium was replaced with fresh medium containing different concentrations of 4-OP (10, 30, 50, or 100 μM/mL). Cell viability assay was performed at multiple time points (24, 48, and 72 h). Assay reagent was added (10 μL per well) and incubated for 30 min. The incubated plate was read on a spectrophotometer (Sunrise™, TECAN) at a wavelength of 490 nm. Cell images were collected for each dose of 4-OP after 72 h.

4.3. Apoptosis detection with TUNEL assay

To evaluate cell death by apoptosis, an in situ cell death detection kit, TMR red (Roche, Germany), was used to quantify DNA and chromatin morphogenic features. The procedures were followed according to the manufacture's guidelines. Cells were cultured on glass slides for 24 h, then exposed to 4-OP (10, 30, 50, or 100 μM/mL) for 48 h. Cells grown on coverslips were washed twice with PBS (Sigma-Aldrich) and fixed with 4% paraformaldehyde in PBS for 60 min at 24℃. Following washing with PBS, these cells were incubated in permeabilization solution (0.1% Trition X-100 in 0.1% sodium citrate) for 2 min on ice. Samples were incubated in 50 μL TUNEL reaction mixture (Roche, Mannheim, Germany) for 60 min at 37 °C in a humidified chamber and in the dark. An in situ cell death detection kit provided the negative control (label solution without terminal transferase, Roche) for the assay; and preincubation of cells with 10 μg/mL DNase I in 50 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, and 1 mg/mL

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BSA for 10 min at room temperature to artificially induce DNA strand breaks served as positive control. Samples were incubated with or without 1 μg/mL 6-diamidino-2-pheylindole (DAPI) in PBS for 10 min and coverslips were applied with mounting solution (Dako, Carpinteria, CA, USA; S3025) and analyzed under fluorescence microscopy (Nikon, Tokyo, Japan).

4.4. Isolation of RNA and quantitative PCR

Total RNA was extracted from TM4 cells using a RNeasy Mini Kit (Qiagen, Hilden, Germany) with on-column DNase treatment (Qiagen). Complementary DNA was synthesized from 1 μg of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) with the Oligo(dT)30 primer according to the manufacturer's instructions. Target gene PCR amplification was carried out for 30 cycles of 30 s at 95 °C, 10 s at 57 °C, and 20 s at 72 °C. Primers were designed using Primer3 (http://frodo.wi.mit.edu). The QPCR was achieved using a total volume of 20 μL, containing 10 ng of cDNA and 1 pM of each primer, in a reaction buffer containing iQ SYBR Green Supermix (170–8880; Bio-Rad Laboratories). The cycle threshold values were normalized against GAPDH gene expression, a denaturation and polymerase activation step at 94 ℃ for 1 min and then 40 cycles consisting of 94 ℃ for 10 s, 57 ℃ for 10 s, and 72 ℃ for 20 s. The primers used to detect porcine transcripts are listed in Table 1.

4.5. Western blot analysis

Whole cell lysates were prepared using RIPA buffer (Thermo Fisher Scientific #89900) supplemented with protease inhibitor cocktail (Roche, #1836153). Protein samples containing equal quantities of protein were subjected to 4% to 20% Mini-TGX (Bio-Rad, Hercules, CA, USA; #456–1096) gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Membrane nonspecific binding was blocked by incubation of the membranes in blocking solution (1% bovine serum albumin (BSA) in tris buffered saline (TBS)) for 1 h at 22 °C, and then membranes were incubated overnight at 4 °C with a primary antibody diluted in TBST (20 mM Tris-HCl with pH 7.5, 150 mM NaCl, and 0.1% Tween-20). The following primary antibodies were used: phospho-Bad (1:1000 dilution; Cell signaling, #5284T), Bad (1:1000 dilution; Cell Signaling, #9239T), caspase-3 (1:1000 dilution; Cell Signaling, #9665T), cleaved caspase-3 (1:1000 dilution; Cell Signaling, #9661T), cytocrome c (1:1000 dilution; Abcam, #ab76107) and β-actin (1:1000 dilution; Santa Cruz Biotechnology, #sc47778). Membranes were washed in TBST and incubated for 1 h with anti-rabbit and anti-mouse IgG and HRP-linked antibody (1:10000 dilutions; Jackson Immuno-Research Laboratories) in TBST. Blots were visualized using Pierce ECL western blotting substrate and HyBlot CL autoradiography film (Denville Scientific, Metuchen, NJ, USA; # E3018).

4.6. Immunofluorescence

TM4 cells were seeded on 12 mm glass coverslips (BD Biosciences, Franklin Lakes, NJ) at a density of 2 × 10^5 cells per coverslip and allowed to attach for 1 d prior to treatment with 10, 30, 50, or 100 μM/mL of 4-OP for 24 h. After washing once with cold PBS, cells were fixed with 4% paraformaldehyde and blocked with 1% BSA in PBS containing 0.2% Triton X-100. Samples were subsequently incubated with mouse anti-cytocrome c antibody (Santa Cruz Biotechnology, sc-13156, 1:200) diluted in blocking solution overnight at 4 °C. After washing 3 times, the samples were incubated with secondary antibody (Alexa Fluor 488 anti-mouse IgG; 1:1000) diluted in blocking buffer (1% BSA in PBS) for 1 h. Then samples were washed once with warm PBS and incubated for 30 min with 100 nM of MitoTracker® red CMXRos (M7512, Life Technologies, Carlsbad, CA, U S A) and washed 3 times. Nuclei were counterstained with TO-PRO-3 (Life Technologies) and DAPI (Sigma-Aldrich). Samples were mounted with mounting medium (Sigma-Aldrich) and images were taken under a confocal microscope (Carl Zeis, Oberkochen, Germany; LSM 700).

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4.7. Statistical analysis

The SPSS statistical package, version 15.0 for Windows (IBM Corp, Somers, NY, USA) was used for data analysis. All the data were expressed as mean ± standard error. The differences between controls and experimental samples were evaluated by one-way ANOVA, followed by Tukey`s honestly-significant difference test. Significance levels of 0.05 and 0.01 were applied during data analysis using Student`s t-test, and different significance levels have been indicated (* $P < 0.05$). A significance levels of 0.05 was applied during data analysis using ANOVA.

Competing Interests

The authors declare that they have no competing interests.

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