Restoring mitochondrial function and normalizing ROS-JNK/MAPK pathway exert key roles in glutamine ameliorating bisphenol A-induced intestinal injury

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Funding information
National Key Research and Development Program of China, Grant/Award Number: 2018YFD0500402 and 2016YFD0700201; 111 Project, Grant/Award Number: B16044

Abstract
Bisphenol A (BPA) is toxic to the reproductive and nervous system, even carcinogenic in humans and animals. However, few studies focused on effects of BPA on the intestinal tract. Here, we detected BPA-induced injuries on intestinal mucosa and explored a reliable approach to counteract BPA effects. C57BL/6J mice were gavage BPA or BPA accompanied with ingestion of 4% (w/w) of glutamine for 4-wks. In vitro, IEC-6 cells were treated with 0.4 mmol/L BPA for 6 hours mimicking acute injury and 0.2 mmol/L BPA for 12 hours followed with or without the inclusion of 4 mmol/L glutamine for 12 hours to determine cell renewal, mitochondrial function and ROS-JNK/MAPK pathway upon moderate BPA exposure. As results, BPA exposure caused severe intestinal injury, and disturbed intestinal epithelial cell proliferation and apoptosis, accompanied with mitochondrial malfunction and activated JNK/MAPK pathway as well. Notably, glutathione metabolism was implicated in BPA-induce injury. Glutamine could well rescue cell renewal and mitochondrial function from BPA exposure-induced injuries. In conclusion, we demonstrated impaired effect of BPA exposure on intestinal functions, which could be well counteracted by glutamine partly via restoring mitochondrial function and normalizing ROS-JNK/MAPK pathway. Thereby, we provided a novel application of glutamine to rescue intestinal injury.

KEYWORDS
apoptosis, glutathione, intestinal mucosa, mitochondrial malfunction

Abbreviations: Bbox1, γ-butyrobetaine dioxygenase; BPA, bisphenol A; BW, body weight; C3, complement C3; Ccnk, cyclin-K; CD244, natural killer cell receptor 2B4; Cyp2b10, cytochrome P450 2B10; Cyp2e29, cytochrome P450 2C29; Cyp2e66, cytochrome P450 2C66; DCFH-DA, 2, 7-dichlorofluorescein-diacetate; DEPs, differentially expressed proteins; Dock2, dedicator of cytokinesis protein 2; ERK, extracellular signal-regulated kinase; FC, fold change; FDA, food and drug administration; Fgb, fibrinogen beta chain; Fgg, fibrinogen gamma chain; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Glu, glutamine; GSH, glutathione; Gstα2, glutathione S-transferase A2; Gstm, glutathione S-transferase Mu; IL-1β, interleukin 1β; JNK, jun N-terminal kinase; Krt10, keratin type I cytoskeletal 10; Krt76, keratin type II cytoskeletal 76; Lrg1, leucine-rich HEV glycoprotein; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; MMP, mitochondrial membrane potential; Nrf1, Nrf1 protein; Orm1, alpha-1-acid glycoprotein 1; Plxna1, plexin-A1; ROS, reactive oxygen species; Rpf2, ribosome production factor 2 homolog; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SOD, superoxide dismutase; Sos1, son of sevenless homolog 1; TEER, transepithelial electrical resistance; TMT, tandem mass tags; TNFα, tumor necrosis factor α; ZOs, zonula occludens.

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1 | INTRODUCTION

The small intestine functions in digestion and absorption, meanwhile defending against pathogens and toxicants. Intestinal epithelial integrity serves as a fundamental barrier between enteric cavity and the internal of the body to guarantee intestinal function.\(^1\) Intestinal epithelial integrity is maintained by continuous cell renewal, while disordered cell renewal would injure intestinal mucosal barrier function.\(^2\) The compromised epithelial barrier can activate the immune system and initiate inflammation and resultant intestinal diseases.\(^3\) The intestine may suffer various luminal stresses including toxicants, which can cause the intestinal mucosa in oxidative stress,\(^4\) exhibiting intracellular reactive oxygen species (ROS) accumulation, intestinal epithelial cell renewal disorder, barrier malfunction, and various diseases, such as gastrointestinal cancer, inflammatory bowel disease (IBD), and enterocolitis.\(^5,6\) Therefore, it is significant to maintain intestinal epithelial integrity and cellular redox homeostasis in keeping intestinal health.\(^7,8\)

Bisphenol A (BPA), as a synthetic xenoestrogen, is widely used in manufacturing plastic packaging, consumer products, and drink bottles,\(^9\) and it can leach from plastics into food and water. Thus, humans are routinely subjected to the ingestion of food, beverages, and water contaminated by BPA.\(^10\) It has been well documented that BPA can cause reproductive toxicity,\(^11-13\) neurotoxicity, and hepatic damage\(^14,15\) as well as carcinogetic effect.\(^16\) The intestinal tract is the border line against exogenous toxicants entering bodies. Although BPA was implicated in intestinal injury and malfunction,\(^17,18\) the mechanism(s) of BPA disturbing intestinal mucosa homeostasis and function remains unclear.

Glutamine (Gln), as a precursor of glutathione (GSH), is the major energy fuel of enterocytes.\(^19\) It had been shown to attenuate apoptosis via the activation of the IRE1α-XBP1\(^20\) and glutathione-related redox homeostasis in porcine enterocytes.\(^21\) Moreover, dietary glutamine can improve intestinal barrier function,\(^22\) maintain the abundance of tight junction proteins,\(^23\) and reduce intestinal inflammation.\(^24\) In addition, glutamine is an important mitochondrial tricarboxylic acid cycle substrate, which can protect mitochondrial function and maintain cell survival.\(^25\)

Mitochondrial function is a key player in enterocyte differentiation and in coordinating stress response, immunity, cellular metabolism, and apoptosis.\(^26\) Nevertheless, the effect of alimentary glutamine on damaged mitochondrial function remains unclear.

Therefore, the present study was aimed to investigate the effect of BPA exposure on intestinal health and disclose the mechanism underlying BPA exposure injures intestinal mucosa, and exploit the possible nutritional intervention to rescue intestinal injury.

2 | MATERIALS AND METHODS

2.1 | Mice and experimental design

The animal experiment was carried out in accordance with the Chinese Guidelines for Animal Welfare and Experimental Protocol, and the protocols were approved by the Animal Care and Use Committee of China Agricultural University (ID: SKLAB-B-2010-003).

Male 6-week old C57BL/6J mice with body weight (BW) of 20.57 ± 0.49 g were housed in temperature- and humidity-controlled cages in an institutional animal care facility with 12 hours light and dark cycles. Mice were allowed free access to diet and water for one week before the start of the experiment, and then, randomly allocated into three groups with 10 mice per group to receive oral gavage vehicle (corn oil, 10 μL/g BW/d) (control), gavage 500 mg/kg BW/d BPA (BPA), or gavage BPA accompanied with ingestion of 4% (w/w) of glutamine diet (BPA + Gln) daily for 4-wks.\(^27\) The dose of BPA in the study was designed according to previous study.\(^28\) It has been shown that the mean level of BPA daily exposure of populations ranges from 0.875 to 1.449 μg/kg BW/d.\(^29\) The maximum concentration of environmental BPA ranges among soil of 10.5 μg/g, air of 135.8 μg/m\(^3\), water of 370 μg/mL, and food of 1.7 μg/g.\(^30\) The dose of 5 mg/kg BW/d BPA exposure was determined by the Food and Drug Administration (FDA) as an appropriate level for no observed adverse effects for systemic toxicity.\(^31\) In accordance with this provision, we calculated the exposure dose for mice according to the body surface area-dose conversion formula, which yielded nearly 50 mg/kg BW/d. Regarding the concern about intestinal health for humans occupationally exposed to high levels of BPA, the dose of 500 mg/kg BW/d (based on geometric progression) was adopted in this study.

Mice in the control and BPA group were offered a standard chow diet (AIN-93), while mice in BPA + Gln group were fed a diet containing 4% (w/w) of Gln, in which Gln replaced equivalent amount of casein as described previously.\(^27\) The control diet contained 19.6 g/kg Gln, and Gln diet contained 54.7 g/kg Gln. The composition of experimental diets was listed in Table S1. Mice were allowed to feed and water ad libitum. Mouse survival rate was determined throughout the entire period of experiment. At the end of experiment, mice were sacrificed for harvesting intestinal samples. The small intestine was collected and flushed with ice-cold PBS, and length was measured. Approximately 3 cm middle jejunum was fixed in 4% (w/v) of paraformaldehyde for analyzing intestinal morphology. Briefly, the fixed tissues were embedded in paraffin, sliced to 5 μm thickness using a microtome device and stained with hematoxylin and eosin for light microscopic examination. At least three digital pictures were taken for villin and crypt morphology measurement by an experienced researcher blinded to the experimental
2.2  |  Tandem mass tags (TMT)-based quantitative proteomic analysis of jejunal mucosal

The jejunal mucosa was lysed by lysis buffer (1% (w/v) of SDS, 8 mol/L urea, 1:15) with the protection of a Halt protease inhibitor cocktail (Thermal Fisher Scientific Rochford, IL, USA). The lysed solution was centrifuged at 13,000 g for 30 minutes at 4°C, and supernatants were collected to test protein concentration using BCA Protein Assay Kit (HX18651, Huaxingbio Science, Beijing, China). Sample quality was tested with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Protein sample was digested routinely. Briefly, each sample containing 100 μg of protein was reduced, alkylated, and digested with trypsin, and then, incubated at 37°C overnight. The resulting peptide mixture was labeled using the 10-plex TMT reagent according to the manufacturer’s instructions (90111, Thermo Fisher Scientific, Waltham, MA, USA). Concretely, each TMT reagent was fully dissolved in 50 μL acetonitrile, and then, the reagent was added to peptide mixture of samples, which were labeled as (control-1)-126, (control-2)-127N, (control-3)-127C, (BPA-1)-128N, (BPA-2)-128C, (BPA-3)-129C, (BPA + Gln-1)-130N, (BPA + Gln-2)-130C, and (BPA + Gln-3)-131, respectively. Subsequently, hydroxylamine was added and the reaction proceeding for 15 minutes at room temperature. Finally, all the peptide mixtures were combined together and vacuum dried.

The peptides of all samples were resuspended with loading buffer (5 mmol/L ammonium hydroxide solution containing 2% (v/v) of acetonitrile, pH = 10), and separated by high pH reverse phase liquid chromatography (Acquity Ultra Performance LC, Waters Inc, Milford, MA). In brief, the gradient elution was performed on high pH RPLC column (ACQUITY UPLC BEH C18 Column 1.7 μm, 3 mm × 150 mm, Waters Inc, Milford, MA, USA) with the gradient increased at 200 μL/min for 66 minutes. Twenty fractions were collected from each sample and were pooled resulting in 10 total fractions per sample. Experiments were performed on a Q Exactive mass spectrometer that was coupled with Easy-nLC 1200. The peptide mixture (2 μg) was loaded onto a the C18-reverse phase column in buffer A (2% (v/v) of acetonitrile and 0.1% (v/v) of formic acid) and separated with a linear gradient of buffer B (80% (v/v) of acetonitrile and 0.1% (v/v) of formic acid) at a flow rate of 300 nL/min. Q Exactive mass spectrometer was operated in the data-dependent mode to switch automatically between MS and MS/MS acquisition. Survey full-scan MS spectra (m/z 350-1300) were acquired with a mass resolution of 70 K, followed by twenty sequential MS/MS scans with a resolution of 35 K. In all cases, one microscan was recorded using dynamic exclusion of 18 seconds. Three biological repeats were performed for each group.

2.3  |  Cell viability, proliferation, and apoptosis

IEC-6 cells (rat intestinal epithelial cells) were seeded in 96-well cell culture microplates. When reached 80% confluence, cells were starved for 6 hours, and then, cultured in medium with 0, 0.1, 0.2, 0.3, 0.4, or 0.5 mmol/L BPA for 6 or 24 hours. Moreover, cells were pretreated with 0.2 mmol/L BPA for 12 hours, and then, treated with 0, 1, 2, or 4 mmol/L glutamine for another 12 hours. Cell viability was measured using CCK-8 kit (R11053, Guangzhou RiboBio Co., Guangzhou, China) according to manufacturer’s instruction. The proliferating cells were stained with Apollo by red, and cell nucleus was stained with Hoechst 33342 by blue. Cell proliferation rate was calculated as the proportion of red to blue. Cells were treated with 0.4 mmol/L BPA for 6 hours and proliferation was measured using EdU cell proliferation assay kit (R11053, Guangzhou RiboBio Co., Guangzhou, China) according to manufacturer’s instruction. The proliferating cells were treated with 0.4 mmol/L BPA for 6 hours and stained with annexin V-FITC/propidium iodide to measure apoptosis by flow cytometry (CytoFLEX, Beckman Coulter Inc, Fullerton, CA).

2.4  |  Transepithelial electrical resistance (TEER)

Cells were seeded in cell culture transwells with 5 x 10^4 cells per well (the membrane area was 0.33 cm^2, pore size was 0.4 μm), which were placed in 24-well cell culture plates. When resistance approached constant, cells were treated with 0.2 mmol/L BPA for 24 hours. Moreover, cells were pretreated with 0.2 mmol/L BPA for 12 hours, then, treated with 4 mmol/L glutamine for another 12 hours. TEER was determined by an EVOM volt-ohmmeter connected to a 12 mm Endohm unit (World Precision Instruments Inc, Sarasota, FL). Data were expressed relative to those of the control group.

2.5  |  Cells redox state

Cells were cultured either in the presence or absence of 0.4 mmol/L BPA for 6 hours to evaluate the influence of acute BPA exposure on redox homeostasis, and then, harvested for analyzing intracellular superoxide dismutase.
(SOD) and malondialdehyde (MDA) using commercially available SOD and MDA assay kits (A001-3 and A003-4, Jiancheng Bioengineering Co., Nanjing, China) according to the manufacturer's instructions on an ultraviolet-visible spectrophotometer (450 nm and 530 nm, respectively). Moreover, intracellular ROS were detected using fluorescent dye 2, 7-dichlorofluorescein-diacetate (DCFH-DA) and the fluorescence intensity was detected by fluorescent microscope. Cells were treated with 0.2 mmol/L BPA for 24 hours as the consequence of moderate BPA exposure on redox status, and then, harvested for determining GSH and GSSG content by GSH/GSSG kit (S0035, Beyotime Biotechnology Co., Shanghai, China) on an ultraviolet-visible spectrophotometer (415 nm). All data were expressed relative to the corresponding control.

2.6 Mitochondrial membrane potential (MMP)

IEC-6 cells were cultured either in the presence or absence of 0.4 mmol/L BPA for 1 or 4 hours, and then, stained with JC-1 by using MPP detection kit (C2006, Beyotime Biotechnology Co., Shanghai, China) according to manufacturer's specification. Briefly, cells were incubated with JC-1 for 20 minutes at 37°C in a CO2 incubator and observed the MMP by fluorescence microscopy. As the potential of mitochondrial membrane shifts from the high to the low, the fluorescence color of JC-1 changes from red aggregates to green monomer. The decrease in MMP was measured by the proportion of green to red and normalized by the control.

2.7 Western Blot analysis

Cells were harvested after treated with 0.4 mmol/L BPA for 6 hours or 0.2 mmol/L BPA for 12 hours, followed by the treatment of 4 mmol/L Gln for 12 hours. Then, harvested cells were lysed by RIPA (HX1862, Huaxingbio Science, Beijing, China) with protease inhibitor mixture cocktail (HX1683, Huaxingbio Science, Beijing, China) and phosphatase inhibitor mixture (HX1684, Huaxingbio Science, Beijing, China). Cell protein was extracted for analyzing the abundance of tight junction proteins, Bax, pro-caspase-3, and key proteins involved in the mitogen-activated protein kinases (MAPK) signaling pathways. Simultaneously, mucosal protein was used to validate the reliability of proteomic analysis by association analysis of the abundances of selected differentially expressed proteins (DEPs) quantified by Western Blot and proteomic analysis. Equivalent amount of protein (30 μg) from jejunal mucosa or cells was loaded onto SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (IPVH00010, 0.45 μm, Merck & Co., Inc. Huntenton, NJ, USA). The membranes were incubated with corresponding primary antibodies, and then, with DyLight 800-labeled secondary antibodies, followed by detection with Odyssey Clx (LI-COR Biotechnology, Lincoln, NE, USA) and quantified using Alphalmager software (2200). All Western Blot experiments were conducted with three biological repeats and repeated three times. All results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin and data were expressed as the relative values to those of control group. The antibodies used in this study were listed in Table S2.

2.8 Quantitative real-time PCR analysis

Cells were treated with 0.4 mmol/L BPA for 6 hours, and then, harvested for quantitative real-time PCR analysis. Total RNA were extracted from cells or mouse jejunal mucosa using Hipure Total RNA Mini Kit (R4114, Magen Co., Guangzhou, China), and used to synthesize cDNA using PrimeScript RT reagent kit (RR037A, TaKaRa Bio Inc, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed with quantitative real-time PCR master mix (RR390A, TaKaRa Bio Inc, Japan) in an analytik jena qTOWER 2.2 real-time PCR system (Analytik Jena, Jena, Germany) in triplicate and repeated at least three times. β-Actin and GAPDH were used as the internal control. Relative mRNA expression was calculated by 2−ΔΔCt method. The sequences of gene-specific primers used were listed in Table 1.

2.9 Cell mitochondrial stress test assay with Seahorse Bioscience

IEC-6 cells seeded in XFe96 cell culture microplates were pretreated with 0.2 mmol/L BPA for 6 hours, and then, treated with 4 mmol/L Gln for another 12 hours. Cells were washed three times with pre-warmed XF assay medium (XF base medium supplemented with 4 mmol/L glutamine and 25 mmol/L glucose (pH 7.4)), and final volume was set to 175 μL/well with assay medium. The plate was incubated at 37°C for 1 hours prior to the assay. Moreover, the XF sensor cartridge was hydrated with Seahorse XF calibrant at 37°C overnight. Oligomycin, FCCP, rotenone, and antimycin A were prepared with pre-warmed XF assay medium and loaded on injection channels A, B, and C with final concentration 2, 1, and 1 mmol/L into the hydrated XF sensor cartridge. After calibrated, the cartridge was loaded on cell culture microplate to detect oxygen consumption rate (OCR) by Seahorse XFe96 Analyzer (Agilent Technologies Inc, CA, USA).
2.10 Statistical analysis

MS/MS spectra were searched by ProteinDiscoverer Software 2.1 against uniprot-mouse database (79954s, 20160909). The highest score for a given peptide mass (best match to that predicted in the database) was used to identify parent proteins. Peptide spectral matches were validated based on q-values at a 1% false discovery rate. Protein was identified by at least one unique peptide. Student’s t test function in R was used to calculate P value of significant difference between samples. In this study, DEPs screening criteria was fold change (FC) > 1.20 or < 0.83, with P < .05. Goatools (https://github.com/tanghaibao/GOatools) and KOBAS (http://kobas.cbi.pku.edu.cn/home.do) were implemented to identify significantly enriched GO terms and KEGG pathways using Fisher’s exact test. The Type-1 error was reduced with FDR correction by bonferroni (multiple hypothesis test method). The P value needs to be adjusted by multiple hypothesis tests. GO terms and KEGG pathways with corrected P value ≤ .05 are considered significantly enriched in DEPs.

Statistical analysis of data obtained from Western Blot, quantitative real-time PCR and cellular index measurement were performed with one-factor ANOVA of SAS (version 9.4, SAS Institute, Cary, NC, USA). Results were expressed as means ± SEMs. Differences were considered significant at P value ≤ .05. The figures were drawn by GraphPad Prism (version 6, La Jolla, CA, USA).

3 RESULTS

3.1 Mouse survival and small intestinal morphology

All mice in control group survived, while only 50% mice in BPA group survived by the end of the experiment (P < .05) (Figure 1A). Also, BPA exposure decreased small intestinal length (P < .05) (Figure 1B) and disturbed jejunal mucosal morphology indicated by disordered villi and impaired crypt (Figure 1C). No mice died in BPA + Gln group during the entire experimental period. Alimentary supplementation of glutamine appeared to alleviate the small intestinal injury...
induced by BPA exposure in terms of intestinal length and morphology (Figure 1).

### 3.2 Global proteomic profile of intestinal mucosa

The procedure of quantitative proteomic analysis of jejunal mucosa was shown in Figure S1A. A total of 302,712 spectrums were detected, and 39,509 peptides were characterized with 5,840 quantified proteins (Figure S1B). The accumulation distribution curve of proteins with 95% reliability coverage was shown in Figure S1C.

A total of 70 DEPs constituting 2.7% of all quantified proteins were identified between the control and BPA group (Table 2). All DEPs were exhibited by volcano diagram in Figure S2A. Among these DEPs, 30 DEPs were up-regulated while 40 DEPs were down-regulated in mice exposed to BPA relative to the controls. The DEPs were classified into cell renewal (18.6%), immunity (25.7%), redox (24.3%), and others (31.4%) according to their annotated physiological functions (Figure S2B).

The reliability of the results of proteomic analysis was routinely valuated by Western Blot analysis, it showed the high reliability of proteomic results in the study ($R^2 = .92$, Figure S3).

In GO function annotation, top 10 significantly clustered biological processes were presented in Figure 2A ($P < .05$), and 17 DEPs involved were listed in Figure 2B. Among these DEPs, the response to nitrosative stress, detoxification of nitrogen compound, and nitrobenzene metabol abl process associate with the BPA metabolism; the responses both of humoral immunity (the positive regulation of humoral immune response and positive regulation of B cell mediated immunity), and cellular immunity (the regulation of CD8-positive, alpha-beta T cell activation and positive regulation of endocytosis) implied that humoral and cellular immunity were implicated in intestinal injury induced by BPA exposure. Notably, the glutathione metabolic process was also significantly enriched upon BPA exposure ($P < .05$).

In KEGG pathway analysis, 10 signaling pathways were significantly enriched ($P < .05$) (Figure 2C,D). Among these pathways, four pathways including the chemical carcinogenesis, drug metabolism-cytochrome P450, metabolism of xenobiotics by cytochrome P450 and steroid hormone biosynthesis were implicated in the cluster of BPA metabolism and human diseases. In addition, pathways including the complement and coagulation cascades, arachidonic acid metabolism, staphylococcus aureus infection, retinol metabolism and linoleic acid metabolism associated with immunity were significantly enriched upon BPA exposure ($P < .05$).
### TABLE 2  Differentially expressed proteins in jejunal mucosa between the control and BPA group mice

| Accession | Description | FC   | P value | Regulation | Function                                           |
|-----------|-------------|------|---------|------------|----------------------------------------------------|
| **Cell renewal** |             |      |         |            |                                                   |
| A2AR50    | Ras-specific guanine nucleotide-releasing factor RalGPS1 | 0.623 | .009    | Down       | Involved in cytoskeleton organization               |
| Q9JY2     | E3 ubiquitin-protein ligase Hakai | 0.678 | .005    | Down       | Cell-cell adhesion                                 |
| Q62245    | Son of sevenless homolog 1 | 0.681 | .030    | Down       | Epidermal growth factor receptor signaling pathway |
| P70206    | Plexin-A1 | 0.756 | .019    | Down       | Remodel of the cytoskeleton and cell migration      |
| P02535    | Keratin, type I cytoskeletal 10 | 0.758 | .050    | Down       | Epidermis development and epithelial cell differentiation |
| Q9JJ80    | Ribosome production factor 2 homolog | 0.770 | .026    | Down       | Related to Ki67                                     |
| Q9RT1T4   | Septin-6 | 0.776 | .036    | Down       | Regulation of cell cycle, differentiation and division |
| Q3UV17    | Keratin, type II cytoskeletal 2 oral | 0.795 | .022    | Down       | Epidermis development                               |
| Q07763    | Natural killer cell receptor 2B4 | 0.652 | .045    | Down       | Participating in adaptive and innate immune response |
| Q8VDW7    | Interferon-inducible GTPase-like | 0.684 | .025    | Down       | GTP binding                                        |
| D3TNY4    | ATPase IMAP family member 4 | 0.729 | .008    | Down       | Playing a role in regulating lymphocyte apoptosis  |
| Q811E7    | Myosin IF | 0.738 | .046    | Down       | Actin binding                                      |
| Q8KX4     | NCK associated protein 1 like | 0.762 | .021    | Down       | Controlling lymphocyte development, activation, proliferation and homeostasis, erythrocyte membrane stability, as well as phagocytosis and migration by neutrophils and macrophages |
| D3Z312    | Tumor necrosis factor alpha-induced protein 8 | 0.773 | .046    | Down       | Acting as a negative mediator of apoptosis          |
| Q32MD7    | Regulator of G-protein signaling 10 | 0.791 | .039    | Down       | Involved in inflammation                            |
| A6X919    | Probable C-mannosyltransferase | 0.793 | .042    | Down       | Related to complement reaction                      |
| Q8C3J5    | Deducator of cytokinesis protein 2 | 0.827 | .038    | Down       | Regulation of T proliferation                       |
| P21614    | Vitamin D-binding protein | 1.225 | .041    | Up         | Enhancement of the chemotactic activity of C5 alpha for neutrophils in inflammation and macrophage activation |
| Q61129    | Complement factor I | 1.257 | .020    | Up         | Innate immune response                              |
| P01027    | Complement C3 | 1.345 | .006    | Up         | Activation of the complement system, participating in inflammatory response |

(Continues)
| Accession | Description | FC  | P value | Regulation | Function                                                                 |
|-----------|-------------|-----|---------|------------|---------------------------------------------------------------------------|
| A6X935    | Inter alpha-trypsin inhibitor, heavy chain 4 GN = Itih4 | 1.438 | .006    | Up         | Involved in inflammatory response to trauma and response to cytokine     |
| Q3TGR2    | Fibrinogen beta chain GN = Fgb | 1.456 | .004    | Up         | Wound repair to stabilize the lesion and guiding cell migration during re-epithelialization |
| Q91X72    | Hemopexin GN = Hpx | 1.474 | .009    | Up         | Regulation of humoral immune response                                   |
| G0YP42    | Anti-human Langerin 2G3 lambda chain | 1.519 | .015    | Up         | Involved in immunity                                                    |
| Q3UER8    | Fibrinogen gamma chain GN = Fgg | 1.540 | .004    | Up         | Involved in wound healing and cellular response to interleukin         |
| Q60590    | Alpha-1-acid glycoprotein 1 GN = Orm1 | 1.640 | .016    | Up         | Regulation of immune system process                                       |
| Redox     | Q99K73      | Nrf1 protein GN = Nrf1 | 0.682 | .037    | Down                    | Controlling of nuclear genes required for respiration, heme biosynthesis, and mitochondrial DNA transcription and replication |
| Q5FW98    | Cytochrome c oxidase subunit 7B, mitochondrial GN = Cox7b | 0.765 | .008    | Down       | Involved in mitochondrial respiration                                   |
| Q3U0N0    | Putative uncharacterized protein GN = Gzma | 0.793 | .050    | Down       | Negative regulation of oxidoreductase activity                            |
| Q80XI4    | Phosphatidylinositol 5-phosphate 4-kinase type-2 beta GN = Pip4k2b | 0.800 | .043    | Down       | Phosphatidylinositol 5-phosphate 4-kinase synthesis                      |
| Q80W21    | Glutathione S-transferase Mu 7 GN = Gstm7 | 1.211 | .018    | Up         | GSH binding                                                             |
| P10648    | Glutathione S-transferase A2 GN = Gsta2 | 1.215 | .020    | Up         | GSH binding                                                             |
| Q924Y0    | Gamma-butyrobetaine dioxygenase GN = Bbox1 | 1.241 | .038    | Up         | Catalyzing the formation of L-carnitine from gamma-butyrobetaine        |
| Q3TEF1    | Glutamate–cysteine ligase catalytic subunit GN = Gclc | 1.244 | .032    | Up         | GSH synthesis                                                           |
| G5E8M7    | Glutathione S-transferase Mu 6 GN = Gstm6 | 1.245 | .014    | Up         | GSH metabolism                                                          |
| G3X9Y6    | Aldo-keto reductase family 1, member C19 GN = Akr1c19 | 1.247 | .008    | Up         | Oxidoreductase activity                                                  |
| P15626    | Glutathione S-transferase Mu 2 GN = Gstm2 | 1.255 | .008    | Up         | GSH metabolism                                                          |
| P19639    | Glutathione S-transferase Mu 3 GN = Gstm3 | 1.260 | .017    | Up         | GSH binding                                                             |
| P10649    | Glutathione S-transferase Mu 1 GN = Gstm1 | 1.290 | .039    | Up         | GSH binding                                                             |
| Q6PJ91    | Gstm7 protein GN = Gstm7 | 1.296 | .047    | Up         | GSH metabolism                                                          |
| Q5GLZ0    | Cytochrome P450 2C66 GN = Cyp2c66 | 1.348 | .041    | Up         | Epoxigenase P450 pathway                                                 |
| Q9WUD0    | Cytochrome P450 2B10 GN = Cyp2b10 | 1.461 | .010    | Up         | Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen |
| Q64458    | Cytochrome P450 2C29 GN = Cyp2c29 | 1.698 | .011    | Up         | Arachidonic acid metabolism                                              |
| Other     | B9EHJ3      | Tight junction protein ZO-1 GN = Tjp1 | 0.810 | .027    | Down                     | Tight junction                                                          |
**Figure 2** Top 10 significantly clustered GO biological processes and total significantly enriched KEGG pathways in jejunal mucosal proteome between the control and BPA exposure mice. A, Top 10 significantly clustered GO biological processes. The horizontal line represents rich factor for GO biological processes. The size of circle represents the number of differentially expressed proteins (DEPs) involved in the processes. The color represents log10 ($P$ value). B, DEPs involved in the terms of (A) were hierarchical clustered based on fold change (FC) (BPA vs control). Heatmap were generated by MeV (4.9.0). C, The significantly enriched KEGG pathways. The horizontal line represents rich factor for KEGG pathways. The size of circle represents the number of DEP involved in the pathways. The color represents log10 ($P$ value). The pathways were classified as BPA metabolism and human diseases, redox, and immunity. D, Interaction of pathways and DEPs involved in the terms of (C). The blue diamond represents KEGG pathways in (C), and the red ovals represent up-regulated DEPs upon BPA exposure, respectively. The red, blue, and green elliptical circles represent BPA metabolism and human diseases, redox, and immunity, respectively. The networks were visualized by Cytoscape (v3.2.1). DEPs, differentially expressed proteins; FC, fold change.
Remarkably, the glutathione metabolism pathway belonging to redox item was significantly enriched as well ($P < .05$).

### 3.3 | Intestinal epithelial cell renewal, apoptosis, and intestinal physical barrier function

A total of 13 DEPs (18.6%) were identified in intestinal mucosal relevant to cell renewal (Table 2). Cyclin-K (Ccnk), a member of cyclin family, promoting cell cycle and cell division, was down-regulated upon BPA exposure. Son of sevenless homolog 1 (Sos1), a binding protein of Ras involved in EGFR-SOS1-Ras signal pathway regulating intestinal epithelial cell growth, was down-regulated as well. Accordingly, the epidermis development was significantly clustered in GO biological processes ($P < .05$) (Figure 2A), and in which Keratin type I cytoskeletal 10 (Krt10), keratin type II cytoskeletal 2 oral (Krt76) and Sos1 were down-regulated in mouse intestinal mucosa in response to BPA exposure (Figure 2B).

The effects of BPA on epithelial cell renewal, cell viability, and proliferation were evaluated in vitro. The cell viability was reduced upon BPA exposure at 6 hours and 24 hours both in a dose-dependent manner compared with the control ($P < .05$) (Figure 3A,B). Based on these results, 0.4 mmol/L BPA treated for 6 hours or 0.2 mmol/L for 24 hours were chosen for the following experiments. The proliferation of cells expressed as the EdU-positive cells relative to total cells was decreased in BPA treated group compared with the control ($P < .05$) (Figure 3C,D).

As shown by flow cytometry, cell apoptosis was increased upon BPA exposure compared with the control in vitro ($P < .05$) (Figure 3E,F). Consistently, BPA exposure increased the protein abundance of Bax and decreased the protein abundance of procaspase-3 ($P < .05$) (Figure 3G,H). Additionally, the mRNA expression of Bcl2 was decreased, while that of Bax and caspase-12 were increased upon BPA exposure ($P < .05$) (Figure 3I).

Furthermore, proteomic analysis showed that tight junction protein ZO-1 was down-regulated in small intestinal mucosa of mice exposed to BPA (Table 2). Accordingly, the protein abundances of ZO-2, occludin, and claudin-7 were decreased upon BPA exposure in vitro (Figure 4A,B), and TEER was reduced as well, which were compared with control group ($P < .05$) (Figure 4C). These results evidenced that BPA exposure impaired intestinal physical barrier function.

### 3.4 | Intestinal immune response

As mentioned above, GO function annotation revealed that BPA disturbed both humoral and cellular immunity of the small intestine ($P < .05$) (Figure 2A,B). KEGG pathway enrichment analysis figured out five pathways associated with intestinal mucosal immunity (Figure 2C,D). Notably, DEPs, complement factor I (Cfi), complement C3 (C3), and fibrinogen beta chain (Fgb), were involved in the complement and coagulation cascade and the staphylococcus aureus infection. According to the function annotation, Cfi is involved in the innate immune response, C3 activates the complement system and participates in inflammatory response, and Fgb is involved in wound repair to stabilize the lesion and guide cell migration during re-epithelialization. All of them were up-regulated upon BPA exposure (Figure 2D). Moreover, cytochrome P450 2B10 (Cyp2b10), cytochrome P450 2C29 (Cyp2c29), and cytochrome P450 2C66 (Cyp2c66), mediating the production of lipid metabolites that involved in inflammation, were up-regulated upon BPA exposure.

Compared with the control, BPA exposure significantly increased mRNA expression of pro-inflammatory cytokines interleukin 1β (IL-1β), IL-6, and tumor necrosis factor α (TNFα), while decreased mRNA expression of anti-inflammatory cytokine IL-10 ($P < .05$) (Figure S4) in mouse intestinal mucosa.

### 3.5 | Intestinal redox status and mitochondrial function

Approximately 24.3% of DEPs were implicated in redox balance (Table 2). Simultaneously, the glutathione metabolism was significantly enriched in response to BPA exposure by both GO function annotation (Figure 2A) and KEGG pathway analysis ($P < .05$) (Figure 2C). It is noteworthy that most DEPs involved in BPA metabolism, including Glutathione S-transferase A2 (Gst2), Glutathione S-transferase Mu 1 (Gstm1), Gstm2, Gstm3, and Gstm7, belonging to glutathione metabolism enzymes were up-regulated in the intestinal mucosa of mice exposed to BPA relative to the control (Figure 2D). In addition, most DEPs involved in mucosa redox reactions, belonging to glutathione S-transferases (GSTs), were also up-regulated upon BPA exposure (Figure 2D).

Furthermore, we demonstrated that BPA exposure significantly decreased SOD activity and the ratio of reduced glutathione/ oxidized glutathione (GSH/GSSH) (Figure 5A,B), while increased MDA content relative to the control in vitro ($P < .05$) (Figure 5A).

Among DEPs, cytochrome C oxidase subunit 7b (Cox7b), participating in the mitochondrial oxidation-reduction process, was significantly down-regulated in mouse jejunal mucosa upon BPA exposure ($P < .05$) (Table 2). Consistently, intracellular ROS content was increased upon BPA exposure compared with those of the control in vitro ($P < .05$) (Figure 5C,D). It is notably that MPP was reduced upon BPA exposure for 1 hour and 4 hours relative to the control in vitro ($P < .05$) (Figure 5E-H).
We detected whether MAPK signaling cascades, including extracellular signal-regulated kinase (ERK), p38 and Jun N-terminal kinase (JNK), were implicated in intestinal injury upon BPA exposure. Western Blot analysis demonstrated that the phosphorylation of ERK1/2 and p38 were not altered, while phosphorylation of JNK was significantly elevated upon BPA exposure relative to the control in vitro ($P < .05$) (Figure 6).
3.7 | Glutamine attenuates BPA-induced intestinal mucosal injury in vivo and in vitro

A total of 48 DEPs between the control vs BPA were healed in mice offered the diet containing 4% of Gln (Figure S5), and among them, the expression pattern of seven DEPs were completely reversed in BPA + Gln group. These seven DEPs were classified into the cell renewal, immunity, and redox state items, respectively (Table 3). In detail, γ-butyrobetaine dioxygenase (Bbox1), belonging to the redox cluster and catalyzing the formation of L-carnitine, is important for mitochondrial energy metabolism;33 dedicator of cytokinesis protein 2 (Dock2), a critical protein in the activation, and migration of leukocytes, plays a key role in immune surveillance;34 plexin-A1 (Plxna1) regulates cytoskeletal dynamics, cell proliferation, and differentiation.35 It is notably that among these seven DEPs, Bbox1 was up-regulated upon BPA exposure and reversed in mice in BPA + Gln group, whereas the rest were down-regulated by BPA exposure and reversed in mice in BPA + Gln group.

Furthermore, it was evidenced that glutamine supplementation to cells upon BPA exposure decreased intracellular ROS content (Figure 7C,D) and increased procaspase-3 abundance while decreased Bax abundance (P < .05) (Figure 7E,F), which was accompanied by the normalization of JNK signaling (Figure 7E,F).

3.8 | Interaction between BPA exposure and glutamine supplementation on mitochondrial respiration

To detect the mechanism underlying interaction between BPA exposure and glutamine supplementation on intestinal injury, we measured the relevant parameters of cellular mitochondrial respiration. We found BPA exposure dramatically decreased OCR in terms of the basal respiration, ATP production, maximal respiration, spare capacity, proton leak, and non-mitochondrial respiration compared with control (P < .05) (Figure 8). ATP production and maximal respiration of cells was reduced approximately 40%, and spare capacity was reduced approximately 70% relative to the control. However, glutamine supplementation could well counteract the adverse effect of BPA exposure on mitochondrial OCR in terms of all above parameters in vitro.
DISCUSSION

In the present study, we displayed the proteomic panorama of small intestinal mucosa upon BPA exposure for the first time. Meanwhile, we showed that BPA exposure damaged mouse survival, intestinal morphology and the homeostasis of signaling pathways concerning cell renewal, BPA metabolism, redox status, and immunity in intestinal mucosa of C57BL/6J mice. It revealed that the small intestine underwent severe damage upon BPA exposure in vivo. Subsequently, we employed IEC-6 cell line, a widely used in mimicking intestinal epithelial cells in vitro study, to evaluate the adverse effects of BPA exposure on cell viability, proliferation, apoptosis, and intestinal physical barrier function in vitro study, and further revealed the mechanism underlying BPA-induced intestinal injuries.

Tight junction consists of tight junction proteins, including claudins, zonula occludens (ZOs), and occludin, and it serves an important role in maintaining paracellular...
We demonstrated that BPA exposure shortened the relative length of the small intestine, damaged intestinal morphology and depressed the expression of ZO-1 in intestinal mucosa of mice exposed to BPA. Accordingly, tight junction proteins ZO-2, occludin, and claudin-7 as well as TEER that usually is used to evaluate epithelial barrier function, were also decreased in vitro, demonstrating BPA exposure seriously injured intestinal physical barrier function.

Small intestinal epithelial cells derive from continuously divided and differentiated stem cells located at the base of crypts, and remain rapid generation and loss. Sos1, as a binding protein of Ras, participates in the promotion of epithelial cell growth via regulating epithelial growth factor receptor signal. Krt10 and Krt76, had been shown to regulate epithelial cell differentiation and proliferation. Ribosome production factor 2 homolog (Rpf2) is a mutual-action relevant protein of Ki-67 that is a marker of cell proliferation. We observed that BPA exposure harassed intestinal epithelial cell renewal, for instance, the process of epidermis development was significantly enriched and DEPs involved in, such as Sos1, Krt10, and Krt76, as well as Rpf2, participating in the regulation of epithelial cell proliferation and differentiation, were significantly down-regulated upon BPA exposure. Consistently, we observed that BPA exposure decreased cell viability and proliferation while increased apoptosis in vitro, which was consistent with results of studies on neural stem cells, colonic epithelial cells, and intestinal goblet cells.

**FIGURE 6** Effects of BPA exposure on MAPK pathways in vitro. IEC-6 cells were treated with 0.4 mmol/L BPA for 6 hours. Protein phosphorylation of ERK (A), JNK (C) and p38 (E) were measured by Western Blot, and AlphaImager software (2200) was used for statistical analysis of Western Blot (B, D, and F). Data are presented as means ± SEMs, n = 3. *P < .05 for comparison between the vehicle and BPA treatment.
Our study provided the evidence that BPA impaired small intestinal cell renewal by depressing cell viability and proliferation and increasing apoptosis. Therefore, we can reasonably deduce that BPA exposure impairs intestinal epithelial cell renewal, and consequently damages intestinal physical barrier function.

BPA exposure has been shown to decrease immune cell viability and disturb immune function. Consistently, we observed that BPA exposure resulted in the significant activation of intestinal immunity. GO functional cluster showed that BPA exposure altered humoral immunity and cellular immunity alike. KEGG pathway enrichment analysis also indicated that intestinal immunity was altered upon BPA exposure. Retinol metabolism and linoleic acid metabolism pathways, had been implicated in immunity and inflammation by KEGG pathway analysis. Moreover, three DEPs of cytochrome P450 family members Cyp2b10, Cyp2c29, and Cyp2c66, serving as hub proteins among KEGG enriched pathways retinol/arachnoid/linoleic acid metabolisms, were up-regulated in intestinal mucosa upon BPA exposure along with altered complement and coagulation cascade and staphylococcus aureus infection pathways. Combining with increased mRNA expression of IL-1β, IL-6, and TNFα, as well as decreased IL-10 mRNA expression in jejunal mucosa, we thereby deduced that BPA exposure disturbed intestinal mucosal immunity and probably triggered inflammation by activating humoral and cellular immunity via altering metabolism of lipids.

Moreover, we demonstrated that BPA exposure gave rise to mitochondrial dysfunction and resultant redox imbalance in terms of MPP, glutathione metabolism, and intracellular ROS, which was consistent with previous studies on tumor and HepG2 cells. Increased intracellular ROS caused by mitochondrial disorder leads to oxidative stress and subsequently cell apoptosis involved in, which was consistent with previous study. It had been reported that increased ROS induced the release of intercellular cytokines and increased intestinal permeability. Therefore, we deduced that BPA exposure might trigger inflammation in the small intestine and accelerate intestinal damage. In addition, cytochrome P450 family was implicated in BPA metabolism and its up-regulation was in concert with the increased intracellular ROS upon BPA exposure. Meanwhile, Cox7b, an enzyme converting electrons to oxygen in mitochondrial respiration, was also down-regulated in intestinal mucosa upon BPA exposure.

In addition, previous studies showed that GSH could protect intestinal epithelial cells from damage induced by toxins, free radicals, and ROS. The dynamic equilibrium of GSH takes part in regulation of cell proliferation, differentiation, and apoptosis. In the present study, we observed that BPA exposure decreased GSH/GSSG and SOD activity, whereas increased MDA content in vitro, along with the promoted expression of glutathione S-transferase in vivo. These results showed that BPA exposure caused intestinal epithelial cell mitochondrial dysfunction, oxidative stress, apoptosis, and consequently impaired intestinal physical barrier function, which was supported by previous study.

### Table 3

| Accession | Description | BPA vs control | BPA + Gln vs BPA |
|-----------|-------------|----------------|------------------|
|           |             | FC  | P value | Regulation | FC  | P value | Regulation |
| P70206    | Plexin-A1 GN = Plxna1 | 0.756 | .019 | Down | 1.210 | .016 | Up |
| D3YT4N    | GTPase Imap family member 4 GN = Gimap4 | 0.729 | .008 | Down | 1.345 | .011 | Up |
| Q8K1X4    | NCK associated protein 1 like GN = Nckap11 | 0.762 | .021 | Down | 1.337 | .045 | Up |
| A6X919    | Probable C-mannosyltransferase DPY19L1 GN = Dpy1911 | 0.793 | .042 | Down | 1.238 | .003 | Up |
| Q8C3J5    | Dedicator of cytokinesis protein 2 GN = Dock2 | 0.827 | .038 | Down | 1.231 | .037 | Up |
| Q3U0N0    | Putative uncharacterized protein GN = Gzma | 0.793 | .050 | Down | 1.416 | .003 | Up |
| Q924Y0    | Gamma-butyrobetaine dioxygenase GN = Bbox1 | 1.241 | .038 | Up | 0.790 | .049 | Down |
As the result of cell proliferation, apoptosis and recovery mechanism, intestinal epithelial cell turnover is governed by the coordination of signaling pathways. MAPK pathways have been well documented to be activated in oxidative stress-induced intestinal cell injury and intestinal inflammation. For instance, the JNK/MAPK pathway was activated by ROS, and subsequently regulated diverse biological functions including apoptosis and epithelial homeostasis in response to environmental stresses. MAPK pathways play a central role in the mediation of cell renewal, redox homeostasis, and immunity, whose regulative function well matches the scope orientated by results of the proteomic analysis.

In the present study, we accordingly demonstrated that increased intracellular ROS was accompanied with the activated JNK/MAPK pathway upon BPA exposure, while neither ERK nor p38 was altered. In previous study, BPA suppressed proliferation and induced apoptosis of colonic epithelial cells through disrupting mitochondrial function and inhibiting the AKT/MAPK pathways. The divergence existed probably due to the different tissues used in two studies. In addition, studies also showed that BPA could induce oxidative stress and activate MAPK signaling pathway in rat liver and Sertoli cells. Therefore, we supposed that BPA exposure induced intestinal injury via mitochondrial dysfunction, increased intracellular ROS accumulation, and cascade activation of ROS-JNK/MAPK signaling pathway as well as disordered cell renewal.

Glutamine, a key energy substrate for enterocytes, had been shown to improve intestinal barrier function, and relieve intestinal injury from malnutrition and oxidative stress. In addition, glutamine is a precursor of glutathione.

**FIGURE 7** Effects of glutamine on BPA-induced injury and JNK activity in vitro. IEC-6 cells were pretreated with 0.2 mmol/L BPA for 12 hours, and followed additional 12 hours-treatment of glutamine or continued BPA exposure. A, Cell viability was determined using Cell Counting Kit-8 (n = 6). B, Transepithelial electrical resistance (TEER) of IEC-6 cells was measured by an EVOM volt-ohmmeter. C, Images of intracellular DCFH-DA-stained reactive oxygen species (ROS) in IEC-6 cells. Scale bars indicate 100 µm. D, Statistical analysis of (C). E, Protein abundance of Bax, procaspase-3, and JNK were measured by Western Blot, and AlphaImager software (2200) was used for statistical analysis of Western Blot (F). Data are presented as means ± SEMs, n = 3. *Means without a common letter differed, P < .05
which maintains intestinal epithelial cellular redox balance against oxidative stress, and reacts with toxins to form non-toxic GSH-toxin conjugates. Here, we demonstrated that glutamine supplementation significantly attenuated small intestinal damage and rescued the survival rate of mice exposed to BPA. Dynamic global proteome pattern also showed that glutamine supplementation ameliorated the adverse impact of BPA exposure on intestinal proteomic profile to a large extent. More than two-third of total DEPs (BPA vs control) were healed by glutamine supplementation, especially among the seven DEPs involved in cell renewal, immunity, and redox, were completely counteracted. Moreover, we also evidenced that glutamine mitigated the adverse impact of BPA exposure on epithelial cell viability, TEER and cell apoptosis.

**FIGURE 8** Effects of glutamine on BPA-induced mitochondrial respiration in vitro. IEC-6 cells were pretreated with 0.2 mmol/L BPA for 6 hours, and then, treated with glutamine for another 12 hours. Oxygen consumption rate (OCR) was measure by Seahorse Bioscience. A, mitochondrial respiration curve; (B) basal respiration; (C) ATP production; (D) maximal respiration; (E) spare respiratory; (F) proton leak; (G) non-mitochondrial respiratory. Data are presented as means ± SEMs, n = 6. a-d Means without a common letter differed, P < .05.
in vitro. This is consistent with previous studies, in which glutamine maintained cell viability and barrier function, and attenuated apoptosis in porcine intestinal epithelial cells.\textsuperscript{21,23} Therefore, we draw the conclusion that glutamine could indirectly prevent intestine from BPA-induced injury.

To detect the mechanism that glutamine supplementation alleviated intestinal injury induced by BPA exposure, we traced the effect of glutamine on key points, where BPA exposure damages intestinal mucosa. Combining the impairment of BPA on mitochondrial function and the activation of ROS-JNK/MAPK signaling pathway, we demonstrated that glutamine could significantly restore mitochondrial function in terms of basal respiration, ATP production, maximal respiration, and proton leak, accompanied with normalizing ROS-JNK/MAPK pathway.

In conclusion, we thereby elucidated that BPA exposure could lead to serious intestinal physical barrier malfunction through mitochondrial dysfunction, ROS-JNK/MAPK pathway activation and consequent epithelial cell renewal disorder, together with intestinal immune irritation. Particularly, our study highlighted that glutamine supplementation could well encounter the adverse effect of BPA exposure on the small intestine partly via restoring mitochondrial function and normalizing the ROS-JNK/MAPK pathway.

ACKNOWLEDGMENTS
The authors thank Caiyun Huang, Yubo Wang, Lu Gong, and Xin He for helping collect samples. We also thank Dr Bing Dong for critical reading of the manuscript. This work was supported by National Key Research and Development Program of China (2018YFD0500402, 2016YFD0700201) and 111 Project (B16044).

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
J. Yin designed and oversaw research; N. Jiao and X. Piao took part in experiment design; N. Jiao, D. Xu, L. Q. Wang and L. Wang performed research; K. Qiu and J. Yin contributed new reagents or analytic tools; N. Jiao and J. Yin conducted bioinformatics analyses of proteomic and data analysis; N. Jiao and K. Qiu drafted the tables and figures; J. Yin and N. Jiao wrote the manuscript.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

**How to cite this article:** Jiao N, Xu D, Qiu K, et al. Restoring mitochondrial function and normalizing ROS-JNK/MAPK pathway exert key roles in glutamine ameliorating bisphenol A-induced intestinal injury. *The FASEB Journal*. 2020;34:7442–7461. [https://doi.org/10.1096/fj.201902503R](https://doi.org/10.1096/fj.201902503R)