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Signaling related with biphasic effects of bisphenol A (BPA) on Sertoli cell proliferation: A comparative proteomic analysis

Li-Chen Ge [a,1], Zhuo-Jia Chen [b,1], Hao Liu [c], Kun-Shui Zhang [d], Qiao Su [e], Xiang-Yu Ma [a], Hong-Bin Huang [b], Zhen-Dong Zhao [f], Yu-Ye Wang [a], John P. Giesy [g], Jun Du [a,*], Hong-Sheng Wang [a,*]

[a] Department of Microbial and Biochemical Pharmacy, Guangdong Provincial Key Laboratory of New Drug Design and Evaluation, School of Pharmaceutical Sciences, Sun Yat-sen University, No. 132 Waihuandong Road, University Town, Guangzhou 510006, China
[b] Department of Pharmacy, Sun Yat-sen University Cancer Center State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangzhou 510060, China
[c] Cancer Research Institute and Cancer Hospital, Guangzhou Medical University, Guangzhou 510095, China
[d] Department of Pharmacy, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 107 Yanjiang West Road, Guangzhou 510120, China
[e] Laboratory Animal Center, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, China
[f] School of Chinese Materia Medica, Guangdong Food and Drug Vocational College, No. 321 Longdongbei Road, Guangzhou 510520, China
[g] Department of Veterinary Biomedical Sciences & Toxicological Center, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

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ABSTRACT

Background: Biphasic effects on cell proliferation of bisphenol A (BPA) can occur at lesser or greater exposures. Sertoli cells play a pivotal role in supporting proliferation and differentiation of germ cells. The mechanisms responsible for inverse effects of great and low concentrations of BPA on Sertoli cell proliferation need further study.
Methods: We utilized proteomic study to indentify the protein expression changes of Sertoli TM4 cells treated with 10^{-8} M and 10^{-5} M BPA. The further mechanisms related to mitochondria, energy metabolism and oxidative stress were investigated by qRT-PCR and Western-blotting analysis.
Results: Proteomic studies identified 36 proteins and two major clusters of proteins including energy metabolism and oxidative stress expressed with opposite changes in Sertoli cells treated with 10^{-8} M and 10^{-5} M BPA, respectively, for 24 h. Exposure to 10^{-5} M BPA resulted in greater oxidative stress and then inhibited cell proliferation, while ROS scavenger NAC effectively blocked these effects. Exposure to 10^{-8} M BPA caused higher intercellular ATP, greater activities of mitochondria, and resulted in significant proliferation of TM4 cells, while oligomycin A, an inhibitor of ATP synthase, abolished these growth advantages.
Conclusions: Our study demonstrated that micromolar BPA inhibits proliferation of Sertoli cells by elevating oxidative stress while nanomolar BPA stimulates proliferation by promoting energy metabolism.
General significance: Micromolar BPA inhibits cell proliferation by elevating oxidative stress while nanomolar BPA stimulates cell proliferation by promoting energy metabolism.

1. Introduction

Bisphenol A (BPA) is one of the largest volume chemicals produced worldwide. It can leach into food and beverages from plastic containers and therefore accumulate in bodies of humans [1]. As a known endocrine disruptor chemical (EDC), results of multiple studies have indicated that BPA can influence multiple endocrine related pathways [2]. However, the debate over whether BPA poses a threat to human health at concentrations observed in tissues of humans has been discussed for the past decade, particularly the risks of low doses of BPA [3]. Researchers claimed that the effects of BPA occur at both lesser and greater concentrations and might be absent at intermediate concentrations. Therefore, there was a need to elucidate the mechanism responsible for the concentration-dependent effects of BPA and other EDCs.

Sertoli cells, one of the somatic constituents of the testis, are the primary supporting cells creating the structural and physiological environments necessary for development of cells during spermatogenesis [4]. The number of Sertoli cells determines the number and activities of spermatozoa produced. Recent studies indicated that Sertoli cells are known targets of a variety of toxicants [5]. Results of several studies have illustrated that environmentally-relevant concentrations of BPA significantly impaired spermatogenesis not only in experimental animals [6] but also in humans [7]. However, there were few data about the effects and mechanisms of BPA on proliferation and functions of Sertoli cells.

* Corresponding authors.
E-mail addresses: dujun@mail.sysu.edu.cn (J. Du), whongsh@mail.sysu.edu.cn, hswang2000@gmail.com (H.-S. Wang).
[1] These authors contributed equally to this work.

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Our recent study revealed that nanomolar concentrations of BPA significantly increased proliferation of cells and both GPR30 and ER α/β were involved in this process. Furthermore, GPR30 are able to activate ERK via the EGFR pathway after stimulated by BPA [8]. Considering that there was no explanation for the stimulatory effects of the nanomolar while inhibition effects of micromolar on cell proliferation for either BPA or other toxic compounds, the present study investigated possible mechanisms responsible for these inverse effects on the basis of proteomic studies.

2. Materials and methods

2.1. Reagents

All reagents used in 2-DE were bought from Bio-Rad (Hercules, CA, USA). All chemicals were of reagent grade or better and purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted. Monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against Cyclin D1 was purchased from Bioworld Technology, Inc (Minneapolis, MN, USA). Horseradish peroxidase-conjugated secondary antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All compounds were solubilized in dimethyl sulfoxide (DMSO). A steroid-free medium containing DMSO (0.5% *v*/*v*) was used as the control.

2.2. Cell culture

TM4 cells (mouse Sertoli cell line, American Type Culture Collection, Manassas, VA, USA) were cultured in phenol red-free DMEM nutrient mixture F-12 HAM (Sigma-Aldrich, St. Louis, MO, USA) containing 5% heat-inactivated fetal bovine serum (FBS), 5% heat-inactivated horse serum and 1.2 g/l sodium bicarbonate supplemented with 10 μg/ml penicillin–streptomycin. The cells were incubated at 37 °C in a 5% CO_2 atmosphere. Both the plastic items used for the experiments and the water used to prepare the reagents were pretreated by enhanced sonochemical degradation to reduce any potential background BPA [9].

2.3. Cell viability assay

Proliferation and viability of cells were detected by use of previously described procedures [10]. Briefly, cell viability was evaluated by use of the CCK-8 kit (Dojindo Molecular Technologies, Gaithers burg, MD, USA) according to the manufacturer's instructions. The 50% inhibitory concentration (IC_{50}) was calculated using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).

2.4. Proteomic analysis of proteins in TM4 cells

TM4 cells were exposed to vehicle (0.5% DMSO), 10^{-8} M or 10^{-5} M BPA for 24 h. Two-dimensional gel electrophoresis (2-DE), protein visualization, and image analysis were performed essentially as described in the Supplementary Materials (SM). The detailed procedures for in-gel tryptic digestion of proteins and MALDI-TOF-MS/MS analysis were also described in the SM.

2.5. Cell cycle and apoptosis analysis

Cells were plated at a density of 1×10^6 per well on six-well plates. After treatment with 10^{-8} M or 10^{-5} M BPA for 24 h or 48 h, both the suspension and the adherent cells were collected for cell cycle and apoptosis analysis (see the SM).

2.6. Mitochondrial density, mitochondrial membrane potential ($\Delta\Psi m$), ROS, GSH, and ATP determination

TM4 cells were spread wells of 96-well plates and treated with 10^{-8} M or 10^{-5} M BPA for 24 h unless otherwise specified. Mitochondrial density was detected with Mitotracker Green (1:1000; Molecular Probe) according to the manufacturer's instructions. The mitochondrial membrane potential ($\Delta\Psi m$) was assessed by using a mitochondrial membrane potential assay kit with JC-1 according to the manufacturer's instructions. Fluorescence was detected with a multiple fluorescence reader. Reactive oxygen species (ROS) were monitored with the oxidation sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), as previously described [11]. Total concentrations of glutathione (GSH) were measured by the enzymatic recycling method using glutathione reductase and 5′,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Luminescence was used to determine the relative concentration of ATP in cells exposed to BPA. The detailed procedures for intracellular ROS, GSH and ATP concentrations were described in the SM.

2.7. Quantitative real-time PCR

Quantitative real-time PCR was performed as previously described [12]. Briefly, after exposure to BPA for 24 h, TM4 cells were washed and total mRNA was extracted with a TRIZOL reagent. PCR was run on an iCycler (Bio-rad, Hercules, USA) using validated primers and SYBR Premix Ex Taq II (Takara, Japan) for detection. The cycle number when the fluorescence first reached a preset threshold (Ct) was used to quantify the initial concentration of individual templates for expression of mRNA of genes of interest. Primer pairs were as follows: GAPDH, forward 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse 5′-TGG TGA AGA CGC CAG TGG A-3′; VADC2, forward 5′-AAC AAA GGA TTT GGC TTT GG-3′ and reverse 5′-CGA GTG CAG TTG GTA CCT GA-3′; PRDX4, forward 5′-AAG GCT TGG AGA GTG ATG AAC GGT-3′ and reverse 5′-TCC TTC CCA ATA AGG TGC TGG CTT-3′; Gdi2, forward 5′-AGC CCT GGC ATC TAG CTT AAT G-3′ and reverse 5′-TGG CAA CAT AAA CCA GGA ACT TC-3′; GLUT3, forward 5′-CTC TTC AGG TCA CCC AAC TAC GT-3′ and reverse 5′-CCG CGT CCT TGA AGA TTC C-3′; LDH A, forward 5′-CAT TGT CAA GTA CAG TCC ACA CT-3′ and reverse 5′-TTC CAA TTA CTC GGT TTT TGG GA-3′.

2.8. Western blotting analysis

Cells were lysed in cell lysis buffer, and then lysates were cleared by centrifugation and denatured by boiling in Laemmli buffer. Aliquots of protein were separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. Following blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated with the primary antibody at 1:1000 dilution overnight at 4 °C and then incubated with a horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 2 h at room temperature, and detected with the Western Lightning Chemiluminescent detection reagent (Perkin-Elmer Life Sciences, Wellesley, MA). Results of densitometric analyses of Western blots, obtained using ImageJ software, are presented as relative optical density (%) to the control (GAPDH).

2.9. Statistical analysis

All values were reported as mean ± SD of three independent experiments unless otherwise specified. Data were analyzed by two-tailed, unpaired Student's *t*-test after data were checked for normality and homogeneity of variance between two groups and by one-way ANOVA followed by Bonferroni test for multiple comparison involved. The statistical analyses were performed using SPSS 17.0 for Windows. A p-value of <0.05 was considered to be statistically significant.

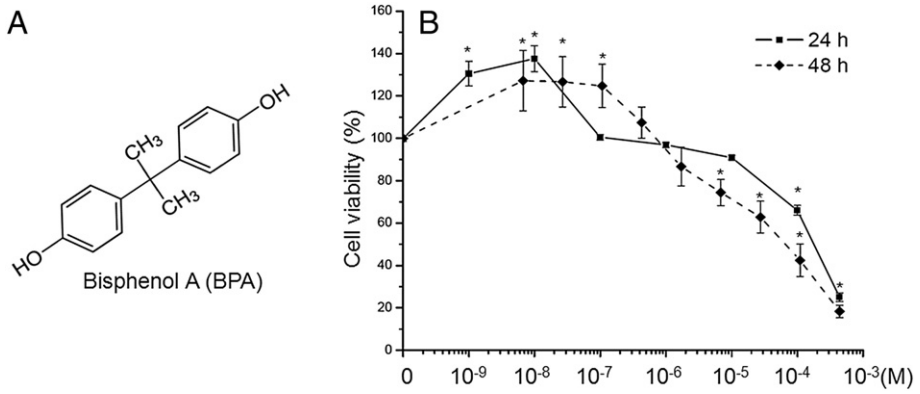

Fig. 1. Effects of increasing concentrations of BPA on the proliferation of Sertoli TM4 cells. (A) Chemical structure of BPA. (B) Cells were treated with various concentrations (10^{-8} to 10^{-3} M) of BPA for 24 h and 48 h, and then cell viability was assessed by CCK8 kit assay. Data are presented as means ± SD of three independent experiments. *$p < 0.05$ compared with control.

3. Results

3.1. Effects of BPA on cell viability

When treated with concentrations of BPA (Fig. 1 A) ranging from 10^{-8} to 10^{-3} M for 24 h and 48 h, we found that micromolar BPA inhibited while nanomolar BPA stimulated proliferation of Sertoli TM4 cells (Fig. 1 B). Proliferation was >30% more than control in cells exposed to 10^{-8} M BPA for 24 or 48 h, while concentrations of BPA greater than 10^{-6} M inhibited proliferation of TM4 cells. This result indicated that lesser concentrations of BPA stimulated while greater concentrations of BPA inhibited proliferation of TM4 cells. The IC_{50} values of BPA to TM4 cells were 1.5×10^{-4} M and 5.0×10^{-5} M for 24 and 48 h, respectively. Therefore, due to the significant opposite effects on proliferation of TM4 cells, 10^{-8} M and 10^{-5} M were chosen as concentrations to represent nanomolar and micromolar concentrations of BPA for further studies, respectively.

3.2. 2-DE and MALDI-TOF MS/MS analysis of BPA-induced proteome alterations in TM4 cells

A 2-DE-based comparative proteomic analysis was performed to investigate the protein changes underlying these opposite effects. After exposure to BPA for 24 h, significantly ($p < 0.05$) 40 differentially expressed protein spots were observed in two-dimensional gel images of control, 10^{-8} M, and 10^{-5} M BPA-treated (Fig. S1). The fold difference is represented by the ratio of the intensity value of the BPA-treated group to the value of the control group (Table 1; Fig. S1). Most of identified proteins, particularly for the groups of energy metabolism, oxidative stress and cell proliferation, were concentration-dependent with opposite changes in expression of proteins Gid2, MDH, APRT, FABP, ATPaseB, IDHα, PRDX4, RBBP4, Sept2α, and RP in cells treated with the lesser and greater concentration of BPA. Expressions of some differently expressed proteins incubated with 10^{-8} M BPA were normal in cells treated with 10^{-5} M BPA. For example, exposure to 10^{-8} M BPA induced a strong decrease in expression of proteins associated with electron transferring flavoprotein, alpha polypeptide (ETFα) (0.14% to control), and GST-Pi (0.20% to control). While exposure to 10^{-5} M BPA did not significantly change expression of those proteins. Conversely, some proteins differently expressed in cells exposed to 10^{-5} M BPA were normally expressed in cells exposed to 10^{-8} M BPA. For example, exposure to 10^{-5} M BPA induced a strong decrease in expression of the following proteins: adenine phosphoribosyltransferase (APRT) (0.15% to control), fatty acid-binding protein (FABP) (0.15% to control), histone-binding protein RBBP4 (RBBP4) (75.3% to control), septin-2 isoform α (Sept2α) (0.31% to control), protein phosphatase 1 (PP1) (0.33% to control), myosin light chain, as well as regulatory B-like (MLC) (0.25% to control). Alternatively, exposure to 10^{-8} M BPA had limited effects on expression of those proteins. These results provided important clues for the stimulatory effects of the lesser concentrations of BPA, but inhibitory effects of the greater concentration of BPA on proliferation of TM4 cells.

3.3. Validation of differentially expressed proteins

To further verify the up- or down-regulation of the identified proteins, the Western blotting analysis and real-time PCR were performed on the selected differentially expressed proteins. The same pattern of expression was observed between the 2-DE analysis and Western blotting analysis and real-time PCR (Fig. S2). These comparisons confirmed up-regulation of ARP3 (actin-related protein 3) and regulation of VDAC2 in cells treated with 10^{-8} M or 10^{-5} M BPA, respectively (Fig. S2 A). Furthermore, the opposite regulations of VDAC2, PRDX4, and Gdi2 mRNA to 10^{-5} M and 10^{-8} M BPA treatment were confirmed by use of real-time PCR assay (Fig. S2 B).

3.4. Functional categories of identified proteins

To understand the biological relevance of the changes in protein expression in response to 10^{-5} M and 10^{-8} M BPA treatment, the PANTHER classification system was used to classify the 36 identified proteins. Those identified proteins were classified into 8 categories according to their functions during biological processes (Fig. 2 A). Energy metabolism, oxidative stress, cell structure and motility-related proteins, cell proliferation and apoptosis-related proteins accounted for the major proportion among the detected proteins.

Protein–protein interactions among the identified proteins were also predicted based on the STRING system. Notably, two major clusters of interacting proteins were highlighted by STRING (Fig. 2 B), including oxidative stress-related proteins and energy metabolism related proteins. Results of this analysis suggested that oxidative stress and energy metabolism might mediate the opposite effects observed on proliferation in cells exposed to the lesser or greater concentrations of BPA.

3.5. Effects of BPA on cell cycle and apoptosis

Effects of lesser (10^{-8} M) or greater (10^{-5} M) concentrations of BPA on cell cycle arrest and apoptosis were determined. Treatment of TM4 cells with 10^{-8} M BPA for 24 h or 48 h resulted in significantly greater proportion of cells in S phase relative to the control (Fig. 3 A). However, treatment with 10^{-5} M BPA for 24 h or 48 h resulted in a significantly lesser proportion of cells at the S phase and more cells in G2/M phases (Fig. 3 A). Annexin V-FITC/PI double staining assay was performed to investigate the induction of apoptosis by BPA. Results showed that very limited effects were observed for both the lesser (10^{-8} M) and greater

Table 1
Identification of differentially expressed proteins in TM4 cells treated with BPA by MALDI-TOF- MS/MS.

Spot no. [a]	Protein name	Gene symbol	Accession no. [b]	MW (kDa)[c]	pI [d]	Matched peptides	Protein score [e]	Coverage (%) [f]	% change (treated × 100 / control)[g]	
									Nanomolar	Micromolar
Energy metabolism										
9	Guanosine diphosphate (GDP) dissociation inhibitor 2	Gdi2	gi|148700277	52,056	6.12	22	552	51	150 ± 6.39	59.3 ± 2.91
15	Malate dehydrogenase	MDH	gi|254540027	36,659	6.16	9	346	29	127 ± 3.44	58.0 ± 2.96
19	Adenine phosphoribosyltransferase	APRT	gi|118601013	19,883	6.31	10	405	62	119 ± 1.47	0.15 ± 0.01
21	Fatty acid-binding protein	FABP	gi|6754450	15,470	6.14	16	395	86	123 ± 2.70	0.15 ± 0.01
22	mCG17741, isoform CRA_b	mCG17741	gi|148682553	8999	7.67	6	180	58	52.8 ± 1.65	52.0 ± 2.72
24	Mitochondrial inner membrane protein isoform 3	Immt3	gi|358439528	82,792	6.26	36	616	48	114 ± 2.18	71.9 ± 4.67
26	Vacuolar adenosine triphosphatase subunit B	ATPaseB	gi|1184661	56,948	5.57	22	490	43	152 ± 9.42	76.3 ± 1.52
30	Electron transferring flavoprotein, alpha polypeptide	ETFα	gi|13097375	35,360	8.62	17	322	45	0.14 ± 0.02	103 ± 5.65
32	Electron transfer flavoprotein subunit beta	ETFβ	gi|38142460	27,834	8.24	11	384	39	0.31 ± 0.02	0.31 ± 0.02
Oxidative stress										
7	Isocitrate dehydrogenase 3 (NAD+) alpha	IDHα	gi|148693875	35,021	5.86	23	626	53	1.03 ± 0.05	188 ± 6.14
17	Peroxiredoxin-4 precursor	PRDX4	gi|7948999	31,261	6.67	9	550	40	191 ± 11.3	0.44 ± 0.02
18	Thioredoxin-dependent peroxide reductase	PRDX3	gi|6680690	28,337	7.15	7	266	27	0.43 ± 0.03	0.08 ± 0.01
20	Cu/Zn superoxide dismutase	SOD	gi|226471	15,923	6.03	6	113	38	74.2 ± 1.21	62.6 ± 1.72
33	Chain A, 1.8 angstroms molecular structure of mouse liver class pi glutathione S-transferase complexed with S-(P-nitrobenzyl) glutathione and other inhibitors	GST-Pi	gi|576133	23,634	8.13	4	266	26	0.20 ± 0.02	96.7 ± 4.39
34	Peroxiredoxin-1	Prx-1	gi|6754976	22,390	8.26	15	637	58	72.4 ± 2.21	62.7 ± 2.08
Cell proliferation, cell cycle, and apoptosis										
6	Cytochrome b-c1 complex subunit 1	UQCRC1	gi|46593021	53,446	5.81	23	609	46	47.3 ± 3.31	57.5 ± 4.32
1	Histone-binding protein RBBP4	RBBP4	gi|5032027	47,911	4.74	17	420	50	109 ± 5.54	75.3 ± 2.38
11	Septin-2 isoform a	Sept2α	gi|6754816	41,727	6.10	24	595	60	116 ± 2.14	0.31 ± 0.03
13	Ribosomal protein	RP	gi|13277927	34,336	5.91	20	676	54	148 ± 4.39	0.24 ± 0.03
DNA/RNA processing										
8	Heterogeneous nuclear ribonucleoprotein H	hnRNP H	gi|10946928	49,454	5.89	19	600	44	112 ± 7.25	67.9 ± 3.57
27	Elongation factor 2	EF2	gi|33859482	96,222	6.41	24	505	22	138 ± 2.08	180 ± 9.14
Protein metabolism and modification										
25	Glycine–tRNA ligase	glyQS	gi|93102417	82,624	6.24	26	840	38	116 ± 1.37	81.5 ± 2.06
3	Prolyl 4-hydroxylase subunit alpha-1	Phyα1	gi|33859596	61,157	5.62	16	655	42	156 ± 8.29	165 ± 3.86
14	Protein phosphatase 1	PP1	gi|148705446	36,091	6.59	19	470	57	115 ± 5.05	0.33 ± 0.02
16	26S proteasome, non-ATPase subunit	PSMD	gi|2505940	34,711	6.18	18	285	43	79.8 ± 2.09	60.6 ± 1.18
Protein folding										
2	Hspd1 protein	Hspd1	gi|76779273	59,559	8.09	29	1110	58	55.6 ± 1.55	64.6 ± 3.95
4	Peptidyl-prolyl cis-trans isomerase FKBP4	PPIase-FKBP4	gi|6753882	51,939	5.54	26	456	53	157 ± 4.25	204 ± 13.4
37	Peptidyl-prolyl cis-trans isomerase A	ppiA	gi|6679439	18,131	7.74	12	495	64	76.2 ± 3.31	67.0 ± 2.08
Cell structure and motility										
5	Actin-related protein 3	ARP3	gi|23956222	47,783	5.61	31	630	61	148 ± 11.8	159 ± 7.28
12	F-actin-capping protein subunit alpha-2	Capzα2	gi|6671672	33,118	5.57	11	269	38	163 ± 12.1	160 ± 5.13
35	Transgelin-2	TAGLN2	gi|30519911	22,552	8.39	19	479	81	11.5 ± 0.56	31.3 ± 2.36
36	Cofilin-1	Cfl2	gi|6680924	18,776	8.22	12	191	75	77.8 ± 3.86	55.4 ± 1.81
38	Destrin	Dstn	gi|9790219	18,852	8.14	5	156	29	60.8 ± 2.69	66.8 ± 3.84
39	Protein S100-A6	S10A6	gi|6755392	10,101	5.30	9	219	60	3.83 ± 0.20	6.51 ± 0.59
40	Myosin light chain, regulatory B-like	MLC	gi|71037403	19,940	4.67	12	554	62	131 ± 5.27	0.25 ± 0.02
Signal transduction										
31	Voltage-dependent anion-selective channel protein 2	VDAC2	gi|6755965	32,340	7.44	3	141	11	123 ± 7.05	81.5 ± 3.74

[a] Spot numbers correspond with 2-DE gel as shown in Fig. S1.
[b] Accession number in NCBInr database.
[c] MW (kDa): molecular mass of predicted protein.
[d] pI: pI of predicted protein.
[e] Protein score: In MASCOT, the score for an MS/MS match is based on the absolute probability (*P*), and the observed match between the experimental data and database sequence is a random event. The reported score is −10 log (*P*). So during a search, if 1.5×10^5 peptides fell with the mass tolerance window the precursor mass, and the significance threshold was chosen to be 0.05, this would translate into a score threshold of 65.
[f] Percentage of predicated protein sequence covered by matched sequence.
[g] Fold change is expressed as a radio of the Vol% between of treated/control cells, and each value represents the mean value ± SD of three independent experiments.

(10^{-5} M) concentrations of BPA (Fig. S3). Exposure to 10^{-8} M BPA resulted in greater expression of PCNA and anti-apoptotic Bcl-2 protein, while exposure to 10^{-5} M BPA resulted in lesser amounts of cyclin D1, PCNA, Bcl-2, and procaspase 3 proteins (Fig. 3 B). Exposure to either 10^{-8} M or 10^{-5} M BPA had no significant effects on expression of cleaved caspase 3. The results suggested that an intrinsic and mitochondrial-related pathway is involved in the opposite effects of greater or lower concentrations of BPA on TM4 cell proliferation.

3.6. Effects of BPA on mitochondrial density and membrane potential (ΔΨm).

Since the intrinsic, mitochondrial-related pathway was suggested to be involved in the effects of BPA on cell proliferation, mitochondrial density was measured by use of Mitotracker green (a marker of mitochondria) in TM4 cells. Exposure to 10^{-5} M BPA for 24 h resulted in significantly lesser density of mitochondria, while 10^{-8} M BPA had

no significant effects compared to the control group (Fig. 4 A and B). This result further confirmed that dysfunctions of mitochondria are involved in the inhibitory effects of greater concentrations of BPA on TM4 cells.

Maintenance of membrane polarity ($\Delta\Psi m$) is critical and a good indicator of cell viability. We found that greater concentration of BPA (10^{-5} M) treatment for 24 h caused a significant loss of $\Delta\Psi m$ in TM4 cells. However, exposure to the lesser concentration of 10^{-8} M BPA caused an elevated $\Delta\Psi m$ (Fig. 4 C). These results suggested that mitochondrial-mediated pathways are involved in both the inhibitory effects of the greater concentration and stimulatory effects of the lesser concentration of BPA on cell proliferation.

3.7. Micromolar BPA inhibited cell proliferation via increasing oxidative stress

Based on the results of proteomic study, mitochondrial-related proteins such as Immt3, ETFα/β, and UQCRC1 were influenced by exposure to BPA. Dysregulation of these proteins resulted in accumulation of ROS and dysfunction of mitochondria [13]. Mitochondrial density and $\Delta\Psi m$ were significantly decreased in TM4 cells exposed to 10^{-5} M BPA. Therefore, roles of oxidative stress on proliferation of cells exposed to greater concentrations of BPA were further investigated. Concentrations of ROS were rapidly increased by exposure to 10^{-5} M, but not 10^{-8} M BPA at 4 h (Fig. 5 A). The elevation of ROS can be detected in TM4 cells after 24 h treatment with 10^{-5} M BPA (Fig. 5 B). GSH is a major antioxidant and protects cells from oxidative stress. The depletion of GSH was necessary for an initial increase in ROS production [14]. We found that exposure of cells to 10^{-5} M BPA for 24 h led to a 27% depletion while 10^{-8} M BPA led to a 35% elevation of intracellular GSH (Fig. 5 C). These results are consistent with the greater concentrations of BPA inducing production of ROS in TM4 cells.

To confirm that the greater concentration BPA inhibited proliferation of cells via oxidative stress, TM4 cells were treated with 10^{-5} M BPA for 24 h in the presence or absence of N-acetylcysteine (NAC, 5 mM), a potent scavenger of ROS, and then cell viability was determined by use of the CCK-8 kit. The presence of NAC completely reversed the inhibitory effects of 10^{-5} M BPA on cell proliferation (Fig. 5 D). Furthermore, blocking production of ROS by NAC resulted in increases of cyclin E1 and Bcl-2 (Fig. 5 E), which can trigger TM4 cells to enter into the S phase of the cell cycle and promote cell proliferation. The variation of cyclin A, cyclin B and cyclin D1 induced by 10^{-5} M BPA was also erased by the treatment of NAC. There is no significant effect on the protein expression of TM4 cells treated with NAC (5 mM) alone (data not shown).

3.8. Nanomolar BPA stimulated cell proliferation by promoting energy metabolism

Exposure to 10^{-8} M BPA increased the density of mitochondria (Fig. 4 A) and $\Delta\Psi m$ of TM4 cells (Fig. 4 B), elevated GSH concentrations by 36% relative to that in control cells (Fig. 5 C), and decreased concentrations of ROS (Fig. 5 A). Proteomic study revealed that 10^{-8} M BPA significantly down regulated the expression of mitochondrial-related proteins such as Immt3, ETFα/β, UQCRC1, and SOD. Therefore, the

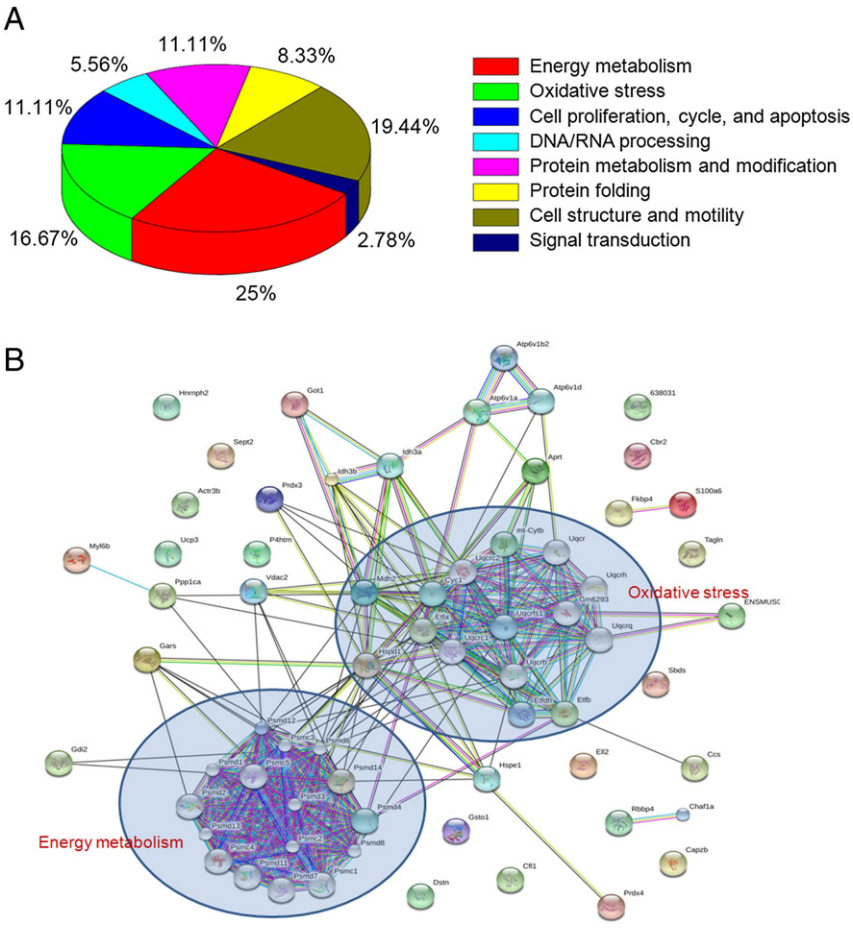

Fig. 2. Functional classification and distribution of all identified proteins. (A) Eight protein groups were categorized based on the putative biological functions of identified proteins and the percentages of each protein group were indicated. (B) The protein–protein interaction network of the identified proteins. The network containing 36 identified proteins was mapped using the STRING system based on evidence with different types.

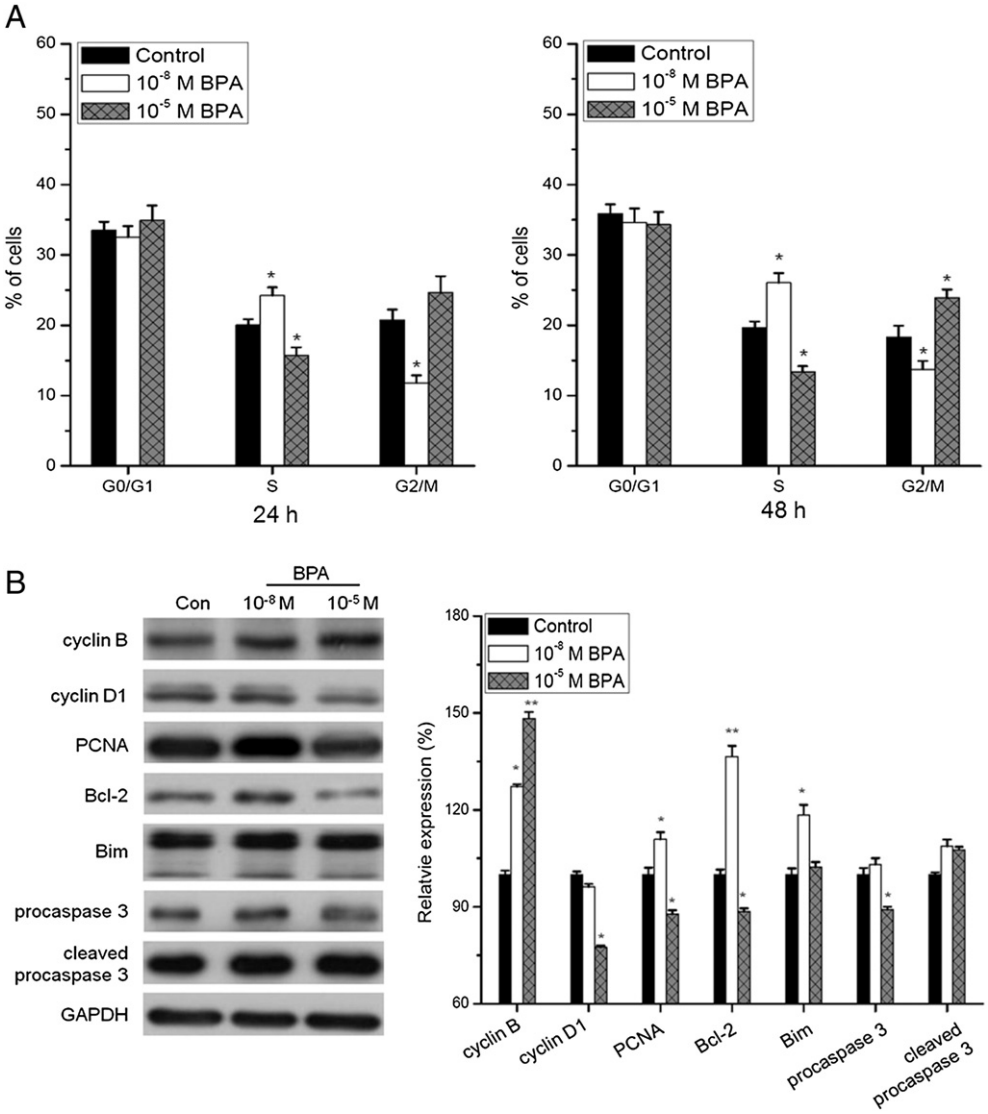

Fig. 3. Effect of lesser and greater concentrations of BPA on the cell cycle of TM4 cell. (A) Cells were treated with 10^{-8} M and 10^{-5} M BPA for the indicated time, fixed in ethanol, and then stained with propidium iodide (PI). The DNA contents of TM4 cells treated with BPA for 24 h (left) or 48 h (right) were determined by flow cytometry. The percentage of cells in each phase of the cell cycle (sub-G1, G0/G1, S and G2/M) were calculated using FlowJo Analysis Software. (B) Effects of BPA on the expression of cell cycle and apoptosis related proteins. TM4 cells were treated with 10^{-8} M and 10^{-5} M BPA for 24 h, then cell cycle and apoptosis related protein expression levels were analyzed by Western blotting analysis. $^{*}p < 0.05$ compared with control; $^{**}p < 0.01$ compared with control.

roles of mitochondrial related energy metabolism of TM4 cells exposed to 10^{-8} M BPA was further evaluated.

Exposure to 10^{-8} M BPA for 24 h significantly increased concentrations of ATP by 80% relative to that of the control cells (Fig. 6 A). To verify the role of ATP concentrations in 10^{-8} M BPA induced cell proliferation, 0.5 μg/ml or 5 μg/ml oligomycin A, an inhibitor of ATP synthase, was added to the incubation mixture. Neither 0.5 μg/ml nor 5 μg/ml oligomycin A had a statistically significant effect on proliferation of TM4 cells. However, both 0.5 μg/ml and 5 μg/ml oligomycin A totally abolished the greater proliferation of TM4 cells caused by 10^{-8} M BPA (Fig. 6 B). Furthermore, blocking production of ATP by oligomycin A attenuated 10^{-8} M BPA-induced up regulation of cyclin A, cyclin B and Bcl-2 and resulted in less expression of cyclin D1 and cyclin E1 (Fig. 6 C), which would inhibit TM4 cells from entering into the G1 phase of the cell cycle and therefore suppress cell proliferation. There is no significant effect on the protein expression of TM4 cells treated with oligomycin A (0.5 μg/ml or 5 μg/ml) alone (data not shown).

The effects of 10^{-8} M BPA on the expression of key enzymes in cell energy metabolism were detected. Glucose transporter 3 (GLUT3) is a protein that facilitates uptake of glucose across the plasma membrane and is responsible for the rate-limiting step of glucose metabolism [15]. It is usually associated with transport of glucose in cells with high-energy demands [16]. Lactate dehydrogenase A (LDH A), the predominantly expressed isoform in testis, catalyzes conversion of pyruvate to lactate and plays key roles in cell anaerobic respiration [17]. In this study, exposure to 10^{-8} M BPA resulted in significant up-regulation of mRNA of GLUT3 (Fig. 6 D) while down-regulation of that of LDHA (Fig. 6 E). The effects lasted for more than 48 h. These results suggested that exposure to 10^{-8} M BPA stimulated the uptake and consumption of glucose by increasing expression of GLUT3, which can inhibit anaerobic respiration by decreasing production of lactate, and then ultimately promoted cell proliferation.

4. Discussion

The mechanisms responsible for the opposite effects of nanomolar and micromolar BPA on cell proliferation remained to be illustrated. In the present study, the proteomic study was performed to illustrate the opposite effects of lesser (10^{-8} M) and greater (10^{-5} M) concentrations of BPA on Sertoli TM4 cell proliferation. Two major clusters of

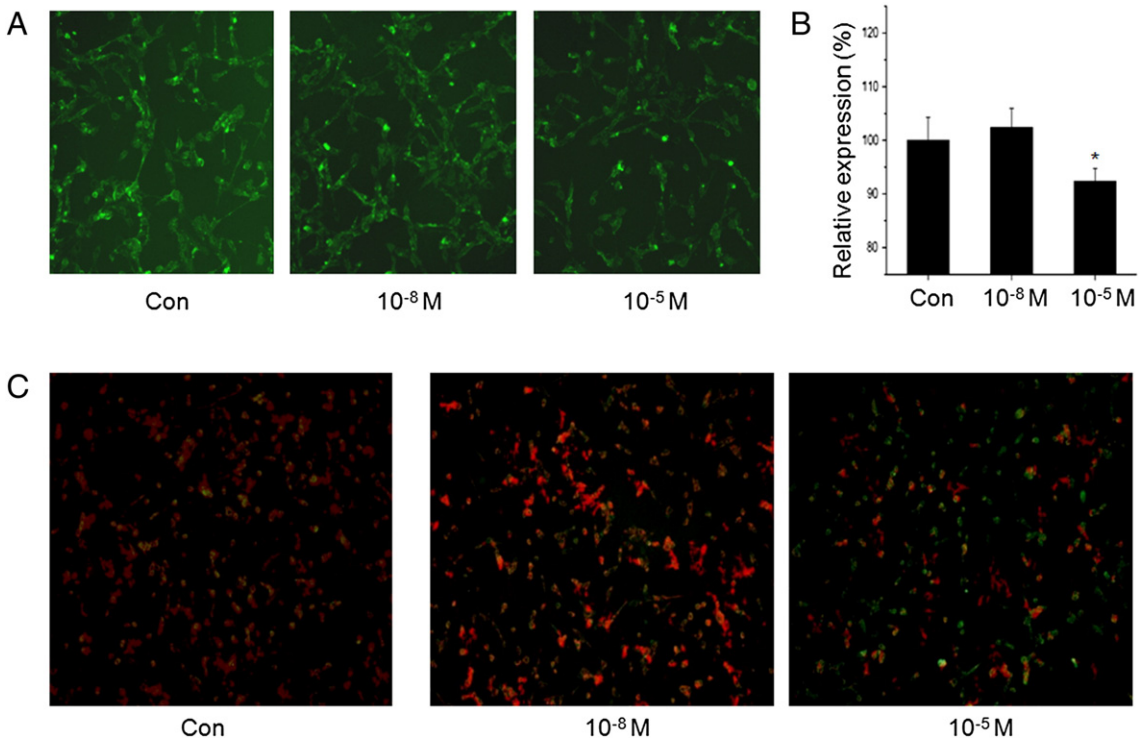

Fig. 4. Effects of BPA on mitochondrial density and mitochondrial membrane potential ($\Delta\Psi m$). TM4 cells were treated with 10^{-5} M or 10^{-8} M BPA for 24 h. (A) The microscopy of mitochondria in nanomolar and micromolar BPA treated TM4 cells with Mitotracker Green. (B) Quantification of mitochondria in BPA treated TM4 cells by flow cytometry with Mitotracker Green. (C) The mitochondria-specific dye, JC-1 was excited at 488 nm by argon-ion laser sources and detected separately at 530 and 590 nm for JC-1 monomer with green fluorescence and JC-1 aggregates with red fluorescence, respectively. Apoptotic cells mainly show green fluorescence, while healthy cells show red fluorescence (yellow when merged). $^{*}p < 0.05$ compared with control.

proteins, energy metabolism and oxidative stress related proteins were highlighted among all 36 differently expressed proteins. Most of the proteins, particularly for the groups of energy metabolism, oxidative stress and cell proliferation, such as Gid2, MDH, APRT, Immt3, FABP, ATPaseB, IDHα, PRDX4, RBBP4, Sept2α, and RP, exhibited opposite responses when cells were exposed to the lesser or greater concentrations of BPA. These results suggested that these two clusters of proteins might play a vital role in mediating the opposite effects of lesser and greater concentrations of BPA on TM4 cell proliferation. The proteomic studies have been performed in the mammary glands of rat offspring exposed to BPA in utero [18], mouse immune organs [19], and abalone (*Haliotis diversicolor supertexta*) [20]. Genome wide microarray has been performed previously in testicular Sertoli cells using a high dose of BPA (200 μM), which revealed cell injury by BPA accompanying endoplasmic reticulum (ER) stress [21]. Of special interest are proteins identified in this study that have previously been reported as proteomic biomarkers of BPA, such as heat shock cognate protein and peroxiredoxin. Our present study identified more different protein spots (36) than other studies and revealed that energy metabolism and oxidative stress related proteins are the two main clusters of protein responsible for lesser and greater concentrations of BPA on TM4 cell proliferation. The difference of proteomic profiles among the present and previous studies might be due to that ability of BPA to exert effects on protein expression depends on the species and strain of animals or cells, time of exposure, susceptibility of target organs or cells, does of exposure, and the sensitivity of proteomic methods.

The results of proteomic study that proteins which are related to energy metabolism, oxidative stress and cell proliferation exhibited opposite responses provided important clues to illustrate the mechanisms responsible for opposite responses of lesser or greater concentrations of BPA. As to the proteins up-regulated by 10^{-8} M BPA in TM4 cells, Gdi2 (GDP-dissociation inhibitor 2) has been reported to promote the proliferation of colorectal cancer cells and protect cancer cells against drug-induced apoptosis [22]. MDH (malate dehydrogenase) can promote the prostate cancer cell proliferation and decrease docetaxel sensitivity [23]. FABP (fatty acid-binding protein) promotes proliferation and invasion of human intrahepatic cholangiocarcinoma and glioblastoma cells [24], while knockdown of FABP4 in endothelial cells significantly reduced cell proliferation [25]. The activities of ATPaseB are well documented to associate with cell growth and proliferation, and ATP bioluminescence has been used to measure cell proliferation and cytotoxicity [26]. PRDX4 (peroxiredoxin 4) is a newly discovered antioxidative protein, which exerts its protective function against oxidative damage by scavenging ROS in the extracellular space [27]. Sept2α (septin-2 isoform α) has been reported to high expression in cancer cells and its phosphorylation is important for hepatoma carcinoma cell proliferation [28]. Ribosome biogenesis plays an essential role in proliferation of cells; therefore, the up-regulation of RP (ribosomal protein) by 10^{-8} M BPA indicated greater proliferation of cells [29]. hnRNP H has been reported to promote the cellular growth and proliferation [30]. Metabolite profiling identifies a key role for glycine in rapid proliferation of cancer cells, and up-regulation of glyQS (glycine–tRNA ligase) suggested greater proliferation of cells in TM4 cells [31]. Dephosphorylation of AKT by PP-1 (protein phosphatase 1) significantly modulates its functions in regulating the expression of downstream genes, promoting cell survival and modulating differentiation [32]. Over expression of VDAC2 (voltage-dependent anion-selective channel protein 2) specifically complexes with the inactive form of Bak and selectively blocks Bak-dependent but not Bax-dependent apoptosis [33]. Therefore, most of proteins up-regulated by 10^{-8} M BPA are positively correlated with cell proliferation. As to proteins increased by high concentrations while decreased by nanomolar BPA, ETFα and ICDH3α have been reported to negatively associate with cell proliferation and engage in regulation of apoptosis [34]. Generally, the results of the comparative proteomic analysis revealed that nanomolar concentrations of BPA active and up-regulate the proliferation promoting protein to trigger the TM4 cell proliferation. Conversely greater concentrations of BPA down-regulate them and inhibit cell proliferation.

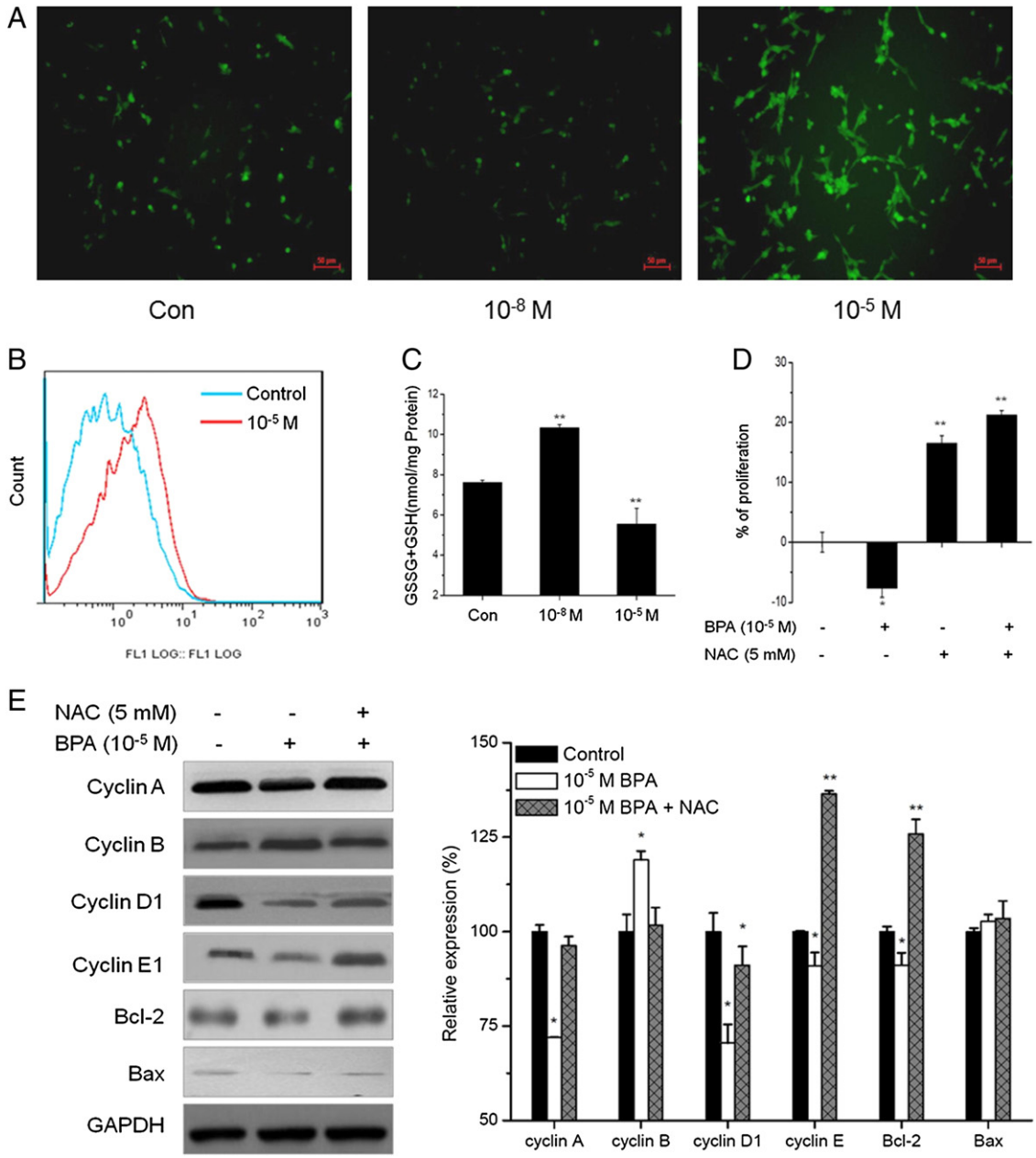

Fig. 5. Micromolar BPA inhibited cell proliferation via increasing oxidative stress. (A) ROS generation in 10^{-5} M or 10^{-8} M BPA treated TM4 cells for 4 h. The ROS levels were marked with DCF-DA and measured by confocal microscopy. (B) 10^{-5} M significantly increased the ROS generation in TM4 cells after exposure for 24 h. ROS levels were flow cytometry with DCF-DA as an indicator. (C) Total glutathione levels (GSSG and GSH) in TM4 cells after treatment with 10^{-5} M or 10^{-8} M BPA for 24 h. (D) Cells were treated with 10^{-5} M BPA and 5 mM NAC alone or together for 24 h. The cell viability was detected with CCK-8 kit assay. (E) The protein expression of cell cycle and apoptosis related proteins in TM4 cells treated with 10^{-5} M BPA alone or the combination with 5 mM NAC for 24 h. Data are presented as means ± SD of three independent experiments. *$p < 0.05$ compared with control; *$p < 0.05$ compared with control; **$p < 0.01$ compared with control.

Some proteins, such as ribosomal protein, cytochrome b-c1, and RGGP4, which are related to cell proliferation, cell cycle and apoptosis [35], were up-regulated by 10^{-8} M BPA while down-regulated by 10^{-5} M BPA (Table 1). This suggested that lesser or greater concentrations of BPA might have different effects on the cell cycle and apoptosis. This was confirmed by flow cytometry analysis that 10^{-8} M BPA significantly increased the proportion of cells in the S phase while 10^{-5} M BPA significantly increased the proportion of cells in the G2/M phase. There is no data available about the effects of BPA on the cell cycle of Sertoli cells. It has been reported that 10^{-8} and 10^{-6} M BPA promoted cells to enter into the G2/M phase in human normal breast cells HBL-100 [36]. This difference might be due to the different cell line and BPA concentrations and needs further study. The data revealed that there was no significant apoptosis that occurred in TM4 cells treated with both 10^{-8} M and 10^{-5} M BPA, suggesting that micromolar BPA inhibiting the TM4 cell proliferation is not via induction of apoptosis. No significant change of apoptotic cell number was also observed in the luminal and glandular epithelia of the uterus [37] and MCF-7 cells [38] after BPA exposure. Previous studies also suggested that BPA can increase cell necrosis compared to the DMSO control in human endometrial endothelial cells (HEECs) [39] and mice granulose cell [40]. The conflicting results might largely be due to the millimolar concentrations of BPA utilized by most apoptotic studies.

Several proteins were regulated in the same trend by both 10^{-8} M and 10^{-5} M BPA in TM4 cells, such as the up-regulation of EF2, Phyα1, PPIase-FKBP4, and Capzα2, or the down-regulation of mCG17741, ETFβ, UQCRC1, PRDX3, SOD, PSMD, Hspd1, ppiA, TAGLN2, cofilin-1, destrin, and protein S100-A6; most of them are

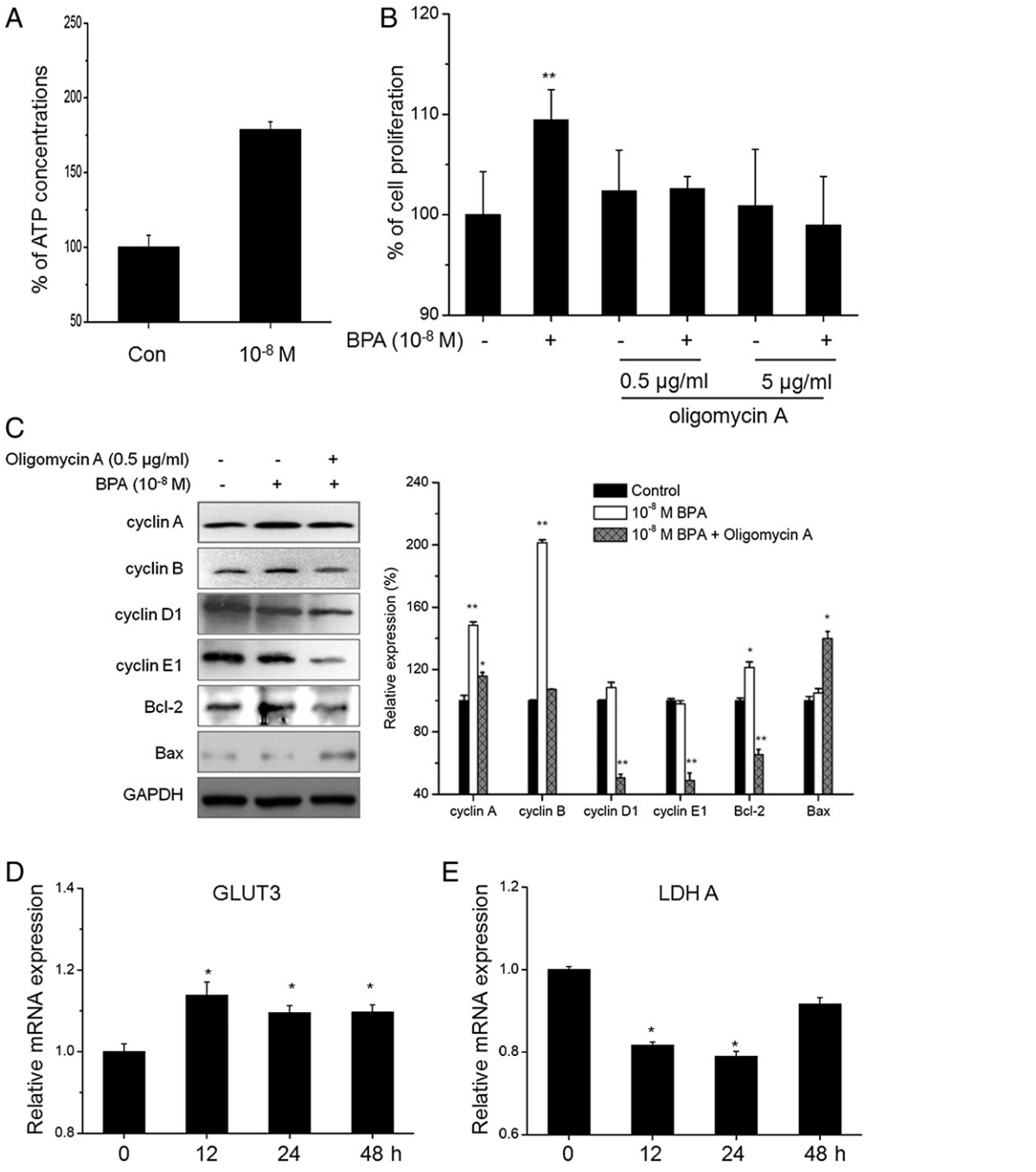

Fig. 6. Nanomolar concentration of BPA stimulated cell proliferation via promoting energy metabolism. (A) TM4 cells were treated with 10^{-8} M BPA, and then ATP concentrations were measured by use of an ATP assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instruction. (B) TM4 cells were treated with 10^{-8} M BPA alone or combination with 0.5 or 5 μg/ml oligomycin A for 24 h. The cell viability was detected with a CCK-8 kit assay. (C) The protein expression of cell cycle and apoptosis related proteins in TM4 cells treated with 10^{-8} M BPA alone or the combination with 0.5 μg/ml oligomycin A for 24 h. RT-PCR analysis of mRNA expression of GLUT3 (D) and LDH A (E) of cells treated with 10^{-8} M BPA for 0, 12, 24, and 48 h, respectively. Data are presented as means ± SD of three independent experiments. The control value was set as 1. $^{*}p < 0.05$ compared with control; $^{**}p < 0.01$ compared with control.

related to cell structure, interaction, and motility [41]. BPA has been reported to influence the migration and cell junction of Sertoli cells [42]. Whether these proteins are responsible for or involved in BPA-induced variation of tight of blood–testis–barrier needs further study. Furthermore, 26S proteasome (PSMD) was down regulated by both 10^{-5} M (60.6 ± 1.18%) and 10^{-8} M (79.8 ± 2.09%) BPA, suggesting that proteasome is a potential target of BPA in TM4 cells. The down regulation of proteasome by micromolar BPA could lead to accumulation of ubiquitinated proteins, and subsequently inhibit cell proliferation [43].

The proteomic study revealed that nanomolar and micromolar BPA had opposite effects on most of energy metabolism and oxidative stress related proteins. Further study suggested that micromolar BPA increased depolarized mitochondria. Mitochondria are the major source of cellular ROS, which will result in oxidative stress. The loss of $\Delta\Psi m$ was recognized as a crucial characteristic of apoptosis involving the mitochondrial pathway [44]. Recently, studies have indicated that excessive ROS induced by exogenous agents cause oxidative mitochondrial damage and inactivation of redox-sensitive molecules, and inhibited the cell proliferation [45]. These data suggested that intrinsic

mitochondrial-mediated pathways are involved in the regulation effects of 10^{-8} M and 10^{-5} M BPA on cell proliferation.

Our data revealed that micromolar BPA inhibited Sertoli cell proliferation by inducing ROS generation and inhibiting enzymes in the respiratory chain or the antioxidases in the antioxidant system. The results of the proteomic study showed that 10^{-5} M BPA increased the expression of isocitrate dehydrogenase 3 (NAD+) alpha (complex I) and isocitrate dehydrogenase 3 (NAD+) alpha in the mitochondrial electron transport chain, which are considered as the major sources of ROS [46]. Indeed, ROS generation was significantly increased in TM4 cells upon 10^{-5} M BPA treatment. Overproduction of ROS can inflict serious damage to cell lipids, proteins and DNA, and these generated ROS in cells can cause the cellular functional disorder and result in the cell death [45]. Furthermore, glutathione S-transferase, which localizes in mitochondria and protects against oxidative stress [47], was decreased in 10^{-5} M BPA treated cells. The antioxidant NAC effectively blocked the inhibition effects of 10^{-5} M BPA on TM4 cell proliferation and abolished the down regulation of 10^{-5} M BPA to cyclin A, cyclin E1 and Bcl-2. The results were consistent with those of a previous study that 50 or 100 μM BPA induced Neuro2a and GC-1 cell injury through reactive ROS via the mitochondrial signaling pathway [48]. Therefore, the adverse effects of greater concentrations of BPA on male reproduction may be due to induction of oxidative stress in sperm [49] and Sertoli cells revealed in the present study.

The results of the present study also illustrated that lesser concentrations of BPA up-regulated the mitochondrial activities, triggered the glucose metabolism, enhanced ATP concentrations, and therefore stimulated proliferation of TM4 cells. The lesser concentration (10^{-8} M) of BPA significantly stimulated proliferation of TM4 cells and increased the S phase in the cell cycle. The proteomic study and mitochondrial analysis suggested that lesser concentration BPA can elevate the activities of mitochondria. The results revealed that 10^{-8} M BPA markedly increased ATP concentrations in Sertoli cells. The increase of cellular ATP content can promote cell cycle regulator activation and trigger cell proliferation [50]. Both 0.5 and 5 μg/ml oligomycin A, a complex V inhibitor of ATP synthase, abolished the growth advantages provided by 10^{-8} M BPA, and completely reversed the BPA induced up-regulation of Bcl-2, cyclin D1 and cyclin B, confirming our hypothesis that nanomolar BPA stimulated cell proliferation depends on cellular energy promoting effects. GLUT3, which allows the energy independent transport of glucose across the hydrophobic cell membrane, can increase the glucose uptake and further promote cell proliferation and reduce cell apoptosis [51]. LDH A, which catalyzes the pyruvate to lactate, has been illustrated to suppress mammary gland tumor cells, which show a diminished proliferation in vitro and an impaired tumor growth in vivo [52]. Low concentration (10^{-8} M BPA) treatment resulted in significant up-regulation of mRNA of GLUT3 while down-regulation of LDHA, suggesting that the lesser concentration of BPA can stimulate the glucose uptake and consumption by increasing the expression of GLUT3, inhibit the anaerobic respiration by decreasing the lactate production, and therefore promote TM4 cell proliferation.

Sertoli cells are the primary supporting cells creating the structural and physiological environments necessary for development of cells during spermatogenesis [4]. Regulation of spermatogenesis by Sertoli cells is dependent on its functions and cell number. Among all health problems caused by BPA, male fertility influenced by BPA gets increasing concerns [42]. Our study revealed that both low and great concentrations of BPA can modulate the proliferation of Sertoli cells, which might be one of the important reasons for male infertility caused by BPA [53].

In conclusion, the current study characterized the proliferation stimulatory effects of nanomolar BPA and inhibition effects of micromolar BPA to TM4 cells. With the instruction of proteomic analysis, it was concluded that greater concentrations of BPA can up-regulate the amounts of ROS in cells, decrease the mitochondrial density and activities, and therefore inhibit cell proliferation. Inversely, nanomolar BPA promotes the energy metabolism and elevates ATP concentrations, therefore triggers proliferation of TM4 cells. This study not only provided new insight to illustrate the mechanisms responsible to the opposite effects of lesser and greater concentrations of BPA, but also provided further direction for investigation of the dose-effects of other endocrine disruptor chemicals.

Acknowledgments

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Appendix A. Supplementary data

Details of methods and three figures are available at the supplementary materials (SM). Supplementary data to this article can be found online at doi:http://dx.doi.org/10.1016/j.bbagen.2014.05.018.

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