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Research Paper

Low concentration Tetrabromobisphenol A (TBBPA) elevating overall metabolism by inducing activation of the Ras signaling pathway

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ABSTRACT

Tetrabromobisphenol A (TBBPA), one of the most common flame retardants, affects neurodevelopment, disrupts the endocrine system, and increases the possibility of tumorigenesis. This study investigates the cytotoxic effects, genetic effects, and metabolic effects from exposure to low concentration TBBPA. The cell exposure was measured by mimicking the residual TBBPA concentrations in human plasma, specifically in occupational populations. Our results revealed that long-term TBBPA exposure, especially at 1 nM concentration, significantly promoted the proliferation of HepG2 cells. Furthermore, long-term TBBPA exposure can double the levels of reactive oxygen species (ROS) released from mitochondria, thereby increasing Adenosine Monophosphate activated Protein kinase (AMPK) gene expression level to promote cellular proliferation. However, ROS can also mediate the apoptosis process through the mitochondrial membrane potential (MMP). The RNA-seq analysis confirmed that the Ras signaling pathway was activated by the growth factor to mediate cell detoxification mechanism, increasing lipid and vitamin metabolic rate. Our work uncovers a cellular mechanism by which long-term exposure to low concentration TBBPA can induce the activation of the Ras signaling pathway and demonstrates potential metabolic disorder in the human hepatic cells upon plasma TBBPA exposure.

1. Introduction

Tetrabromobisphenol A (TBBPA), a reactive brominated flame retardant (BFR), is widely used in fire prevention these days (Liang et al., 2019b; Alexander et al., 2011). However, it is released into the environment during manufacturing, usage, recycling, and dismantling processes. The mean concentrations of TBBPA in dust from residential areas, industrial areas, commercial areas, parks, and countryside in Chongqing city, China, are reported to be 26.7, 25.0, 21.3, 14.9, and 9.02 ng/g, dw (dry weight), respectively (Lu et al., 2018). The TBBPA concentration in the indoor environments of Taizhou city, China (3435 ng/g, dw) is roughly 1.2 times higher than the outdoors due to electronic waste (E-waste) dismantling activities and the use of appliances and furniture (Wu et al., 2016). Besides, exceptionally high TBBPA concentrations of 5.50–23.8 $\times$ 10^3 ng/g have also been measured in an e-waste dismantling industrial park in southern China (Liu et al., 2020).

What's more, TBBPA of 2.5–580 pg/g lw (lipid weight)) was also detected in the plasma of random population from Sweden, American, Belgium, Romanian, Belgian, and China (Jakobsson et al., 2002; Johnson-Restrepo et al., 2008; Dufour et al., 2017; Shi et al., 2013; Li et al., 2020; Dirtu et al., 2010). Even higher TBBPA concentrations were detected in the plasma of occupational exposure groups like e-waste dismantling workers (1.09–3.99 ng/g, lw) and computer technicians (0.54–1.84 ng/g, lw) in Sweden, and e-waste dismantling workers (0.64–1.80 ng/g, lw) and circuit board producers (0.10–0.80 ng/g, lw) in Norway (Covaci et al., 2009). Plasma levels of TBBPA are higher in occupational exposure groups than those in the general population. Although previous researches indicated that the half-life of TBBPA in Bos taurus and Wistar Han IGS rats is short (Knudsen et al., 2018; Shin et al., 2020), high exposure concentrations and long exposure times in the workplace could still lead to the in vivo TBBPA accumulation. Therefore, the toxicity of TBBPA, especially at the occupational population level in plasma, is worth investigating.

TBBPA was upgraded to Group 2A (probably carcinogenic to

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humans) by the International Agency for Research on Cancer (IARC) in 2016 (Grosse et al., 2016). Dunnick et al. found that the incidence of hepatoblastoma was 5.5-fold greater in rats in the 250 mg/kg TBBPA exposure groups (22%) than the incidence in the control groups (4%) (Dunnick et al., 2015; National Toxicology, 2014). Further, they revealed long-term exposure to TBBPA induced activation of interferon (IFN) and metabolic network, indicating that TBBPA might be carcinogenic through IFN (Dunnick et al., 2017). As well known, hepatocellular carcinoma (HCC) is one of the most frequently diagnosed cancers and the third most common cause of cancer-related deaths worldwide (Sun et al., 2018). According to the European Union Risk Assessment Report, the highest ^{14}C labelled TBBPA radioactivity levels were found in the liver at 8 h post-dosing (ECB, 2006). However, little is known about the potential carcinogenic and cancer progression risk from exposure to TBBPA in liver cancer.

According to studies based on normal cell lines, TBBPA can cause TM4 Sertoli cell death (lethal concentration of 50%, LC_{50} = 18 μM) (Ogunbayo et al., 2008; Yin et al., 2018; Szychowski and Wojtowicz, 2016; Strack et al., 2007). Cells exposed to 1–100 μM TBBPA would increase the cytotoxicity, such as growth inhibition (Yin et al., 2018), increased ROS (Choi et al., 2017), imbalanced Calcium (Zieminska et al., 2017), and reduced ATP synthesis (Nakagawa et al., 2007). Although previous studies used TBBPA concentrations higher than the environment and human plasma, their results provide an indispensable reference for our low concentration exposure research.

Based on TBBPA concentration detected in occupational population level in plasma (Covaci et al., 2009), simulating equivalent exposure concentration (1.838–73.3 nM) seemed closer to the actual situation, helping to further dig up its health risks, specifically the risk of human cancer progression. Low concentration TBBPA (nM) causes neither pathological change in tissues nor cell death (Choi et al., 2017; Cannon et al., 2019; Liang et al., 2019a; Park et al., 2019). However, the cytotoxicity of long-term exposure to low concentration TBBPA on cell metabolism has still not been attempted yet, although similar studies involving the effect of bisphenol A on promiscuous sexual orientation have been verified in zebrafish, mice, and other animal models (Poimenova et al., 2010; Porrini et al., 2005; Chen et al., 2016a, 2016b).

This study investigates the chronic exposure of low concentration TBBPA at the occupational population level, using HepG2 cell line as the research model, and analyzing reactive oxygen species (ROS) level, apoptosis measurements, RNA-sequencing analysis, and gene expression level verification. This study explores physiological and metabolic changes, clarifying the cytotoxic effects, genetic effects, metabolic effects, and their correlation mechanism induced by low concentration TBBPA exposure.

2. Materials and methods

2.1. Materials

TBBPA enters the body mainly through diet, breath, and skin contact. After long-term exposure, part of TBBPA gets degraded or expelled from the body, while part of it continues to accumulate. According to the European Union Risk Assessment Report, the highest ^{14}C labelled TBBPA radioactivity level in the liver was detected 8 h post-dosing (ECB, 2006). The purpose of this study is to explore the health risk of TBBPA at the actual plasma concentration. TBBPA accumulates mostly in the liver; therefore, representative liver cells were selected as the research object. The human hepatocellular liver carcinoma cell line (HepG2) was generously provided by the Guangzhou Medical University. All other chemicals and reagents used in this work are summarized in Supporting Information (SI).

2.2. Cell culture and TBBPA exposure

The cell line was grown in DMEM medium supplemented with 10% fetal calf serum (FBS) and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 humidified cell culture incubator.

Based on the exposure concentrations of TBBPA at the occupational population level in plasma (Covaci et al., 2009), two exposure concentrations of TBBPA were selected to simulate occupational exposures at Low and High concentrations of 1 and 81 nM, respectively. The stock solution of TBBPA was prepared in dimethyl sulphoxide (DMSO). The final DMSO concentration in the medium was 0.1%. The medium containing TBBPA changed after the cells stuck to the culture plate wall and was changed daily. Triplicate experiments were conducted for each exposure group of Low or High concentration TBBPA.

2.3. Cell proliferation activity test

Cell proliferation activity was detected with Real-Time Cell-based Analyzer (xCELLigence RTCA S16, Agilent, China), placed in a 5% CO_2 humidified cell culture incubator. Growth curves were constructed using disposable E-plates with 16 wells containing integral sensor electrode arrays. A detailed procedure is also provided in the SI. HepG2 cells were cultured in a 6-well plate for TBBPA exposure. The cell cycle analysis was performed using the method reported by Herman-Antosiewicz and Singh (2005), and a detailed procedure is also provided in SI.

2.4. Apoptosis and lactate dehydrogenase (LDH) assay

The cells were stained using the cell apoptosis detection Kit (Beyotime) according to the manufacturer's instructions. The cell pellet was washed twice with a phosphate-buffered solution (PBS). The 1 × Binding buffer was then used as the dyeing buffer and added to the cell suspension to achieve a final concentration of 1×10^6 cells/mL. Then propidium iodide (PI) was added in the dark. After 30-min incubation in the dark, the cells were immediately analyzed using Cyan flow cytometer and ModFit software.

HepG2 cells were cultured in 6-well plate overnight in 5% CO_2 humidified cell culture incubator. The existing medium was changed into a new medium containing TBBPA (1 or 81 nM at Low or High concentration), with 0.1% DMSO as the negative control. After 24 h cultivation, the supernatants were transferred into a 96-well plate, and their absorbance was measured at 450 nm using a microplate reader (Thermo Fisher, USA). The degree of cell membrane damage was calculated from the ratio of LDH released from sample to the total protein extracted from cells in a homologous well. The quantification procedure of the total protein is provided in SI.

2.5. Wound healing assay

HepG2 cells were seeded into 6-well culture plates and allowed to grow to 90% confluence. Similar-sized wounds were introduced to the monolayer cells using sterile pipette tips. Wounded monolayer cells were washed 3 times by PBS to remove cell debris. TBBPA (1 or 81 nM at Low or High concentration) was then added with DMSO (0.1%, v/v) as the negative control. The wound closure speed was monitored and photographed with an inverted microscope (Olympus, Beijing) after 12 and 24 h, respectively. The relative wound closure speeds of treated cells against the control were calculated using the following formula:

(the wound width of treated cells at 0 h – the wound width at 24 h) / (the wound width of the control cells at 0 h – the wound width at 24 h) × 100.

2.6. Mitochondrial reactive oxygen species (mitoROS) measurement

HepG2 cells were exposed to TBBPA (1 or 81 nM) and 0.1% DMSO as the negative control. After 24 h cultivation, the mitoROS levels were measured with MitoSOX Red Mitochondrial Superoxide Indicator. The cells were incubated with HBSS (Hank's Balanced Salt Solution)/Ca/Mg solution containing 5 μM MitoSOX reagent for 10 min at 37 °C in dark,

followed by cleaning with PBS. The MitoSOX (Ex580nm/Em510nm) fluorescence was measured using flow cytometry.

2.7. Mitochondrial membrane potential (MMP) examination

MMP was measured using the JC-1 assay kit according to the manufacturer's protocol. The detailed procedure is provided in SI.

2.8. RNA isolation and relative assay

Total RNA was extracted from HepG2 cells using the TRIzol reagent. cDNA was obtained by reverse-transcription through MonScript™ RTIII All-in-One Mix with dsDNase (Wuhan, China). Real-time q-PCR was performed using TB Green® Premix Ex Taq™ II with CFX96 Touch System. The real-time q-PCR procedure is also provided in SI, and the primer sequences are shown in Table S1.

RNA-seq analysis was also performed following standard procedures. Sequencing of total RNA from HepG2 cells treated with TBBPA, DMSO, or DMEM was performed in Majorbio Company (Shanghai, China). Based on the quantitative results of expression levels, the genes with differential expression between two groups of Low and High concentrations were analyzed. The difference analysis software was edgeR, and the screening threshold was $|\log2FC = \geq 1$ & p adjust < 0.05. The free online platform of Majorbio Cloud Platform was used for the data analysis.

2.9. Statistical analysis

Results are expressed as average ± SD. Statistical significance was evaluated using the Student's *t*-test. The P-value was determined, and the significance level was set at $P < 0.05$. Microsoft Excel was used to perform all statistical analysis.

3. Results

3.1. Low concentration TBBPA promoted HepG2 cell proliferation

The occupational population (8 h work per day) is exposed to an average TBBPA concentration of 3435 ng/g, dw (Lu et al., 2018), following which TBBPA gets accumulated in the body through breathing, skin contact, and even diet. After debromination and derivatization in vivo, TBBPA level in plasma was obtained as 1–80 nM (Jakobsson

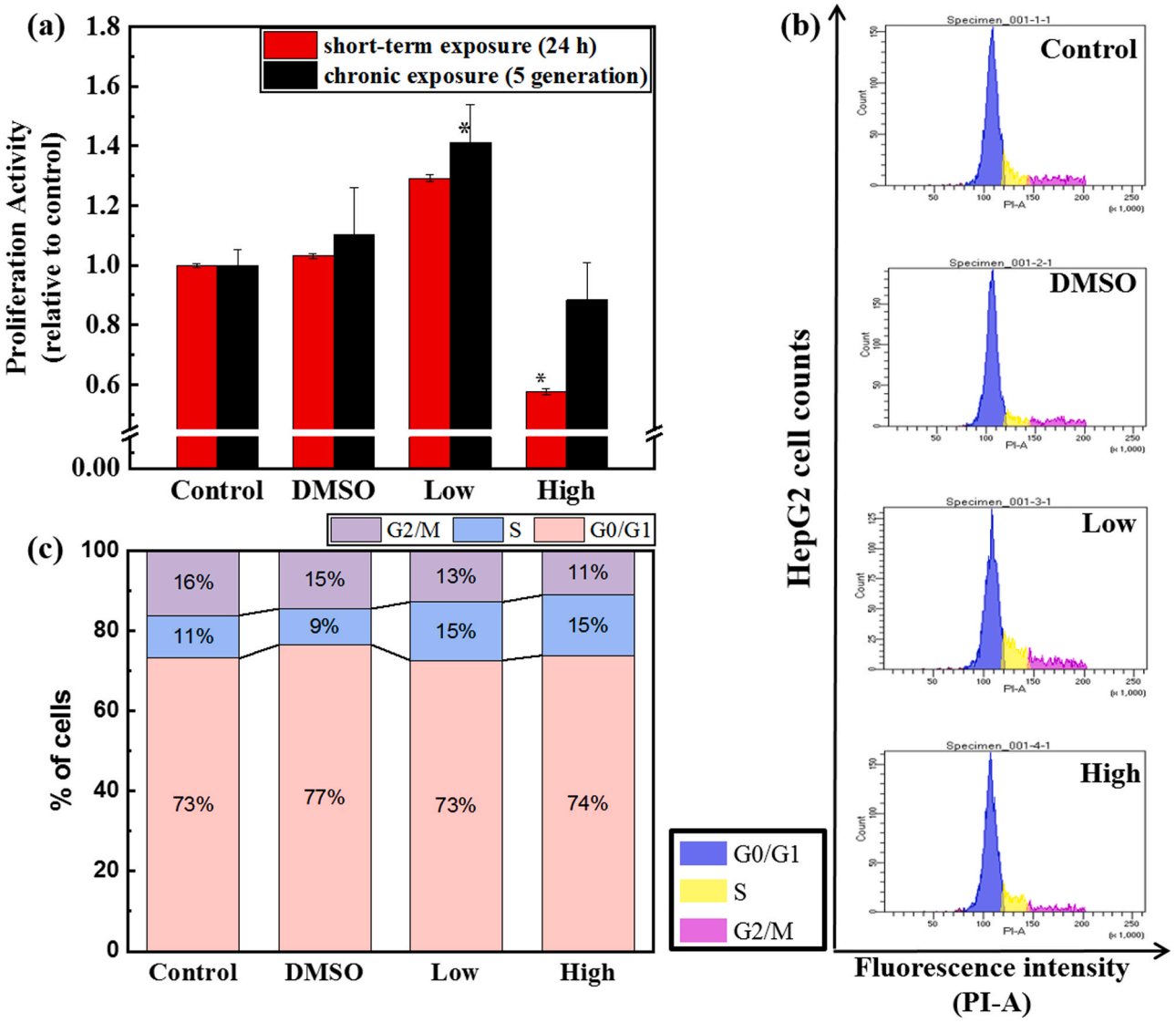

Fig. 1. Cell activity assay. (a) Cell growth of HepG2 cells treated with TBBPA. *$p < 0.05$ ($n = 3$) as compared with matched controls, by student's *t*-test. ((b) and (c)) Cell cycle progression in HepG2 cells. Control means DMEM treated samples, and DMSO stands for negative control. Low and High group represent TBBPA exposure at 1 and 81 nM, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., 2002). HepG2 cells were chosen in this study to characterize the toxicity to the hepatic metabolic system from exposure to low-levels of TBBPA.

First, HepG2 cells were exposed to Low and High concentrations of TBBPA. We found that the cell proliferation activity got promoted from 16.9% to 30.8% times of the Control when exposed to low levels of TBBPA (0.33–27 nM) within 24 h, while 42.3% and 52.2% restrained with high levels of TBBPA (81–243 nM) (Fig. S1A). To simulate long-term exposure mode of the occupational population, five generations of TBBPA exposure experiments were carried out, in which 1 and 81 nM TBBPA were chosen as the representatives of Low and High concentration groups, respectively. As Fig. 1a illustrates, long-term exposure to 1 nM TBBPA increased the proliferation activity by 41.1%, roughly 11.4% more than the 24 h exposure. However, the proliferation activity of 81 nM TBBPA was still 11.7% restrained, almost 30.6% of it was restored.

Besides, in order to intuitively reflect the activity of cells caused by TBBPA exposure, a wound-healing assay was also conducted. In line with the proliferation activity assay, TBBPA at the tested concentrations of 1 nM enhanced the cell wound healing by 34.3%, 16.8%, and 1.7% at 6, 12, and 24 h, respectively, from the start of the scratch (Fig. S2). Besides, the cell wound healing of HepG2 was enhanced by 26.7%, 34.3%, and 0.6% at 6, 12, and 24 h of TBBPA exposure, respectively. Combined with the restrained proliferation activity, we speculated that the migration of HepG2 was enhanced with TBBPA exposure. After 24 h exposure, the difference in the scratch closure rate of them and the Control group was insignificant, probably due to the high cell density and the cells not being in the logarithmic growth phase.

For further characterizing the proliferation ability after TBBPA exposure, a cell cycle progression assay was conducted. According to cell cycle progression analysis, at G0 and G1 phase, the genome is diploid before DNA synthesis, which has a relatively low fluorescence intensity and can be observed as a blue peak in Fig. 1b. No significant differences were observed in G0/G1 phases compared with the Control, indicating that TBBPA exposure neither blocked cell cycle progression nor affected the RNA synthesis. With the start of DNA synthesis, the genome ranges between diploid and tetraploid, and the fluorescence intensity also varies between two peaks, highlighted in yellow color in Fig. 1b. TBBPA's effect on DNA synthesis rate was estimated during S-phase from the number of cells in the S-phase at different exposure concentrations. The ratio of cells in the S phase increased by 36.3% after TBBPA exposure (Fig. 1c), indicating that the TBBPA-treated cells were under higher cell division state, thereby confirming a higher proliferation activity. After DNA synthesis, the protein and RNA synthesis begin (G2-phase), followed by mitosis (M-phase), represented by the purple peak in Fig. 1b. Owning to higher ratio in S-phase while steady at G0-phase, it is evident that the mitosis was shortened by 18.8–31.3% in the TBBPA treated-samples, fastening the cell to enter another cell cycle.

These findings demonstrated the low concentration TBBPA exposure can increase cell proliferation, which might be the cellular response to foreign substances and can accumulate with prolonged exposure times. Besides, cells with long-term TBBPA exposure are also prone to get higher scratch closure rate by promoting proliferation and migration. However, the cellular mitosis was less integrated than before, which may affect the genetic consistency of the cells after the division.

3.2. MitoROS levels elevated to maintain cellular stability

MitoROS in HepG2 was measured to investigate the role of oxidative stress from TBBPA exposure. As Fig. S1B presents, no significant effect was observed on mitoROS production in HepG2 cells after 24 h exposure. However, with prolonging the exposure time, the mitoROS levels were increased to 11.9% and 4.5% at 1 and 81 nM levels, respectively

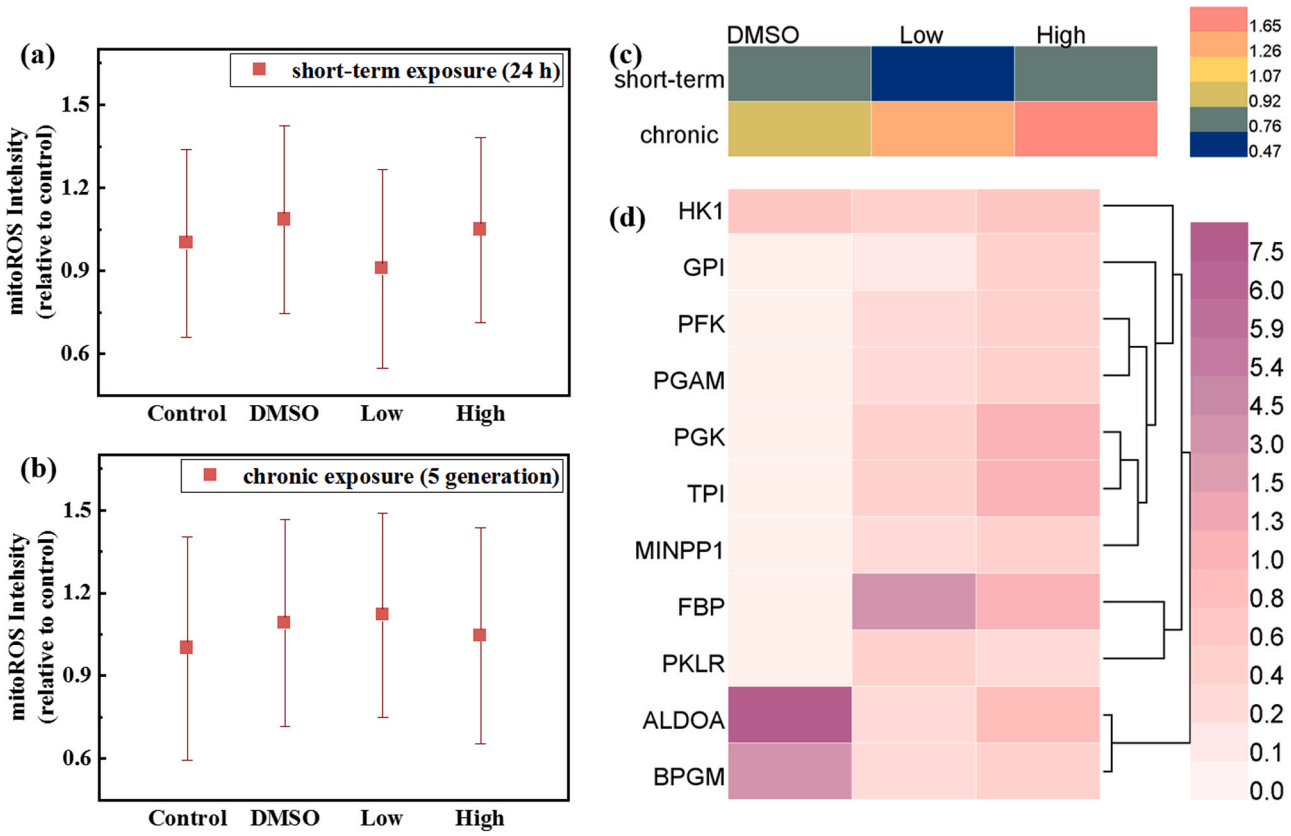

Fig. 2. TBBPA induces ROS generation in mitochondria of HepG2 cells treated with (a) 24 h and (b) five generation, respectively, and the fluorescence intensity of MitoSOX in mitochondria were analyzed with Flow Cytometer (FCM); (c) The q-PCR test for AMPK expression; (d) Heatmap depicting glycolysis related genes expression in HepG2 cells.

(Fig. 2b). Even at this low concentration, TBBPA could slightly stimulate mitoROS production. Although the effect was minimal, these could accumulate with an increase in the exposure time.

It has been well documented that high AMPK (Adenosine Monophosphate activated Protein Kinase) levels can change target cells from an anabolic to a catabolic state, followed by high OXPHOS (OXidative phosphorylation) and ROS levels. In turn, mitoROS could also activate AMPK (Nejabati et al., 2020). Then, the adaptive cellular response provokes stress resistance and improves life span. To investigate whether the AMPK was activated, the gene expression level of AMPK was also measured. As Fig. 2c shows, no significant difference was observed in the AMPK expression levels in HepG2 cells between the

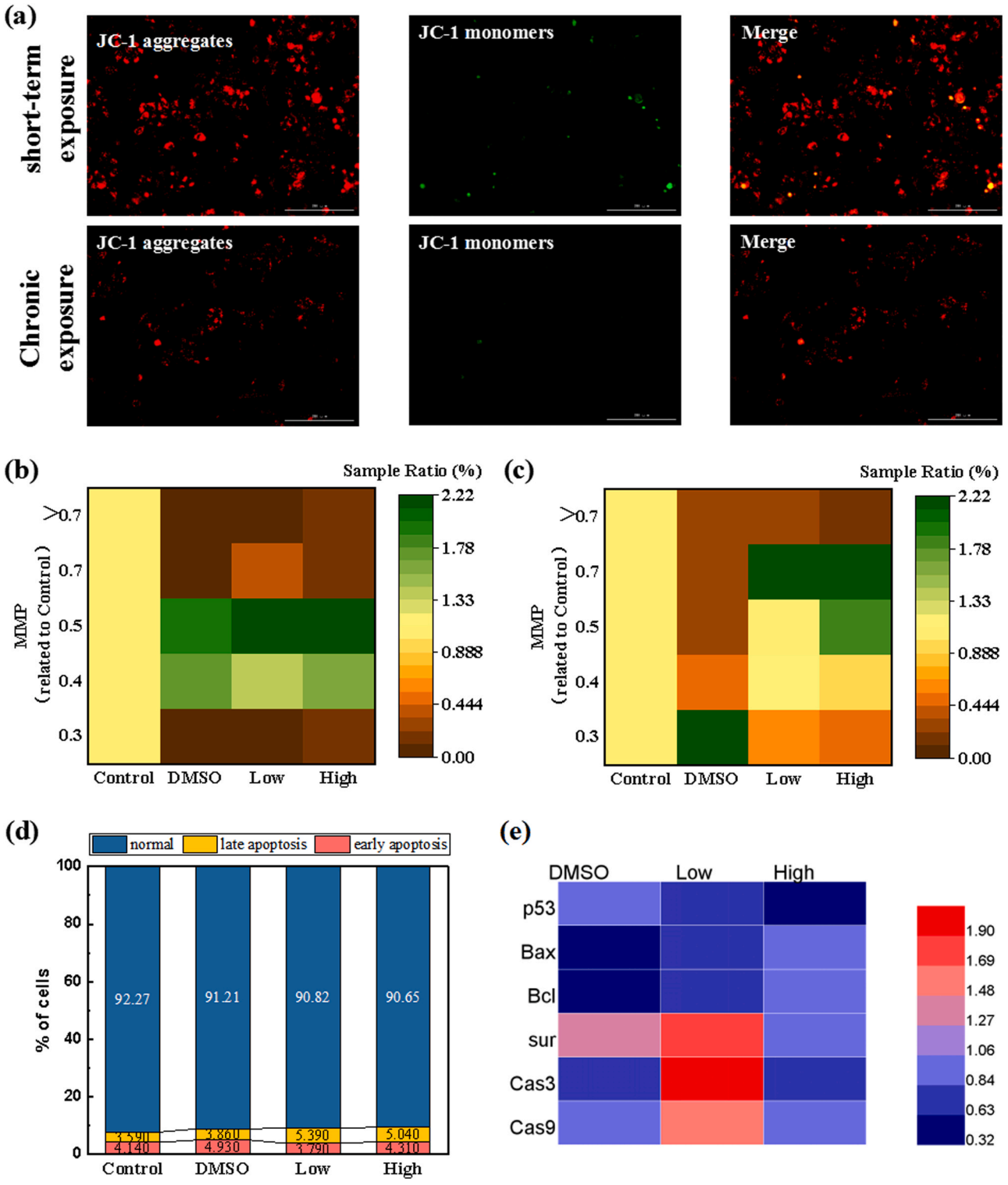

Fig. 3. TBBPA induces the change in mitochondrial membrane potential of HepG2 cells. The fluorescence intensity of JC-1aggregates and JC-1 monomers in mitochondria were analyzed with laser confocal microscope. (a) Immunofluorescence for JC-1 aggregates and JC-1 monomers in mitochondria. Scale bar, 200 μm; HepG2 cells were treated for (b) 24 h and (c) five generation, respectively. (d) Histogram show apoptotic cells ratio by TBBPA. (e) Heatmap depicting apoptosis related genes expression in HepG2 cells.

negative control (DMSO) and TBBPA exposure groups after 24 h. In contrast, the AMPK pathway was activated, as evidenced by a 63% and 86% increase in the AMPK expression after long-term 1 and 81 nM TBBPA exposure, consistent with the increase of mitoROS levels.

To ascertain whether the activated AMPK pathway could promote metabolic rate, the Glycolysis-related gene expression was further measured during the TBBPA exposure. As the ROS was mainly related to mitochondria, the based glycometabolism (e.g., glycolysis) was mainly considered. As Fig. 2d shows, within the two TBBPA-exposure groups, while two genes (ALDOA and BPGM) were significantly down-regulated by 87.3–95.1%, most of the remaining genes were slightly up-regulated. Specifically, the most significantly up-regulated gene was found to be FBP, which was up-regulated 246 times at 1 nM TBBPA exposure level. Likewise, gene PFK was 435-times up-regulated at 81 nM exposure level. Altogether, the above data support that TBBPA could invoke adaptive cellular response by tenderly increasing ROS levels, which is agreement with the activation of the AMPK signaling pathway and improving proliferation and longevity of HepG2 cells through glycolysis.

3.3. Low concentration TBBPA mediated cellular apoptosis

Considering that both mitoROS and OXPHOS are related to mitochondria, the mitochondrial membrane potential (MMP) was also measured to evaluate the mitochondrial status in TBBPA exposure cells. As Fig. 3b shows, the heatmap represented the ratio of the aggregates and the monomeric form of JC-1, using which the percentage of mitochondrial depolarization was measured. Within 24 h exposure, the majority of MMP ratio was around 0.4–0.5 after TBBPA exposure, 1.37–2.21 times higher than the Control group. Similar to the ROS results, with prolonging exposure time, the majority of MMP ratio also increased to 0.7 (Fig. 3c), indicating that TBBPA exposure could promote MMP. The continuously accumulated MMP indirectly suggested that more energy was generated from mitochondria, causing faster respiration and energy conversion after TBBPA exposure.

Furthermore, the relationship between the increase of MMP and cell apoptosis was also clarified. After TBBPA exposure, the cells in green gate (forward angular light scattering) representing the cells at early apoptosis stage decreased, while the cells in blue gate (both forward and lateral angular scattering) representing late apoptosis stage increased (Fig. S4). As Fig. S1D presents, after 24 h exposure, no significant effect was observed on apoptosis in HepG2 cells at 0.33–81 nM TBBPA exposure. However, after long-term TBBPA exposure, the percentage of late apoptotic cells was found to be 1.5- and 1.4-fold than at 1 and 81 nM TBBPA exposure, respectively. In contrast, the percentage of early apoptosis cells was on the opposite (Fig. 3d), which was also confirmed by the gene expression assay (Fig. 3e). That is, the genes that regulate the release of chemical signals like caspase-3 and caspase-9 were found to be 1.65–2.61 times up-regulated. However, the pro-apoptosis gene of p53 was down-regulated by 28.7% and 43.6%, and the anti-apoptosis gene of Bcl was up-regulated by 53.9% and 79.6% at 1 and 81 nM TBBPA exposure, respectively. These results demonstrated that TBBPA exposure not only led to mitochondrial hyperactivity, which is the opposite of apoptosis, but can also activate pro-apoptosis gene, thereby promoting apoptosis procession. Combined with the effect on proliferation activity, it can be seen that low concentration TBBPA could mediate cellular apoptosis through two different mechanisms, leading to an increase in the ratio of abnormal cells.

3.4. Low concentration TBBPA affected the gene expression

To delineate the effect of TBBPA exposure on HepG2 cells, RNA-seq analysis was further performed, and the differences between samples and some enrichment pathways involved with samples were discovered by bioinformatics analysis. The samples were grouped into the Control, DMSO, Low and High concentration groups according to HepG2 cells cultured with only medium, medium with DMSO, medium with 1 nM TBBPA, and medium with 81 nM TBBPA, respectively. The principal component analysis (PCA) revealed that the sample similarity between the four groups was farther apart (Fig. 4a), meaning that each of these pairs had a large difference from each other, and TBBPA could significantly influence the gene expression. Furthermore, the correlation between two TBBPA treatment groups was much stronger than between the Control and either TBBPA treatment groups (Fig. 4b), confirming the rationality of our experimental design. By Venn analysis through RSEM, 13,711 genes were found to be getting co-expressed among four differently treated groups (Fig. S5A). A group was established based on the co-expression genes, and the difference of gene expression levels and the gene differential expression (DEG) was further screened out. Compared with DMSO group, 208 genes were up-regulated, and 132 were down-regulated in the Low concentration group. In contrast, 217 genes were up-regulated and 125 genes down-regulated in High concentration group (Fig. 4c). To determine the specific physiological changes after TBBPA exposure, the co-expression genes were divided into four categories based on the expression levels in two Low and High TBBPA exposure groups compared with the DMSO group. From the Venn analysis based on these four categories, 46 genes were both up-regulated compared with DMSO, while 18 genes were down-regulated (Fig. 4d).

Furthermore, functional annotation analysis of DEGs in both Low and High concentration groups was carried out based on the eggNOG database. As Fig. S5B shows, compared with DMSO group, based on the seven classes of KEGG metabolic pathways, which consist of Metabolism, Genetic Information Processing, Environmental Information Processing, Cellular Processes, Organismal Systems, Human Diseases, and Drug Development, respectively. In the first six classes, 5, 1, 2, 4, 10, and 8 pathways were involved in both Low and High concentration groups. Among these, the pathways involving more than three genes were Folding, sorting and degradation, Signal transduction, Cell growth and death, Endocrine system, Immune system, Cancer overview, bacterial related Infections disease, viral related Infections disease, and substance dependence. After TBBPA exposure, the expression level of metabolism and organismal systems-related genes were changed based on GO classification statistical results. Some genes related to cellular processes, biological regulation, cell component, organelle component, membrane component, and binding process were up-regulated (Fig. S5C). In contrast, some genes related to cellular process, biological regulation, cell component, organelle component, and binding process were down-regulated (Fig. S5D). In all, TBBPA at the occupational population level in plasma can invoke cell stress reactions. In this process, endocrine system related genes were up-regulated, followed by the signal transduction related genes, explaining the enhanced proliferation activity, up-regulation of Glycolysis-related genes, and increased MMP (Figs. 1a, 2d, 3b, and 3c).

To further clarify the specific function of the DEGs, the genes significantly up-regulated and down-regulated in the Low and High TBBPA exposure groups of 1 or 81 nM were further chosen. After verifying the up- and down-regulation state through q-PCR, 29 distinct genes whose gene expression levels were different from both the Control and negative control (DMSO) groups were screened out. The gene expression of 12 genes was testified up-regulated, while 17 genes were testified down-regulated (Fig. 4e). Among them, the ACSL5, RPL44, and PLCXD3 genes were the top three up-regulated genes with an up-regulation range of 5.50–136.24 times. Meanwhile, the CYP2B6, CYP4F3, and RPL31 genes were also the top three down-regulated genes, ranging from 9.43 to 105.05 times. As Fig. 4e shows, after TBBPA exposure, the genes related to metabolism stage changed, with 1.25–136.24 times up-regulated lipid metabolism genes ACSL5, AOX1, and PLCXD3, but 1.08–21.88 times down-regulated vitamin metabolism genes CYP2B6, SLC19A2, and CYP4F3, indicating the metabolic pathways most affected by low concentration TBBPA exposure. It is known that CYP (cytochrome P450) enzymes can metabolize exogenous compounds in liver microsomes (Hedrich et al., 2016). We believe the toxicity under chronic exposure of TBBPA was caused by multiple

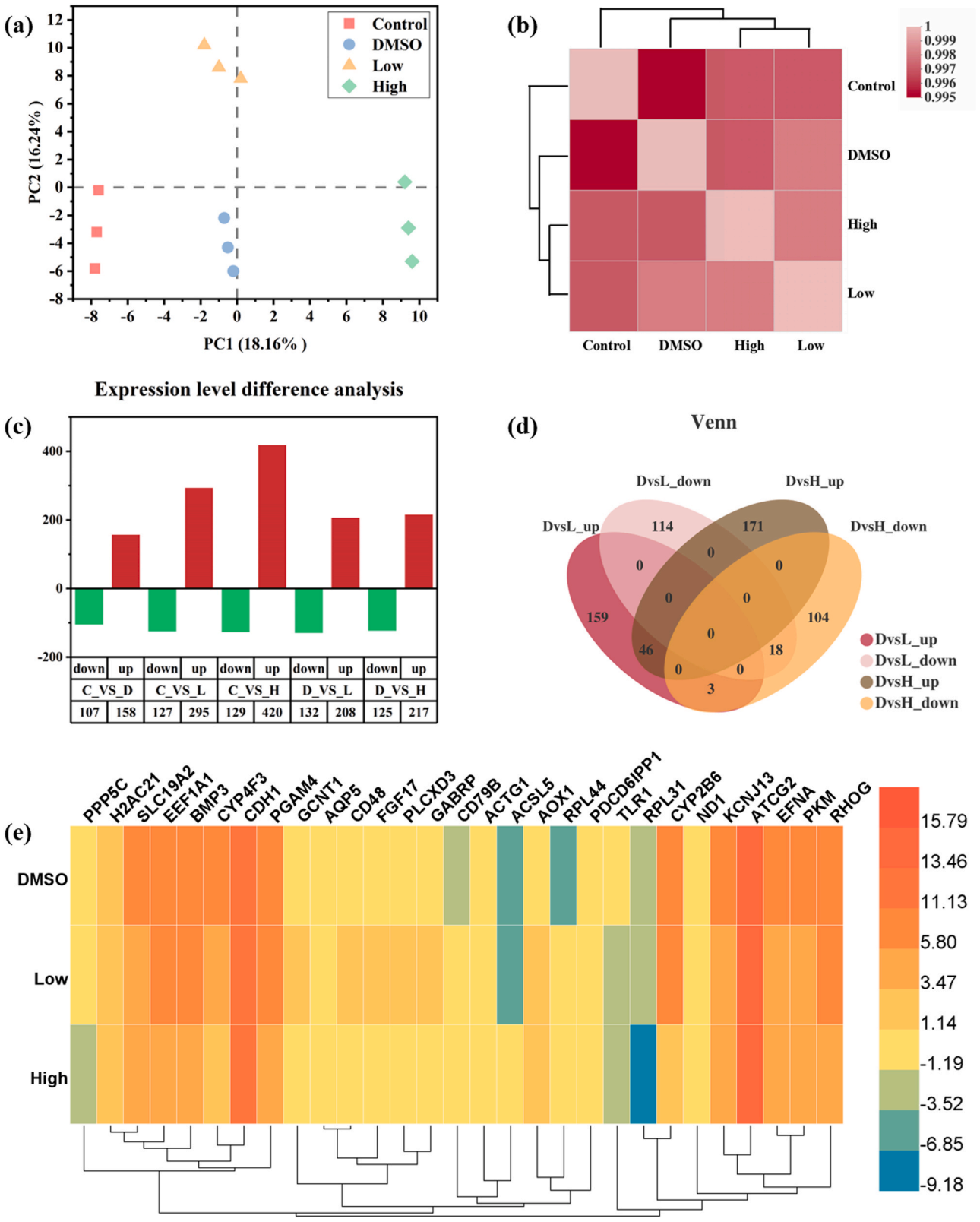

Fig. 4. The bioinformatics analysis to gene expression profiles. (a) The PCA showing PC1 and PC2 for all RNA-seq data of TBBPA-Low and TBBPA-High. The distance between two samples indicates the similarity of different samples. The increasing distance means the growing difference. (b) Correlation analysis result for RNA-seq. (c) The expression level difference analysis. (d) The Venn diagram describes the co-expression and special expression genes among four groups based on the expression level difference analysis. (e) The q-PCR test verification the significantly differentially expressed genes.

substances, including TBBPA and its derivative metabolites under metabolic processes such as oxidative debromination, reductive debromination, oxidative CYP enzyme-mediated biotransformation, Phase II conjugation (Hakk and Letcher, 2003). Based on the down-regulation of CYP2B6 and CYP4F3 after TBBPA exposure, we speculated that the process of TBBPA metabolism was impeded. Besides, BMP3, a gene-mediated nucleotide metabolism through mediating gene expression involving gonadal growth and embryonic differentiation, was also 4.87 times down-regulated at 81 nM TBBPA exposure levels. In comparison, CD48 capable of inducing gene expression that releases growth factor through CaN was slightly up-regulated. These opposing outcomes again verify that after TBBPA exposure, the cells could induce two different pathways to deal with TBBPA toxicity, risking genomic disruptions and leading to metabolism abnormalities. Meanwhile, 1.24–105.05 times down-regulated genes of RPL31 and EEF1A1 as well as 7.88- to 8.76-fold up-regulated RPl44 gene, all participated in protein biosynthesis and metabolism process, indicating that the rate of protein biosynthesis and metabolism process were all affected after TBBPA exposure.

As Fig. 4e shows, in keeping with MMP result, GABRP was also found to be induced hyperpolarization with 4.16-fold up-regulation at 1 nM TBBPA exposure level. One of the outcomes of this result was the inhibition of apoptosis. Consistent with this result, the gene TLR1 and ppp5c mediated apoptosis pathways through the gene MyD88 and gene MAPK, respectively, were also found to be slightly down-regulated. This can further explain the above-mentioned heatmap result that one of the pro-apoptosis genes were down-regulated (Fig. 3e). Unexpectedly, consistent with GO annotations analysis (Fig. S5E), cellular skeleton-related genes like ACTG2 and RHOG were also 1.55- to 4.04-fold down-regulated. Besides, RHOG could also mediate actin-β through Rac signal pathway (Klems et al., 2020), thereby down-regulating ACTG2. Besides skeleton protein, the expression of genes coding transmembrane protein like KCNJ13, SLC19A2, and AQP5 were all found to be 1.36–6.76 times down-regulated after TBBPA exposure (Fig. 4e), indicating that TBBPA could impair the stability of cellular skeleton and the transmembrane transport of the cell membrane.

In addition, mitochondrial complex I related gene *ND1* and ion transport protein-related gene KCNJ13 were also found to be 1.16- to 6.76-fold down-regulated in both Low and High concentration TBBPA treated groups, while the glucose uptake related gene of GCNT1 was up-regulated (Fig. 4e). As is well known, the mitochondria of eukaryotes consist of five complexes, together affecting normal functioning of glycolysis, electron transport chain, energy metabolism, and oxidative stress (Martinez-Reyes et al., 2016). Therefore, we can conclude that TBBPA exposure can regulate mitochondrial function in HepG2 cells, evidenced by MMP promotion, ROS generation, glucose ingestion, and glycolysis-related gene upregulation according to the results shown in Figs. 2–4.

3.5. Low concentration TBBPA activated Ras signaling pathway

From RNA-sequencing results, two growth factors, FGF17 and EFN5A, were found with different expression genes. Their regulation levels were up-regulated and down-regulated, respectively (Fig. 4e),

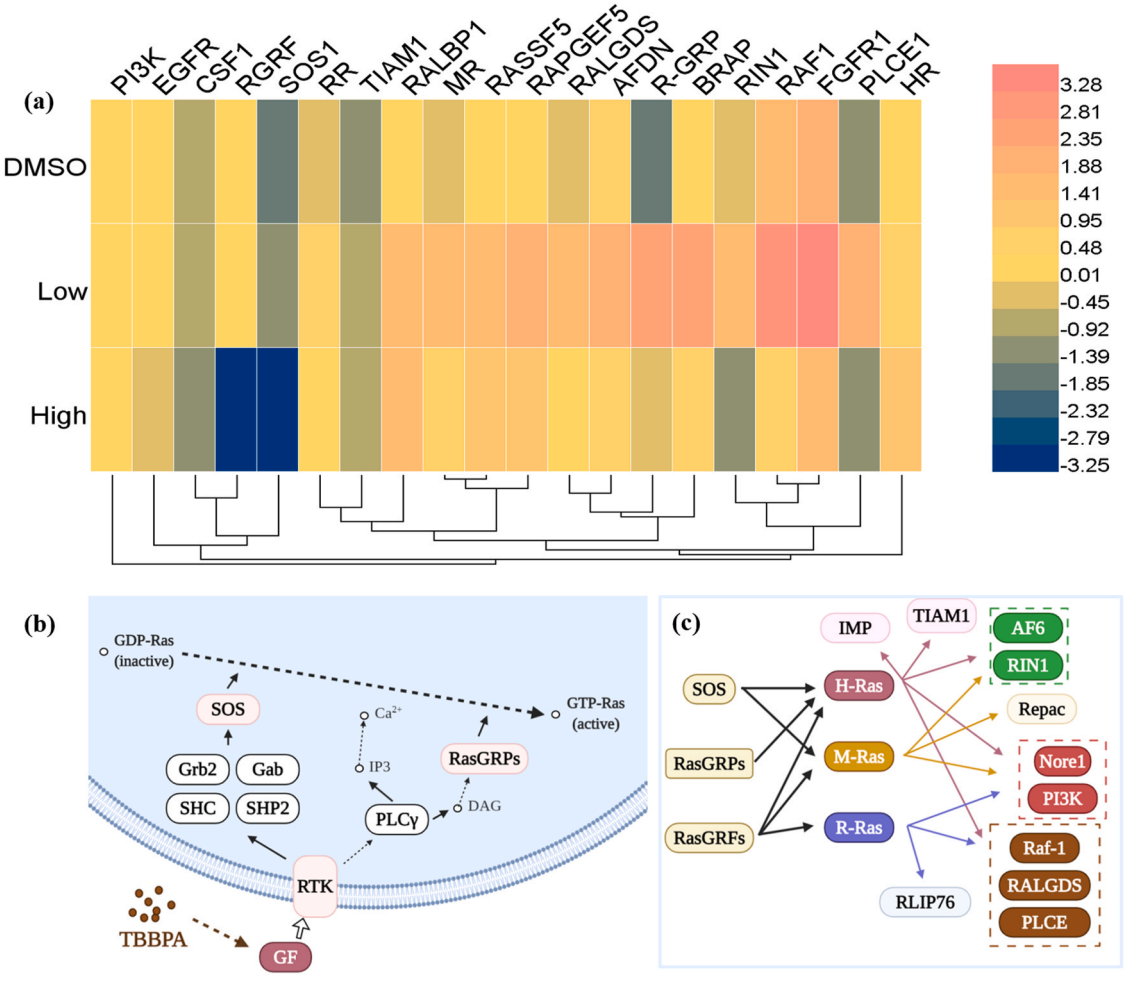

Fig. 5. (a) Heatmap depicting all targeted genes related to Ras signaling pathway in HepG2 cells. (b) Ras upstream signaling pathway. (c) Ras downstream signaling pathway (Nore1 also known as RASSF5, AF6 also known as AFDN, IMP also known as BRAP, RLIP76 also known as RALBP1).

after TBBPA exposure. The enriched KEGG term analysis revealed that FGF17 and EFN5A were both involved in the activation of Ras signaling pathway, in which they mainly pass signals into cellular through membrane receptor proteins like CSF1R, FGFR1, and EGFR (collectively known as RTK) (Fig. 5b). FGFR1 was found to be 3.10-fold up-regulated in 1-nM TBBPA exposure group, while CSF1R and EGFR were found to be 1.40–1.60 times down-regulated in 81-nM TBBPA exposure group (Fig. 5a).

To further figure out final results of these superimposed effects upon their co-downstream gene Ras (Steelman et al., 2020; Zhao et al., 2020; Agrawal et al., 2020), the gene expression of three major forms of Ras (Fragoza et al., 2019) (H-Ras, M-Ras, and R-Ras) and the corresponding downstream genes were further measured. From q-PCR test (Fig. 5a), H-Ras, M-Ras, and R-Ras were found to be 1.3–1.8, 1.7–3.2, and 1.5–2.0 times up-regulated after TBBPA exposure, respectively, proving that TBBPA could induce the up-regulation of these three kinds of Ras genes. R-Ras was activated by RasGRFs, which are not mediated through GF and its corresponding receptor. Thus, the genes downstream of H-Ras and M-Ras were considered. As shown, Nore1, TIAM1, AF6, BRAP, RALGDS, and RLIP76 were found to be 1.17–4.03 times up-regulated in both 1-nM and 81-nM TBBPA exposure groups. Among them, five genes of Nore1, TIAM1, AF6, BRAP, and RALGDS were all found to be regulated by either H-Ras or M-Ras. Therefore, based on the q-PCR assay results, we could affirm a high correlation between low concentration TBBPA and Ras signaling pathway. Low concentration TBBPA exposure could induce cellular Ras signaling pathway through the growth factor FGF17. Although a few genes in the Ras signaling pathway were down-regulated through other mechanisms, the total expression situation showed the leading role of FGF17 in this pathway, and the corresponding outcome of the cellular toxicity caused by TBBPA exposure was confirmed herein.

4. Discussion

Since TBBPA has been used as BFRs, several studies have been conducted about toxicity of itself and its derivatives (Yu et al., 2019). Existing in vivo (mice or zebrafish) or in vitro (cells or liver microsome) studies mainly focused on the toxicity at a sub-lethal TBBPA concentration. It can be concluded from the existing studies that low concentration TBBPA exposure would cause neither death nor acute illness. However, low concentration level exposure might disrupt normal physiological and metabolic activities leading to chronic diseases like endocrine, neurodevelopmental or immune system disorders. Given the lack of studies on TBBPA, it is crucial to explore the effects of TBBPA on human beings with occupational population-level exposure in plasma.

This study mainly focuses on the chronic impact of low concentration TBBPA (nM) in vitro. The actual residual concentrations detected in human plasma ranged from tens to hundreds of nM depending upon the exposure level (Covaci et al., 2009). To simulate occupational exposure mode, long-term exposure (also called chronic exposure) experiments were conducted, where HepG2 cells were exposed to low concentration TBBPA for four to five generations. The culture medium containing TBBPA was changed once a day.

The proliferation activity of HepG2 was monitored after TBBPA exposure. The cellular proliferation activity responded most sensitively to 1 nM TBBPA exposure (Fig. S1). The proliferation effect accumulated with prolonged exposure time (Fig. 1a), which disagreed with previous research results that a TBBPA concentration higher than 1 μM can cause a decrease in the cell activity and eventually the cell death (Ogunbayo et al., 2008; Strack et al., 2007), while in line with recent research that demonstrated that TBBPA could promote the proliferation of endometrial cancer cells (Guan et al., 2021; Jin et al., 2021). In keeping with the proliferation, AMPK was activated, improving proliferation and longevity of HepG2 cells through glycolysis (Fig. 2c-d) (Nejabati et al., 2020). Besides, Nore1, one of the downstream genes of Ras, was up-regulated (Fig. 5a), which help to resist the processes of cell-cycle arrest and apoptosis (Li et al., 2018). However, while cell proliferation was promoted, cells were rushed into another cell cycle. The cellular mitosis was simpler than that supposed to be, affecting the genetic consistency of the cells after the division. Herein, the proliferation effect of TBBPA probably is not a short-term stress, and even at this level the toxicity of TBBPA is still worth evaluating.

In line with the migration capabilities of Ishikawa cells, an endometrial cancer cell line, promoted by low concentration TBBPA and its derivatives (Su et al., 2020; Lyu et al., 2020), the scratch closure rate was found to be promoted by TBBPA (Fig. S2), indicating that TBBPA promotes the malignant migration of cancer cells. We also chose 1 and 81 nM as representative Low and High concentration groups to study the cytoplasmic membrane toxicity of TBBPA. Results showed that exposure to TBBPA for 24 h could cause cell membrane damage. However, the damage was also repaired within the exposure duration (Fig. S3). Our 24 h exposure data were consistent with a previous study (Szychowski and Wojtowicz, 2016). Paleo et al. found a conserved mechanism known as “Plasma membrane repair”, which helps cells to survive an injury (Paleo et al., 2020). The level of LDH released in culture media is probably due to stress after 24 h TBBPA exposure, which can be repaired by regulating cell function. The membrane of HepG2 could be repaired even under the long-term stimulation of TBBPA. These studies indicated that low concentration TBBPA exposure may not only cause cytotoxicity, but also evoke the self-saving mechanism of HepG2 cells. However, the RNA-sequencing results further confirmed that the cellular skeleton and the transmembrane transport of the cell membrane-related genes were down-regulated (Fig. 4e), demonstrating that the membrane injury was still genetically regulated.

ROS generation in the cells might be involved in cytotoxicity, apoptosis, and autophagy (Zorov et al., 2014). The majority of ROS are produced by mitochondria, as reported in early reference (Hamanaka and Chandel, 2010). ROS produced by mitochondria (mitoROS) can enter cytoplasm either through free diffusion or voltage-dependent anion channels, playing an important role in signal transduction and maintaining intracellular activities (Mittler et al., 2011). In our study, low concentration TBBPA exposure caused little ROS generation in HepG2 cells in 24 h (Fig. 2a), which is consistent with a previous study (Kuiper et al., 2007) stating that long-term exposure could lead to the accumulation of TBBPA concentration and its effects resulting in the increase of the mitoROS release level when exposure time was prolonged. Our results also confirmed that ROS generation at a relatively low level could activate the AMPK signal and promote cell proliferation and survival (Fig. 2c). Thus, increasing exposure time could increase ROS production and impose a higher cytotoxicity risk, impacting tumor progression (Chandel and Tuveson, 2014).

MitoROS overproduction, the reduction of MMP, and the decreased ATP are three major pathological states when mitochondrial damage occurred (Nejabati et al., 2020; Wang et al., 2020). A similar cumulative effect on MMP has also been observed after long-term exposure to low concentration TBBPA (Fig. 3a). Accordingly, in combination with mitoROS release in a relatively low degree, promotion of MMP, and activated OXPHOS, the mitochondria were speculated to be in a more active state leading to speed up the metabolism. Thereby, a low degree of ROS generation prolonged the lifespan of the cells and resisted toxic injury. High MMP was confirmed to provoke the pathway opposite to apoptosis to offset some of the damages, possibly leading to a high apoptosis state. However, the percentage of apoptotic cells in the total cells still increased after the exposure (Fig. 3d and 3e), confirming that TBBPA had at least two different mechanistic effects on the cell viability, leading to two opposite effects. This also suggested that the self-saving mechanism of HepG2 cells was not strong enough to offset apoptosis (Nejabati et al., 2020; Meng et al., 2020).

During RNA-sequencing, 29 DEGs were identified affected by low-level TBBPA in HepG2. Bioinformatics analysis, consistent with previous studies, revealed that DEGs were involved in regulating multiple biological processes and molecular functions, such as hyperpolarization,

apoptosis, transmembrane transport, mitochondrial function, and glucose uptake (Figs. 2 and 3). Furthermore, lipid-related metabolic activity was enhanced, evidenced by the up-regulated ACSL5, AOX1, and PLCXS3 genes. In contrast, the vitamin-related metabolic activity was inhibited, proven by the down-regulation of cyp2b6, slc19a2, and cyp4f3 genes. It has been reported that the triacylglycerol biosynthesis was promoted while gluconeogenesis process was inhibited in response to Phenanthrene (Phe) treatment through Ras/MAPK/PI3K/akt/FOXO signaling pathways, thereby disturbing the sugar and lipid metabolism (Jiang et al., 2021). Besides, except for vitamin metabolism, CYP (cytochrome P450) enzymes can metabolize exogenous compounds and help cellular metabolization of TBBPA, further reducing its toxicity (Hakk and Letcher, 2003). As a result, the process of TBBPA metabolism was impeded, cells were continually stimulated by TBBPA. The oxidative stress and inflammatory effects persisted, providing growth signals that promote the proliferation of malignant cells (Iliopoulos et al., 2009). However, metabolic activity related to nucleotide and protein was affected but unclear because some of genes were up-regulated while others were downregulated (Fig. 4e). Combined with the DEGs related to nucleotide metabolism without linking with a specific pathway, the sequencing results prove that TBBPA at the occupational population level in plasma can affect gene expression and elevate overall metabolism.

Furthermore, two crucial genes, FGF17 and EFNA, were found upstream of these different expression genes, both of which are growth factors that could pass a signal through the adequate receptor following Ras activation (Fig. 5b). Ras is one of the oncogenes. The occurrence of lung cancer, pancreatic cancer, and colorectal cancer is closely related to its mutation, and the patients with cancer are more liable to possess a higher mutation possibility (Khan et al., 2019). Ras (also known as p21) protein is located inside cell membrane and plays an essential role in signaling cell growth and differentiation. It is a type of guanosine triphosphate (GTP) binding protein (a coupling factor of cellular information transfer), which regulates information transfer through the mutual transformation of GTP and guanosine diphosphate (GDP) (Thurman et al., 2020). However, the activation mechanism of Ras signaling pathway and the self-saving mechanism of healthy hepatic cells following exposure to organic pollutants are still unclear. A study arrived at a similar result that (Yang et al., 2021) TBBPA can activate the FGF10 signaling and promote expression of genes related to cell cycle progression, proving that after TBBPA exposure at the hepatoblast stage, the cells would especially evoke cytokine the growth factor to prolong the life span of the cancer cells. These findings will help reveal the self-saving mechanism and stress injury effect on HepG2 cells with low concentration TBBPA exposure, leading to an overall elevation of the mechanism as a consequence.

CRediT authorship contribution statement

Lirong Lu: Methodology, Formal analysis, Writing - original draft. **Junjie Hu:** Methodology, Data curation. **Guiying Li:** Writing - review & editing. **Taicheng An:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.125797.

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