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Nonylphenol induces apoptosis in rat testicular Sertoli cells via endoplasmic reticulum stress

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ABSTRACT

Nonylphenol (NP) is a widely distributed environment contaminant and has been documented to disrupt testicular development and decrease male fertility. Amongst possible targets of this compound are testicular Sertoli cells, which play a crucial role in supporting and nourishing sperm cells. In the present study, we found that NP treatment could cause dramatic morphological changes as well as decreased cell viability of Sertoli cells, while the following Annexin V–PI staining demonstrated that NP treatment led to increased proportion of cell apoptosis, which was evidenced again by the detection of condensation and marginal changes of chromatins using Hoechst staining and transmission microscopy observation. In addition, increased intracellular Ca^{2+} levels and changes of endoplasmic reticulum (ER) ultrastructure were also observed in NP-treated groups, indicating the action of NP on ER. The subsequent data showed that the expressions of ER-stress signaling targeted genes GRP78 and gadd153 were elevated, suggesting the activation of ER-stress signal pathway. Furthermore, the detection of ER-stress related proteins by western blotting revealed that the expression of gadd153 was upregulated by NP, whereas the expressions of GRP78 and ERp57 were both first upregulated and then inhibited. Taken together, it is suggested that NP can induce ER stress in Sertoli cells, which may plays an important role in the induction of apoptosis.

1. Introduction

Alkylphenol polyethoxylates (APEOs), the major group of nonionic surfactants, have been used extensively as components of cleaners, detergents and emulsifiers (Hawrelak et al., 1999), with their annual world production estimated 360,000 tons in 1988 (Nimrod and Benson, 1996). Alkylphenol are synthesized as the substrates for the manufacture of APEOs and they are also the primary degraded products of APEOs in the environment. Because of the large-scale use of these compounds, it is generally recognized that APEOs and their respective alkylphenol are a major determinant of organic material in sewage (Giger et al., 1987). In the family of APEOs, nonylphenol polyethoxylate (NPEOs) approximately takes up a proportion of 80% (de Voogt et al., 1997); therefore, their degraded product nonylphenol (NP) holds a vital position in the environment contamination. In recent years, NP has been detected from aquatic area in China and many other countries with concentrations ranged from 0 to 41.3 mg/L (Li et al., 2004; Shao et al., 2005; Vitali et al., 2004). Due to the hydrophobic characteristic of NP, it can be absorbed by aquatic wildlife species, especially by fish and accumulated in adipose tissue (Coldham et al., 1998); then it will enter the ecosystem via the food chain and pose great hazard to the health of human and animals. It has been reported that 0.2–0.3 ng/ml for NP and 0.1–0.2 ng/ml for OPs were observed in human plasma sample from healthy volunteers (Kawaguchi et al., 2004).

Recent evidence suggests that exposure of human and animals during pre- and postnatal development to environmental chemicals may be responsible, at least in part, for a reported decline in sperm count (Toppari et al., 1997). While this hypothesis remains to be tested directly, it has been well documented in fish and rodents that administration of NP profoundly impairs testicular function as evidenced by reduced testis size, low circulating testosterone, disturbed testicular structure and suppressed spermatogenesis (Cardinali et al., 2004; Nagao et al., 2001; Tan et al., 2003).

As we all know, normal onset of spermatogenesis and the eventual production of a sufficient sperm number to insure fertility depends at least in part on Sertoli cells, which exert important functions in supporting and nourishing germ cells as well as the constitution of blood–testicle barrier. Therefore, specific impairment of Sertoli cells will produce a parallel dysfunction in sperm production. The study of Weber demonstrated that chronic aqueous

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exposure to nonylphenol caused increased Sertoli cell apoptosis in adult medaka (Weber et al., 2002). In another study, Miura et al. used the eel testicular organ culture system to testify that NP exposure could induce hypertrophy of Sertoli cell (Miura et al., 2005). All the above studies were consistent with the report of Monsees that Sertoli cells were the target of many reproductive hazards (Monsees et al., 2000). Though, the underlying mechanisms of NP action on Sertoli cells are not fully understood. The study of Hughes illustrated NP could inhibit testis endoplasmic reticulum (ER) Ca^{2+} pumps, rendering the disturbance of intracellular Ca^{2+} homeostasis and then leading to cell death (Hughes et al., 2000). Recent studies identify that ER as an important subcellular compartment implicated in apoptotic execution (Rao et al., 2004). The ER, which is the site for folding and assembly of proteins, lipid biosynthesis, vesicular traffic, and cellular Ca^{2+} storage, is sensitive to alterations in homeostasis. Several stimuli, including alterations of Ca^{2+} homeostasis and accumulation of unfolded proteins in the ER, can cause ER stress (Kadowaki et al., 2004), and prolonged ER stress will lead to apoptosis.

In the present study, we demonstrated that NP displays apoptosis-inducing effect on rat testicular Sertoli cells. To ascertain whether ER stress signal pathway has vital role in NP-induced apoptosis, we first investigated the changes of intracellular Ca^{2+} levels and the ultrastructure alteration of ER. Then the gene and protein expression levels of several important proteins which function a lot in the ER stress signal pathway were also examined.

2. Materials and methods

2.1. Reagents

NP (4-nonylphenol) with 98% analytical standard was from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Dulbecco's modified Eagle's medium-Ham's F-12 medium (DMEM-F12 medium), penicillin, streptomycin sulfate, trypsin and collagenase I were purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). $C_8H_{17}N_2O_4SNa$ (HEPES sodium salt) and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Amresco Inc. (Solon, OH, USA). Fluo 3 AM was obtained from AnaSpec Inc. (San Jose, CA, USA). Annexin V/PI apoptosis assay kit was purchased from Invitrogen Co. (Eugene, Oregon, USA). Hoechst and staining kit and DAPI were bought from Beyotime Company of China. Total RNA extraction

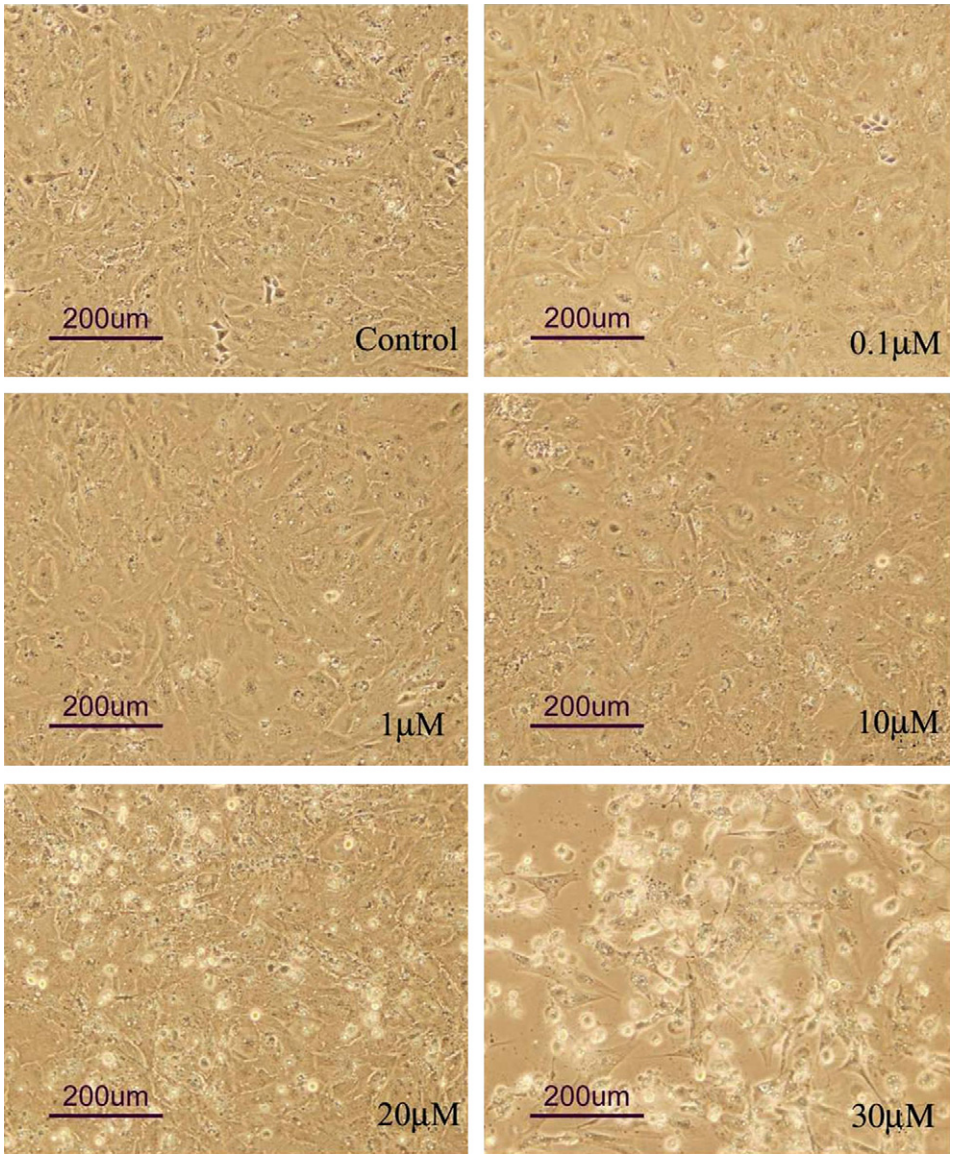

Fig. 1. Morphological changes of Sertoli cells after NP treatment. (A) Phase-contrast microphotographs of Sertoli cells. After being incubated with NP at 0, 0.1, 1, 10, 20 and 30 μM for 24 h, Sertoli cells were photographed under phase-contrast microscope. Bar = 200 μm. (B) Histology staining pictures of Sertoli cells. Cells in the control and 30 μM NP-treated groups were stained using the method of hematoxylin and eosin staining. Bar = 100 μm.

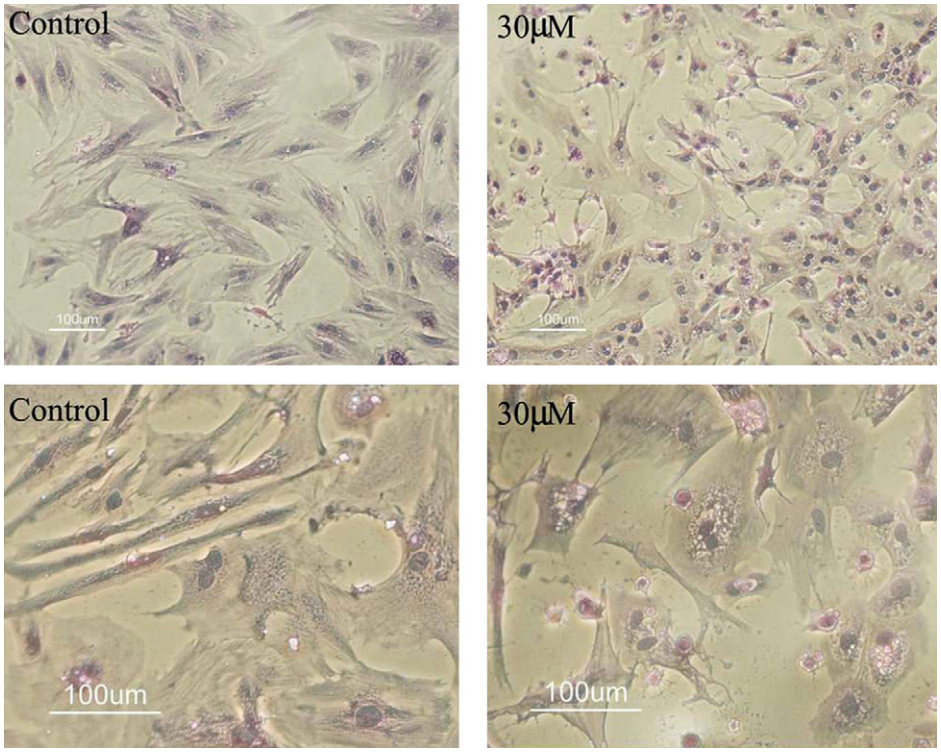

Fig. 1. (*Continued*).

reagent (TRNzol) was purchased from TianGen Biotech Co. (Beijing, China). All the other regents used in RT-PCR were products of Promega (Madison, WI, USA). BCA protein assay kit and the enhanced chemiluminescence detection kit were from Pierce (Rockford, IL, USA). Anti-GRP78 and gadd153 antibodies were from Santa Cruz Biotechnology, Inc. (CA, USA), while anti-ERp57 antibody was from Stressgen Bioreagents, Inc. (Ann Arbor, MI, USA). HPR-conjugated monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GADPH) was bought from Kangchen bio-tech Co. (Shanghai, China) fluorescein labeled goat anti-mouse IgG was got from KPL company (Gaithersburg, MD, USA).

The culture medium used was DMEM-F12 (1:1) supplemented with 4 mM glutamine, 15 mM Hepes, 6 mM L-(1)-lactic hemicalcium salt hydrate, 1 mM sodium pyruvate, antibiotics (final concentrations: penicillin, 100 IU/ml; streptomycin sulfate, 100 μg/ml).

2.2. Primary culture of rat testicular Sertoli cells

Sprague–Dawley Rats were purchased from Nanjing Medical University and kept in accordance with NIH Guide for the Care and Use of Laboratory Animals. Sertoli cells were isolated from rats at the age of 30 days according to the method of Steinberger with some modifications (Steinberger et al., 1975). Testes were removed and decapsulated. The seminiferous tubules were dispersed gently using forceps, but not fragmented, and then washed twice in PBS. The settled seminiferous tubules were incubated with 0.25% trypsin at 37 °C, shaking at 50 oscillation/min for about 30 min. The supernatant, which contained interstitial cells, was decanted. The tubules were washed three times and then incubated in a solution containing 0.1% collagenase at 37 °C, shaking at 80 oscillations/min for 40 min. The preparation then was filtrated through a 100-mesh stainless steel filter, and collected for centrifugation at 1000 rpm for 6 min. The pellet subsequently was washed three times. Cells were resuspended in DMEM-F12 medium containing 5% fetal bovine serum (FBS) and seeded onto culture flasks, maintaining in a humidified atmosphere of 95% air, 5% CO_2 at 34 °C. After 2 days culture, Sertoli cells attached to the bottom of flasks with only tiny dendrites protruding, but most of germ cells suspended in the medium and can be removed by changing the medium. Two days later, the medium was changed again for the second purification and the Sertoli cells grow quickly to form a monolayer in new medium.

2.3. Histology staining (hematoxylin and eosin staining)

Sertoli cells were cultured in 2.5 cm × 2.5 cm coverslips and treated with vehicle and 30 μM NP for 24 h. Then, cells were fixed using acetone and stained with a standard hematoxylin and eosin staining (HE staining).

2.4. Cell proliferation assay

Cell viability was determined and quantified by 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, Sertoli cells transplanted into 96-well plates were exposed to vehicle or different concentrations of NP for 24 h. Afterwards, 20 μl MTT solution was added to each well and incubated at 37 °C for 4 h. Formazan crystals formed were dissolved by adding 100 μl DMSO and the absorbance was measured on an automated microplate reader (Bio-Rad, Japan) at 570 nm.

2.5. Apoptosis analysis with Annexin V–FITC and PI staining

Cells at the density of 2×10^5/ml were incubated with NP for 24 h and then harvested. Specific binding of Annexin V–FITC was carried out by incubating the cells for 15 min at room temperature in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) containing a saturating concentration of Annexin V and PI.

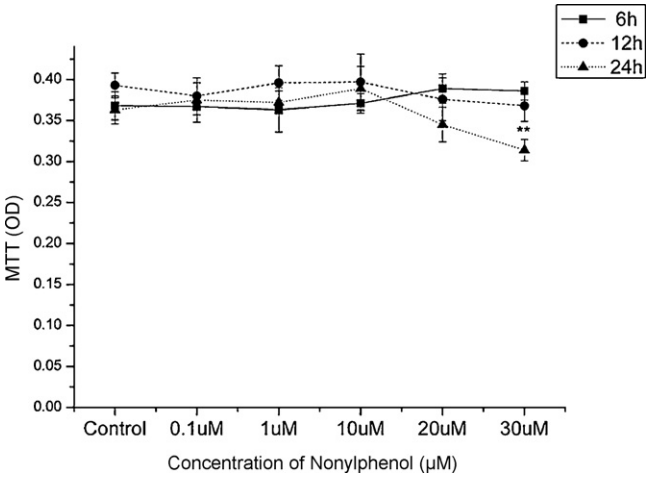

Fig. 2. Effect of NP on the viability of cultured Sertoli cells. Sertoli cells were treated with 0, 0.1, 1, 10, 20 and 30 μM NP for 6, 12 and 24 h respectively, and the viability of cells was determined by MTT. Data are presented as mean ± SD. Statistically different from the control is marked with asterisk (**$P < 0.01$).

After incubation, the cells were pelleted and analyzed in a FACScan flow cytometer (Becton-Dickson, San Jose, CA, USA).

2.6. In situ labeling of apoptotic cells

For examining nuclear morphology, Sertoli cells were transplanted into 96-well plates and treated with graded concentrations of NP. After incubation, cells were washed in PBS, incubated with a DNA dye, Hoechst 33342 according to the protocol of the kit. The result of staining was visualized under a fluorescent microscope that was excited at a wavelength of 350 nm and measured at 460 nm.

2.7. Intracellular free Ca^{2+} detection

Acetoxymethyl ester of Fluo3 (Fluo-3 AM) is a cell permeant probe used extensively for intracellular Ca^{2+} detection. In our experiment, Intracellular Ca^{2+} detection of Sertoli cells was performed after cells were exposed to NP for indicated times. In brief, cells were loaded with 4 μmol/L Fluo-3 AM in physiological buffer (PSS: 136 mM NaCl, 6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.4) at 37 °C for 50 min. The resulting fluorescence as the indicator of Ca^{2+} concentration was observed under the fluorescent microscopy (Nikon, Chiyoda-ku, Tokyo, Japan) at 488 nm excitation wavelength and analyzed by the software SimplePCI.

2.8. Transmission electron microscopy

Sertoli cells treated with vehicle and NP were collected and washed with PBS. Afterwards, cells were fixed with 2.5% cold glutaraldehyde for 2 h and then in 1% osmic acid for 1 h. Samples were dehydrated in a series of acetone and embedded in Epon 812 by standard procedures. 70 nm ultra-thin sections were prepared, stained

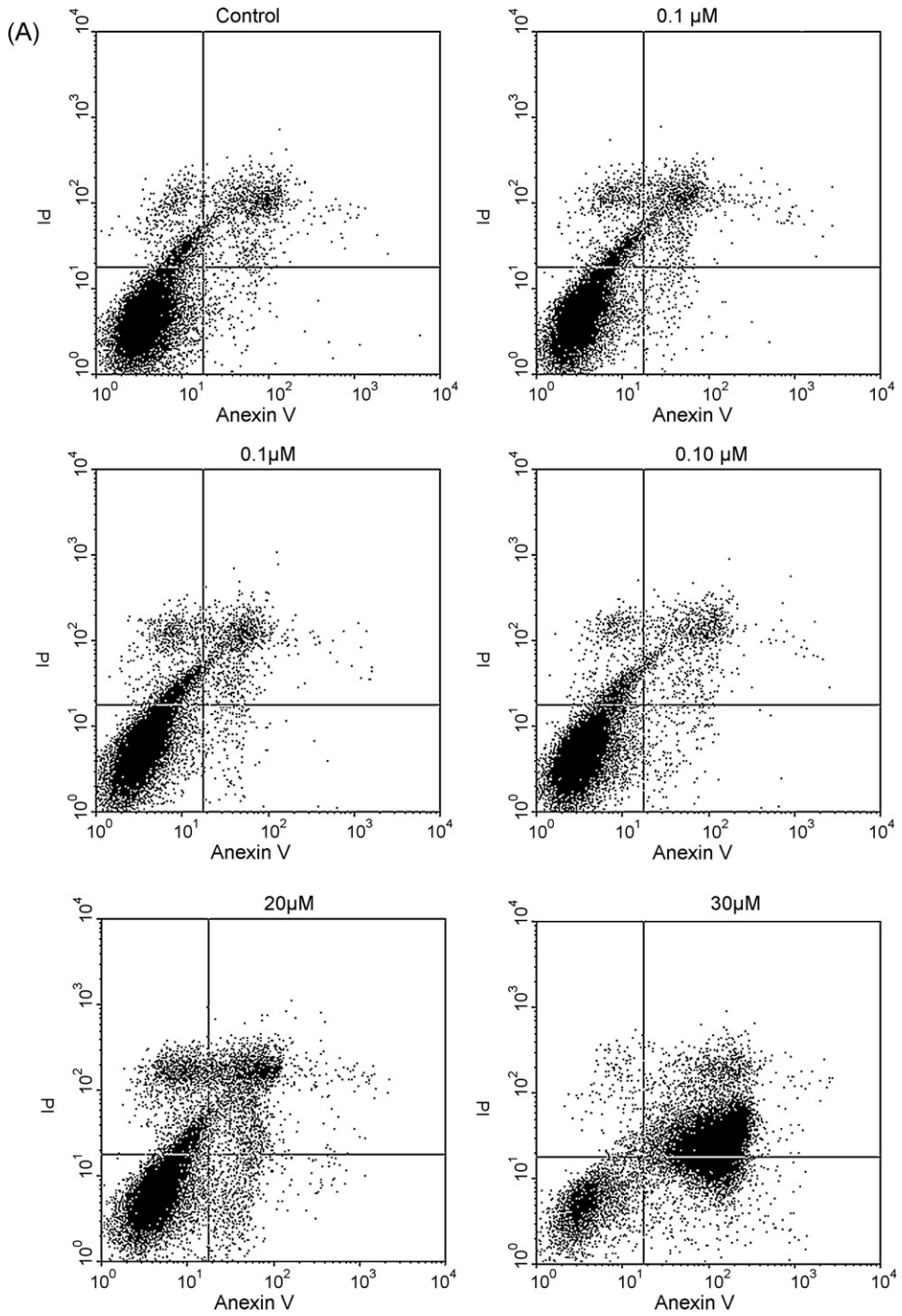

Fig. 3. AnnexinV–FITC and PI staining for the detection of apoptosis. After exposure to graded concentrations of NP for 24 h, Sertoli cells were collected for AnnexinV–FITC and PI staining followed by flow cytometry analysis. (A) Flow cytometric plots. (B) Flow cytometric analysis result. Data are presented as mean ± SD. Statistically different from the control is marked ($^*P < 0.05$, $^{**}P < 0.01$).

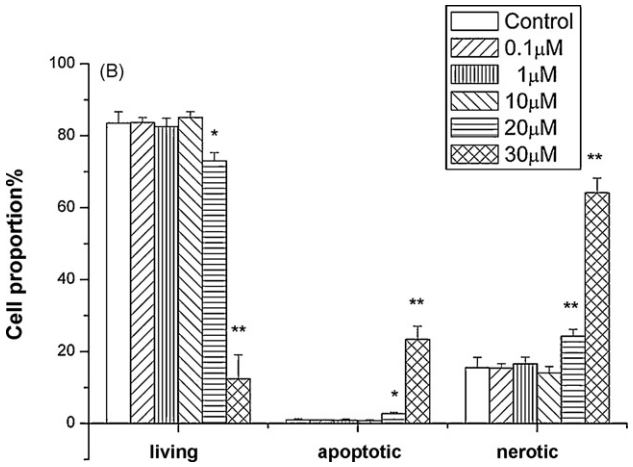

Fig. 3. (*Continued*).

with both uranyl acetate and lead citrate, and assessed using JEM 1200 electron microscope.

2.9. RT-PCR analysis

After Nonylphenol treatment, Sertoli cells were lysed by addition of TRIzol Reagent. Total RNA was prepared according to manufacturer's protocol. RNA was quantified spectrophotometerically by absorbance at 260 and 280 nm. Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to analyse the expression of mRNA for GRP78 and gadd153. The condition for reverse transcription and PCR steps were performed as previously reported with some modification. The respective sense and anti-sense primers chosen by Primer3 program were 5′-TCACCCACACTGTGCCCATCTATGA-3′and 5′-CATCGGAACCGCTCATTGCCGATAG-3′ for β-actin (300 bp), 5′-AGCCCACCGTAACAATCAAG-3′ and 5′-CCTGTCCCTTTGTCTTC-AGC-3′ for GRP78 (184 bp), 5′-TCAGATGAAATTGGGGGCAC-3′ and 5′-TTTCCTCG-TTGAGCCGCTCG-3′ for gadd153 (340 bp). The number of cycles was optimized to ensure product accumulation in the exponential range. Amplified products were separated by electrophoresis on 1.5% agarose gel and documented using Gel Doc EQ

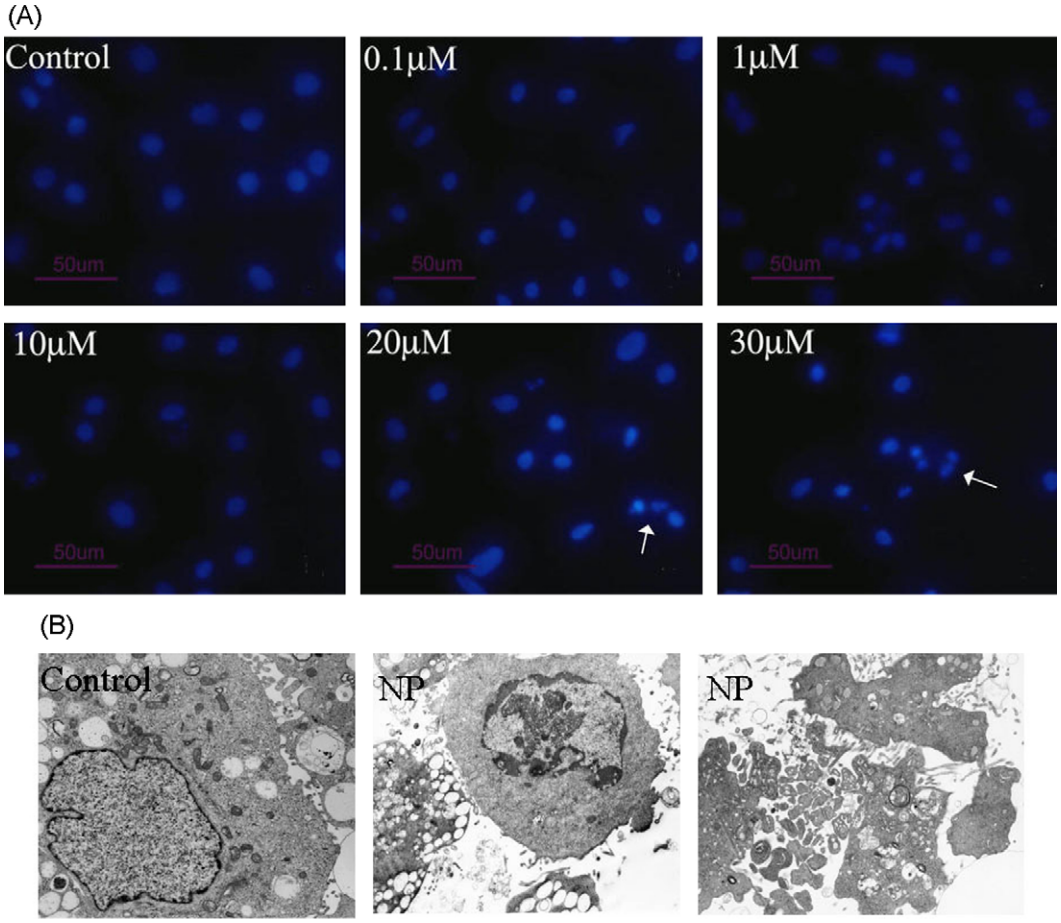

Fig. 4. NP-induced nuclear morphological alterations. (A) The graded concentrations of NP were added to Sertoli cells for 24 h. Then, cells were washed and stained with Hoechst 33342, and examined under a fluorescence microscope. Condensed and fragmented nuclei were marked with arrows. (B) Cells were incubated in the absence (control) or presence (NP) of 30 μM NP for 24 h, and then prepared for electron microscopic examination.

image station (Bio-Rad, Milan, Italy) and then digitized with the quantity one software. Band intensities for the respective gene were normalized against β-actin in the same sample.

2.10. Western blotting

Cells were washed twice with cold PBS and lysed in extraction buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100 and 0.1% SDS) for 30 min on ice. The lysates were centrifuged at 13,000 × *g* for 15 min, the supernatants collected and protein concentration determined by Bradford protein assay. Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis and electroblotted onto PVDF membrane by standard procedures. Transferred blots were incubated sequentially with blocking agents (5% non-fat milk in PBS–tween), primary anti-GRP78, gadd153 and ERp57 antibodies and peroxidase conjugated secondary antibodies. The detection of signal was performed with an enhanced chemiluminescence detection kit.

2.11. Immunofluorescent staining

Sertoli cells were cultured in 48-well plates and treated with NP (30 μM) for indicated time. Immunofluorescent staining of gadd153 was performed as described below. Sertoli cells were first fixed with methanol, and then blocked in 1% BSA for 2 h. After being incubated with gadd153 primary antibody for 2 h at 37 °C (1:100 dilution in 1% BSA for gadd153), cultures were treated with a fluorescein labeled goat anti-mouse IgG (1:80 dilution in 1% BSA). Nuclei were stained with DAPI (1 μg/ml). Images of fluorescence were captured using fluorescent microscopy (Nikon, Chiyoda-ku, Tokyo, Japan).

2.12. Statistical analysis

Data are presented as the mean ± SD for indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls post-test. *P*-values less than 0.05 were considered significant.

3. Results

3.1. NP induced morphological changes of Sertoli cells

The microphotographs of Sertoli cells treated with different concentrations of NP for 24 h were presented in Fig. 1A. It can be noticed

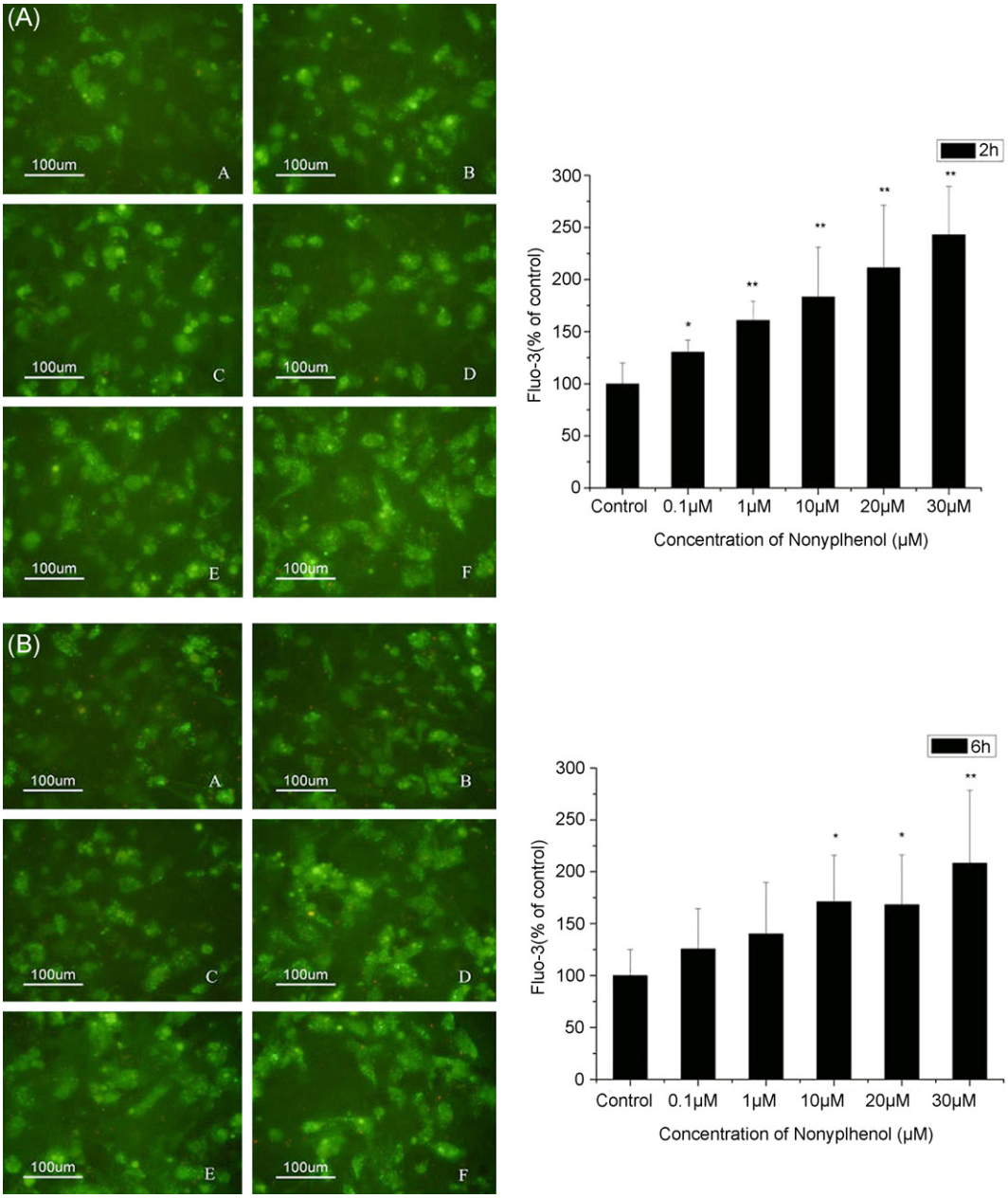

Fig. 5. Intracellular free Ca^{2+} alteration following NP exposure. Sertoli cells planted in 96-well plates were treated with 0, 0.1, 1, 10, 20 and 30 μM NP for 2 h (A) and 6 h (B), and then loaded with Fluo-3 AM. The resulting fluorescence of Fluo-3 indicated the Ca^{2+} level. Data are presented as mean ± SD. Statistically different from the control is marked with asterisk (*$P < 0.05$, **$P < 0.01$).

that NP at low concentrations (0.1, 1 and 10 μM) did not induce evident morphological changes of Sertoli cells; however, Sertoli cells exposed 30 μmol/L NP showed remarkable morphological changes, including cellular shrinkage and cell rounding. To display the morphological changes more clearly, Sertoli cells in the control and 30 μM NP-treated groups were stained with the standard method of hematoxylin and eosin staining, and the result was shown in Fig. 1B. As we can see, Sertoli cells in the control (A and C) were proportional and well distributed, with the cytoplasm spreading broadly, while in NP-treated groups (B and D), Sertoli cells became sparse, and presented obvious morphological changes, such as cytoplasm rarefaction, cell membrane rupture, cytoskeletal collapse and nucleus shrinkage, which were all the common characters of cell death.

3.2. Effect of NP on cell viability

The result of cell viability assay was presented in Fig. 2. As can be seen, Sertoli cell viability showed no significant variations between control and NP-treated groups after 6 and 12 h treatment. However, with the exposure time increased to 24 h, cell viability began to decline at 20 μM NP and significantly decreased at 30 μM as compared with the control, which was consistent with the morphological observations.

3.3. Examination of NP-induced apoptosis

We first determined whether NP induces apoptosis in Sertoli cells by AnnexinV–FITC and PI staining. The result of flow cytometry analysis revealed that the proportion of apoptotic cells was significantly increased at 20 and 30 μM NP groups (Fig. 3). Furthermore, nuclear staining with Hoechst 33342 and electron microscopic examination demonstrated that in comparison with the normal nuclear morphology of control cells, significant morphological changes such membrane blebbing, condensation and marginal changes of chromatins and apoptotic body formation were observed in 20 and 30 μM NP treated groups, which were all the standard characteristics of apoptosis (Fig. 4).

3.4. Intracellular free Ca^{2+} studies

The result shown in Fig. 5 illustrated that NP induced a dose-dependent elevation of cytosolic free Ca^{2+}. The difference between the NP treated groups and the control was significant after 2 h exposure. When exposure time increased to 6 h, Ca^{2+} concentrations in NP cells were still higher than that in the control, but only 10, 20 and 30 μM treatments exhibited significant differences as compared with the control.

3.5. NP-induced ultrastructure alterations of the ER

To examine the effect of NP on ER, transmission electron microscope was used to analyze the ultrastructure alteration of ER. As displayed in Fig. 6, Swelling ERs accompanied by an enlargement of lumen were frequently observed in NP treated Sertoli cells, which indicates that ER was significantly influenced by NP treatment.

3.6. Effect of NP on ER stress-related gene expression

The effect of NP on ER stress-related gene (gadd153 and GRP78) expression was studied by using RT-PCR analysis. As displayed in Fig. 7A, low concentrations of NP (0.1, 1, 10 μM) show a weak but not significant stimulating effect on gadd153 and GRP78 gene expression, while 20 μM NP dramatically increased the expression of gadd153 and GRP78 both after 12 h and 24 h treatment. The time course of the 30 μM NP action on gadd153 and GRP78 expression was revealed in Fig. 7B, from which we can see that the expression of GRP78 was significantly increased from 3 to 18 h, whereas gadd153 expression was remarkably upregulated from 6 to 18 h.

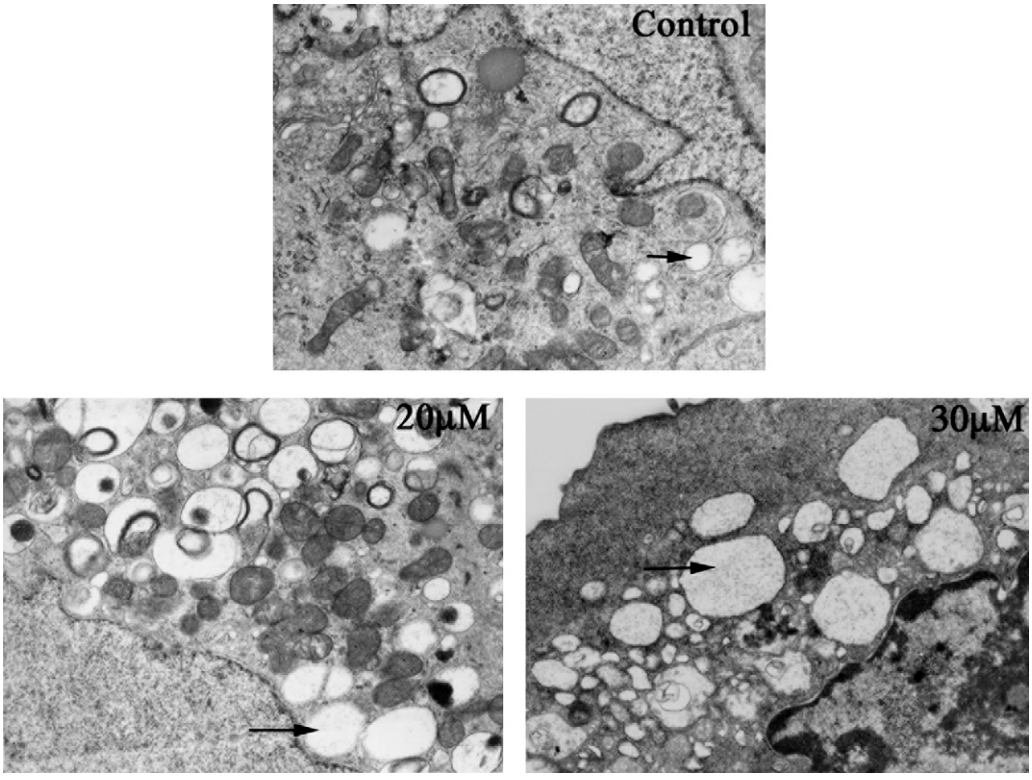

Fig. 6. NP-induced alterations in the ultrastructure of the ER in Sertoli cells. After NP treatment for 24 h, Sertoli cells were processed and examined under a transmission electron microscope as described in Section 2. Arrow indicates the expansion of ER.

3.7. Effect of NP on ER stress-related protein expression

In order to determine the ER stress-related protein changes following NP exposure, we used western blotting to analyze the expression of gadd153, GRP78 and ERp57. The result demonstrated that 20 and 30 μM NP treatment for 3, 6 and 12 h all exhibited stimulating effect on gadd153 protein expression in comparison to the control. However, the expression of GRP78 and ERp57 after 20 and 30 μM NP treatment shown a different tendency, that was an immediate upregulation (3 h) and subsequent decrease (6 h) as compared with the control (Fig. 8).

3.8. Immunofluorescent staining of gadd153

To examine the relationship between gadd153 expression and cell apoptosis, Sertoli cells were treated with 0 and 30 μM NP for 12 h and then subjected to DAPI staining and immunofluorescent staining of gadd153. The result in Fig. 9 demonstrated that Sertoli cells in the control group showed weak gadd153 immunoreactivity and normal nuclear morphology, while in NP-treated group, increased gadd153 immunoreactivity accompanied with condensation and fragmentation of nucleus (apoptotic symbol) were frequently observed, suggesting that gadd153 expression was closely related to cell apoptosis.

4. Discussion

Despite the wide distribution of NP in the environment and its great hazard to the reproductive health of human and animals, the detailed mechanism of NP toxicity has not been fully elucidated. In the present study, we first demonstrate that NP treatment lead to increased apoptosis of primary cultured rat testicular Sertoli cells. To investigate the detailed mechanism, we find that NP causes disturbance of intracellular Ca^{2+} homeostasis and ultrastructure changes of ER in Sertoli cells. Furthermore, our results indicate that several gene and protein markers of ER stress are changed after NP exposure. These findings suggest that NP can cause ER stress in rat testicular Sertoli cells, which may underlie the NP-induced apoptosis to Sertoli cells.

NP as an important environment contaminant has been demonstrated to induce apoptosis in several cell types. It was reported by Kim et al. that NP was able to trigger apoptosis in human embryonic stem (hES) cells (Kim et al., 2006). Besides, it was also demonstrated that NP could induce thymocyte apoptosis (Yao et al., 2006). In this study, we identified that NP treatment also led to remarkably increased apoptosis in Sertoli cells. However, there was still no definite explanation about the underlying mechanism. Kim found that Fas/FasL system played vital role in NP induced hES cell apoptosis, while Yao reported that caspase-3 activation and mitochondrial depolarization were involved in NP induced thymocyte apoptosis (Kim et al., 2006; Yao et al., 2006).

Recently, several researchers have focused on ER-stress induced cell apoptosis (Chiang et al., 2005; Yeh et al., 2007). For example, it was suggested in Yeh's study that genistein induced apoptosis in human hepatocellular carcinomas via ER-stress. Two distinct mechanisms are involved in the process: the accumulation of unfolded or misfolded protein and the Ca^{2+} signaling, which can interact with each other (Shen et al., 2004). In the present study,

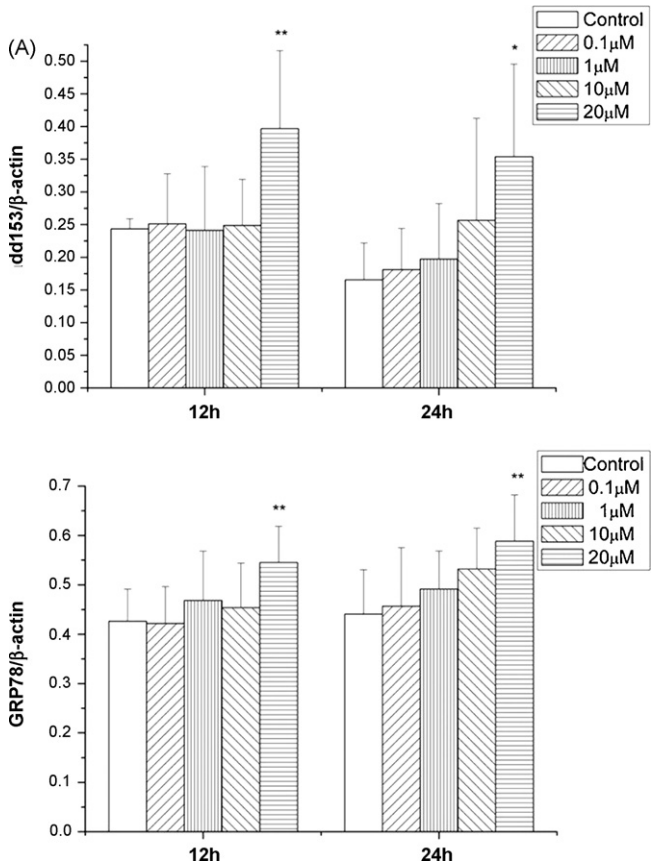

Fig. 7. NP upregulated gene expressions of GRP78 and gadd153. (A)Sertoli cells were treated with graded concentrations of NP(Control, 0, 0.1, 1, 10 and 20 μM) for 12 and 24 h, and then lysed for RNA extraction. The changes of gadd153 and GRP78 expression were analyzed by RT-PCR. Amplified products were separated by electrophoresis on 1.5% agarose gel and documented using GelDoc image station and then digitized with the quantity one software. Band intensities for the respective gene were normalized against β-actin in the same sample. (B) Time course of the action of 30 μM NP on gadd153 and GRP78 gene expression, sample preparation and analysis was the same as the description above. Data are presented as mean ± SD. Statistically different from the control is marked (* and # $P < 0.05$, ** and ## $P < 0.01$).

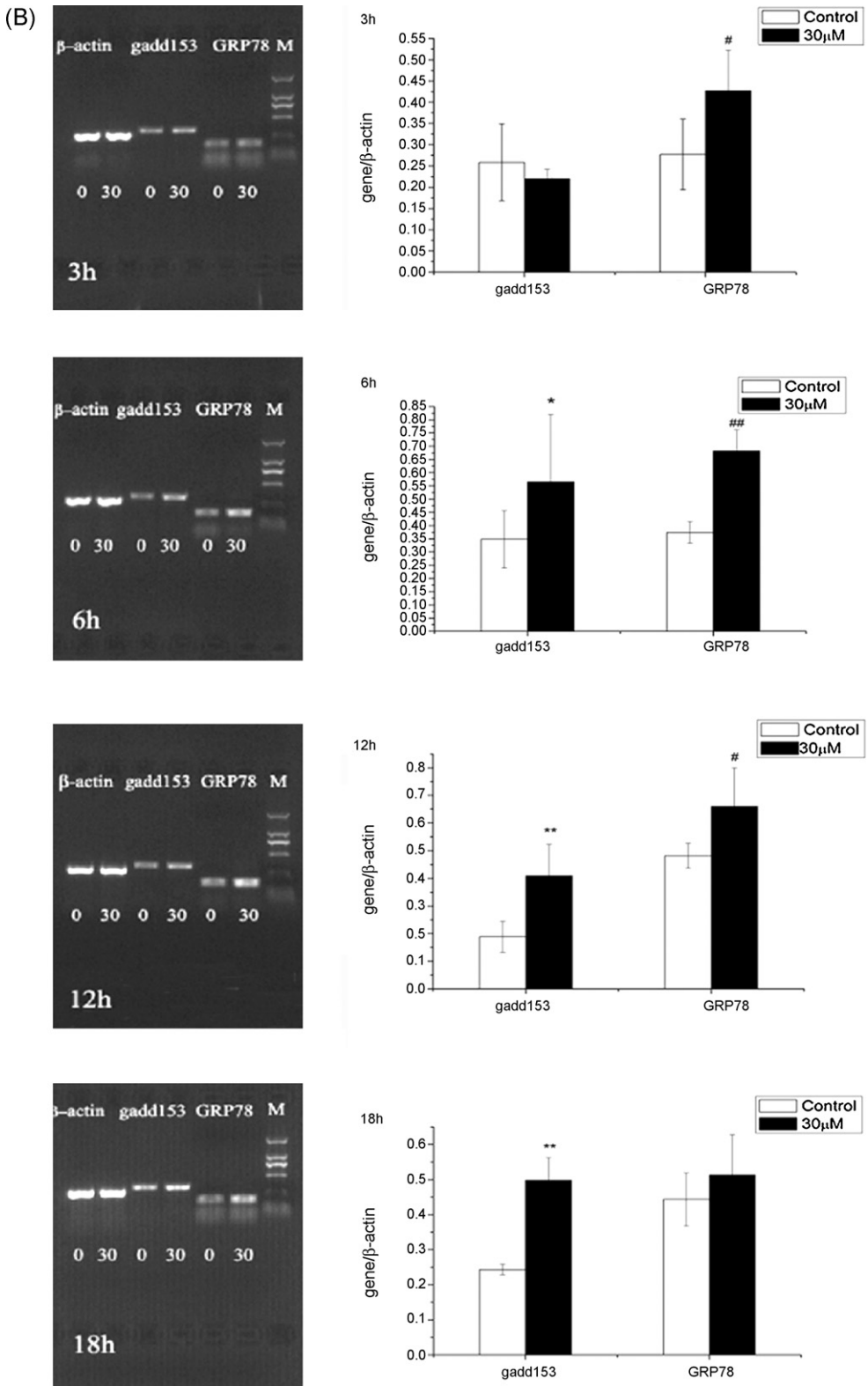

Fig. 7. (*Continued*).

increased intracellular Ca^{2+} elevation in Sertoli cells following NP treatment was first visualized, indicating the alteration of Ca^{2+} homeostasis were implicated in NP-induced effect. The result was consistent with Wang's study that NP could cause Ca^{2+} elevation in osteosarcoma cells (Wang et al., 2005). Besides, during our experiment, we chose the short time treatment (2 and 6 h) to show that Ca^{2+} signaling changes were the earlier trigger of cell apoptosis.

The ER is crucial for Ca^{2+} signaling. It can act as a sink for Ca^{2+} that enters the cell through channels in the plasma membrane, but

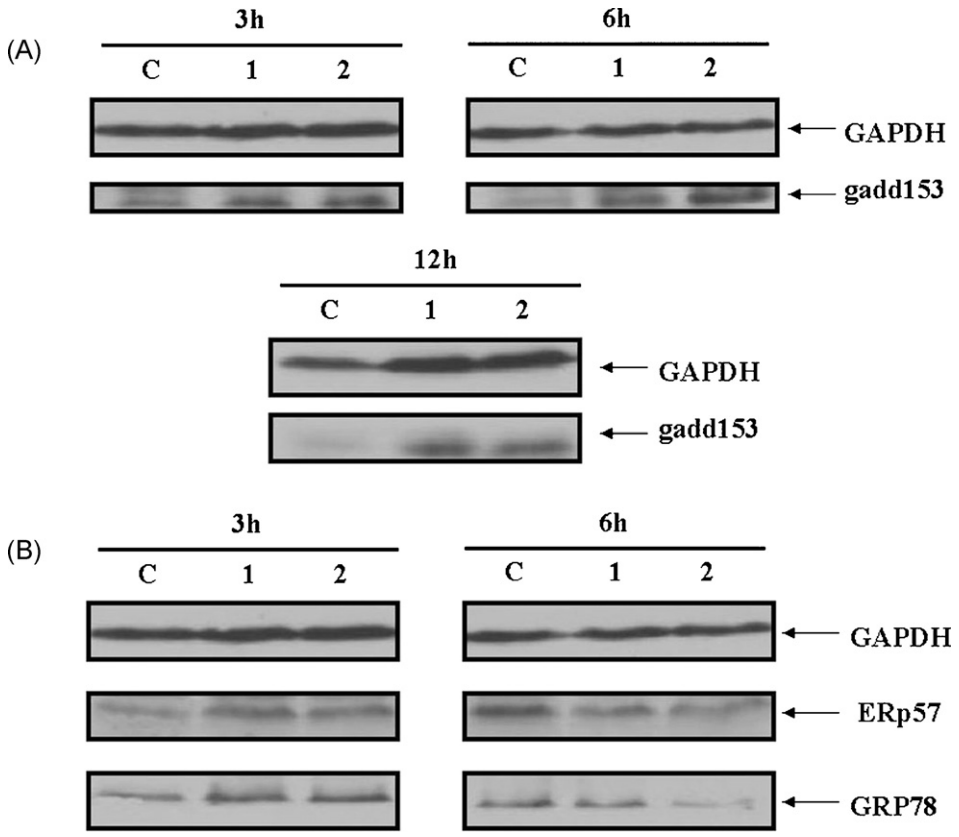

Fig. 8. Detection of gadd153, GRP78 and ERp57 protein expression following NP treatment. Sertoli cells were treated with graded concentrations of NP (control, 20 and 30 μM) for indicated time intervals, and then lysed for protein collection. The changes of gadd153, GRP78 and ERp57 expression were analyzed by western blotting. Proteins were separated by 12% SDS–polyacrylamide gel electrophoresis, transferred to PVDF membrane and immuno-reacted with indicated antibody.

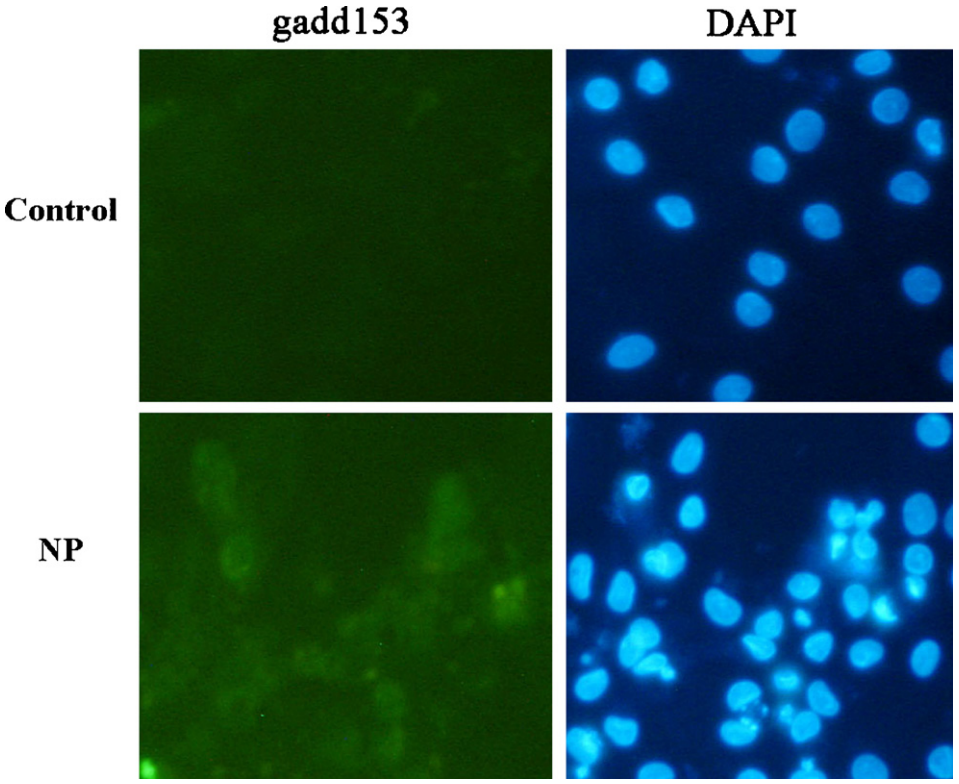

Fig. 9. Immunofluorescent staining to show the localization of gadd153 in apoptotic cells. Sertoli cells treated with 0 and 30 μM NP for 12 h were subjected to DAPI staining (showing nucleus) and immunofluorescent staining of gadd153, and then visualized under fluorescent microscope.

can also be a source for Ca^{2+} release into the cytosol in response to intracellular messengers. According to Hughes, Ruehlmann and Khan's studies, NP-induced Ca^{2+} homeostasis might be attributed to the inhibiting effect of NP on endoplasmic reticulum Ca^{2+} pumps and channels (Khan et al., 2003; Ruehlmann et al., 1998), and our previous data also revealed that NP treatment led to the alterations of Ca^{2+}-ATPase activity (not shown). On the other side, the normal function of the ER requires appropriate concentrations of free Ca^{2+} within the ER lumen, and disturbance of Ca^{2+} homeostasis will hamper the activation of Ca^{2+}-dependent chaperones and impair protein folding (High et al., 2000), which will render accumulation of unfolded and misfolded protein in the ER and cause ER stress (Banhegyi et al., 2007). In the present study, swelling ERs accompanied by an enlargement of lumen were observed in NP-treated cells, indicating that NP disturbed the function of ER and caused its morphological changes.

Glucose regulated protein (GRP78) and protein disulfide isomerase (ERp57) are both ER resident proteins and playing pivotal functions in correct protein folding in the ER (Chichiarelli et al., 2007; Lee, 2005; Zapun et al., 1998). Besides, GRP78 is crucial in directing ER-stress signaling. In unstressed cells, GRP78 bind to the lumenal domains of several ER stress sensors such as IRE1, PERK and ATF6 (Bertolotti et al., 2000; Liu et al., 2000; Liu et al., 2002; Morris et al., 1997). As unfolded proteins accumulate in the ER lumen, GRP78 disassociates from these ER stress sensors to help protein folding. Consequently, released IRE1 and PERK undergo activation and result in the opening of ER-stress signaling pathway, which may in turn improve the expression of the target gene GRP78. In our study, we found that GRP78 mRNA expression was severely enhanced by 20 and 30 μM NP treatment, suggesting the activation of ER-stress signaling pathway. The protein expression of GRP78 and ERp57 was different from the gene expression. They were both first elevated after short time treatment and then decreased, with the effect of 30 μM NP more evident. In the Wang study of ER stress induced by thiamine deficiency, he also found that the GRP78 expression displayed a first elevation and the following decrease (Wang et al., 2007). We speculate that cells under ER stress will first increase GRP78 and ERp57 expression to help protein folding and prevent accumulation of misfolded proteins. However, prolonged ER stress will cause damage to the cell as well as the structure of ER, rendering the decreased GRP78 and ERp57 expression.

gadd153 is another specific marker of ER stress response. It is the first identified pro-apoptotic transcription factor during the activation of ER stress signaling pathway, which can forms stable heterodimers with C/ERP family members and controls expression of a set of stress-induced genes involving in apoptosis (Friedman, 1996; Zinszner et al., 1998). Here, we demonstrated that the gene expression of gadd153 was significantly increased both after 20 and 30 μM NP treatments, another evidence of NP-induced ER stress in Sertoli cells. The protein expression of gadd153 exhibited a similar trend, i.e. the continuing increase following NP exposure, which was distinguished from that of GRP78 and ERp57. Furthermore, the result of immunofluorescent staining revealed weak gadd153 staining in control living cells but stronger immunoreactivity in NP-induced apoptotic cells, which suggested that expression of gadd153 was closely related to cell apoptosis.

Taken together, we drew the conclusion that NP could cause disturbance of Ca^{2+} homeostasis and the normal function of ER, rendering the prolonged activation of ER stress signaling pathway and finally leading to Sertoli cell apoptosis via increased gadd153 expression.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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