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Synergistic endocrine disruption and cellular toxicity of polyethylene microplastics and bisphenol A in MLTC-1 cells and zebrafish

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The study investigates the synergistic endocrine disruption and cellular toxicity resulting from co-exposure to polyethylene microplastics (PE-MPs) and bisphenol A (BPA) in zebrafish and MLTC-1 cells. Previous research has extensively examined the individual effects of PE-MPs and BPA on endocrine systems and cellular health. However, the specific interactions and combined toxicological impacts of these two common environmental pollutants remain underexplored, particularly in terms of their synergistic effects on endocrine pathways and cellular viability. To fill this knowledge gap, we characterized PE-MPs using scanning electron microscopy and Raman spectrometry and exposed MLTC-1 cells to PE-MPs, BPA, or combinations of both. The results showed that co-exposure to 100 μg/mL PE-MPs and 100–150 μmol/L BPA for 48 h significantly decreased cell viability, increased apoptosis rates, induced G2/M cell cycle arrest, reduced mitochondrial membrane potential, and altered the transcriptional expression of genes related to steroidogenesis. Specifically, co-exposure upregulated the *Ar* while downregulating *Lhr* and *3β-Hsd*, with these effects being more pronounced than those observed with single exposures. In a complementary in vivo study, adult zebrafish were exposed to environmentally relevant concentrations of PE-MPs (1 mg/L) and BPA (1.5 μg/L) for 28 days. This co-exposure resulted in significant increases in the GSI and alterations in the gene expression associated with the HPG axis. In male zebrafish brains, genes such as *Gnrh2*, *Esr1*, and *Ar* were downregulated, while in female brains, *Gnrh3*, *Esr1*, and *Ar* also exhibited downregulation. In male testes, *Star*, *Cyp11a1*, and *Hsd11b2* were upregulated, whereas *Cyp19a1a*, *Hsd3b*, *Hsd20b*, and *Hsd17b3* were downregulated. In contrast, female ovaries showed upregulation of *Cyp11a1*, *Cyp17*, *Cyp11b*, *Hsd3b*, *Hsd20b*, and *Hsd17b3*, while *Cyp19a1a* was downregulated, indicating a sex-specific endocrine disruption. Overall, the findings reveal that co-exposure to PE-MPs and BPA induces synergistic toxic effects both in vitro and in vivo, which underscores the importance of studying the effects of combined pollutants to better assess environmental health risks.

Keywords PE-MPs, BPA, Synergistic endocrine disruption, MLTC-1, Zebrafish

In recent years, the rapid increase in microplastics (MPs) production has contributed to global plastic output exceeding 300 million metric tons annually. Inefficient waste management has led to substantial plastic pollution, with large plastic fragments breaking down into MPs (particles < 5 mm) through processes like photodegradation and mechanical disintegration[1,2]. Additionally, MPs are directly manufactured for industrial and consumer applications, infiltrating various environmental compartments, including freshwater, marine, terrestrial, and atmospheric systems[3,4]. Polyethylene (PE) is one of the most prevalent MPs, commonly found

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in aquatic environments due to its durability and low cost, with concentrations ranging from 0.1 to 6162.5 p/L in freshwater[5,6].

Bisphenol A (BPA), a widely recognized endocrine disruptor used in polycarbonate plastics and epoxy resins, is extensively studied for its interference with hormonal regulation[7]. BPA is prevalent in aquatic ecosystems, largely due to wastewater effluents, landfill leachates, and leaching from plastic waste. BPA concentrations range from 10 to 1000 ng/L in estuarine and coastal waters, with higher levels detected in industrial wastewaters[8]. BPA can leach from MPs and nanoplastics, thereby contributing to environmental contamination[9,10]. More importantly, the physicochemical properties of MPs, particularly their strong hydrophobicity and large specific surface area, facilitate the adsorption of BPA from aquatic environments, consequently enhancing its bioavailability and environmental persistence. These two mechanisms - the leaching capacity from plastic particles and the adsorption capability of MPs - synergistically contribute to the ubiquitous co-occurrence of BPA and MPs in both environmental matrices and biological organisms, indicating potential synergistic toxicological interactions[11].

The toxicological impacts of MPs include tissue damage, endocrine disruption, altered enzyme activity, oxidative stress, and behavioral changes in organisms[12,13]. Recent studies have detected MPs in human semen samples, suggesting that these particles can pass through the epididymis and seminal vesicles[14]. Additionally, mass spectrometry analyses have identified MPs in human testis samples, further confirming their presence in reproductive tissues[15]. Although the impact of MPs on Leydig cell steroidogenesis and apoptosis remains poorly understood, the detection of MPs in human semen and testicular tissue has raised significant concerns regarding their potential reproductive toxicity. Additionally, the adsorption of pollutants like phthalate esters and benzo[a]pyrene onto MPs may enhance their toxicity, potentially affecting organisms through the food chain[16,17]. While some studies suggest MPs can amplify pollutant toxicity, others show no significant effects from co-exposure[18]. Recent findings indicate that PE-MPs can transport pollutants like phthalates and benzo[a] pyrene into organisms, leading to adverse effects on growth, reproduction, and health[19–21]. The smaller the MPs, the greater their surface area and ability to transport pollutants into tissues[22]. Therefore, co-exposure to MPs and BPA may alter toxicity levels in aquatic organisms, underscoring the need to investigate their combined effects[23,24].

The co-occurrence of PE-MPs and BPA may have synergistic or additive toxic effects, particularly on steroidogenesis and cellular functions in hormone-producing cells. Understanding BPA's presence on microplastics is crucial for assessing potential reproductive health risks. MLTC-1 cells, derived from mouse Leydig cell tumors, are commonly used in reproductive endocrinology research due to their homology with human steroid hormone synthesis enzymes[25,26]. The zebrafish, an ideal model organism for toxicological research, is widely used to study the endocrine-disrupting effects of pollutants on the hypothalamus–pituitary–gonad (HPG) axis[27]. To date, studies combining both in vitro and in vivo approaches to provide a comprehensive understanding of the combined effects of PE-MPs and BPA on steroidogenesis and cellular viability remain underexplored in previous studies. Therefore, this study aims to fill these gaps by examining the combined toxicity of PE-MPs and BPA. We hypothesize that co-exposure to PE-MPs and BPA will result in synergistic toxic effects on steroidogenesis, apoptosis, and cell viability in MLTC-1 cells and zebrafish. Specifically, we expect to observe significant alterations in gene expression related to the hypothalamus–pituitary–gonad (HPG) axis and reproductive health endpoints.

To achieve this, we conducted both in vitro and in vivo experiments. In the in vitro study, we assessed the combined effects of PE-MPs and BPA on steroidogenesis, apoptosis, and cell viability in MLTC-1 cells. We measured the expression of key steroidogenic enzymes and intracellular signaling molecules to elucidate the potential interaction mechanisms between PE-MPs and BPA. In the in vivo study, adult zebrafish were exposed to environmentally relevant concentrations of BPA alone and in combination with PE-MPs for 28 days. We analyzed biomarkers such as the gonadosomatic index (GSI) and gene expression related to the HPG axis to evaluate the endocrine-disrupting effects of both substances individually and together. Our findings provide novel insights into the synergistic mechanisms of PE-MPs and BPA, highlighting their combined impact on steroidogenesis and cellular viability. These results have important implications for environmental health risk assessments and future regulatory strategies.

Materials and methods

Materials

Murine Leydig tumor cells (MLTC-1) were sourced from the Chinese Academy of Sciences Cell Bank. PE-MPs, with an average diameter of 20 μm, were acquired from Dongguan Junxin Plastic Co., Ltd., China. Bisphenol A (BPA), with the Chemical Abstracts Service Registry Number 80-05-7, was obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd.

Composition and morphology characterization of the microplastics

The microscopic morphology of the surface of microplastics was characterized using a scanning electron microscope (ZEISS Sigma 300, Germany). The samples were directly attached to conductive adhesive and sputter-coated with gold using a Oxford Quorum SC7620 splash coating spray for 45 s using an ion sputter (with a sputtering current of 10 mA). The morphology of the microplastics was observed using SEM at an accelerating voltage of 3 kV.

The composition of the microplastics was tested by using a microscopic Raman spectrometer (Horiba LabRAM HR Evolution, Japan). Placed the sample on the glass slide and move it under the test lens for optical focusing; then performed secondary laser focusing to form a good photon collection; selected the laser and switch, change the laser power for testing, seted the integration time and the grating size according to the test

results. The laser wavelength was 785 nm. Spectral data was collected and tested ranging from 200 cm^{-1} to 4000 cm^{-1}.

Cell culture and treatment protocol

MLTC-1 cells were maintained in RPMI 1640 medium (Gibco, America, Cat. No. 21870100) supplemented with 10% FBS (Bioind, Israel, Cat. No. 04-001-1 A) and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 incubator. Cell viability was assessed using the CCK-8 assay kit (TransGen, China, Cat. No. FC101-02). Cells were seeded at a density of 1×10^5 cells/well in 96-well plates. A preliminary viability assay established that 100 μg/mL PE-MPs were non-cytotoxic and thus selected for subsequent experiments.

To ensure a uniform distribution, the stock solution of PE-MPs was mixed using a vortex mixer and sonicated briefly. Working solutions were prepared by diluting the stock in culture media, with the DMSO concentration maintained below 0.1% to avoid DMSO-induced cytotoxicity. A vehicle control with an equivalent DMSO concentration was also included.

Cells were initially exposed to varying concentrations of BPA (0, 1, 10, 100, 150, 200, and 250 μmol/L) and PE-MPs (0, 10, 100, 1000 μg/mL) for durations of 24, 48, and 72 h to conduct dose-response studies. The cell viability assay was conducted at all exposure time points (24, 48, and 72 h) to comprehensively evaluate the cytotoxic effects of the treatments over different durations. Based on the viability data obtained, single and combined exposure experiments involving 100 μg/mL PE-MPs and BPA concentrations of 0, 100, 150, 200, and 250 μmol/L were selected for further cell viability studies. Following treatment, 10 μL of CCK-8 reagent was added to each well, and the cells were incubated for an additional 2 h. Absorbance was measured at 450 nm using a SpectraMAX M2 Multimode Plate Reader, and the relative optical density (OD) was normalized against the untreated control to evaluate the effects of the treatments. Based on the viability data, concentrations of 100 and 150 μmol/L BPA combined with 100 μg/mL PE-MPs were deemed optimal for further experiments. The treatment groups were as follows: (1) Control: a solvent control group treated with 0.1% DMSO; (2) PE 20: an MPs exposure group cultured in medium containing 100 μg/mL PE MPs; (3) B 100: a BPA exposure group cultured in medium containing 100 μmol/L BPA; (4) B 100 + PE 20: an MP-BPA coexposure group treated with 100 μmol/L BPA and 100 μg/mL PE MPs; (5) B 150: a BPA exposure group cultured in medium containing 150 μmol/L BPA; (6) B 150 + PE 20: an MP-BPA coexposure group treated with 150 μmol/L BPA and 100 μg/mL PE MPs.

Experimental animals, acclimation, and exposure

Adult healthy zebrafish (*Danio rerio*) were purchased from Shanghai Feixi Biotechnology Co., Ltd. and acclimated for one week under a controlled photoperiod (14 h light:10 h dark), temperature (28 ± 0.5 °C), and humidity (70%) with daily water changes. The zebrafish used in this study were of standard laboratory strain, with an average body length of approximately 2.7–2.8 centimeters and an average weight of 0.3–0.4 g at the start of the experiment. Drawing from environmental concentrations and findings of prior research[28–30], we have chosen experimental concentrations of PE-MPs of 1 mg/L and BPA of 1.5 μg/L. Following acclimation, healthy zebrafish were randomly allocated into four experimental groups with a 1:1 sex ratio in 5-liter glass aquaria: (1) solvent control (0.1% DMSO), (2) PE-MPs only (1 mg/L), (3) BPA only (1.5 μg/L), and (4) co-exposed to both PE-MPs and BPA (1 mg/L PE-MPs and 1.5 μg/L BPA), All groups contained 0.1% DMSO.

Each group was replicated three times, and the exposure period was 28 days, which is a commonly used exposure period in toxicology studies, ensuring that the results are comparable with existing literature. The exposure solution was refreshed daily to maintain water quality and solute concentrations. Mortality was recorded, and deceased fish were removed. The exposure to BPA or PE-MPs resulted in no noticeable behavioral alterations during exposure period. At the end of the exposure, all zebrafish were anesthetized for approximately 95 s using 200 mg/L tricaine methanesulfonate (MS-222), measured for total body length (tbl) and wet weight (ww), and the growth condition factor (K) was calculated as $K = (ww / (tbl)^3) \times 100$. Organs were harvested, weighed, and stored at -80 °C for RNA extraction and gene expression analysis. The gonadosomatic index (GSI) was calculated as $GSI = (gw / ww) \times 100$, where gw represents the gonad weight of the fish, with the unit being grams (g). The experiments were performed under the relevant laws and institutional guidelines set by the Guiyang University Subcommittee of Experimental Animal Ethics with approval number: GYU-BEE-2022-03. All experimental protocols were approved by the Guiyang University Subcommittee of Experimental Animal Ethics. And all methods are reported in accordance with ARRIVE guidelines.

Cell cycle analysis

The cell cycle analysis was determined using a cell cycle and apoptosis analysis kit (Beyptime, China, Cat. No. C1052). After exposing MLTC-1 cells for 48 h, the supernatant is discarded, and the cells are collected by centrifugation (1500 rpm for 5 min). The cells are then fixed in pre-cooled 70% ethanol for 12 h, after which the supernatant is discarded again. Subsequently, 0.5 mL of propidium iodide staining solution is added to re-suspend the cell pellet. The cells are then incubated at 37 °C in the dark for 30 min. Cell cycle distribution was analyzed using flow cytometry. During the flow cytometry assay, the excitation wavelength was set at 488 nm and the emission wavelength was detected at 617 nm for PI, and the number of events analyzed was standardized to 10,000 events per sample.

Apoptosis detection

The cell apoptosis detection was determined using an annexin V-FITC/PI cell apoptosis detection kit (TransGen, China, Cat. No. FA101-01). After exposing MLTC-1 cells for 48 h, negative control groups (without any dye) and compensation control groups (stained with Annexin V-FITC alone or stained with PI alone) are set up. The supernatant is then discarded, and the cells are digested with trypsin that does not contain EDTA. The cells are

collected and centrifuged (1500 rpm for 5 min). After that, the cells are washed twice with pre-cooled PBS at 4 °C and centrifuged again (1500 rpm for 5 min). The supernatant is discarded, and 100 µL of pre-cooled 1× Annexin V Binding Buffer is added to re-suspend the cells. Finally, 5 µL of Annexin V-FITC and 5 µL of PI are added, gently mixed, and the cells are allowed to react in the dark at room temperature for 15 min. Cells were processed, stained, and analyzed by flow cytometry. For propidium iodide (PI) an excitation wavelength of 488 nm and an emission wavelength of 617 nm, while for annexin V-FITC, the excitation wavelength is 488 nm and the emission wavelength is 520 nm. The number of events analyzed during the flow cytometry assay was set at 10,000 events per sample to ensure sufficient data points for accurate analysis.

Mitochondrial membrane potential (MMP) assessment

The MMP was examined using fluorescence probe Rhodamine 123 (Beyotime Biotechnology, China; Cat. No. C2008S). After exposing MLTC-1 cells for 48 h, the cells are collected by centrifugation at 1500 rpm for 5 min, and the supernatant is discarded. Then, 1 mL of 500 nM Rhodamine 123 staining working solution is added to re-suspend the cells. The cells are then incubated at 37 °C in a cell culture incubator for 30 min. After the incubation period, the cells are centrifuged again at 1500 rpm for 5 min to collect the cell pellet, and then washed twice with pre-warmed cell culture medium. After re-suspending the cells in 1 mL of cell culture medium, they are immediately analyzed using a flow cytometer. During the flow cytometry assay, the excitation wavelength was set at 488 nm and the emission wavelength was detected at 530 nm for Rhodamine 123, and the number of events analyzed was standardized to 10,000 events per sample.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using the TransZol Up RNA Kit (TransGen, China, Cat. No. ER501-01-V2). Subsequently, reverse transcription was performed using a PrimeScript RT Reagent Kit (TaKaRa, China; Catalog Number RR047A). The concentration of the RNA was determined with a NanoDrop spectrophotometer. For the real-time PCR, a protocol was followed that included an initial denaturation step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s for denaturation, 60 °C for 30 s for annealing, and 72 °C for 30 s for extension. After the amplification cycles, a melting curve analysis was performed by gradually increasing the temperature from 65 °C to 95 °C at a rate of 0.5 °C per second while monitoring fluorescence to confirm the specificity of the amplified products. qRT-PCR was performed with specific primers for target genes, the sequences of the primers used were presented in Table 1, and gene expression was normalized to *β-actin* using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Data are presented as mean ± standard deviation (Mean ± SD) from at least three independent experiments. One-way ANOVA and Tukey's Honestly Significant Difference (HSD) test was used for data analysis using SPSS version 19.0, and Tukey's HSD correction was applied for multiple comparisons. Graphs were generated with Origin 8.5 software. Significance was denoted as * for $P < 0.05$ and ** for $P < 0.01$. Comparisons between treatment groups used # for $P < 0.05$ and ## for $P < 0.01$.

Results

Composition and morphology characterization of the microplastics

Scanning electron microscopy was used to characterize the surface of the microplastics. As shown in Fig. 1A, the microplastics appear as irregular particles, with a few in sheet or strip form. They vary in size from several micrometers to over ten micrometers and exhibit diverse surface morphologies. Some surfaces are relatively smooth, while others are rough, featuring wrinkles, pores, cracks, grooves, and some even exhibit a porous structure.

The Raman spectrum of the detected microplastics is shown in Fig. 1B. As can be seen, the raman spectrum of microplastics exhibits numerous characteristic peaks, among which the peaks at 2253 and 2947 cm^{-1} are the most prominent. Notably, the peak at 2947 cm^{-1} is similar to the characteristic peak of PE microplastics.

Effects of MPs and BPA on the viability of MLTC-1 cells

The impact of individual and combined exposures to PE-MPs and BPA on MLTC-1 cell viability was assessed after a 48-hour treatment period. The effect of MLTC-1 cells exposed to PE-MPs for 24, 48, and 72 h on cell viability is shown in Fig. 2A. As depicted, PE-MPs at concentrations below 100 µg/mL had no impact on the viability of MLTC-1 cells. However, exposure to 1000 µg/mL PE-MPs for 48 and 72 h reduced cell viability to 72.65% and 62.47%, respectively. The influence of MLTC-1 cells exposed to BPA microplastics for 24, 48, and 72 h on cell viability is illustrated in Fig. 2B. As shown, with increasing BPA exposure concentrations and prolonged exposure time, the viability of MLTC-1 cells gradually decreased. Exposure to BPA concentrations above 100 µmol/L for 48 h significantly reduced the viability of MLTC-1 cells. As illustrated in Fig. 2C, cells treated with 100 µg/mL PE-MPs alone exhibited no significant deviation in viability from the control group, suggesting that this concentration of PE-MPs is non-toxic to MLTC-1 cells. In contrast, increasing concentrations of BPA (150 µM, 200 µM, and 250 µM) of 48 h induced a significant decrease in cell viability ($P < 0.01$),with a dose-dependent trend observed as BPA concentration escalated from 100 µM to 250 µM. When cells were co-exposed to BPA and PE-MPs, a pronounced reduction in cell viability was observed compared to the control ($P < 0.01$). Notably, the co-exposure groups displayed a more substantial decrease in cell viability than those treated with BPA alone at 100, 150 and 200 µM ($P < 0.01$), down from 88.96 to 55.94%, 68.60% to 44.02, 51.30–26.23%, respectively, indicating a potential synergistic toxicity between BPA and PE-MPs. However, at the highest concentration of BPA (250 µM), cell viability was too compromised to discern any additional effect of PE-MPs.

Given the substantial impact on cell viability at 200 and 250 µM BPA combined with 100 µg/mL PE-MPs, subsequent experiments focused on lower concentrations (100 and 150 µM BPA, and 100 µg/mL PE-MPs) to

Genus	Genes	primer sequence(5' to 3')	GenBank NO.
Zebrafish	*Star*	GCCTGAGCAGAAGGGATTTG CCACCTGGGTTTGTGAAAGTAC	NM_131663.1
	Cyp11a1	GAGGGGTGGACTCGGTTACTT GCAATACGAGCGGCTGAGAT	AF527755.1
	Cyp17	CTGCTCTGTTTAAGCCTGTTCTC GCTGGCACAAATCCATTCATC	AY281362.1
	Cyp19a1a	CGGGACTGCCAGCAACTACT TGAAGCCCTGGACCTGTGAG	NM_131154.2
	Cyp11b	CTGGGCCACACATCGAGAG AGCGAACGGCAGAAATCC	DQ650710.1
	Hsd3b	AGCCCATTCTGCCCATCTT TGCCTCCTCCCAGTCATACC	AY279108.1
	Hsd20b	TGGAGAACAGGCTGAGGTGAC CGTAGTATCGGCAGAAGAGCAT	AF298898.1
	Hsd17b3	ACATTCACGGCTGAGGAGTTT ATGCTGCCATACGTTTGGTC	AY551081.1
	Hsd11b2	CAACCCCAGGTGCGATACTAC GCACGAGGCATCACTTTCTTCT	NM_212720.2
	Gnrh2	GGTCTCACGGCTGGTATCCT TGCCTCGCAGAGCTTCACT	NM_181439.4
	Gnrh3	TGGTCCAGTTGTTGCTGTTAGTT CCTGAATGTTGCCTCCATTTC	NM_182887.2
	Esr-1	ACTCTCACCCATGTACCCTAAGG CGGGTAGTATCCCACTGAAGC	NM_152959.1
	Ar	CCACGAACCCCCGTTTATCT TCCATCCATTCGCCCATCT	NM_001083123.1
	β-actin	TCTGGCATCACACCTTCTACAAT TGTTGGCTTTGGGATTCAGG	AF057040.1
MLTC-1	*3β-Hsd*	AGTGATGGAAAAAGGGCAGGT GCAAGTTTGTGAGTGGGTTAG	NM_001304800.1
	Star	TGGAAAAGACACGGTCATCA CTCCGGCATCTCCCCAAAAT	NM_011485.4
	Cyp11a1	CGTGACCAGAAAAGACAACA AGGATGAAGGAGAGGAGAGC	NM_019779.3
	Ar	AGATGGGCTTGACTTTCCCAGAAAG ATGGCTGTCATTCAGTACTCCTGGA	NM_000044.6
	Insl-3	GCTTCCTCTCCAGGCTTCTCA TGTGGTCCTTGCTTACTGCGA	NM_013564.7
	Lhr	GAGAAGCGAATAACGAGACG AGCCAAATCAACACCCTAAG	NM_013582.2
	Sf-1	TACCCACACTGCGGGGACAAA GGTTGTTGCGGGGCATCTCGT	NM_139051.3
	β-actin	CCAACCGTGAAAAGATGA CAGAGGCATACAGGGACA	NM_001017992.4

Table 1. Primers used in the real-time quantitative PCR analysis.

evaluate their effects on cell cycle, apoptosis, mitochondrial membrane potential, and steroid hormone synthesis pathways in MLTC-1 cells.

Effects of MPs and BPA on the cell cycle of MLTC-1 cells

The cell cycle distribution of MLTC-1 cells following exposure to BPA and PE-MPs is detailed in Fig. 3. Cells exposed to 100 μg/mL PE-MPs alone showed no significant alteration in cell cycle distribution. However, BPA exposure led to a significant reduction in the proportion of cells in the G0/G1 and S phases ($P < 0.01$) and a corresponding increase in the G2/M phase ($P < 0.01$), indicative of G2/M cell cycle arrest. The combined exposure to BPA and PE-MPs resulted in a similar G2/M arrest. Specifically, compared to 150 μM BPA alone exposure, co-exposure at 150 μM BPA with PE-MPs significantly increased the proportion of cells in the G2/M phase ($P < 0.01$), increased from 16.53 to 21.08%, suggesting a synergistic effect on cell cycle disruption.

Effects of MPs and BPA on apoptosis in MLTC-1 cells

Apoptotic responses in MLTC-1 cells to PE-MPs and BPA are depicted in Fig. 4. Single exposure to 100 μg/mL PE-MPs did not significantly affect apoptosis rates. In contrast, 100 and 150 μM BPA alone significantly induced apoptosis, with increased early apoptosis rates of 13.6% and 11.41%, and late apoptosis rates of 11.75% and 19.12%, respectively ($P < 0.01$). The combination of BPA and PE-MPs further significantly elevated the overall apoptosis rate ($P < 0.01$) compared to BPA alone, with a significant increase in late apoptosis rates of 19.18% and 24.14% observed at 100 μM BPA ($P < 0.05$) and 150 μM BPA ($P < 0.01$), highlighting the exacerbation of BPA-induced apoptosis by PE-MPs.

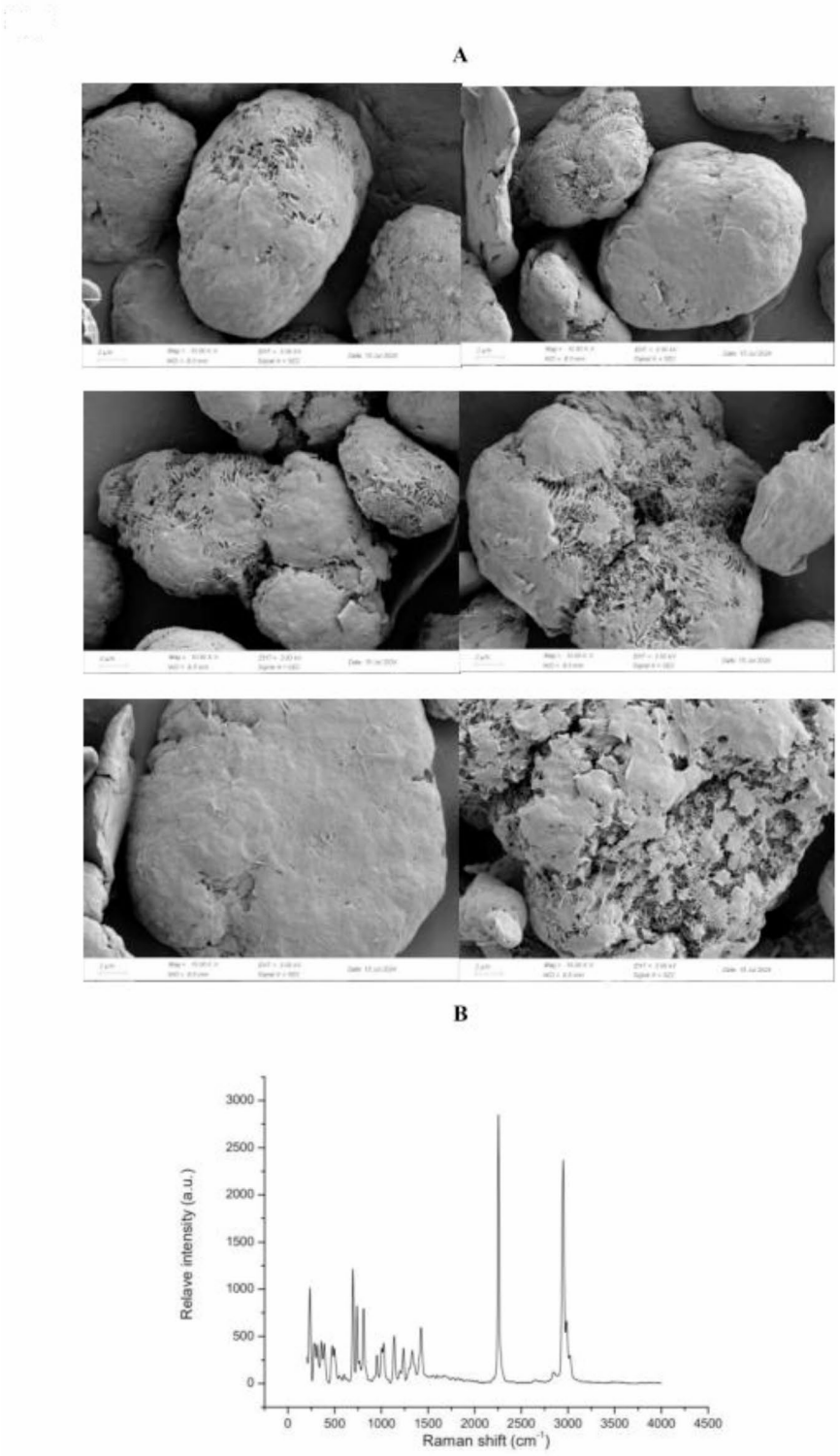

Fig. 1. Composition and morphology characterization of the microplastics. Note: (**A**): Microscopic morphology of the surface of PE-MPs using a scanning electron microscope, Magnification: 10.00 KX; (**B**): The composition of the PE-MPs using a microscopic raman spectrometer.

Effect of MPs and BPA on the mitochondrial membrane potential in MLTC-1 cells

The mitochondrial membrane potential of MLTC-1 cells was evaluated following exposure to PE-MPs and BPA, as shown in Fig. 5. PE-MPs alone at 100 μg/mL did not significantly affect mitochondrial fluorescence intensity. In contrast, BPA exposure resulted in a significant decrease in fluorescence, indicative of reduced mitochondrial membrane potential to 84.55% and 88.06% at 100 μM BPA and 150 μM BPA ($P < 0.01$) compared to control. The

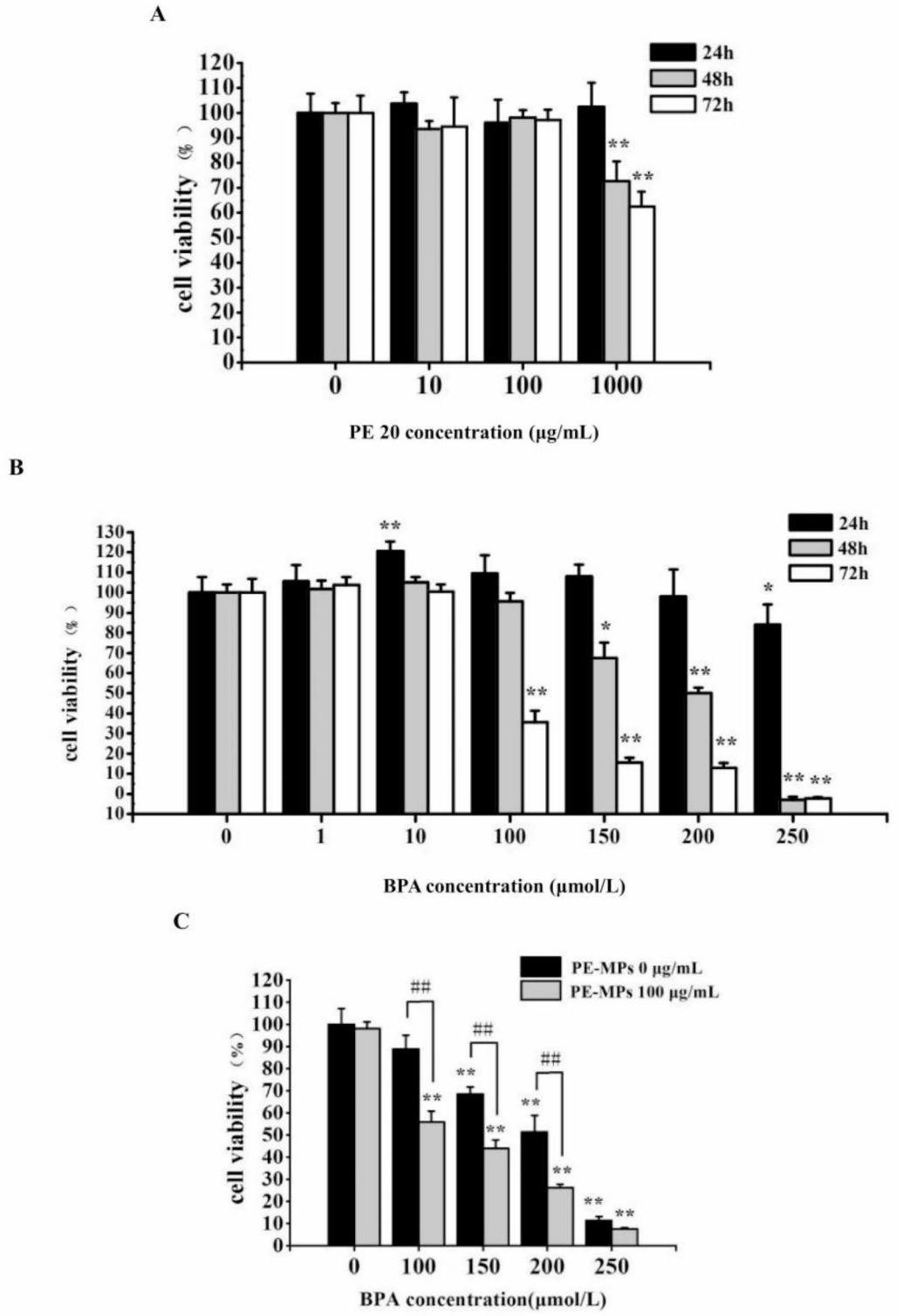

Fig. 2. Effects of BPA and PE-MPs on the cell viablility of MLTC-1 cells. Note: (**A**): Effects of PE-MPs on the cell viablility of MLTC-1 cells; (**B**): Effects of BPA on the cell viablility of MLTC-1 cells; (**C**): Effects of BPA and PE-MPs on the cell viablility of MLTC-1 cells; Results are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences between the exposure group and the control. $\#P < 0.05$, $\#\#P < 0.01$ indicates the significant differences between BPA and BPA + PE-MPs treatment group.

Fig.3

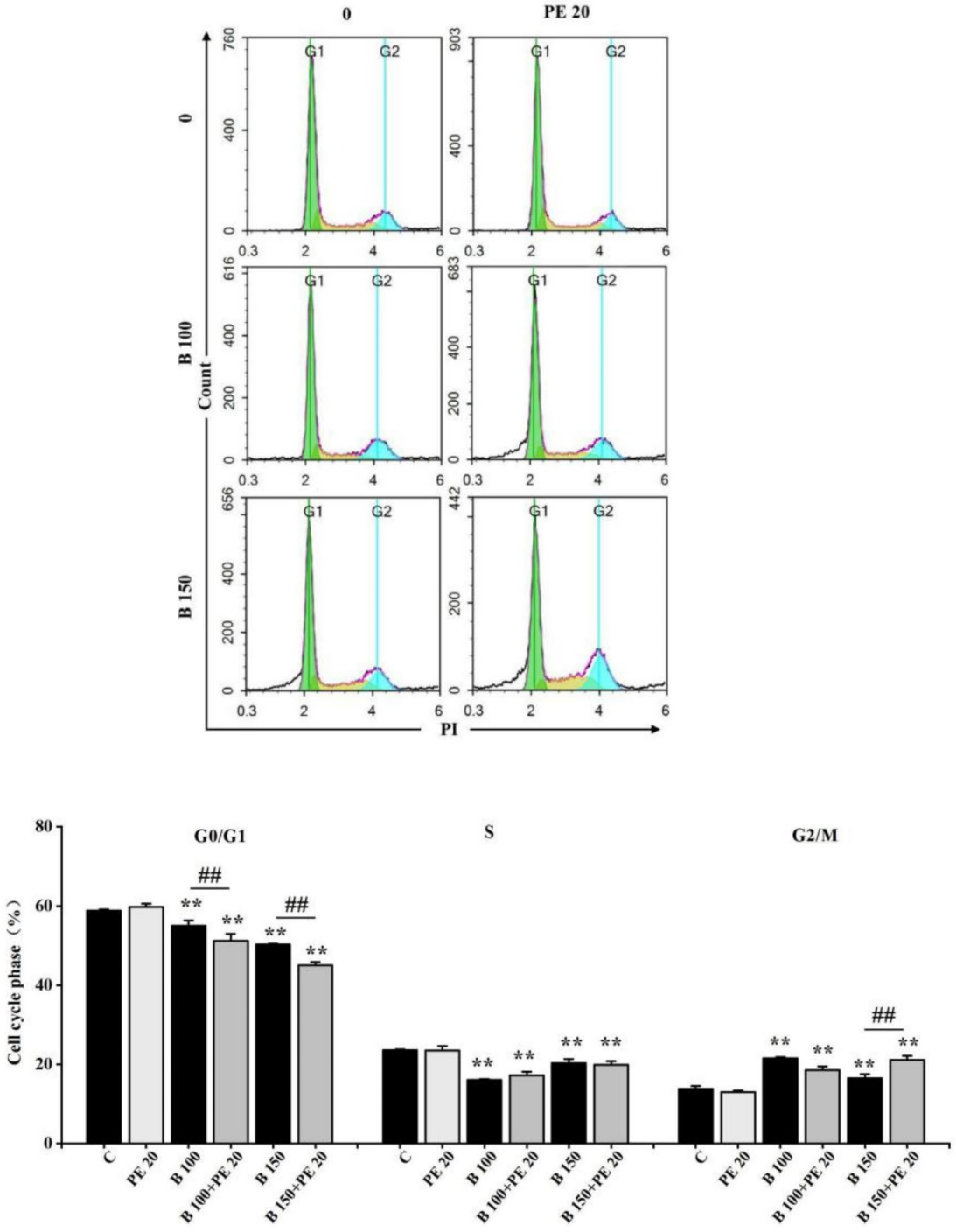

Fig. 3. Effects of BPA and PE-MPs on the cell cycle of MLTC-1 cells. Note: Results are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences between the exposure group and the control. $\#P < 0.05$, $\#\#P < 0.01$ indicates the significant differences between BPA and BPA + PE-MPs treatment group.

Fig.4

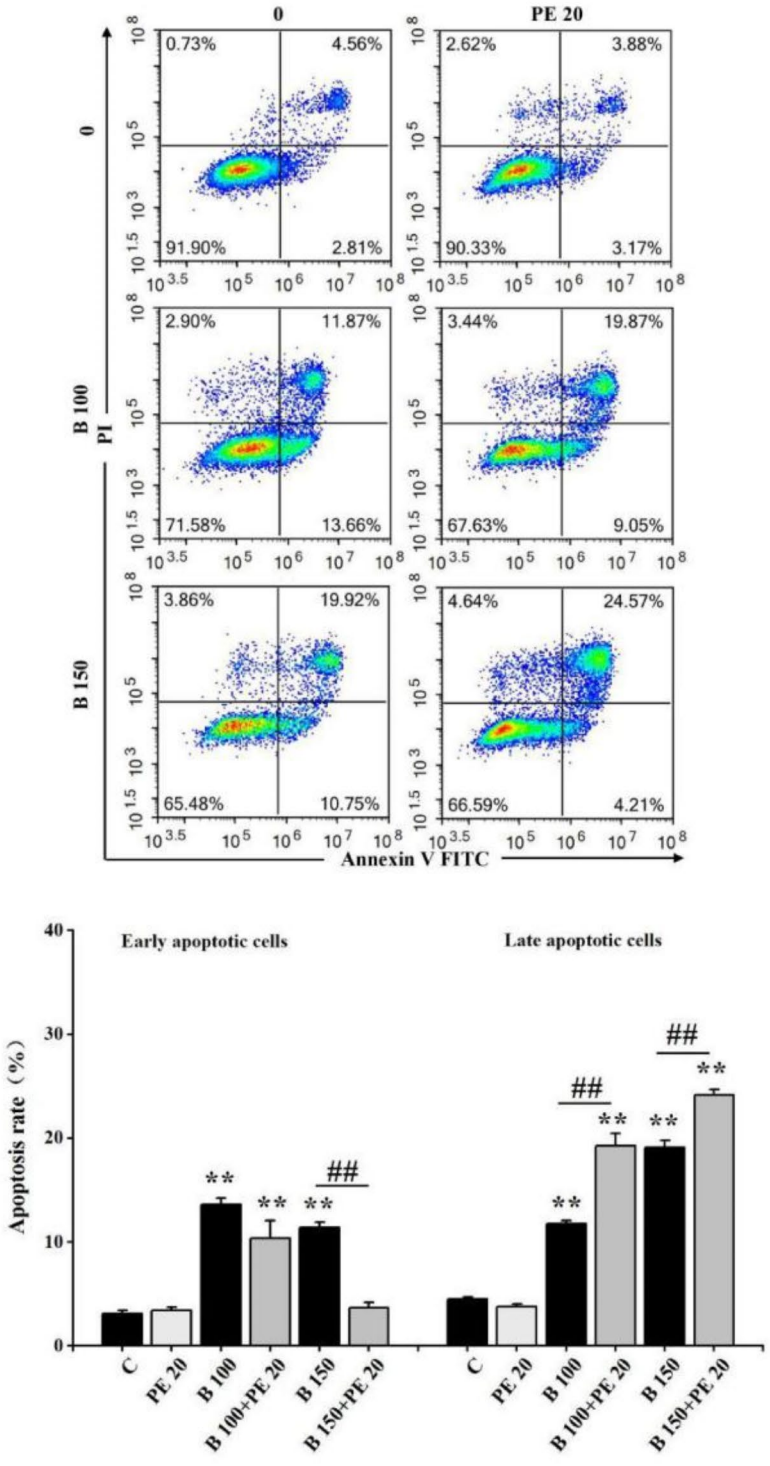

Fig. 4. Effects of BPA and PE-MPs on the apoptosis of MLTC-1 cells. Note: Results are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences between the exposure group and the control. $\#P < 0.05$, $\#\#P < 0.01$ indicates the significant differences between BPA and BPA + PE-MPs treatment group.

Fig.5

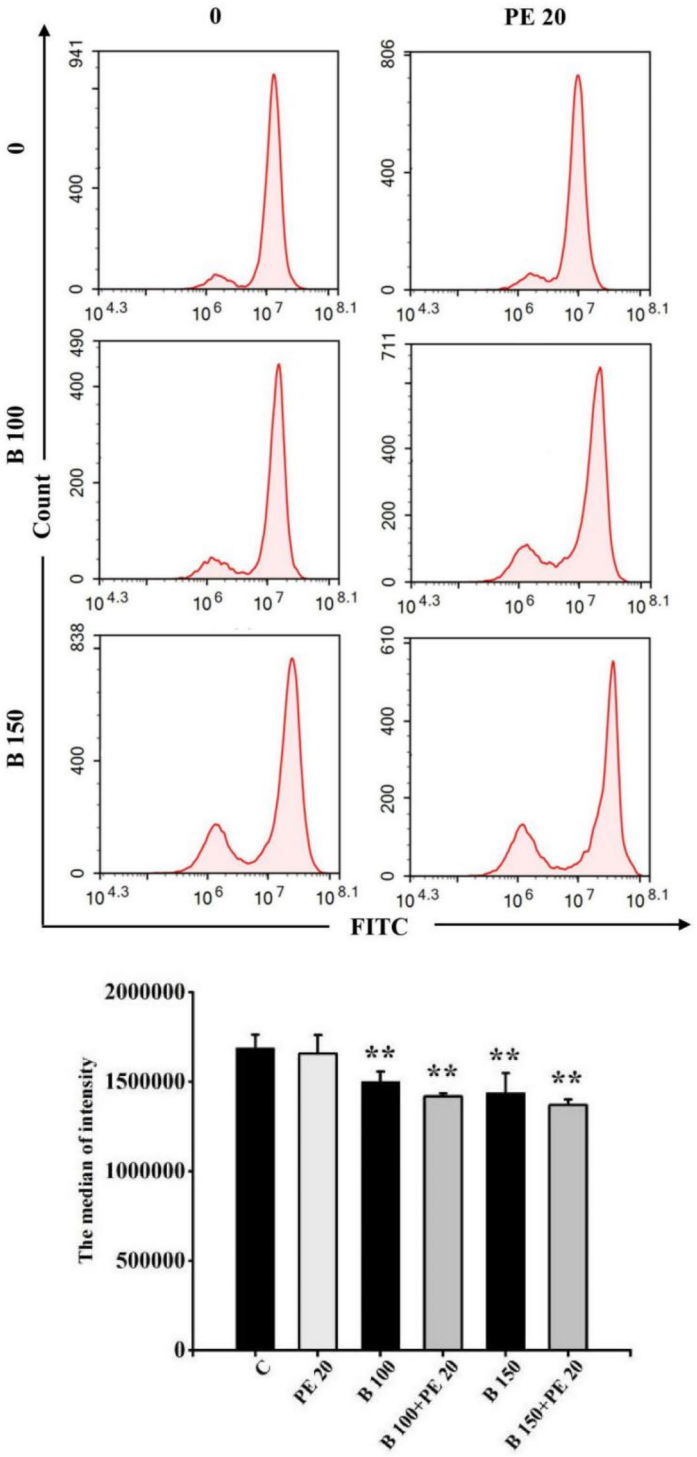

Fig. 5. Effects of BPA and PE-MPs on the mitochondrial membrane potential of MLTC-1 cells. Note: Results are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences between the exposure group and the control. $\#P < 0.05$, $\#\#P < 0.01$ indicates the significant differences between BPA and BPA + PS-MPs treatment group.

combined exposure to BPA and PE-MPs also led to a significant decrease in fluorescence to 84.79% and 80.84% at 100 μM BPA and 150 μM BPA ($P<0.01$) compared to control ($P<0.01$), but the addition of PE-MPs did not further affect the membrane potential in BPA-exposed cells.

Effects of MPs and BPA on gene expression in MLTC-1 cells

The influence of PE-MPs and BPA on gene expression related to the steroid synthesis pathway in MLTC-1 cells is presented in Fig. 6. Exposure to 100 μg/mL PE-MPs alone had no significant impact on the transcription of selected genes. However, BPA at 100 μM and 150 μM significantly altered the transcription of several genes, with upregulation of steroidogenic acute regulatory protein (*Star*) to 2.76 and 2.83 times of control and steroidogenic factor 1 (*Sf-1*) to 2.6 and 2.81 times of control ($P<0.01$) and downregulation of luteinizing hormone receptor (*Lhr*) to 0.55 and 0.35 times of control ($P<0.01$). BPA at 150 μM significantly downregulated the transcription of 3 beta-hydroxysteroid dehydrogenase (*3β-Hsd*) to 0.63 times of control, cytochrome P450 family 11 subfamily a member 1 (*Cyp11a1*) to 0.52 times of control, Insulin-Like 3 (*Insl-3*) to 0.40 times of control, and upregulated androgen receptor (*Ar*) to 1.33 times of control. Co-exposure to BPA and PE-MPs generally downregulated the transcription of *3β-Hsd*, *Cyp11a1*, *Insl-3*, and *Lhr* ($P<0.05$, $P<0.01$) and upregulated *Star*, *Ar*, and *Sf-1* ($P<0.01$). As for *3β-Hsd*, *Ar*, and *Lhr*, compared to BPA alone, co-exposure to BPA and PE-MPs significantly affected the gene expression, indicating a synergistic effect on gene transcription levels.

Impact of MPs and BPA on growth and development parameters of zebrafish

Throughout the 28-day exposure, no mortality was observed across all treatment groups. The effects of PE-MPs and BPA on zebrafish growth and development are shown in Fig. 7. Neither PE-MPs nor BPA, in isolation or combination, significantly affected body weight and length. However, PE-MPs alone significantly increased the GSI in both sexes ($P<0.05$), while BPA alone decreased the K in males ($P<0.01$). Combined exposure to BPA and PE-MPs increased the GSI ($P<0.05$, $P<0.01$) and improved the K in both sexes ($P<0.05$), suggesting significant developmental and endocrine-disrupting effects on zebrafish.

Impact of MPs and BPA on gene expression related to the HPG Axis in zebrafish

The transcriptional responses of genes associated with the HPG axis in zebrafish are detailed in Figs. 8 and 9. In male zebrafish, single exposures to PE-MPs and BPA altered the transcription of gonadotropin-releasing hormone 2 (*Gnrh2*), estrogen receptor 1 (*Esr-1*), *Ar*, and gonadotropin-releasing hormone 3 (*Gnrh3*) genes, with combined exposure further impacting these genes, particularly *Esr-1* ($P<0.01$). In female zebrafish, similar patterns were observed, with significant changes in *Gnrh3*, *Esr-1*, and *Ar* genes following combined exposure ($P<0.01$). These results indicate that both PE-MPs and BPA can significantly disrupt the HPG axis at the transcriptional level, with combined exposure exacerbating these effects. In the gonads of male zebrafish, single and combined exposures to PE-MPs and BPA led to significant changes in the transcription of steroidogenic genes, with upregulation of *Star*, *Cyp11a1*, and hydroxysteroid 11-beta dehydrogenase 2 (*Hsd11b2*), and downregulation of cytochrome P450 family 19 subfamily a member 1a (*Cyp19a1a*), 3β-hydroxysteroid dehydrogenase (*Hsd3b*), 20β-hydroxysteroid dehydrogenase (*Hsd20b*), and 17β-hydroxysteroid dehydrogenase type 3 (*Hsd17b3*) observed in combined exposures ($P<0.05$, $P<0.01$). In female zebrafish, combined exposure upregulated *Cyp11a1*, steroid 17α-hydroxylase and/or 17,20 lyase (*Cyp17*), 11β- hydroxylase (*Cyp11b*), *Hsd3b*, *Hsd20b*, and *Hsd17b3*, while downregulating *Cyp19a1a*, indicating a complex impact on the ovarian steroidogenic pathway.

Discussion

The widespread presence of BPA and MPs poses potential risks to organisms, which may be exposed to both pollutants simultaneously. The toxicity of BPA is likely modulated by the co-existence of MPs, as evidenced by the slight reduction in cell viability at high concentrations (1000 μg/mL) of PE-MPs in Caco-2 and A549 epithelial cells[31]. In our study, 1000 μg/mL PE-MPs significantly reduced MLTC-1 cell viability after 48 h and 72 h, whereas 100 μg/mL had no significant effect at any time point. This indicates that the concentration of 100 μg/mL may be below the threshold required to induce detectable changes, as higher concentrations or lower size of PE-MPs are often necessary to trigger such responses. This is inconsistent with previous finding[32,33], where PE-MPs did not or slightly affect the viability of human cancer cell lines, suggesting a possible variation in cellular sensitivity to PE-MPs across different cell types. The observed discrepancies in the effects of PE-MPs on cell viability can be attributed to several key factors, including cell type, exposure duration, and experimental conditions. Similarly, MLTC-1 cells may be less sensitive to PE-MPs at 100 μg/mL, explaining the lack of significant effects. The significant reduction in cell viability observed upon combined exposure to BPA and PE-MPs in our study indicates a synergistic interaction that enhances cytotoxicity. Previous study reported that polyethylene (0.25-1.0 mg/mL) increased mitochondrial superoxide production in Caco-2 and HT-29 cells but did not affect cytosolic ROS[34]. This suggests that mitochondrial responses to PE-MPs may require higher concentrations. And another study demonstrated that coexposure to microplastics and Bisphenol A exacerbated cellular damage, including reduced HSP90 levels and increased oxidative stress[35]. This implies that PE-MPs alone may not induce significant stress at lower concentrations, but their effects could become apparent under conditions of combined exposure or higher doses. Moreover, the varying size and rough surface morphology of PE-MPs, featuring wrinkles, pores, and cracks, likely increase their capacity to adsorb BPA. This supports the hypothesis that MPs may act as "Trojan horses," facilitating the uptake of BPA and resulting in an elevated intracellular concentration, leading to enhanced cytotoxic effects, a mechanism consistent with previous studies[36,37]. Firstly, PE-MPs have a hydrophobic surface that can adsorb hydrophobic compounds like BPA. This adsorption increases the local concentration of BPA on the MP surface, creating a reservoir of the pollutant that is co-transported into cells. Secondly, PE-MPs can be internalized by cells via endocytosis or phagocytosis,

Fig.6

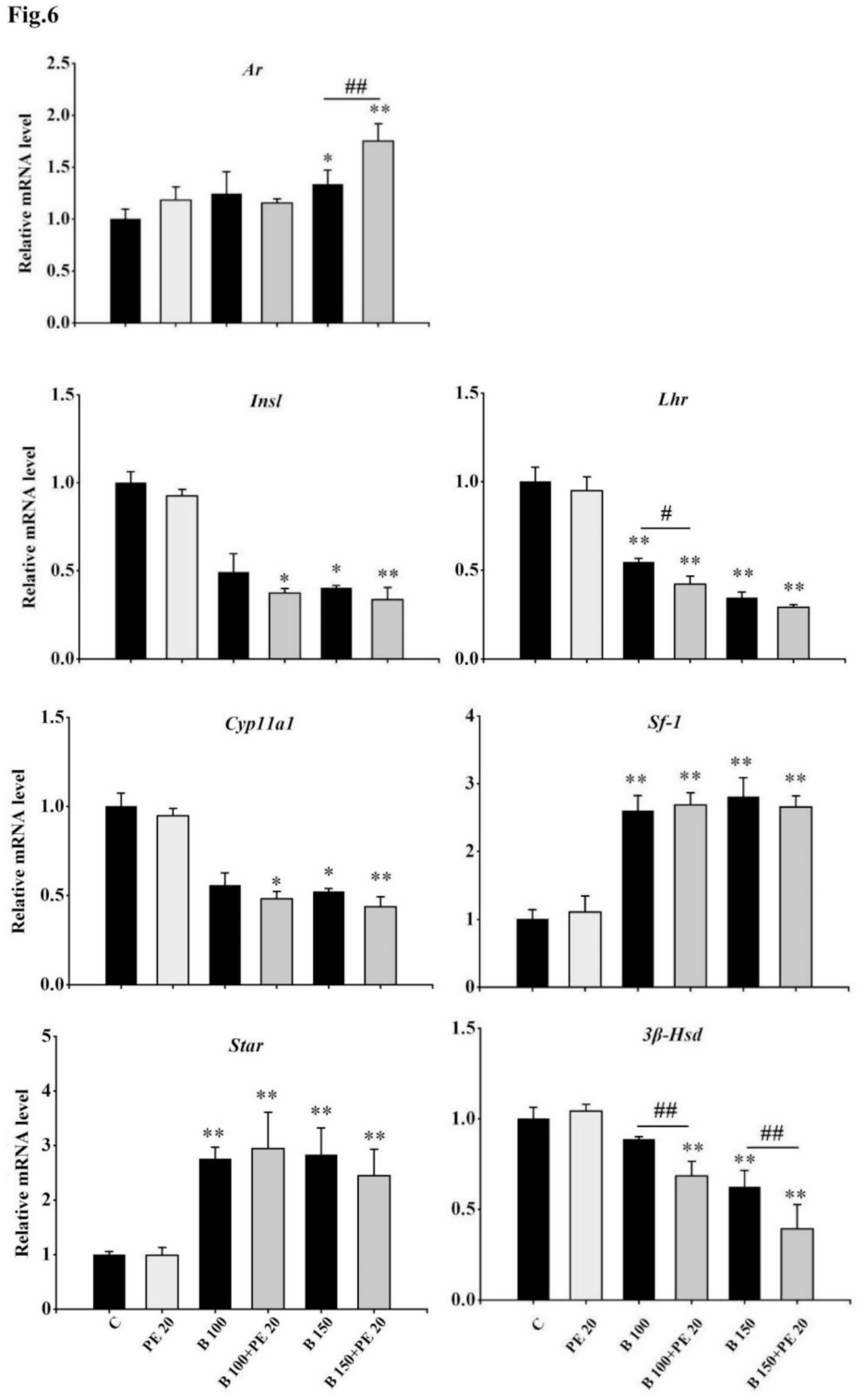

Fig. 6. Effects of BPA and PE-MPs on steroidogenic related gene transcription levels in MLTC-1 cells. Note: Results are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences between the exposure group and the control. $\#P < 0.05$, $\#\#P < 0.01$ indicates the significant differences between BPA and BPA + PE-MPs treatment group.

depending on the cell type and particle size. During this process, the adsorbed BPA is co-internalized, effectively bypassing the cell membrane's selective permeability barriers. Once inside the cell, the acidic and enzymatic environment of lysosomes or other intracellular compartments may promote the release of BPA from the PE-MPs. This localized release can result in higher intracellular concentrations of BPA than would occur through

Fig.7

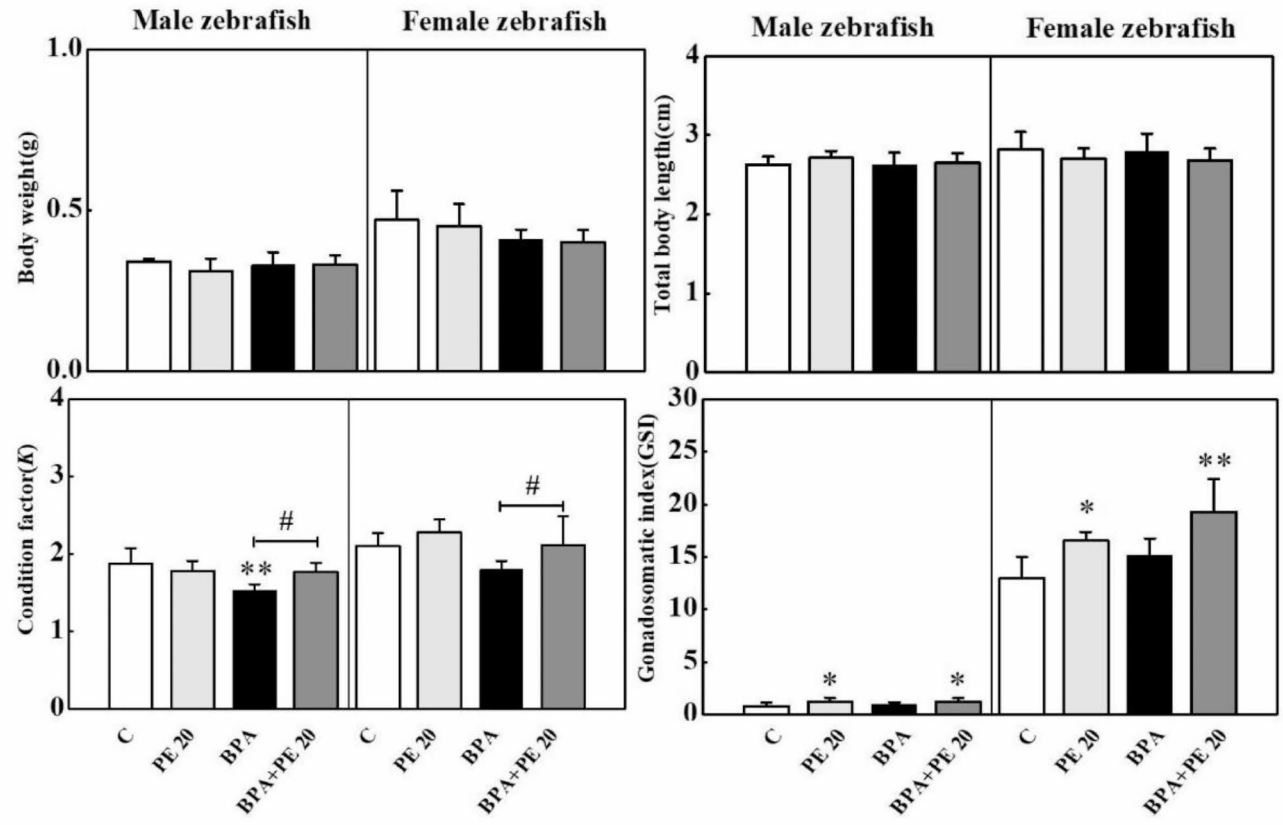

Fig. 7. Effects of BPA and PE-MPs on the growth and development parameters and organ index of adult zebrafish. Note: Results are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences between the exposure group and the control. $\#P < 0.05$, $\#\#P < 0.01$ indicates the significant differences between PE-MPs or BPA and BPA + PE-MPs treatment group.

passive diffusion alone, exacerbating cellular stress and toxicity. The co-transport of BPA with PE-MPs can lead to synergistic effects through exacerbating pro-oxidant and pro-inflammatory responses in kidney proximal tubular cells exposed to both PE-MPs and BPA[35]. This suggests that PE-MPs not only facilitate BPA uptake but also amplify its toxicological impact. However, The addition of PE-MPs did not always exacerbate BPA's effects at higher concentrations, possibly due to a plateau effect in the toxicity response. At high concentrations, BPA may have already saturated its binding sites and triggered maximal cytotoxic effects, leaving little room for further enhancement by PE-MPs. Additionally, it is possible that PE-MPs could have a mitigating effect under certain conditions, potentially through mechanisms such as adsorption of BPA onto their surface, reducing the bioavailability of free BPA molecules. Further studies are needed to explore these interactions in more detail. The observed decrease in cell viability could result from inhibited cell proliferation or the induction of apoptosis and necrosis[38]. Consequently, further investigation into cell cycle progression and apoptosis was undertaken.

Apoptosis pathways have been the primary focus of research in the context of reproductive toxicity induced by MPs in both male and female subjects[39,40], which have documented that exposure to MPs triggers caspase-dependent apoptosis within the testicular tissue of zebrafish, as reported in their study[39]. Extended exposure to polystyrene microplastics (PS-MPs) for a period of 35 days has been observed to cause significant disruptions in mitochondrial membrane potential, accompanied by an upregulation of both inflammatory and apoptotic biomarkers. These alterations have been correlated with our study results of induction of apoptosis and disruptions in mitochondrial membrane potential. The enhanced impact of combined BPA and PE-MPs exposure on cell cycle progression suggests a potential for PE-MPs to modulate cellular responses to BPA. This is in line with previous study[41], which reported synergistic DNA damage and apoptosis induction when SiNPs and B[a]P were co-exposed, highlighting the potential for nanoparticles to alter the cellular response to toxicants. The induction of apoptosis by BPA, as observed in our study, is consistent with previous study[42], who found that BPA and its analogs significantly reduced the viability of stem cells and induced apoptosis. The increased apoptosis rate in combined exposure groups suggests that PE-MPs may exacerbate BPA-induced cell death, potentially through modulating apoptotic pathways. Mitochondrial dysfunction, a known inducer of apoptosis, was significantly affected by BPA, and this effect was further disrupted by PE-MPs, as shown in our study and supported by Zhang Lei[43], who also reported BPA-induced changes in mitochondrial membrane potential.

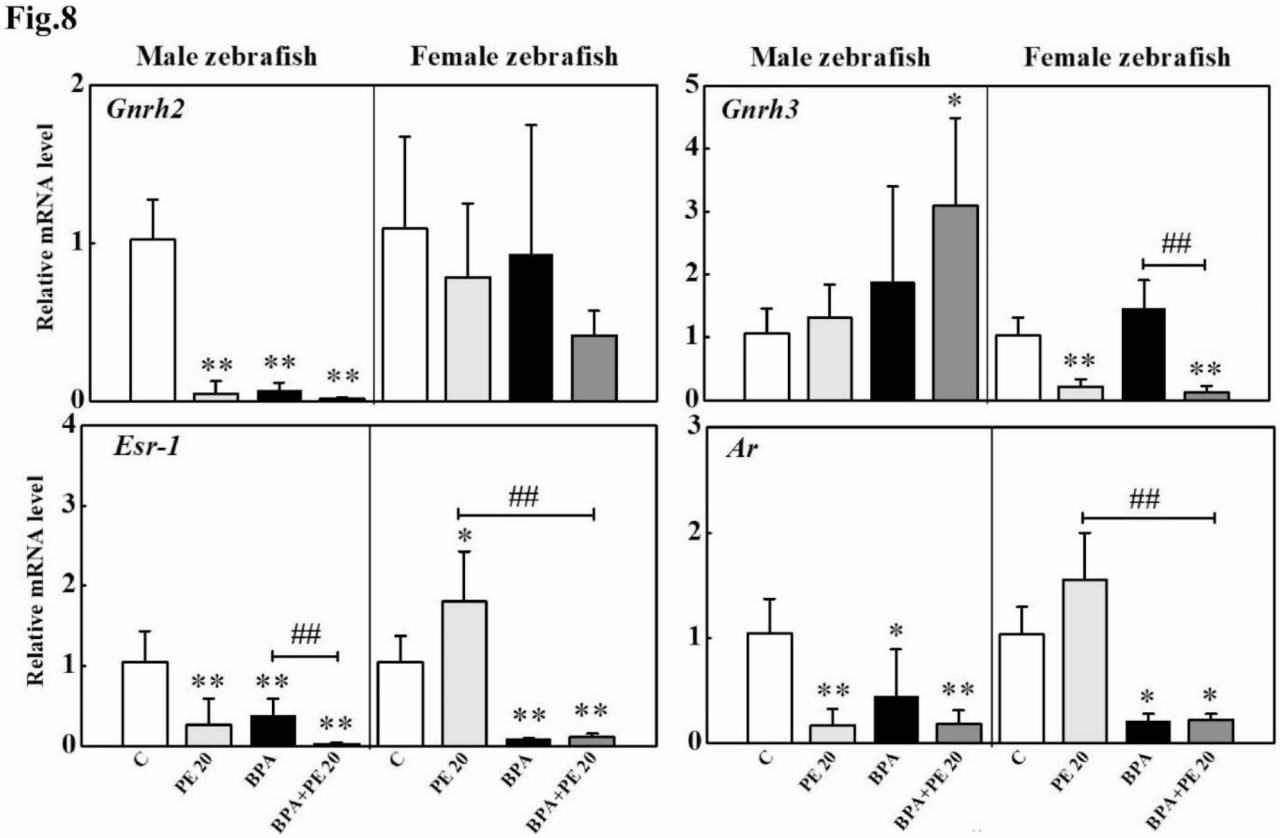

Fig. 8. Effects of BPA and PE-MPs on hypothalamic and pituitary hormones, receptor signaling pathways related gene transcription levels in adult zebrafish. Note: Results are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences between the exposure group and the control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicates the significant differences between PE-MPs or BPA and BPA + PE-MPs treatment group.

Testosterone plays an indispensable role in the process of spermatogenesis and is secreted by the Leydig cells within the testes. The secretion of testosterone is intricately regulated by the steroidogenesis signaling pathway[44,45]. Research evidence indicates that BPA has detrimental effects on reproductive function, impairing steroidogenesis[46–49]. Leydig cells, pivotal in testosterone production, have been extensively utilized in BPA research. Studies have shown that the impact of BPA on these cells is dose-dependent. High-dose BPA exposure has been correlated with a reduction in Leydig cell count and the expression of steroidogenic enzymes in pubertal Wistar/ST rats[50]. This finding was corroborated in adult male Leydig cells subjected to lower BPA dosages[51]. Additionally, in vitro investigations involving foetal testes from humans, mice, and rats have consistently linked BPA exposure to diminished testosterone levels[52]. The impact of BPA and PE-MPs on the expression of genes involved in steroidogenesis is a significant finding of our study. The downregulation of *3β-Hsd* and *Cyp11a1*, and upregulation of *Star*, could interfere with cholesterol conversion to testosterone, as supported by literature showing BPA's impact on steroidogenic activity in rat Leydig cells and mouse testes[53]. This disruption is hypothesized to occur through the inhibition of the *Cyp11a1* enzyme, a pivotal component in the steroidogenic pathway[46]. BPA is postulated to disrupt steroidogenesis through modulation of both the transcriptional and translational expression levels of key steroidogenic enzymes, including *Cyp11a1*, *3β-hsd*, and aromatase[54]. For instance, higher BPA concentrations (40–100 μM) used also induced the upregulation of aromatase expression and oestrogen synthesis[55]. The exposure to BPA and BPA combined with PE-MPs also suppressed the expression of the *Ins1-3* gene, an important regulatory factor in the steroid synthesis process, and significantly upregulated the expression of the *Sf-1* gene. Literature reports indicate that exposure of pregnant mice to Nonylphenol can reduce the mRNA and protein expression of *Ins1-3*, leading to impaired testicular development in male fetal mice[56]. Previous study found that BPA substitute BHPF increased the expression of the *Sf-1* gene to 1.7 times that of the DMSO control group[57]. *Sf-1* is a key transcription factor in steroidogenesis that plays a crucial role by regulating the transcription of steroidogenic genes (*Star* and *Cyp11a1*)[58,59]. The results of this study show that under the influence of BPA and BPA + PE-MPs, the transcriptional level of the androgen receptor gene *Ar* was significantly upregulated, while the transcriptional level of the luteinizing hormone receptor gene *Lhr* was significantly downregulated. Literature reports suggest that rats exposed to 25 mg BPA/kg BW (Body Weight) have a relative increase of 2 times in *Ar* levels. Androgens need to bind to *Ar* to exert their effects, which requires a certain amount of *Ar* to maintain normal levels. An increase in *Ar* expression can enhance the

Fig.9

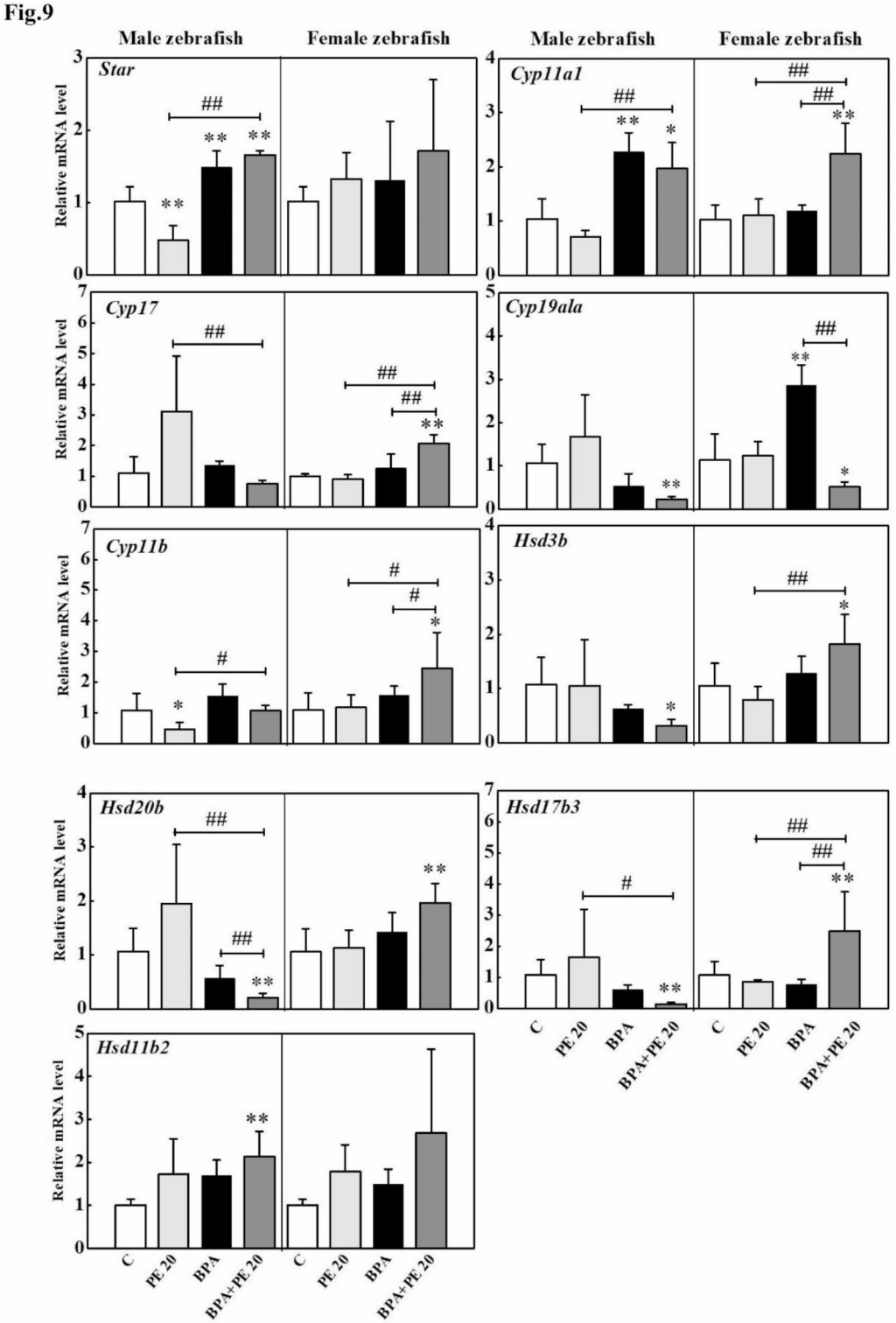

Fig. 9. Effects of BPA and PE-MPs on steroidogenic pathways related gene transcription levels in adult zebrafish. Note: Results are expressed as mean ± standard deviation ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant differences between the exposure group and the control. $\#P < 0.05$, $\#\#P < 0.01$ indicates the significant differences between PE-MPs or BPA and BPA + PE-MPs treatment group.

action of androgens to a certain extent. May Jie[60] found that in male rats, the expression level of the *Lhr* gene was significantly increased in the 0.5 μg/kg BPA group, while it was significantly decreased in both the 50.0 μg/kg BPA and 50.0 mg/kg BPA groups, indicating a complex dose-response relationship in BPA's interference with steroid hormone synthesis, characterized by low-dose stimulation and high-dose inhibition. However, in

this study, single exposure to PE-MPs did not significantly affect the expression of genes related to the steroid synthesis pathway in MLTC-1 cells, possibly due to the lower exposure concentration in this experiment. The combined exposure of BPA and MPs significantly affected the transcriptional levels of some genes compared to single exposure, indicating that the toxicity of MPs also needs to be taken seriously. In summary, BPA exposure can affect the expression of key enzymes, receptors, and regulatory factor genes in the steroid hormone synthesis pathway of MLTC-1 cells. The combined exposure of BPA and PE-MPs shows a synergistic toxic effect on the transcriptional levels of some genes, with potential endocrine-disrupting effects. The significant changes in gene transcription levels under combined exposure indicate a synergistic toxic effect on endocrine function.

The endocrine-disrupting effects of BPA and PE-MPs on the HPG axis in zebrafish, as indicated by changes in *Gnrh2*, *Esr-1*, and *Ar* gene transcription, are of particular concern. Wang et al. conducted research on *Oryzias melastigma* and demonstrated that exposure to PE-MPs can lead to reproductive disruption. This disruption is associated with the modulation of genes linked to the HPG axis[61]. In fish, the HPG axis is pivotal in regulating both reproductive function and steroid synthesis. The interaction between gonadotropin-releasing hormone (GnRH) and its receptor (GnRHR) initiates a cascade of responses that are crucial for the regulation of the expression of key reproductive hormones[62]. In the brain of male zebrafish, PE-MPs exposure groups significantly reduced the transcription levels of *Gnrh2*, *Esr-1*, and *Ar*, could subsequently regulate the synthesis of steroid hormones. The biosynthesis of sex steroids in the gonads begins with the transfer of cholesterol to the mitochondria mediated by the enzyme *Star*. Subsequently, cholesterol undergoes a cascade of processes, facilitated by enzymes such as *Cyp17a1*, *Cyp11a1*, *Hsd3b*, and *Hsd17b*, to be converted into testosterone (T). Finally, the enzyme *Cyp19a1* converts T into estradiol (E2). In this study, it was found that PE-MPs significantly downregulated the transcriptional level of the *Star* gene in the testes of male zebrafish, consistent with the finding[63], where a significant decrease in *Star* expression was observed after exposure to PS-MPs. This may be due to the primary effect of PE-MPs on steroidogenic enzymes being the inhibition of the first rate-limiting enzyme. In bony fish, the biologically active androgen is 11-ketotestosterone (11-KT), which is a key hormone for spermatogenesis and the maintenance of testicular function. In male fish, 11-KT is produced from T through a reaction catalyzed by the enzyme Cyp11b[64]. This study observed that exposure to PE-MPs led to a downregulation in the transcriptional level of the *Cyp11b* gene in the testes of male zebrafish, which could potentially reduce the synthesis of 11-KT in the testes. Furthermore, combined exposure to PE-MPs and BPA suppressed the steroidogenesis pathway by reducing the transcriptional levels of *Hsd3b*, *Hsd20b*, *Hsd17b3*, and *Cyp19a1*. Similar results observed in goldfish larvae, that the transcriptions of *Cyp19a*, *Hsd3β* and *Hsd20β* decreased after PE-MPs exposure 30 days, which could inhibit the steroidogenesis pathway in *Paramisgurnus dabryanus*[65]. In contrast, the combined exposure resulted in the upregulation of *Star*, *Cyp11a1*, and *Hsd11b2*. Unlike male zebrafish, female zebrafish exposed to a combination of BPA and PE-MPs showed a significant upregulation in the transcriptional levels of the genes *Cyp11a1*, *Cyp17*, *Cyp11b*, *Hsd3b*, *Hsd20b*, and *Hsd17b3* in the ovaries, and a significant downregulation in the transcriptional level of the *Cyp19a1a* gene. There was no significant impact on the transcriptional levels of the *Star* and *Hsd11b2* genes. This indicates that the combined exposure to BPA and PE-MPs significantly disrupted the expression of genes related to the steroid synthesis pathway in female zebrafish, demonstrating an interfering effect on the expression of steroid synthesis-related genes in the gonads of both male and female zebrafish. Our study uncovered notable gender disparities in the response of zebrafish to the experimental treatments. Specifically, male zebrafish displayed heightened sensitivity to BPA exposure, manifesting in altered expression of certain genes such as *Gnrh2*, *Star*, and *Cyp11a1*, compared to their female counterparts. Similarly, male zebrafish exhibited increased sensitivity to PE exposure, evident in modified expression of genes like *Gnrh2*, *Ar*, *Star*, and *Cyp11b*, relative to females. Moreover, male zebrafish showed greater sensitivity to combined exposure of BPA and PE, evident in altered expression of genes such as *Gnrh2* and *Star*, compared to females. This finding aligns with previous studies that have documented sex-specific responses to endocrine disruptors in fish species[66]. We postulate that these gender differences may be ascribed to the distinct hormonal profiles and physiological attributes of male and female zebrafish. Furthermore, the differential expression of genes implicated in hormone regulation and metabolism, behavior, and immune response may also play a role in the observed gender-specific responses. Males may have different metabolic pathways for processing BPA and PE-MPs, leading to higher accumulation and greater disruption of endocrine pathways. Additionally, differences in immune response and behavior could affect how males and females interact with these pollutants. For example, males may exhibit more aggressive feeding behavior, leading to higher exposure levels. Further studies are needed to elucidate these gender-specific interactions. The sexually dimorphic response to PE-MPs exposure in zebrafish suggests that males may be more sensitive to such pollutants, although further research is needed to confirm this observation.

Furthermore, the study's findings showed that the toxic effects of BPA were markedly exacerbated by the simultaneous presence of MPs. This exacerbation could be attributed to the interactions between BPA and MPs, including due to the absorption of BPA on MPs, co-presence of MPs may facilitate the entry of BPA into the body and cell, resulting in aggravated accumulation of BPA, which were also reported in previous studies[24,35]. Based on above results, we concluded that combined exposure to PE-MPs and BPA could disturb the steroidogenesis pathway and lead to the subsequent reproductive dysfunction in zebrafish.

The comparison between in vivo and in vitro results offers valuable insights into the intricate interactions between biological systems and environmental factors. Our in vitro studies revealed the induction of gene expression of *Star* and downregulation of *Hsd3b*, which were corroborated by the in vivo findings in male zebrafish. Nonetheless, there were also notable disparities between the two experimental setups. For instance, in vivo exposure to PE-MPs resulted in upregulation of *Cyp11a1*, which was not observed in the in vitro experiments. This discrepancy may be attributed to the presence of additional factors in the in vivo environment, such as the immune response and the complex interplay of multiple organ systems. The discrepancies in *cyp11a1* expression between in vivo and in vitro systems likely arise from systemic immune and hormonal influences, microenvironmental differences, metabolic and oxidative stress conditions, exposure duration, and species- or

tissue-specific responses. These factors highlight the complexity of translating in vitro findings to in vivo contexts and underscore the need for complementary approaches to fully understand the mechanisms underlying PE-MP toxicity.

The mechanisms of action of BPA in isolation and in conjunction with PE-MPs exhibit intricate patterns that profoundly impact cellular processes and the steroidogenesis pathway in MLTC-1 cells as well as in zebrafish models. When BPA acts solely, it engages with androgen receptors, namely *AR* and *Lhr*, in MLTC-1 cells, triggering a cascade that involves upregulation of steroidogenesis-related genes such as *Star* and *Sf-1*, while concurrently downregulating key steroidogenic enzymes like *Cyp11a1* and *3β-Hsd*. This dysregulation leads to hormonal imbalances and perturbations within the endocrine system, which can instigate an array of cellular responses, including accelerated apoptosis, pronounced G2/M cell cycle arrest, diminished mitochondrial membrane potential, and ultimately, compromised MLTC-1 cell viability, exacerbating endocrine disruptions. The presence of PE-MPs accentuates BPA's effects on the steroidogenesis pathway-related gene expression and cellular dynamics in MLTC-1 cells. In male zebrafish, BPA exposure alone attenuates the expression of genes critical for neuroendocrine regulation (*Gnrh2*, *Esr1*, and *Ar*) in the brain, while simultaneously enhancing the expression of *Star* and *Cyp11a1* in the testis, contributing to endocrine perturbations and hormonal imbalances. In the context of PE-MPs, BPA's influence becomes more pronounced, with further suppression of *Gnrh2*, *Esr1*, and *Ar* expression in the brain accompanied by a surge in *Gnrh3* expression. Additionally, there is an upregulation of *Star*, *Cyp11a1*, and *Hsd11b2* in the testis, accompanied by downregulation of *Cyp19a1a*, *Hsd20b*, *Hsd3b*, and *Hsd17b3*, culminating in an elevation of the GSI, indicating altered gonadal development and/or function. These findings underscore the complex and synergistic interplay between BPA and PE-MPs in disrupting critical biological processes. In female zebrafish, exposure to BPA alone was observed to elicit a reduction in the transcriptional levels of *Esr1* and *Ar* genes within the brain, subsequently enhancing the expression of *Cyp19a1a* in the ovary. This cascade of events led to hormonal imbalances and disruptions within the endocrine system. In contrast, when BPA was administered in conjunction with PE-MPs, a more intricate pattern of gene expression alterations emerged. Specifically, BPA in the presence of PE-MPs suppressed the expression of *Gnrh3*, *Esr1*, and *Ar* genes in the brain, while paradoxically increasing *Gnrh3* expression, likely indicative of a complex regulatory feedback loop. Furthermore, in the ovary, this combined exposure upregulated the expression of multiple steroidogenic enzymes, including *Cyp17*, *Cyp11b*, *Hsd3b*, *Hsd20b*, *Hsd17b3*, and *Cyp11a1*, while downregulating *Cyp19a1a*. These alterations culminated in an increase in the GSI, a measure of reproductive development and function. Thus, the mechanisms of action underlying BPA's effects, both independently and in the context of PE-MPs, are intricate and multifaceted, capable of modulating cellular processes and disrupting the steroidogenesis pathway in both in vitro models (e.g., MLTC-1 cells) and in vivo zebrafish models. Similarly, exposure to polystyrene nanospheres has been shown to exacerbate homosolate-induced estrogenic effects in the in vitro MCF-7 cell model and disrupt the HPG axis in vivo zebrafish models, with observed gender-specific response patterns[67]. Notably, these effects exhibit both in vitro-in vivo discrepancies and sex-specific patterns, highlighting the need for comprehensive and nuanced investigations into the environmental health implications of these contaminants.

Conclusion

The study provides evidence for the synergistic toxic effects of BPA and PE-MPs on cellular processes and endocrine function. The findings underscore the need for a comprehensive understanding of the environmental health risks posed by these pollutants, particularly in the context of their combined exposure. Thus, both in vitro and in vivo studies have suggested that BPA and PE-MPs exposure alters steroidogenesis pathways. Future research should focus on in vivo models to validate these findings and explore long-term ecological consequences, as well as develop mitigation strategies to address the impacts of BPA and PE-MPs pollution. Future research should focus on confirming the observed interactions between BPA and PE-MPs using additional in vivo models to validate our findings. Examining other microplastic types (e.g., polystyrene, polypropylene) and additional environmental contaminants (e.g., heavy metals, pesticides) in combination with BPA would provide a more comprehensive understanding of their synergistic effects. Additionally, long-term exposure studies and investigations into the underlying molecular mechanisms, such as the role of specific receptors and signaling pathways, are needed to fully elucidate the endocrine-disrupting effects of these pollutants.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

T.Y. and R.Y. were responsible for writing the main manuscript and preparing Figs. 2, 3, 4, 5, 6, 7, 8 and 9. T.Y. and Y.L. contributed to Fig. 1 and conducted microplastics composition and morphology characterization assays. R.Y., S.H. and J.L. were involved in cell viablility, cycle, apoptosis, mitochondrial membrane potential, and gene expression assay. Data analysis was performed by T.Y. and R.Y. The results were validated by T.Y. and R.Y. H.L. and C.L. provided research direction and guidance for the study.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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