Binaprofen induces zebrafish liver injury via the mitochondrial pathway

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Abstract. Binaprofen ($C_{18}H_{23}NO_5$) is a drug not commercially available that causes liver injury; however, the underlying mechanism is unknown. The aim of the present study was to determine the mechanism underlying binaprofen-induced liver injury at the genetic level. Zebrafish were treated with binaprofen. Serum biomarkers [alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH)], malondialdehyde (MDA) and glutathione (GSH) content analysis, liver cell morphology examination, DAPI staining, electron microscopy, microarray analysis and reverse transcription-quantitative (RT-q)PCR were performed 12, 24 and 48 h post-treatment to analyze the mechanism underlying binaprofen-induced liver injury. Following exposure to binaprofen, zebrafish serum levels of ALT, AST and LDH increased; MDA content of liver tissue increased and GSH content decreased. Liver cells exhibited mild to moderate vacuolization and mitochondria exhibited vacuolization and disrupted cristae. Liver cell apoptosis rate increased. There were 190 common differentially expressed genes at 12, 24 and 48 h. Gene Ontology analysis showed that the function of downregulated genes was primarily associated with 'DNA replication', 'DNA metabolic process', 'cell cycle', 'cell redox homeostasis', 'mitochondrion' and 'lipid transport'. The function of upregulated genes was primarily associated with 'peroxisome proliferator', 'oxidation activity', 'peroxisome' and 'apoptosis'. Pathway analysis showed that downregulated genes were those pertaining to 'cell cycle', 'DNA replication', 'ribosome', 'spliceosome', 'pyrimidine metabolism', 'purine metabolism', upregulated genes were those pertaining to 'PPAR signaling pathway', 'p53 signaling pathway'; RT-qPCR assay supported the microarray results. The mechanism underlying binaprofen-induced liver injury was associated with lipid peroxidation and apoptosis. Binaprofen downregulated genes associated with lipid transport and anti-apoptosis genes, upregulated pro-apoptosis genes and induces liver cell injury via the mitochondrial signaling pathway.

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Introduction

Drug-induced liver injury (DILI) is a toxic side effect of numerous drugs (1-4), including a number of anti-inflammatory and analgesia drugs (5-8). DILI is the commonest reason for withdrawing drugs from the market and/or issuing warnings and modification of use (9). Data from prospective DILI registries suggest that antibiotics remain the most common cause of DILI (10-12). The American DILI Network (DILIN) reported antibiotics to be implicated in 45.4% of cases (13). Other common drug classes reported by the American DILIN (14) are herbal and dietary supplements (16.1%), cardiovascular agents (9.8%), central nervous system agents (9.1%), anti-neoplastic agents (5.5%) and analgesics (3.7%). DILI includes the whole spectrum from asymptomatic elevation in liver tests to acute liver failure (ALF). In fact, DILI remains the most common cause of ALF in the UK (14) and USA (15).

Binaprofen is an anti-inflammatory drug that is not currently in market, and still in clinical study; its chemical structure is $C_{18}H_{23}NO_5$ and it relieves fever and analgesia (16-18). It exhibits a notable analgesic effect. Studies have done about the pharmacological and toxicological effects of binaprofen (19,20), and it has been clinical licensed in China.

Our previous study (21) demonstrated that binaprofen induces liver toxicity and damage similar to acetaminophen (APAP) in zebrafish. APAP is one of the most widely used antipyretic and analgesic drugs in the US. It is reported to be regularly consumed by over 60 million Americans on a weekly basis (22). Though it is safe at therapeutic doses, an overdose can cause severe liver injury and even ALF in humans (23). The mechanisms that underlying APAP-induced liver injury have been extensively studied (24,25). So APAP was chosen as positive drug in this paper. To the best of our knowledge, however, the mechanism underlying liver injury has not yet been revealed. The present study aimed to determine this

Table I. Primer design.

Primer	Sequence, 5'→3'
Zebrafish-Zgc136383-F	GTTCCCATCAATCCAGACGGT
Zebrafish-Zgc136383-R	TGACAGTTCTGCATCAACACATC
Zebrafish-Zgc123120-F	CCAGACACCTCCCCTCATT
Zebrafish-Zgc123120-R	CTCTCCAGCACAACTTCCC
Zebrafish-Eif4ebp3l-F	AAGAAAGCACATCAGAACATAAA
Zebrafish-Eif4ebp3l-R	GAAATCCAGGCAAACGAAA
Zebrafish-Cap3-F	CCGCTGCCCATCACTAGA
Zebrafish-Cap3-R	ATCCTTTCACGACCATCT
Zebrafish-Loc100330641-F	TGAGATTGCTAATGGTGTTGGC
Zebrafish-Loc100330641-R	ACATAGCCGTACCATTGACACTTGC
Zebrafish-Vtg6-F	TGAGTATGCTAATGGTGTGGTTGGC
Zebrafish-Vtg6-R	TGTTCTGCGTCTTCTTGAGGTTGAG
Zebrafish-GAPDH-F	GTGACCCCTTTGCTGTTTCTTT
Zebrafish-GAPDH-R	GGCACGTGGTGCAAACATT

F, forward; R, reverse; Zgc136383, vitellogenin 4; Zgc123120, BCL2/adenovirus E1B interacting protein 4; Eif4ebp31, eukaryotic translation initiation factor 4E binding protein 3; Cap 3, apoptosis-related cysteine peptidase 3; Loc100330641, vitellogenin-like; Vtg 6, vitellogenin 6.

mechanism at the genetic level and provide a basis for potential treatment options.

Materials and methods

Maintenance and breeding of zebrafish. Male and female AB-line, 1-2 g, adult zebrafish (*Danio rerio*, 90-100 days post-fertilization; weight, 1-2 g) were obtained from Southern Medical University, Guangzhou, China. Zebrafish were acclimatized for 2 weeks. The animal protocol was designed according to animal welfare and ethics, which was approved by animal care and use committee of Guangzhou General Pharmaceutical Research Institute (Haizhu, China; approval no. was 2012-005). Fish were maintained in aerated water at 23±1˚C, humidity 65%, pH 7.8±1.0, 0.25 g/l hardness, 12/12-h light/dark cycle and density of 1 fish/l with free access to food and water. Experiments were performed using a total of 150 animals, including 75 male and 75 female.

Zebrafish exposure to binaprofen. Our previous study (21) demonstrated that binaprofen at 0.8 mM and APAP at 4.0 mM cause notable liver damage in zebrafish. Therefore, zebrafish were divided into control (untreated), binaprofen (0.8 mM) and APAP (4.0 mM) groups (n=50/group). Zebrafish were exposed to drug at 22.8˚C for 12, 24 or 48 h, then euthanized via 2-step hypothermal shock, as described in AVMA guidelines (26).

Zebrafish were rapidly killed by immersion in 2-4˚C water for 10-20 sec. Exposure was continued for ≥10 min following loss of operculum movement to ensure death. Followed by rapid chilling, zebrafish tail was transected as previously described (27) and 20 μl blood was collected. Exsanguination was performed to euthanize zebrafish. Rapid chilling followed by exsanguination was the recommended 2-step euthanasia method in AVMA Guidelines for the Euthanasia of Animals: 2020 Edition (28).

Serum biomarkers detection. Blood was collected by exsanguination. Serum samples were collected by tail cutting and capillary collection method (n=5/group). Briefly, the tail was transected from cranial to the caudal fin, 100-μl microcapillaries was used to collect zebrafish blood from the cut surface and blood was placed in 1.5 ml centrifugal tube. A total of 200 μl blood was collected as one sample; there were 5 samples/group. Blood was centrifuged for 10 min at 1,800 x g at 4˚C. Supernatant was collected to detect alanine transaminase (ALT), aspartate transaminase (AST) and lactate dehydrogenase (LDH) (ALT assay kit, cat. no. 130301, AST assay kit, cat. no. 130201and LDH assay kit, cat. no. 130503, all kits from Zhejiang Yilikang biotek Company) by biochemical detection method using a biochemical analyzer (7100; Hitachi, Ltd.).

Malondialdehyde (MDA) and glutathione (GSH) detection. The entire zebrafish liver (n=5/group) was collected and placed in 1.5 ml centrifugal tube. 0.9% NaCl was added and liver tissue was homogenized and centrifuged for 10 min at 4,000 x g at 4˚C. Supernatant was collected to detect MDA and GSH (MDA assay kit, cat. no. 20130515 and GSH assay kit, cat. no. 20130428; both kits from Nanjing Jiancheng Bioengineering Institute) using a microplate reader (Elx800; BioTek China) according to the manufacturer's instructions.

Histological analysis. Liver tissue samples (n=10/group) were fixed in 10% formalin for at 25˚C for 24 h and dehydrated with gradient alcohol. Tissue was immersed in 60˚C paraffin wax for 1 h and moved to -10˚C freezing table for 30 min. Paraffin-embedded samples were sliced (5 μm). Sections were dewaxed with xylene and rehydrated in descending

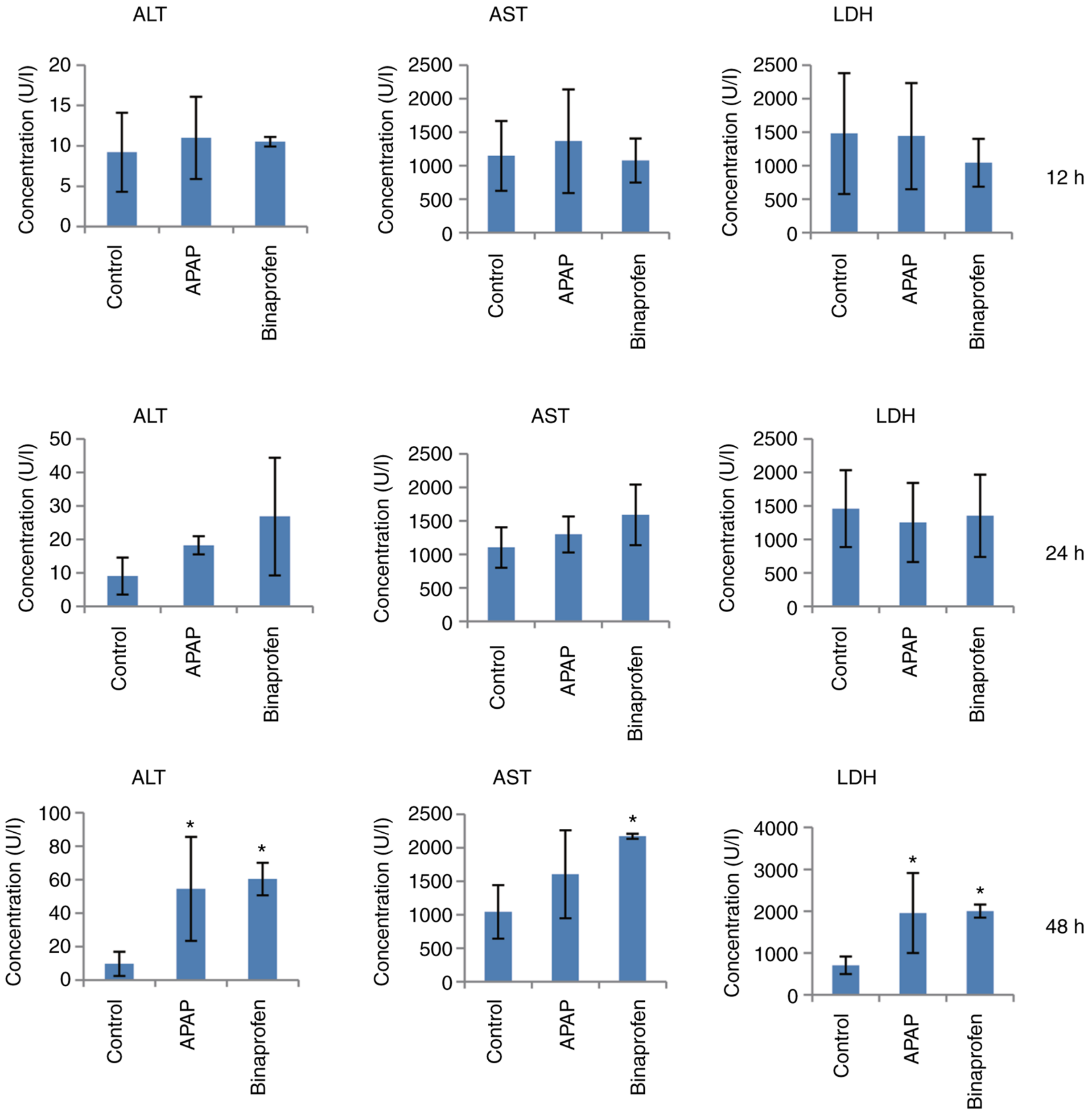

Figure 1. Value of serum biomarkers. ALT, AST and LDH serum levels following exposure to binaprofen or APAP for 12, 24 and 48 h. One-way ANOVA showed no significant difference between treatment groups and control at 12 and 24 h. ALT, AST and LDH increased significantly in binaprofen group at 48 h. *P<0.05 vs. control. ALT, alanine transaminase; AST, aspartate transaminase; LDH, lactate dehydrogenase; APAP, acetaminophen.

alcohol. Slices were dyed with 0.5% hematoxylin aqueous solution at 25˚C for 3 min and 0.5% eosin staining solution at 25˚C for 3 min. 90% neutral balata was used as blocking reagent, slices were blocked at 25˚C for 30 sec. Morphological examination of hepatocytes was performed using a light microscope (BX51; Olympus Corporation) at 40X magnification using image analysis system 11.0 (cellSens Standard; both Olympus Corporation). Samples were scored as previously described (29) by two independent pathologists who were blinded to the experimental groups.

DAPI analysis. Liver tissue slices (n=10/group) were prepared as aforementioned. Slices were stained with 1 μg/ml DAPI at 25˚C for 20 min. Apoptosis of hepatocytes was assessed by fluorescence microscopy (BX51, Olympus Corporation) with fluorescence light, 40x magnification using image analysis system 11.0 (cellSens Standard; both Olympus Corporation, Japan). Five visual fields were randomly selected from each slice to observe the apoptotic cells. Normal cell: complete nucleus and uniform chromatin; Apoptosis cell: nuclear enrichment, deep staining, or crescent-shaped aggregation of nuclear chromatin on one side of the nuclear membrane.

Electron microscopic detection of mitochondria. Liver tissue samples were fixed in 2% osmium tetroxide at 4˚C for 24 h, dehydrated, embedded in Epon/Araldite resin at 4˚C for 1 h and sectioned (70 nm). Samples were stained with premixed solutions of 2% uranyl acetate and lead citrate at 4˚C for 20 min. Ultrastructure examination of hepatocytes

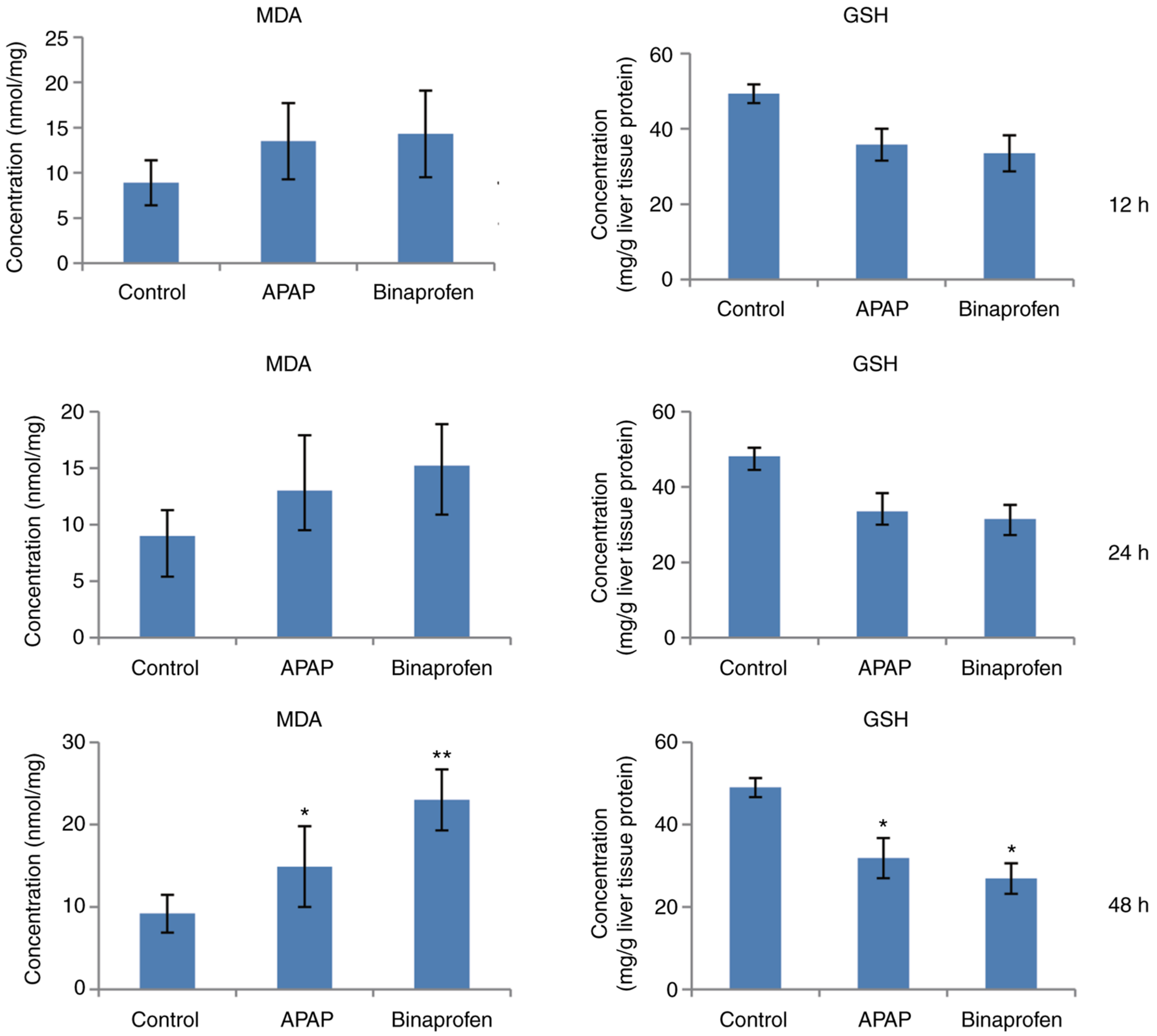

Figure 2. Value of MDA and GSH. MDA and GSH levels in liver tissue following exposure to binaprofen or APAP for 12, 24 and 48 h. One-way ANOVA showed no difference between treatment and control groups at 12 and 24 h. MDA increased and GSH decreased significantly in binaprofen group at 48 h. *$P<0.05$, **$P<0.01$ vs. control. MDA, malondialdehyde; GSH, glutathione; APAP, acetaminophen.

was performed using a transmission electron microscope (JEM-1400; JEOL, Ltd.) with 10x magnification. The morphological changes of hepatocyte mitochondria were examined using image analysis system 11.0 (cellSens Standard; both Olympus Corporation).

Microarray analysis. Livers tissue samples were collected and total RNA was extracted (RNA extraction kit, Qiagen GmbH) and purified. RNA quality was assessed before detection. RNA was hybridized into two gene chip probe arrays (Affymetrix; Thermo Fisher Scientific, Inc.). RNA was used to synthesize double stranded cDNA (iScipt cDNA Synthesis kit; Qiagen GmbH, German) and produce biotin-tagged cDNA. cDNA was fragmented to strands 35-200 bases in length. Fragmented cRNA was hybridized to gene chip array at 45˚C for 16 h using Gene Chip Hybridization Oven640 (Affymerix; Thermo Fisher Scientific, Inc., US). Gene chip arrays were washed using Gene Chip IVT labeling kit (Affymerix; Thermo Fisher Scientific, Inc., US) and stained in SAPE solution at 25˚C for 10 min using Affymetrix Fluidics Station 450 and scanned using Gene Chip Scanner 3000 (both Affymetrix; Thermo Fisher Scientific, Inc., US) with SAPE solution and array holding buffer at 25˚C for 10 cycles.

Gene chip data were analyzed using Gene Chip Operating Software (version 1.4, Thermo Fisher Scientific, Inc.). The criterion of differentially expressed genes was >2-fold change compared with control. Gene Ontology (GO analysis) was performed to determine the function of differentially expressed genes by using Gene Ontology Resource (geneontology.org).

Kyoto Encyclopedia of Genes and Genomes Pathway analysis was performed to determine the pathways associated with differentially expressed genes by KEGG pathway database (https://www.kegg.jp). $P<0.05$ was considered to indicate a statistically significant difference. Common different genes were analyzed using VENN database (bioinformatics.psb.ugent.be).

Reverse transcription-quantitative (RT-q)PCR. Microarray data were validated by RT-qPCR using five samples/group. GAPDH was used as housekeeping for the internal control. A

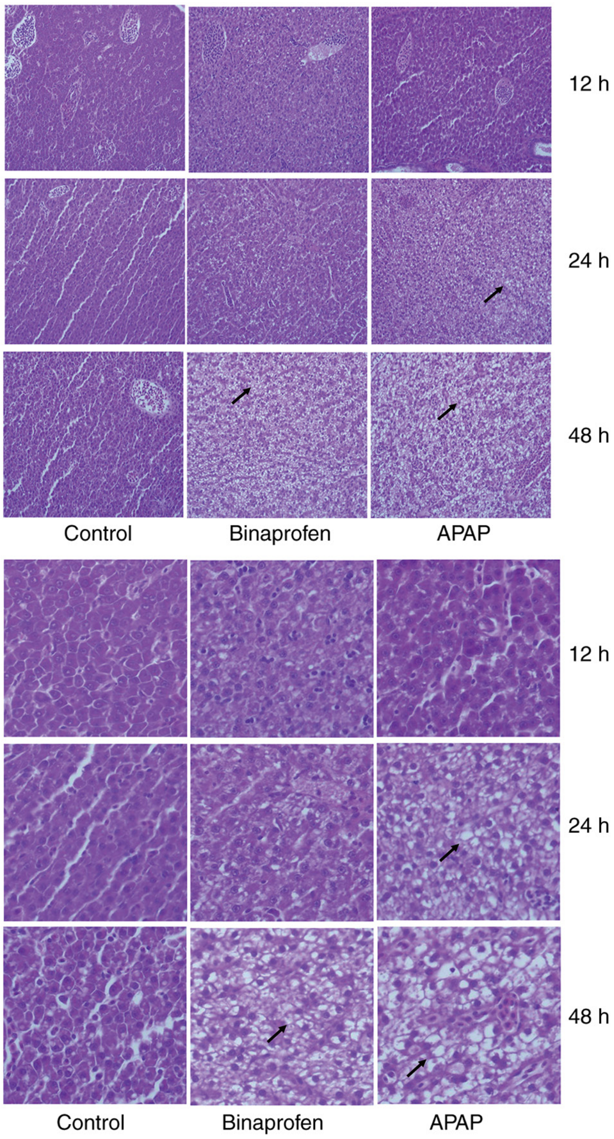

Figure 3. Representative hematoxylin and eosin‑stained micrographs of zebrafish liver cells following exposure to binaprofen or APAP for 12, 24 and 48 h. There was no notable change in zebrafish liver cells following exposure to binaprofen at 12 and 24 h. Half of zebrafish liver samples exhibited mild (arrow) to moderate vacuolization at 48 h in binaprofen group. Magnification, x400 (top) and x800 (bottom). APAP, acetaminophen.

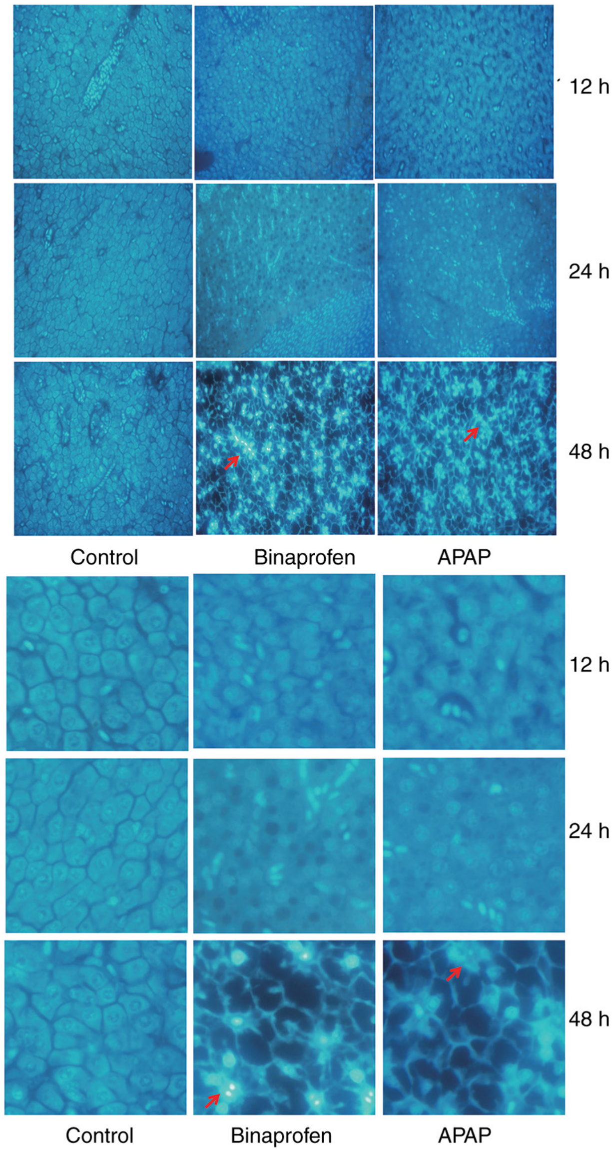

Figure 4. Representative DAPI‑stained micrographs of zebrafish liver cells following exposure to binaprofen or APAP for 12, 24 and 48 h. Zebrafish liver cells exhibited apoptosis at 48 h. Apoptotic cells (arrow) presented with nuclear enrichment, deep staining, or crescent‑shaped aggregation of nuclear chromatin on one side of the nuclear membrane and nucleus broke down to form fragments and disintegrated. Magnification, x400 (top) and x800 (bottom). APAP, acetaminophen.

total of six candidate genes was chosen for RT‑qPCR. Primer 5.0 software (PREMIER Design Lnc.) was used to design primers (Table I). RNA was extracted from liver samples and purified (RNA Simple Total RNA kit; Tiangen Biotech Co., Ltd.). RT‑qPCR was performed by cDNA synthesis (iScipt cDNA Synthesis kit) and amplification using a 2 Real‑time Detection system (2X QuantiFast SYBR Green PCR Master Mix; both Qiagen GmbH). RT kit was used according to the manufacturer's protocol. Thermocycling conditions were as follows: Initial denaturation at 94˚C for 4 min, followed by 45 cycles of 94˚C for 20 sec, 58˚C for 25 sec and 72˚C for 25 sec. Melting curve analysis was performed. Following amplification, quantitative detection was performed using a fluorescence qPCR instrument (cat. no. DA7600; Daan Gene Co., Ltd.). The relative fold change was calculated using the $2^{-\Delta\Delta Cq}$ method (30).

Statistical analysis. SPSS software (13.0; SPSS, Inc.) was used for statistical analysis. The data are presented as mean ± SD (n=5). Variables with normal distribution were analyzed with Student's t‑test (unpaired) for two groups; for multiple groups, one‑way ANOVA followed Tukey's post hoc test was used. Variables with abnormal distribution were analyzed with Mann‑Whitney test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Value of serum biomarkers. Compared with control, APAP increased ALT and LDH levels at 48 h (Fig. 1). Binaprofen treatment increased ALT, AST and LDH levels at 48 h. These results indicated injury or inflammation of liver.

Table II. Number of differentially expressed genes following exposure to binaprofen for 12-48 h.

	Gene count		
Gene regulation	12 h (n=3,673)	24 h (n=3,945)	48 h (n=5,496)
Up	2,499	2,745	1,200
Down	1,174	1,200	1,645

Value of MDA and GSH. Compared with control group, APAP and binaprofen increased MDA and decreased GSH levels at 48 h (Fig. 2). These results indicated increased levels of hepatic oxidative products.

Liver morphological changes from histological analysis. Compared with control group, APAP caused mild vacuolization in 2/10 samples at 12 and 24 h and mild to moderate vacuolization in all samples at 48 h (Fig. 3). Binaprofen caused mild vacuolization in one sample at 12 h and 2 samples at 24 h and mild to moderate vacuolization in 5/10 samples at 48 h. These results indicated morphological changes of liver cell.

Liver cell apoptosis. Control cells exhibited round nuclei with clear edges and uniform staining, while the nuclei of apoptotic cells exhibited irregular edges and concentration of chromosomes (Fig. 4). Liver cells with strong staining accompanied by nuclear contraction and nucleosome fragmentation were considered to indicate apoptosis. In Binaprofen and APAP groups, DAPI staining is bluish-white fluorescent, apoptotic cells presented with nuclear enrichment, deep staining, or crescent-shaped aggregation of nuclear chromatin on one side of the nuclear membrane. The nucleus broke down to form fragments and disintegrated. These results indicated that liver cell apoptosis occurred in binaprofen and APAP groups.

Mitochondrial change. Compared with control, APAP treatment caused endoplasmic reticulum thickening, mitochondrial swelling and vacuolation and ruptured cristae at 48 h (Fig. 5). Binaprofen treatment caused mitochondrial swelling and vacuolation and rupture or disappearance of cristae at 48 h.

Gene expression profiling. Compared with control group, in binaprofen group, 3,673 genes exhibited fold-change ≥2.0 or ≤0.5 in expression levels at 12 h. Of these, 2499 genes were up- and 1,174 genes were downregulated. At 24 h, 3,945 genes exhibited fold-change ≥2.0 or ≤0.5 in expression levels; of these, 2,745 genes were up- and 1,200 genes were downregulated. At 48 h, 5,496 genes exhibited fold-change ≥2.0 or ≤0.5 in expression levels; of these, 3,851 genes were up- and 1,645 genes were downregulated (Table II, Fig. 6). Venn analysis identified 190 common differentially expressed genes at 12, 24 and 48 h. The function of downregulated genes was primarily associated with 'DNA replication', 'DNA metabolic process', 'cell cycle', 'cell redox homeostasis', 'mitochondrion' and 'lipid transport'. The function of upregulated genes was primarily associated with 'peroxisome proliferator', 'oxidation activity', 'peroxisome' and 'apoptosis'. There was a significant increase in Bcl2 and caspase gene expression. Expression levels of 8 genes were

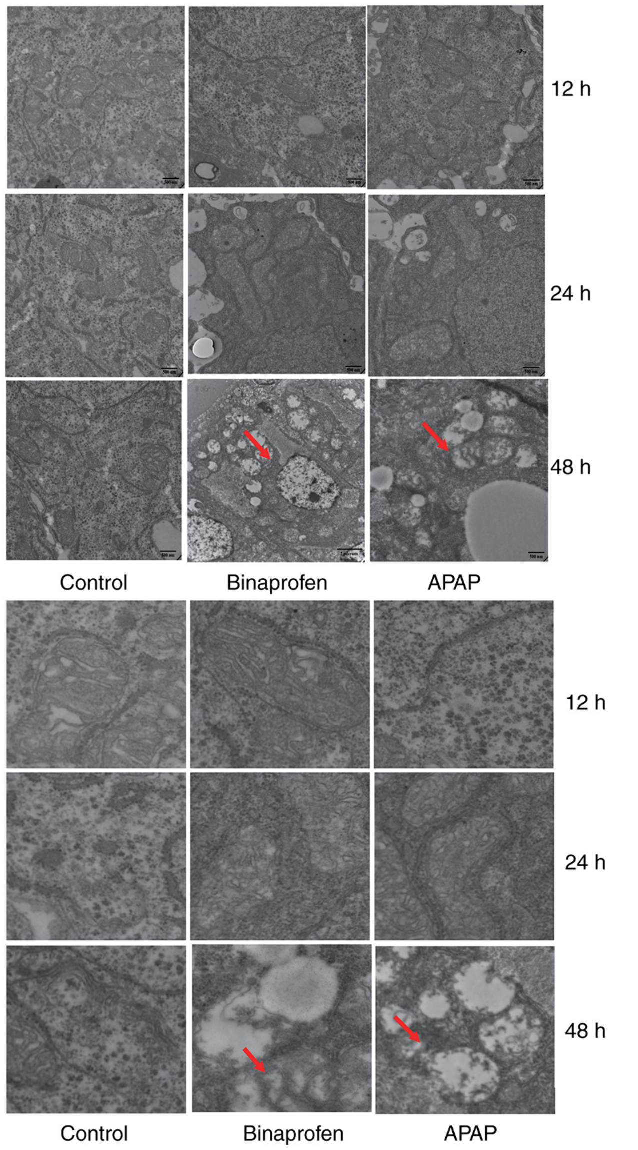

Figure 5. Representative transmission electron microscopy micrographs of zebrafish liver cells following exposure to binaprofen or APAP for 12, 24 and 48 h. Liver cell mitochondria (arrow) are swollen and vacuolarized, arranged in disorder or rupture of the crest at 48 h. Magnification, x30,000 (top) and x60,000 (bottom). APAP, acetaminophen.

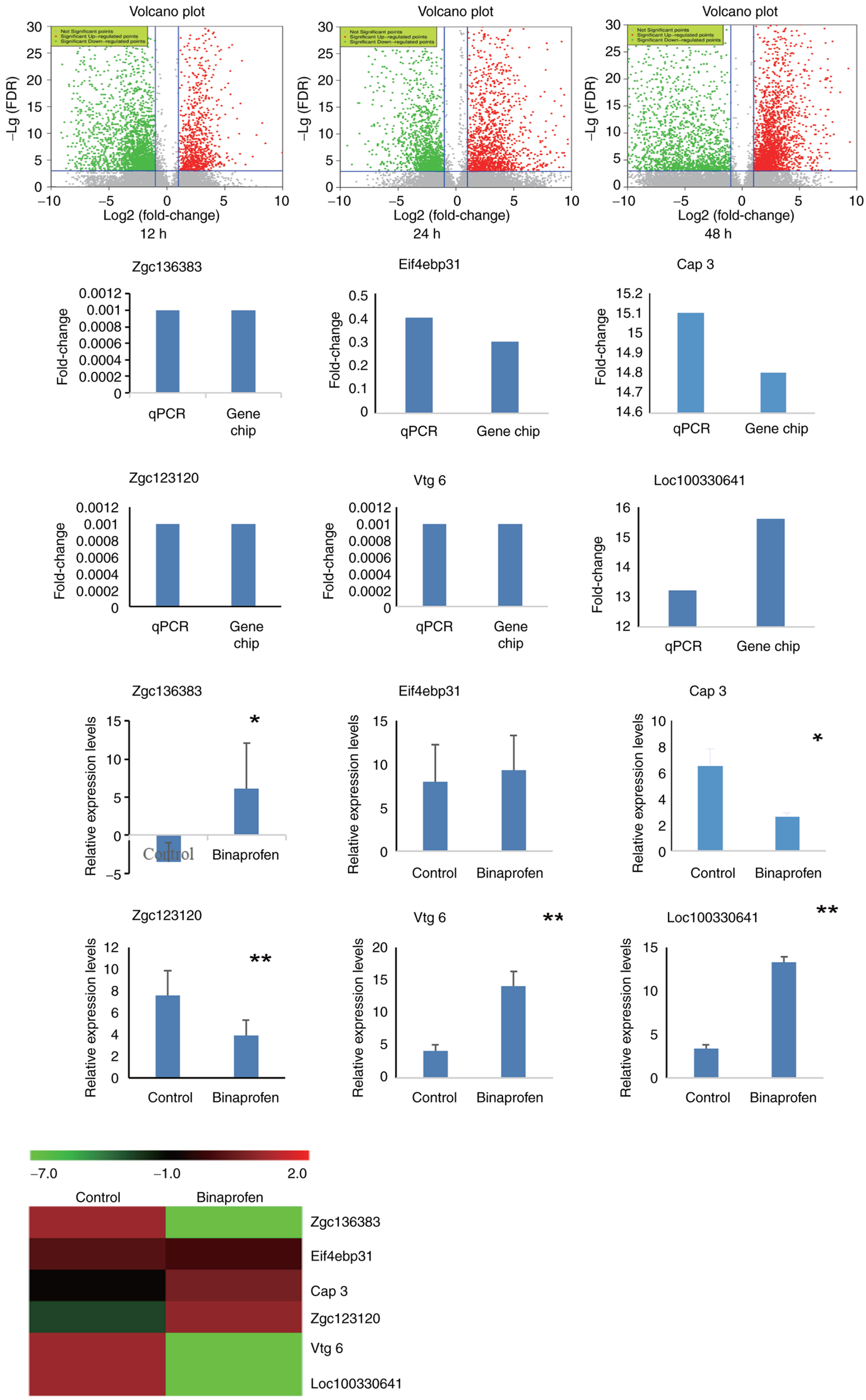

Figure 6. Microarray and RT-qPCR assay of zebrafish liver cells following exposure to binaprofen for 48 h. Volcano graphs showed that 3,673 genes exhibited fold-change ≥2.0 or ≤0.5 in expression levels at 12 h. Of these, 2,499 genes were up- and 1,174 genes were downregulated. At 24 h, 3,945 genes exhibited fold-change ≥2.0 or ≤0.5 in expression levels; of these, 2,745 genes were up- and 1,200 genes were downregulated. At 48 h, 5,496 genes exhibited fold-change ≥2.0 or ≤0.5 in expression levels; of these, 3,851 genes were up- and 1,645 genes were downregulated. Among these, 6 genes were verified by RT-qPCR assay. A total of five samples/group was used to detect zgc136383, Vtg 6, Loc100330641, Eif4ebp31, Zgc123120 and Cap 3. Heatmap of microarray results was consistent with RT-qPCR results. *P<0.05, **P<0.01 vs. control. RT-q, reverse transcription-quantitative; Zgc136383, vitellogenin 4; Zgc123120, BCL2/adenovirus E1B interacting protein 4; Eif4ebp31, eukaryotic translation initiation factor 4E binding protein 3; Cap 3, apoptosis-related cysteine peptidase 3; Loc100330641, vitellogenin-like; Vtg 6, vitellogenin 6.

Table III. Effect of binaprofen on certain differentially expressed genes at 12, 24 and 48 h.

Gene	Fold change			Regulation	Time of greatest differential expression, h
	12 h	24 h	48 h		
Zgc136383	0.001	0.001	0.000	Down	48
Vtg6	0.0008	0.0007	0.0005	Down	48
Loc100330641	0.001	0.001	0.000	Down	48
Eif4ebp31	0.429	0.314	0.301	Down	48
Loc1005352888	0.0004	0.0004	0.0003	Down	48
Loc100534731	0.442	0.414	0.276	Down	48
Zgc123120	11.459	18.673	15.656	Up	24
Cap 3	50.117	50.375	14.833	Up	24

Zgc136383, vitellogenin 4; Zgc123120, BCL2/adenovirus E1B interacting protein 4; Eif4ebp31, eukaryotic translation initiation factor 4E binding protein 3; Cap 3, apoptosis-related cysteine peptidase 3; Loc100330641, vitellogenin-like; Vtg 6, vitellogenin 6, Loc100535288, uncharacterized Loc100535288; Loc100534731, uncharacterized Loc100534731.

Table IV. Gene Ontology analysis of differentially expressed genes following exposure to binaprofen for 12-48 h.

A, Downregulated genes

Term	Gene count		
	12 h	24 h	48 h
DNA replication	32	6	6
DNA metabolic process	71	4	8
Cell cycle	47	8	8
Mitochondrion	27	18	18
Lipid transport	-	15	10

B, Upregulated genes

Term	Gene count		
	12 h	24 h	48 h
Peroxisome proliferator	28	-	-
Oxidation activity	-	4	98
Peroxisome	-	4	4
Apoptosis	12	12	15

-, indicates that there is no obvious up regulation or downregulation.

Table V. Pathway analysis of differentially expressed genes following exposure to binaprofen for 12-48 h.

KEGG ID	Pathway	Gene count		
		12 h	24 h	48 h
dre03030	DNA replication	24	24	29
dre03010	Ribosome	-	28	29
dre03040	Spliceosome	41	6	-
dre04110	Cell cycle	41	41	52
dre00240	Pyrimidine metabolism	31	-	34
dre00230	Purine metabolism	34	-	37
dre03320	PPAR signaling pathway	-	12	18
dre04115	p53 signaling pathway	12	12	13

KEGG, Kyoto Encylcopedia of Genes and Genomes; upregulated and downregulated genes pathways were associated with 'cell cycle', 'DNA replication', 'ribosome', 'spliceosome', 'pyrimidine metabolism', 'purine metabolism', 'PPAR signaling pathway' and 'p53 signaling pathway'.

different at 12, 24 and 48 h; of these, six genes were down- and two were upregulated (Table III).

GO analysis. GO analysis showed that the function of downregulated genes was primarily associated with 'DNA replication', 'DNA metabolic process', 'cell cycle', 'cell redox homeostasis', 'mitochondrion' and 'lipid transport' (Table IV). The function of upregulated genes was primarily associated with 'peroxisome proliferator', 'oxidation activity' and 'peroxisome'.

Gene pathway analysis. Differentially expressed genes were associated with biological process according to GO pathway analysis. Pathway analysis showed that upregulated and downregulated GO pathways were associated with 'cell cycle', 'DNA replication', 'ribosome', 'spliceosome', 'pyrimidine metabolism', 'purine metabolism', 'PPAR signaling pathway' and 'p53 signaling pathway' (Table V).

RT-qPCR. Expression levels of 8 genes changed over time: Loc100535288, Loc100534731, Zgc136383, vitellogenin (Vtg) 6, Loc100330641, Vtg-like eukaryotic translation initiation factor 4E binding protein 31 (Eif4ebp31), Zgc123120 and Cap 3. Because Loc100535288 and Loc100534731 were uncharacterized geneswithout any functional information, the other six genes were selected to verify the results of microarray (Fig. 6). Microarray showed that Zgc136383, Vtg6, Loc100330641, Eif4ebp31 were downregulated and Zgc123120 and Cap 3 were upregulated. RT-qPCR showed that Zgc136383, Vtg 6, Loc100330641, Eif4ebp31 were downregulated and Zgc123120 and Cap 3 were upregulated. Meanwhile, the fold-change was also similar. The results of RT-qPCR and microarray were consistent.

Discussion

Binaprofen is not currently commercially available. Our previous study evaluated the effect of different doses of binaprofen and APAP on liver injury; both induced liver injury to a similar extent (21). The half-maximal lethal concentration (LC_{50}) of binaprofen was 1.2 mM, which was 2 times higher than its maximum recommend dose (19); LC_{50} of APAP was 5.2 mM, which was 1.6 times higher than its people maximum recommend dose (31). Therefore, binaprofen was selected for investigation of the underlying mechanism of toxicity. APAP was used as a positive control because of its known ability to induce liver injury (32).

The present study investigated the mechanism of binaprofen-induced liver injury in zebrafish. Binaprofen increased levels of liver biomarkers ALT, AST and LDH in a time-dependent manner, increased MDA and decreased GSH content. Binaprofen induced hepatocyte vacuolization, as well as mitochondrial swelling, vacuolization and rupture or disappearance of cristae. Binaprofen induced hepatocyte apoptosis. Binaprofen induced altered gene expression at 12, 24 and 48 h. There were 190 common differentially expressed genes at all three timepoints. The function of downregulated genes were primarily associated with 'DNA replication', 'DNA metabolic process', 'cell cycle', 'cell redox homeostasis', 'mitochondrion' and 'lipid transport'. The function of upregulated genes was primarily associated with 'peroxisome proliferator', 'oxidation activity', 'peroxisome' and 'apoptosis'. GO pathways were associated with 'cell cycle', 'DNA replication', 'ribosome', 'spliceosome', 'pyrimidine metabolism', 'purine metabolism', 'PPAR signaling pathway' and 'p53 signaling pathway'. Six genes from microarray were verified, and the results of RT-qPCR were in accordance with microarray results.

Therefore, the experimental results showed that the mechanism of hepatotoxicity was associated with lipid peroxidation and apoptosis.

DILI is associated with inappropriate activation of apoptotic cell death pathways (33-36). Apoptosis serves a key role in progression of liver disease, such as cirrhosis (37,38). Apoptosis is mediated by two central pathways, the intrinsic and extrinsic pathway. The mitochondrial pathway is the intrinsic pathway and begins with permeabilization of the mitochondrial outer membrane (39-42). Release of cytochrome c from mitochondria is key factor to initiate apoptosis (43,44). The released cytochrome c activates caspase-9; this leads to caspase-3 activation, cellular protein cleavage and apoptosis (45). Following binaprofen exposure, there was swelling in mitochondria and increase in DAPI-positive cells with condensed and fragmented nuclei. According to reports (46), DAPI stains apoptotic cells with high labeling efficiency (~100%) and does not change the ultrastructure of organelles. The present study aimed to determine the toxicity of binaprofen, therefore, DAPI was used to detect hepatocyte apoptosis. The results suggested apoptosis occurred; this was confirmed by altered expression of genes associated with apoptosis in the microarray. Moreover, electron microscopy showed liver cell mitochondrial swelling and vacuolization, which indicated that apoptosis was associated with mitochondria. In addition, there was a significant increase in Bcl2 family and caspase gene expression in microarray; this was validated by RT-qPCR. These data suggested that binaprofen induces zebrafish liver injury via the mitochondria-mediated apoptosis pathway.

In the present study, DAPI staining of apoptotic cells was not quantified. TUNEL staining and western blotting were not performed to confirm levels of apoptosis markers; these experiments should be performed in future to quantify apoptosis. Here, it is also found that the signaling pathway of binaprofen-induced liver injury may be associated with PPAR signaling pathway and P53 signaling pathway, we will do further study to clear them.

The mechanism of binaprofen-induced liver injury was associated with lipid peroxidation and apoptosis. Binaprofen induced hepatocyte mitochondrial structural damage and activated apoptosis via the mitochondrial signaling pathway.

Acknowledgements

Not applicable.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus database of National Center for Biotechnology Information repository, ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE199758 (accession no. GSE199758).

Authors' contributions

QG designed and performed experiments. GC performed experiments. HO and QN analyzed the data. RJ conceived the study. RQ and RJ interpreted the data. All authors have read and approved the final manuscript. QG and GC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Guangzhou General Pharmaceutical Research Institute (approval no. 2012-005) and performed in accordance with international guidelines for the care and use of laboratory animals. All zebrafish experiments in this study were performed from 1st of July to 30th of August 2012.

Patient consent for participation

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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