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2-Amino-3-methylimidazo[4,5-f]quinoline induces oxidative stress-mediated DNA damage and apoptosis in multi-organ of zebrafish

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ABSTRACT

2-Amino-3-methylimidazo[4,5-f]quinoline (IQ) is one of mutagenic/carcinogenic heterocyclic amines (HCAs) found mainly in well-cooked meats. As a common HCAs, IQ can pose a health risk to animals as well as humans. However, to date, few studies have explored IQ's toxic effects on systemic health, and its relevant mechanism of toxicity has not been elucidated. In this study, we for the first time determined IQ accumulation and investigated the tissue damage of developing zebrafish. Zebrafish at one-month post-fertilization were exposed to IQ (80 ng/mL) for 35 days. Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis showed that IQ accumulated in multi-organ of zebrafish such as eye, spleen, gonad (ovary or testis), heart, intestine, kidney, muscle, liver, and brain. Our results showed that IQ exposure caused histopathological alterations in IQ-accumulated organs, which could compromise organ function. Exposure to IQ significantly inhibited antioxidant related indicators. Moreover, IQ exposure significantly increased *mTOR* gene levels. The immunohistochemical results further validated that IQ exposure tended to over activate *mTOR*. In addition, IQ exposure significantly reduced the expression of autophagy-related genes, and upregulated the expression of DNA damage and repair-related genes, which also detected the accumulation of fluorescence signal of DNA damage marker γ -H2AX. Furthermore, TUNEL assay showed that IQ exposure caused apoptosis. Our findings revealed that IQ could be taken up by developing zebrafish, and accumulate in multiple tissues, especially across the blood-brain barrier, accumulate in the brain, subsequently causing systemic tissue damage, urging us to pay attention to the control or intervention of IQ exposure and decreasing accumulated impairment to systemic health.

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1. Introduction

Heterocyclic amines (HCAs) are a class of potential carcinogens with high mutagenic activity and are common hazards associated with food thermal processing. They are mainly produced by proteins and amino acids under high-temperature conditions^[1]. The primary origins of HCAs stem from the thermal processing of high-protein foods (primarily

through cooked meat). 2-Amino-3-methylimidazo[4,5-f]quinoline (IQ), as one of the most common HCAs, has a wide range of concentrations in food and we are chronically exposed to this compound in our daily life. For example, the concentration of IQ is 1.7–240.0 ng/g in the cooked meat^[2]. Exposure to endogenous or exogenous genotoxic agents can lead to the formation of structurally modified DNA bases (DNA adducts). In addition to the discovery of HCAs and their metabolites in human urine and feces, HCAs or their adducts have also been detected in human blood^[3], human hair^[4], breast milk^[5], and human tissues^[6]. It is necessary to determine the accumulation of IQ in different organs that could provide a more comprehensive understanding of tissue-specific susceptibility to the toxic effects of IQ.

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The DNA adducts of HCAs are prone to frameshift mutations and point mutations that exert their genotoxicity^[7]. In particular, *in vivo* animal experiments have shown that long-term ingestion of HCAs produces tumors in multi-organ tissues of mice, rats^[8], and monkeys^[9]. The IARC classifies HCAs as either possible (Group 2A) (e.g., IQ) or probable (Group 2B) (e.g., PhIP) human carcinogens^[1], with HCAs like IQ is among the most mutagenic of these compounds and its multi-organ carcinogenic effects have been extensively studied^[9]. However, whether IQ exposure has systemic health effects before cancer development has not been reported. Currently, several toxicological and epidemiological investigations have found that exposure to HCAs exerts adverse biological effects *in vitro* and *in vivo* including DNA damage^[10], genotoxicity^[11], reproductive toxicity^[12], carcinogenicity^[13], oxidative damage^[14], endoplasmic reticulum stress^[15], neurotoxicity^[16], and insulin resistance^[17]. Limited research on HCAs has typically focused on a single or few tissues and their carcinogenicity, whereas cancer is a progressive process that is closely linked to multiple tissue dysfunctions. Despite the persistence and toxicity of IQ, little is known about its accumulation and potential multi-tissue toxicity after prolonged exposure, necessitating a systematic assessment of its impact on developing multi-organ.

In our previous investigation, we observed that IQ exposure resulted in damage to the liver and brain, and reductions in antioxidants^[18–19]. However, the effects of IQ exposure on other tissues and organs have not been reported, and it is reasonable to speculate that IQ may cross the blood-brain barrier and have relevant effects on other tissues and organs. Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) is commonly used for the quantitative analysis of HCAs^[20–22], which can help us detect whether IQ is distributed to other tissues after exposure, ultimately leading to tissue damage. We first performed a pathological analysis, which is widely used to determine the toxic effects. Oxidative stress was next analyzed because of its important implications for numerous events of chronic disease pathophysiology, and the attack of reactive oxygen species (ROS) on pyrimidine plays a key role in mutagenicity^[23]. Further, we analyzed the DNA damage and apoptosis of tissues. Moreover, abnormal activation of the mTOR is associated with apoptosis^[24]. These results suggest that IQ may have a similar effect on different organs and tissues. Understanding the toxic effects of IQ from a systemic perspective is of great significance for the study of protective strategies to alleviate the harm caused by IQ. Therefore, the present study fills the gap in IQ-induced systemic damage and its associated mechanisms.

In the present study, we for the first time determined IQ accumulation in biological samples of zebrafish after 35-day exposure by using UPLC-MS/MS assay to help us understand the toxic actions of IQ *in vivo*. In addition, we performed phenotype, biochemistry and genic-expression analyses to characterize the systematic effects of IQ on various tissues of the developing zebrafish. Specifically, the specificity and generality of IQ exposure in inducing oxidative damage, DNA damage and apoptosis, and in modulating biological signaling were investigated. Overall, this work will provide a wealth of information on the potential risks of IQ exposure and the mechanisms involved.

2. Materials and methods

2.1 Materials

IQ (98%, CAS: 76180-96-6) was obtained from Toronto Research Chemicals (Downsview, Ontario, Canada). Rapamycin was procured from MedChemExpress (Monmouth Junction, NJ, USA). Hematoxylin and eosin (H&E) staining kit and ROS assay kit were purchased from Beyotime Biotechnology (Shanghai, China). The commercial assay kits of catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA), and total antioxidant capacity (T-AOC) were obtained from Jiancheng Bioengineering Institute (Nanjing, China). ChamQ Universal SYBR qPCR Master Mix kit was purchased from Vazyme Biotech (Piscataway, NJ, USA). The antibody for phosphorylation (p)-the mammalian target of rapamycin (mTOR) was purchased from Cell Signaling Technology (Danvers, MA, USA). The HRP-conjugated affinipure goat anti-rabbit IgG (H + L) secondary antibody was purchased from ProteinTech Group (Chicago, IL, USA). An immunohistochemical detection kit was purchased from ProteinTech Group. One step terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay kit was purchased from Meilun Bio. (Dalian, China). The antibody for γ -H2AX was purchased from Cell Signaling Technology (Danvers, MA, USA). The donkey anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody was purchased from Thermo Fisher Scientific Invitrogen (Waltham, MA, USA).

2.2 Animals and experimental design

AB strain zebrafish were used in this study. Culture and exposure of zebrafish were performed according to previous protocols^[15,25]. Our previous works found that exposure to IQ at 8, 80, and 800 ng/mL can significantly induce liver and brain damage^[18–19]. Therefore, in order to facilitate direct comparison and enable a better continuation, this study chose 80 ng/mL of IQ to expose zebrafish aged one-month post-fertilization. Zebrafish typically attain sexual maturity at the 3rd month of their development, so groups do not distinguish between males and females until dissection. A total of 180 fish were randomly assigned to IQ-exposed group and Control group with 6 replicates per group (12 tanks in total). In addition, we set up rapamycin (an mTOR inhibitor) treatment group ($n = 10$) with 3 replicates per group, and the zebrafish in this group were exposed to IQ and treated with rapamycin at the same time. A stock solution of rapamycin (2 mmol/L) was prepared in DMSO and was diluted to a final concentration (2 μ mol/L) using system water (0.015% DMSO in the final solution). The determination of rapamycin dose was based on a previous literature report^[26]. Zebrafish in the rapamycin treatment group (IQ + Rapa) were treated with 2 μ mol/L rapamycin working solution for one hour in addition to 80 ng/mL IQ exposure per day. The exposure solutions were renewed daily. After 35 days exposure and 1 day starvation, fish from the same group were randomly mixed into 1 sample (at least 0.01 g per sample and 3 independent biological replicates). After quick euthanasia in ice water, the eye, spleen, gonad (ovary or testis), heart, intestine, kidney, muscle, liver, and brain tissues of zebrafish were dissected and put into microtubes separately. These microtubes were immediately frozen in liquid nitrogen and then

kept at for subsequent analysis. Five fish were randomly taken from each repeat at the end of the 35-day exposure, quickly euthanized in ice water, then the whole fish soaked in 4% paraformaldehyde, embedded in paraffin, and sectioned in 5- μ m portions for subsequent staining observations. All animal experiments were approved by the animal ethics committee of Nankai University (approval number: 2021-SYDWLL-000459).

2.3 UPLC-MS/MS analysis

The extraction and detection of IQ were determined according to the laboratory method established previously^[20]. The eye, spleen, gonad (ovary or testis), heart, intestine, kidney, muscle, liver, and brain samples were first thoroughly homogenized with 100 μ L of ultra-pure water. The tissue homogenate combined with 0.5 mL of 2 mol/L sodium hydroxide solution and 1 mL dichloromethane within a 1.5 mL centrifuge tube, vortexed for 10 min, centrifuged for 10 min at 10 000 r/min, and the dichloromethane layer was then carefully collected to facilitate solid phase extraction (SPE). The above procedure was repeated 3 times in the sample tube containing precipitate to fully collect the extract, all while maintaining room temperature. The SPE process used an Oasis-MCX column (Waters, Milford, MA, USA). The column was washed with dichloromethane (3 mL). Subsequently, the extract was loaded into the Oasis-MCX cartridge. The cartridge was then rinsed successively with a 0.1 mol/L hydrochloric acid-methanol solution (4:6, *V/V*) in a volume of 3 mL, methanol (3 mL), and ultra-pure water (3 mL). Finally, the retained HCAs were eluted from the cartridge using a mixture of methanol and ammonium hydroxide (9:1, *V/V*) in a volume of 3 mL. The eluent was evaporated to dryness utilizing a nitrogen blower and redissolved in pure methanol (200 μ L). After filtration through a 0.22 μ m organic membrane, the resulting pure extract from the samples was subjected to analysis using UPLC-MS/MS (Waters). The separation was performed on an ACQUITY UPLC BEH C₁₈ reversed-phase column (Waters, 100 mm $\times$ 2.1 mm, 1.7 μ m) at 45 °C. A gradient mixture of acetonitrile (solvent A) and water (solvent B) was used as the mobile phase. The flow rate was 0.5 mL/min, and the injection volume was 3 μ L. The following gradient program was used: 0–0.5 min, 10% A; 10 min, 50% A; 10.1–15.0 min, 10% A. The mass spectrometric parameters were described previously^[20].

2.4 Histological and immunohistochemical analyses

As described in a previous study^[27], the fixed fish samples were dehydrated in increasing concentrations of ethanol and embedded in paraffin. The trimmed paraffin block was sectioned at 5 μ m. After the routine dewaxing and hydration, paraffin sections were stained with H&E staining reagent. After that, each section was observed and images were taken using a light microscope (Nikon, Tokyo, Japan).

For immunohistochemical observation, p-mTOR antibody was used as the primary antibody to determine the cellular localization and expression of p-mTOR in eye, spleen, gonad (ovary or testis), heart, intestine, kidney, muscle, liver, and brain tissues of zebrafish. Briefly, paraffin sections were deparaffinized, rehydrated, and incubated with p-mTOR antibody (diluted 1:100). Then the sections incubated with the secondary antibody, exposed to 3,3'-diaminobenzidine (DAB), and stained with hematoxylin before observed. Images were taken using a light microscope (Nikon).

2.5 Oxidative stress index analysis

Briefly, the eye, spleen, gonad (ovary or testis), heart, intestine, kidney, and muscle samples were first thawed on ice and then homogenized in nine volumes of ice-cold saline and centrifuged for 10 min at 3 000 r/min (4 °C), respectively. The supernatants were subsequently collected and diluted for analysis. The enzyme activities of CAT, GSH-Px and SOD, and the level of GSH and MDA in the tissues of zebrafish were measured using commercial assay kits. Briefly, the changes in absorbance measured at 405 nm were recorded by the ammonium molybdate colorimetric method to calculate CAT activities; the activity of GSH-Px was calculated by colorimetric determination of the absorbance change at 412 nm, which was expressed as the reaction rate of catalyzing GSH; the changes in absorbance measured at 450 nm by the water-soluble tetrazolium salt-1 (WST-1) method to calculate SOD activities; the changes in absorbance measured at 405 nm by the 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) method to calculate GSH contents; the changes in absorbance measured at 532 nm by the thiobarbituric acid (TBA) method to calculate MDA contents; the changes in absorbance measured at 405 nm by the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method to calculate T-AOC capacities. The calculation formula was provided by the kit. The ROS level was determined with a microplate reader (Tecan Spark, Zurich, Switzerland) and a fluorescent DCFH-DA probe of the ROS assay kit. The DCFH-DA probe (10 μ mol/L) was diluted with tissue homogenate and incubated at 37 °C for 20 min, and the fluorescence intensity was measured using an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Oxidative stress index concentration was normalized against the total protein concentration. The changes in absorbance were measured at 562 nm by the bicinchoninic acid (BCA) method to calculate protein concentrations using a protein detection kit (Jiancheng Bioengineering).

2.6 qPCR analysis

Total RNA in the eye, spleen, gonad (ovary or testis), heart, intestine, kidney, muscle, liver, and brain tissues of zebrafish was extracted using TRIzol reagent (Life Technologies, Carlsbad, California, USA). Reverse transcription used SYBR qPCR Master Mix kit for the synthesis of cDNA. The qPCR was performed in a real-time PCR detection system (BioRad, Philadelphia, PA, USA) with a 20 μ L reaction volume containing 10 μ L of 2 $\times$ ChamQ Universal SYBR qPCR Master Mix, 0.4 μ L of 10 μ mol/L each of forward and reverse primers, 2.0 μ L of diluted cDNA template, and 7.2 μ L of RNase-free water. The program consisted of initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The 2^{− $\Delta\Delta$ CT} method was used to calculate the expression levels of each targeting mRNAs by normalized to β -actin. The specific sequences of primers used in this study are shown in Table S1.

2.7 TUNEL assay

Briefly, paraffin sections were stained with TUNEL staining reagent and incubated at 37 °C for 60 min in the dark, washed 3 times with PBS. Finally, the sections were sealed with anti-fluorescence quench capping agent containing 4',6-diamidino-2-phenylindole (DAPI) (Servicebio, Beijing, China). After that, each section was observed and images were taken using a fluorescence microscope (Leica, Wetzlar, Germany).

2.8 Immunofluorescence analysis

Briefly, paraffin sections were soaked in antigen retrieval buffer, boiled by microwave, blocked with 5% BSA, and incubated with primary antibody against γ -H2AX (diluted 1:100) at 4 °C overnight. The stained sections were washed 3 times with PBS, and incubated with a secondary antibody (1:400, Alexafluor 594) at 37 °C for 2 h in the dark, washed 3 times with PBS. Finally, the sections were sealed with anti-fluorescence quench capping agent containing DAPI. The sections were observed and images were taken under a fluorescence microscope (Leica, Wetzlar, Germany).

2.9 Statistical analysis

Data representation and statistical analysis were performed using GraphPad Prism 9 (GraphPad Software Inc.). Graphical abstract was created with BioRender.com. Results were presented as the means or means $\pm$ SD. Statistical significance was determined by Student's *t* test. The significant levels were set at $P < 0.05$.

3. Results

3.1 Detection of IQ in multi-organ of zebrafish after exposure

The quantification of the tissue accumulation IQ UPLC-MS/MS chromatograms was achieved following the 35-day exposure to zebrafish at 80 ng/mL dosage (Fig. 1). Content of IQ were determined in various tissues of zebrafish. Statistical comparison between different tissues regarding IQ accumulation was shown in Fig. 1K. Fig. 1K shows the contents of IQ in zebrafish tissues, which indicated that IQ accumulated in tissues. The bioaccumulation showed higher levels in eye ($12\,180.44 \pm 244.29$ ng/g), spleen ($1\,905.40 \pm 69.26$ ng/g), gonad (testis, 643.87 ± 56.64 ng/g), heart (353.40 ± 23.80 ng/g), intestine (785.31 ± 48.73 ng/g), kidney ($1\,482.27 \pm 84.56$ ng/g), liver ($1\,010.00 \pm 18.54$ ng/g), and the lower level observed in the gonad (ovary, 183.89 ± 4.65 ng/g), muscle (118.89 ± 5.66 ng/g), and brain (160.97 ± 8.22 ng/g). It was discovered in brain and showed that IQ could across the blood-brain barrier. We found that IQ accumulation was higher in internal organs (spleen, kidney, liver, intestine) after IQ exposure than in other organs.

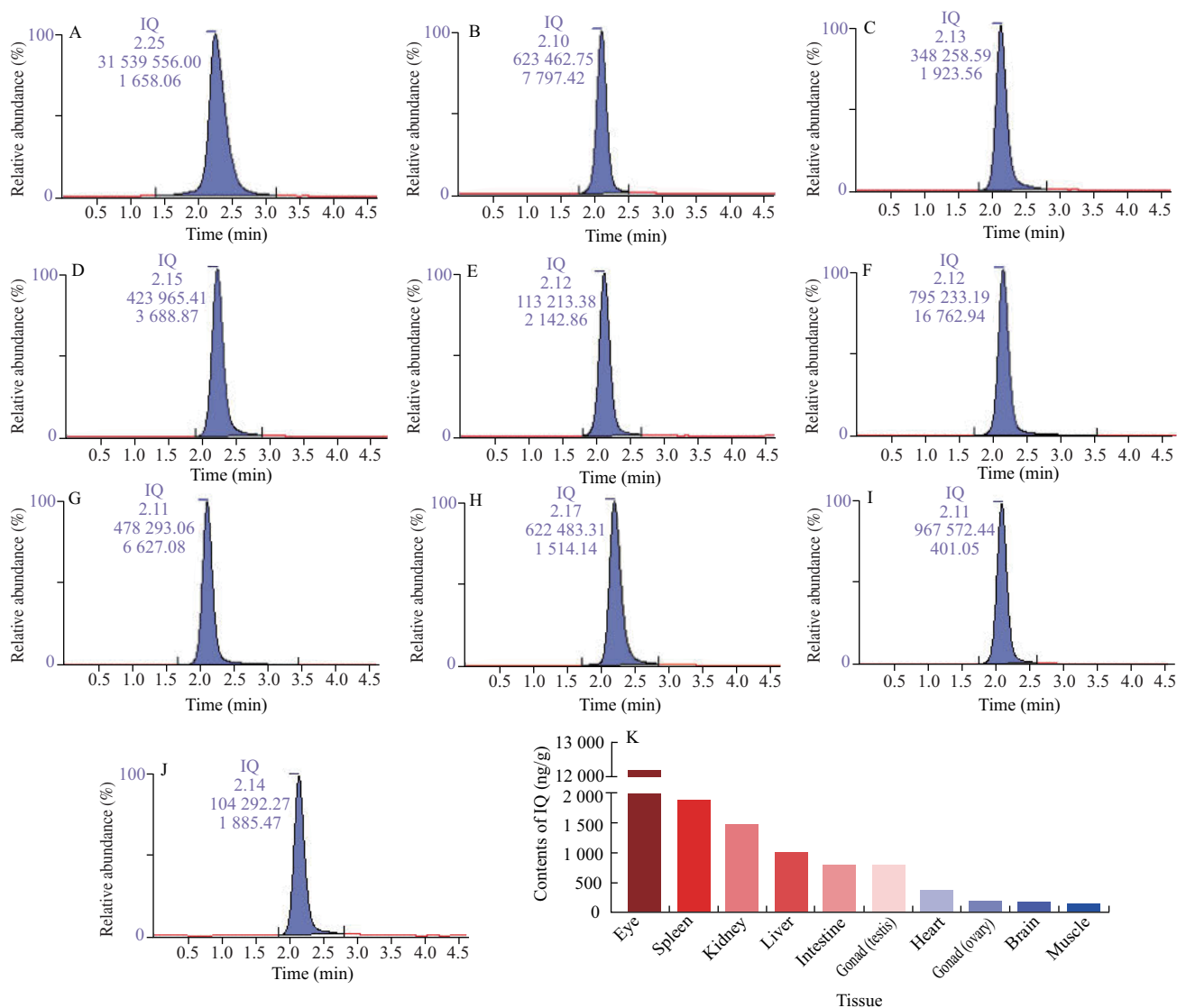


Fig. 1 UPLC-MS/MS analysis of the accumulation of IQ in multiple tissues. Representative chromatograms of the resulting pure extract from the samples of (A) eye, (B) spleen, (C) gonad (ovary), (D) gonad (testis), (E) heart, (F) intestine, (G) kidney, (H) muscle, (I) liver, and (J) brain from zebrafish after IQ exposure. (K) Statistical comparison between different tissues regarding IQ accumulation.

3.2 IQ induced histopathological alterations in multi-organ of zebrafish

We explored the systemic impact of IQ exposure using histological analysis of multi-organ in zebrafish (Fig. 2A). As previously reported for indicators associated with systemic tissue damage^[28–30], compared to the Control group, IQ-treated zebrafish showed a reduction in retinal pigmented epithelium (RPE) width and photoreceptor layer (PRL) width (Fig. 2B), number of spleen cells (Fig. 2C), mature oocyte area (Fig. 2D), mature spermatocyte area (Fig. 2E), and in muscle fibre thickness (Fig. 2I). In contrast, an increment in thickness of the ventricular wall (Fig. 2F), intestine goblet cells per villus (Fig. 2G) and renal tubule area (Fig. 2H) were observed in the IQ-treated zebrafish compared to the Control group.

In addition, tissue cavity enlargement and inflammatory cell infiltration were observed in the liver^[25] of the IQ-exposed zebrafish. Therefore, our results suggest that IQ induces systemic tissue damage in zebrafish.

3.3 IQ induced alterations in the levels of oxidative stress related indicator and genic-expression in multiple tissues of zebrafish

To investigate the effects of IQ on organismal antioxidative status, we measured the levels of oxidative stress related indicator and genic-expression. Antioxidants, both enzymatic and non-enzymatic, were measured to reflect oxidative stress. We also examined the level of MDA (a lipid peroxidation product) and the level of ROS. As shown in Fig. 3, the levels of antioxidants (CAT, SOD, GSH-Px, and GSH), MDA and ROS in multi-organ tissues of zebrafish were detected.

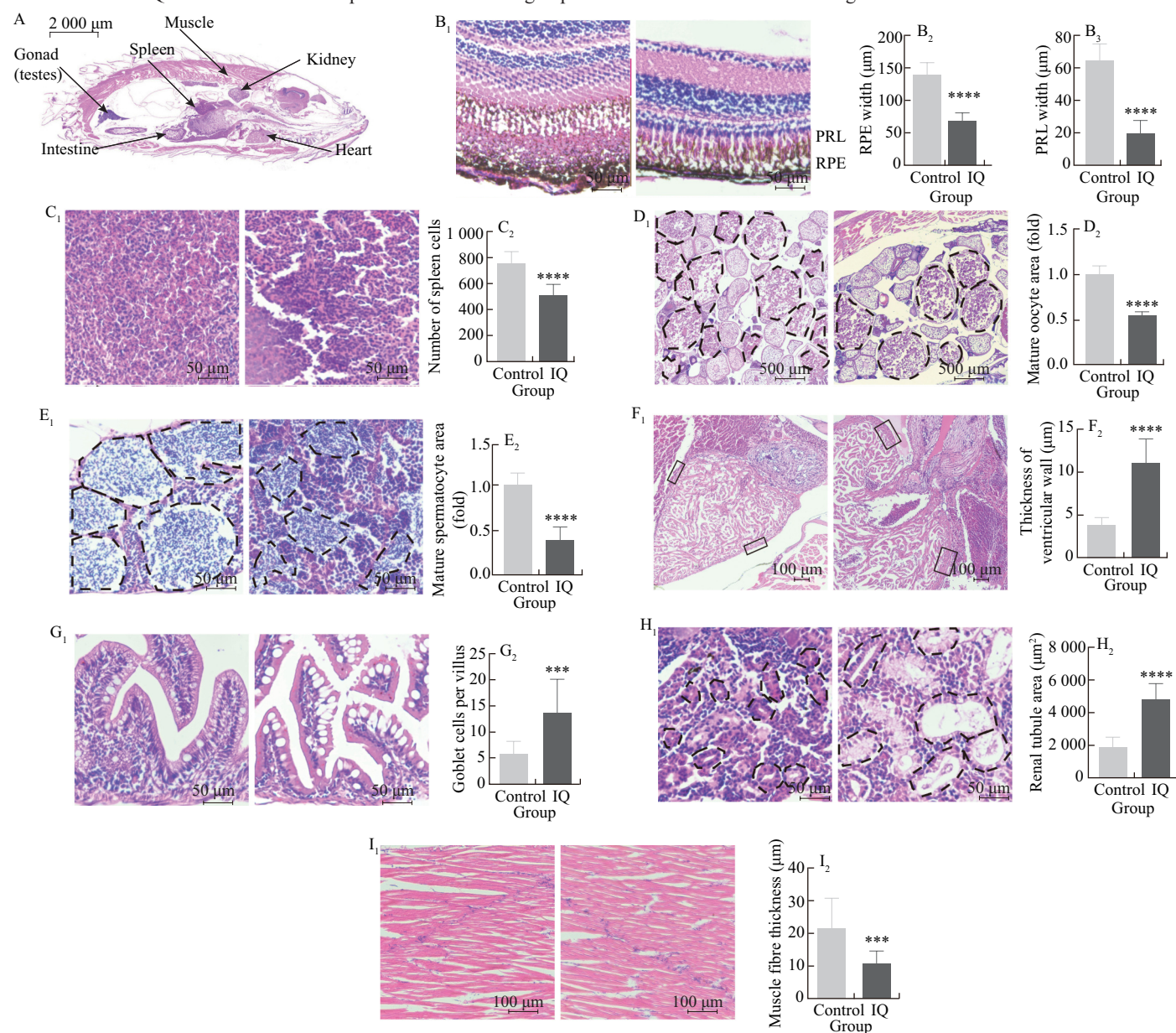


Fig. 2 Effects of IQ on tissue morphology. (A) Representative image of a longitudinal section of a zebrafish stained with H&E. Representative images of (B₁) eye, (C₁) spleen, (D₁) gonad (ovary), (E₁) gonad (testes), (F₁) heart, (G₁) intestine, (H₁) kidney, and (I₁) muscle from zebrafish with or without IQ exposure stained with H&E. Histological quantifications were performed for each tissue, (B₂) RPE width and (B₃) PRL width, (C₂) number of spleen cells, (D₂) mature oocyte area, (E₂) mature spermatocyte area, (F₂) thickness of the ventricular wall, (G₂) goblet cells per villus, (H₂) renal tubule area, and (I₂) muscle fibre thickness, respectively. Data are presented as mean ± SD ($n = 5$), analyzed by Student's t test; *** $P < 0.001$ and **** $P < 0.0001$.

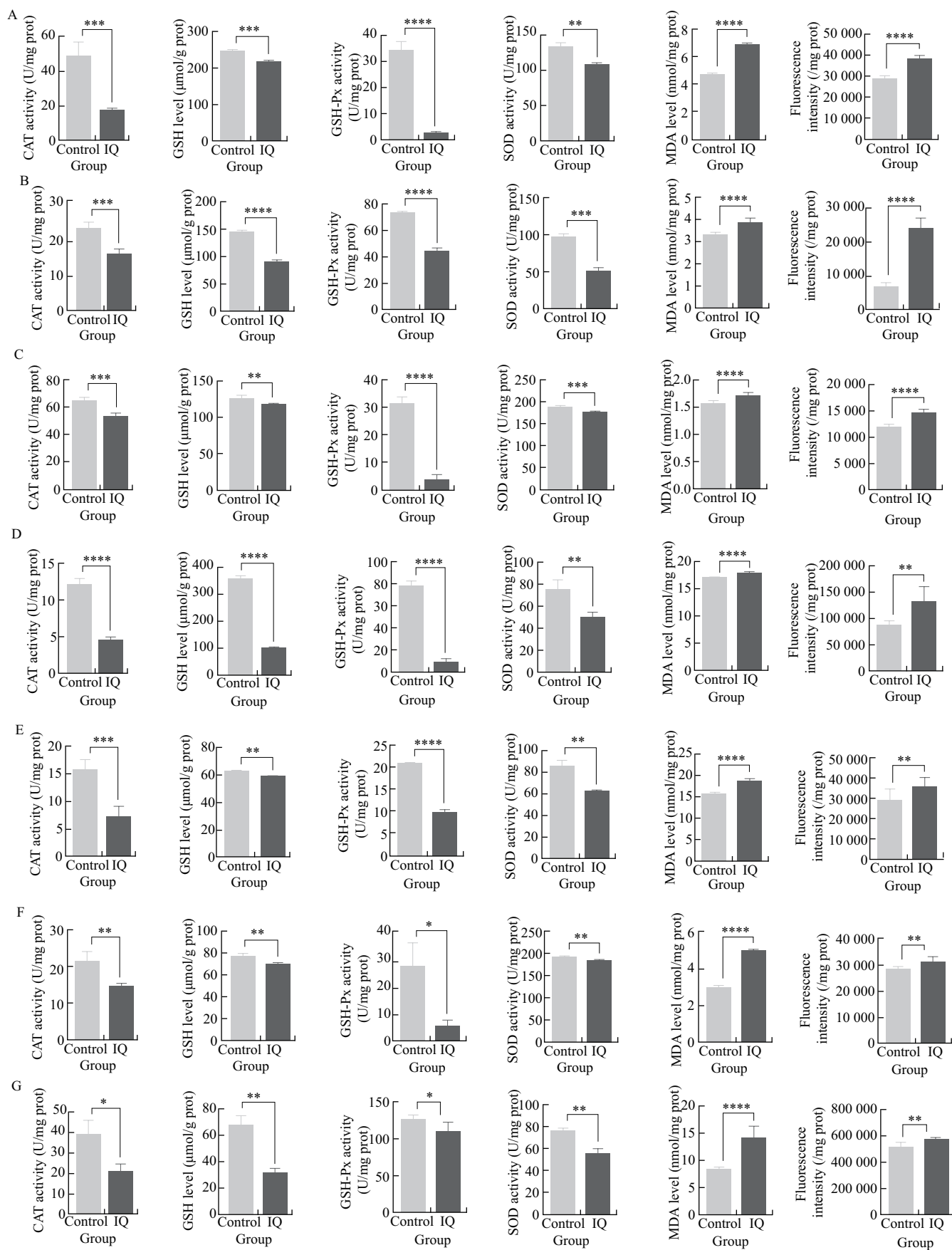


Fig. 3 Effects of IQ on the levels of antioxidant, MDA and ROS in multiple tissues. The levels of antioxidant (CAT, GSH, GSH-Px, and SOD), MDA and ROS in (A) eye, (B) spleen, (C) gonad (ovary), (D) gonad (testis), (E) heart, (F) intestine, (G) kidney, and (H) muscle of zebrafish with or without IQ exposure. Data are presented as mean ± SD (n = 3), analyzed by Student's t test; *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

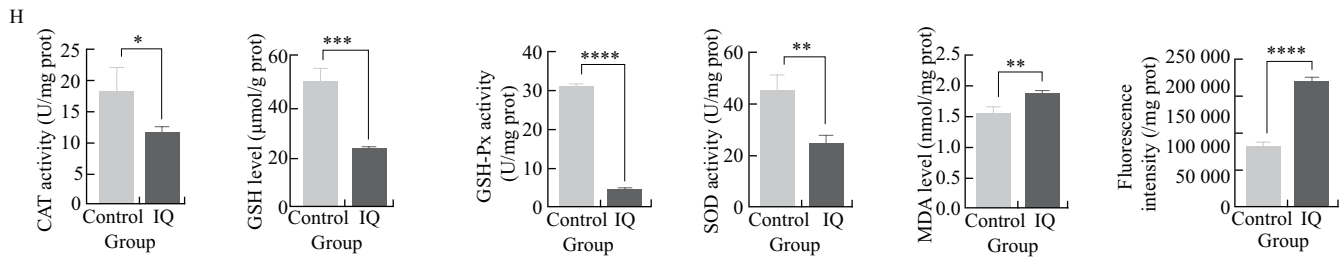


Fig. 3 (Continued)

Compared with the Control group, IQ significantly downregulated the level of antioxidant in the eye, spleen, ovary, testis, heart, intestine, kidney, and muscle of zebrafish (Fig. 3), and significantly increased the levels of MDA and ROS in these tissues, indicating that IQ induced oxidative stress in multiple tissues of zebrafish.

To elucidate the molecular level of IQ-induced systemic tissue oxidative damage, we investigated the mRNA level of genes encoding antioxidants (*cat*, *gpx1a*, *sod1*, *sod2*, glutathione S-transferase pi 1 (*gstp1*), and heme oxygenase 1a (*hmox1a*)). The levels of these genes in multiple tissues of zebrafish are illustrated in Fig. 4. IQ significantly downregulated the mRNA level of antioxidant-related genes in the eye, spleen, ovary, testis, heart, intestine, kidney, and muscle of zebrafish (Fig. 4), in accordance with the decrease in the activities of antioxidants.

The study reported that IQ-exposed zebrafish show reductions in antioxidants in the liver and brain tissues^[19,25]. Altogether, our

data suggest that IQ affects the normal transcription of antioxidant-related genes and oxidative stress parameters in multiple tissues of zebrafish.

3.4 IQ induced mTOR genic-expression in multiple tissues of zebrafish

We previously found that IQ induced liver damage in zebrafish was associated with autophagy inhibition^[15]. We hypothesized that IQ-induced multi-organ damage is closely related to mTOR signaling, which plays an important regulatory role in autophagy. As shown in Fig. 5, the mRNA expression of mTOR was evaluated by qPCR analysis. After 35-day exposure, *mtor* mRNA expression was highly elevated in all the multiple tissues (Fig. 5) of zebrafish exposed to IQ compared to the Control group.

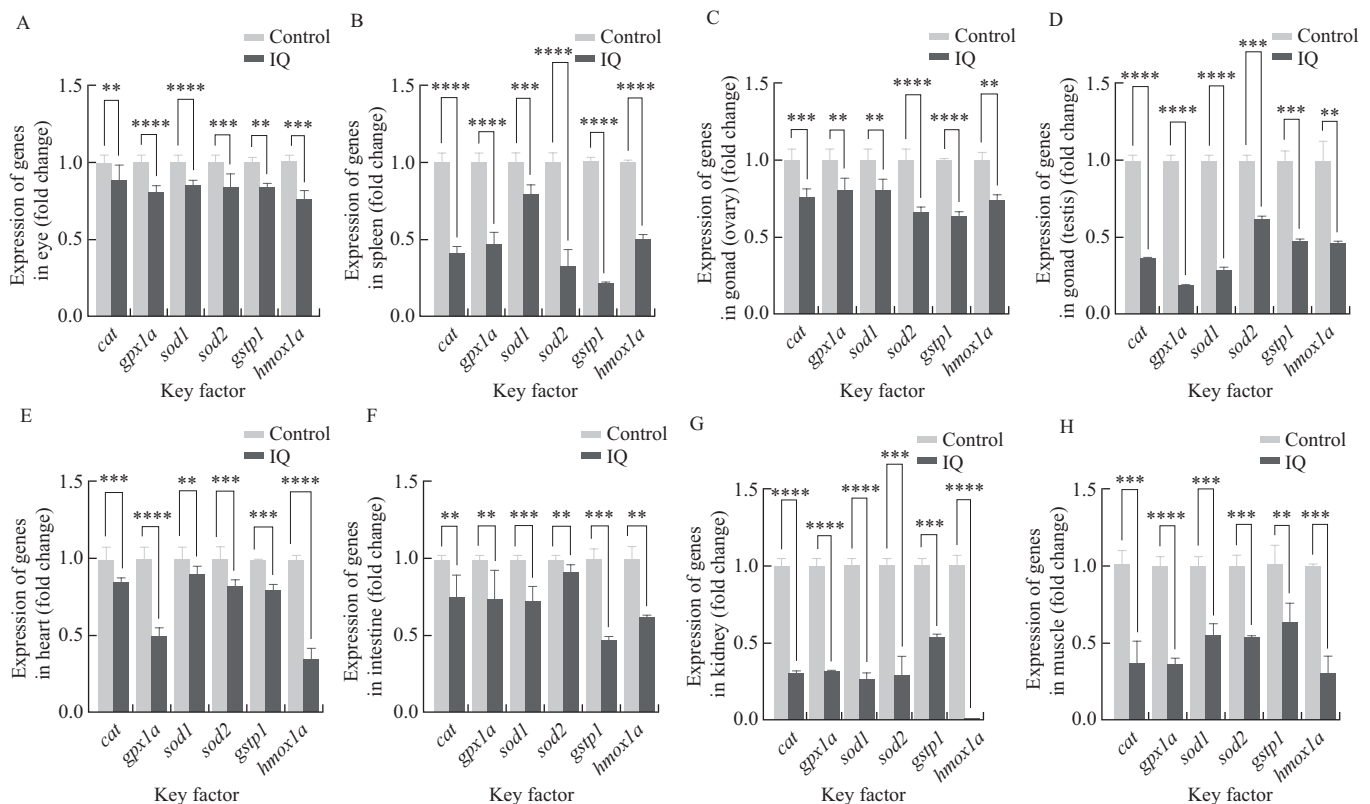


Fig. 4 Effects of IQ on the expression of antioxidant-related genes in multiple tissues. Gene expression of key factors (*cat*, *gpx1a*, *sod1*, *sod2*, *gstp1*, and *hmox1a*) involved in the antioxidant mechanisms in (A) eye, (B) spleen, (C) gonad (ovary), (D) gonad (testis), (E) heart, (F) intestine, (G) kidney, and (H) muscle of zebrafish with or without IQ exposure. Expression of target genes through qPCR analysis using gene-specific primers; β -actin served as the internal loading control for transcript abundance analysis. Data are presented as mean $\pm$ SD ($n = 3$), analyzed by Student's *t* test; ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

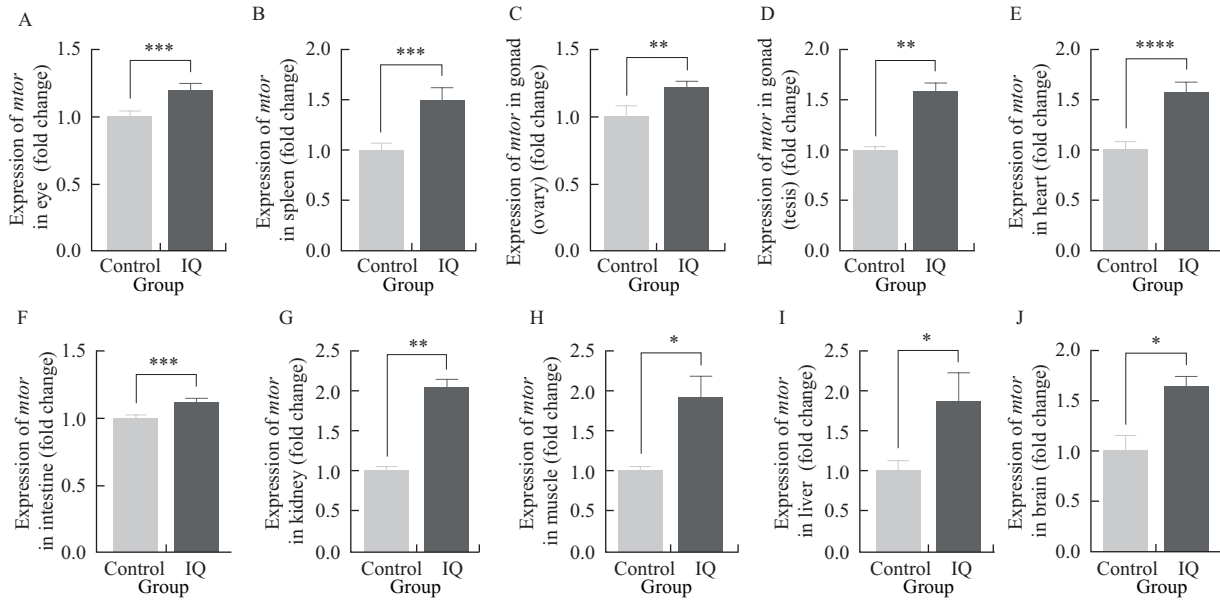


Fig. 5 Effects of IQ on the gene expression of mTOR in multiple tissues. Gene expressions of *mTOR* in (A) eye, (B) spleen, (C) gonad (ovary), (D) gonad (testis), (E) heart, (F) intestine, (G) kidney, (H) muscle, (I) liver, and (J) brain of zebrafish with or without IQ exposure. Expression of target gene through qPCR analysis using gene-specific primers; β -actin served as the internal loading control for transcript abundance analysis. Data are presented as mean $\pm$ SD ($n = 3$), analyzed by Student's t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

3.5 IQ induced mTOR phosphorylation in multiple tissues of zebrafish

Immunohistochemical analysis was performed to detect IQ-induced systematic changes in mTOR phosphorylation protein levels, as shown in Fig. 6. Compared with Controls, IQ induced a significant increase in relative p-mTOR levels in the eye, spleen, ovary, testes, heart, intestine, kidney, muscle, liver, and brain (Fig. 6) of zebrafish, in agreement with the influence of expression pattern at the gene level. These data demonstrate that IQ modulates mTOR phosphorylation in multi-organ tissues, which may be the core mechanism underlying the detrimental effects of IQ.

3.6 IQ induced alterations in the expression of autophagy- and DNA damage and repair-related genes and caused apoptosis in multiple tissues of zebrafish

Given that IQ-induced oxidative stress and mTOR phosphorylation activation are key contributors to multi-tissue damage

in zebrafish, we further examined the expression of autophagy-, DNA damage-, and repair-related genes. The qPCR analysis revealed that all tested autophagy-related genes, autophagy-related 3 (*atg3*), *atg5*, *atg6*, *atg7*, *atg8* and *atg12* were significantly decreased in the eye, spleen, ovary, testes, heart, intestine, kidney, and muscle (Fig. 7) of IQ-exposed zebrafish compared to the Control group; in contrast, DNA damage and repair-related genes, ataxia telangiectasia mutated (*Atm*), cyclin-dependent kinase inhibitor 1 A (*cdkn1a*), damaged DNA binding protein 2 (*ddb2*), growth arrest and DNA-damage-inducible, alpha a (*gadd45aa*), growth arrest and DNA-damage-inducible, gamma a (*gadd45ga*) and tumor protein p53 (*tp53*) were significantly upregulated in these tissues (Fig. 7). TUNEL method was used to detect apoptosis of zebrafish tissues caused by exposure to IQ. The results of TUNEL showed that the incidence of TUNEL-positive cells was obviously higher in multiple tissues of IQ-exposed zebrafish. Collectively, these results indicate that exposure to IQ inhibited autophagy and induced DNA damage and apoptosis in zebrafish multiple tissues.

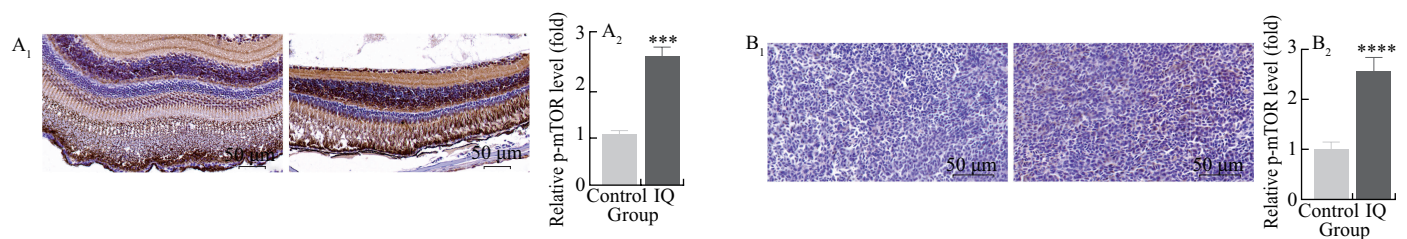


Fig. 6 Immunohistochemical examination of the p-mTOR in multiple tissues. (A₁-J₁) Representative images and (A₂-J₂) statistics of immunohistochemical staining of p-mTOR in (A) eye, (B) spleen, (C) gonad (ovary), (D) gonad (testes), (E) heart, (F) intestine, (G) kidney, (H) muscle, (I) liver, and (J) brain from zebrafish with or without IQ exposure. Data are presented as mean $\pm$ SD ($n = 5$), analyzed by Student's t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

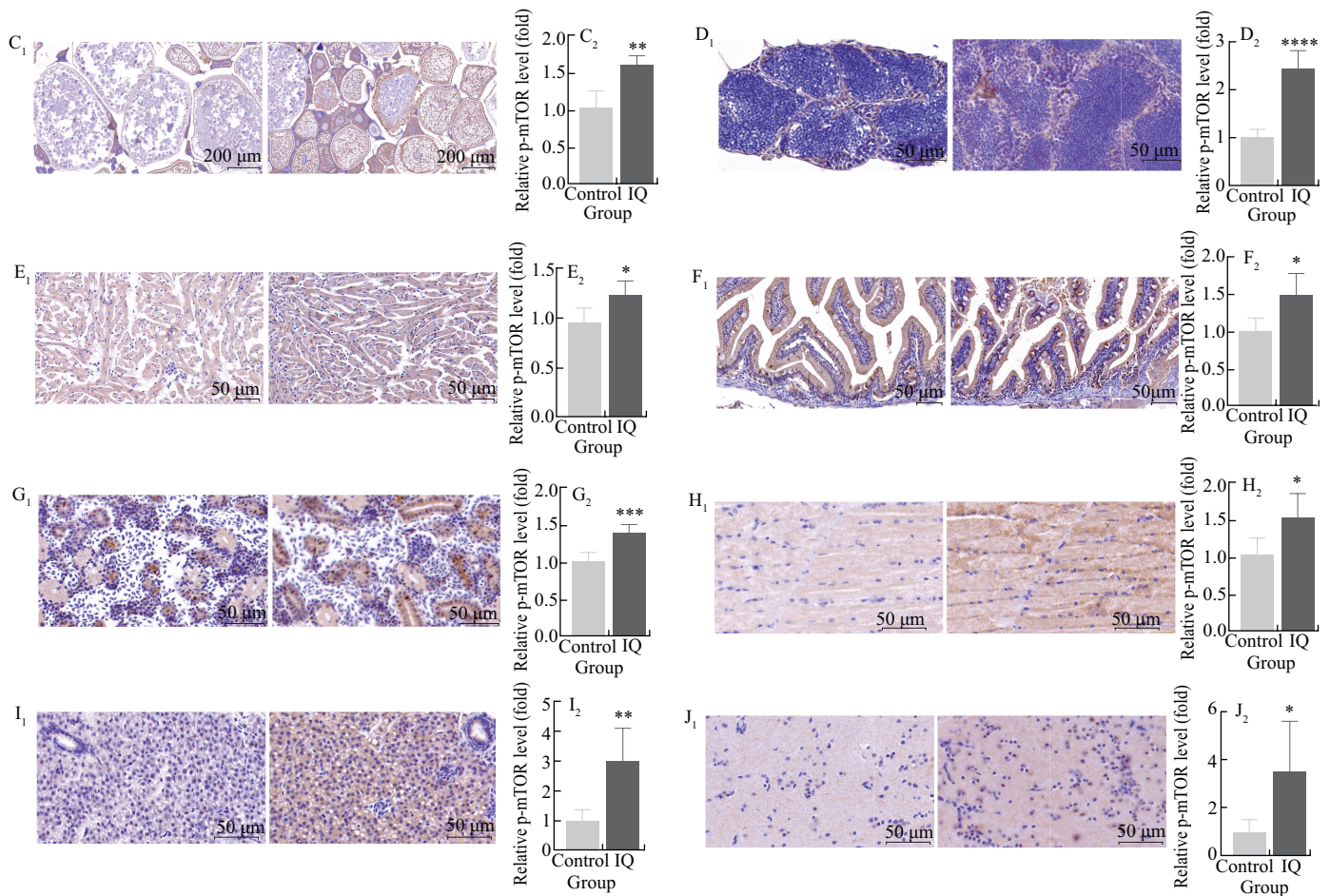


Fig. 6 (Continued)

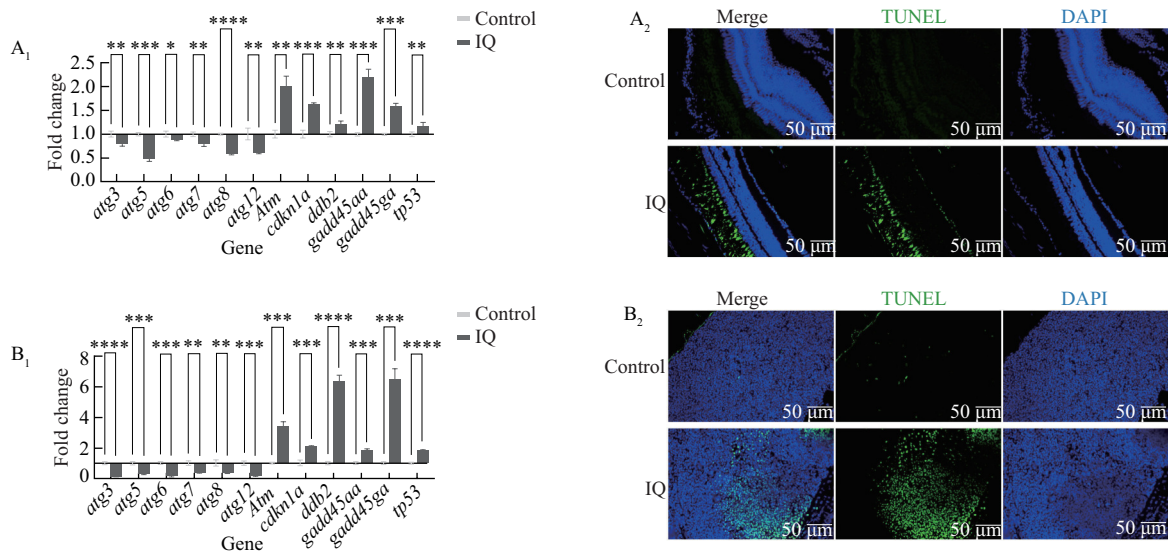


Fig. 7 Effects of IQ on the expression of autophagy- and DNA damage and repair-related genes and apoptosis in multiple tissues. Gene expression of key factors involved in the autophagy and DNA damage and repair in (A₁) eye, (B₁) spleen, (C₁) gonad (ovary), (D₁) gonad (testis), (E₁) heart, (F₁) intestine, (G₁) kidney, and (H₁) muscle of zebrafish with or without IQ exposure; (A₂-H₂) Representative TUNEL staining images of these tissues after IQ exposure. Expression of target genes through qPCR analysis using gene-specific primers; β -actin served as the internal loading control for transcript abundance analysis. Data are presented as mean $\pm$ SD ($n = 5$), analyzed by Student's t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

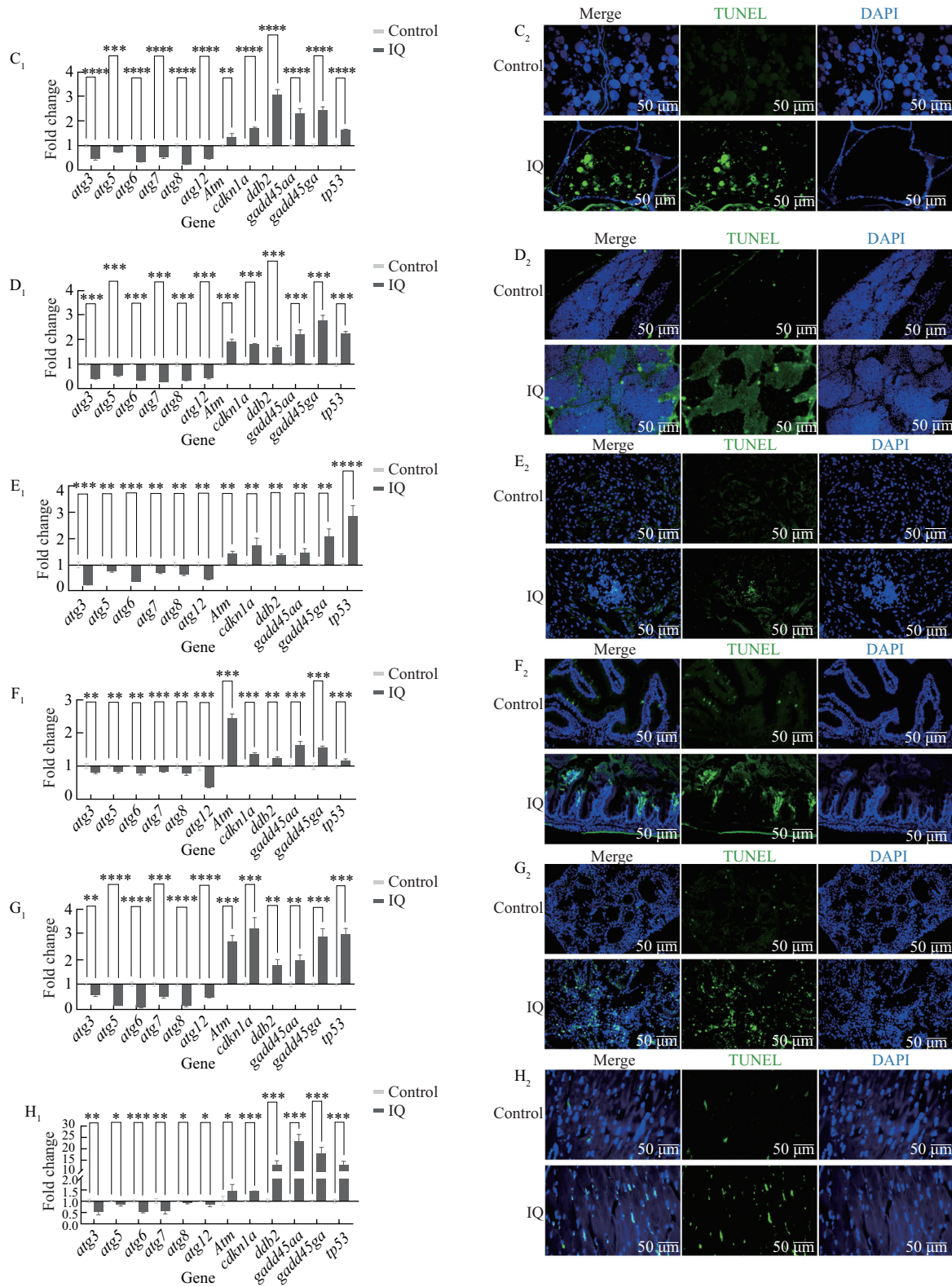


Fig. 7 (Continued)

3.7 IQ induced DNA damage in multiple tissues of zebrafish

Immunofluorescence analysis was performed to confirm IQ-induced DNA damage in zebrafish multiple tissues, as shown in Fig. 8. Compared with controls, IQ induced an obvious increase in

immunostaining of γ -H2AX, a DNA damage marker, in the eye, spleen, ovary, testes, heart, intestine, kidney, muscle, liver, and brain (Fig. 8) of zebrafish. These representative images visually show that exposure to IQ induced DNA damage in multiple tissues of zebrafish.

3.8 Inhibition of mTOR attenuated oxidative stress and apoptosis related markers in multiple tissues of IQ-induced zebrafish

As shown in Fig. 9, the levels of MDA and T-AOC in multi-organ tissues of zebrafish were detected. Compared with the IQ group, IQ + Rapa group showed a decrease in the level of MDA, and increase in the level of T-AOC in the eye, spleen, ovary, testis, heart, intestine, kidney, muscle, liver, and brain (Fig. 9) of zebrafish, indicating that mTOR inhibition was able to restore the altered levels of oxidative stress-related markers in multiple tissues of IQ-induced zebrafish. Under stress, the cell shifts undergo apoptosis instead of autophagy. Therefore, to further investigate

the effect of mTOR inhibition on apoptosis in IQ-induced zebrafish, the gene expression of an autophagy marker *mtor* and apoptotic markers (*Caspase-3*, *Caspase-8*, *Caspase-9*, and *bcl-2*) was studied (Fig. 9). Compared with the IQ group, a decrease in the expression of *mtor* was observed in the IQ + Rapa group in the eye, spleen, gonad, gonad, heart, intestine, kidney, muscle, liver, and brain of zebrafish. Interestingly, the expression of *Caspase-3*, *Caspase-8*, and *Caspase-9* was reduced, and *bcl-2* was upregulated in the IQ + Rapa group in comparison to that of IQ group in multi-organ tissues of zebrafish. These results indicate that inhibition of mTOR could attenuate oxidative stress and apoptosis in IQ-induced zebrafish multiple tissues.

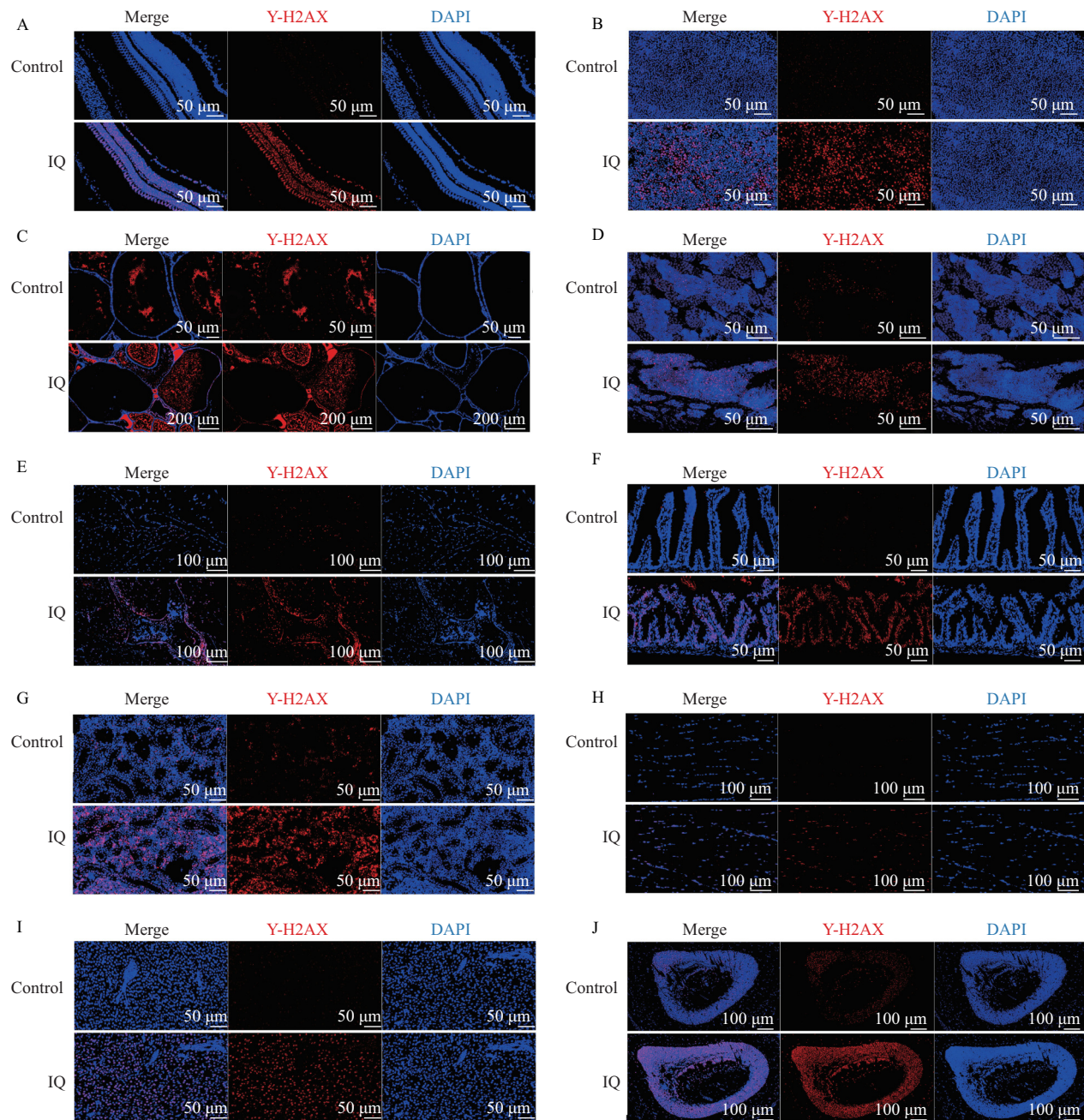


Fig. 8 Immunofluorescence examination of the in multiple tissues. Representative images of immunofluorescence staining of γ -H2AX in (A) eye, (B) spleen, (C) gonad (ovary), (D) gonad (testes), (E) heart, (F) intestine, (G) kidney, (H) muscle, (I) liver, and (J) brain from zebrafish with or without IQ exposure.

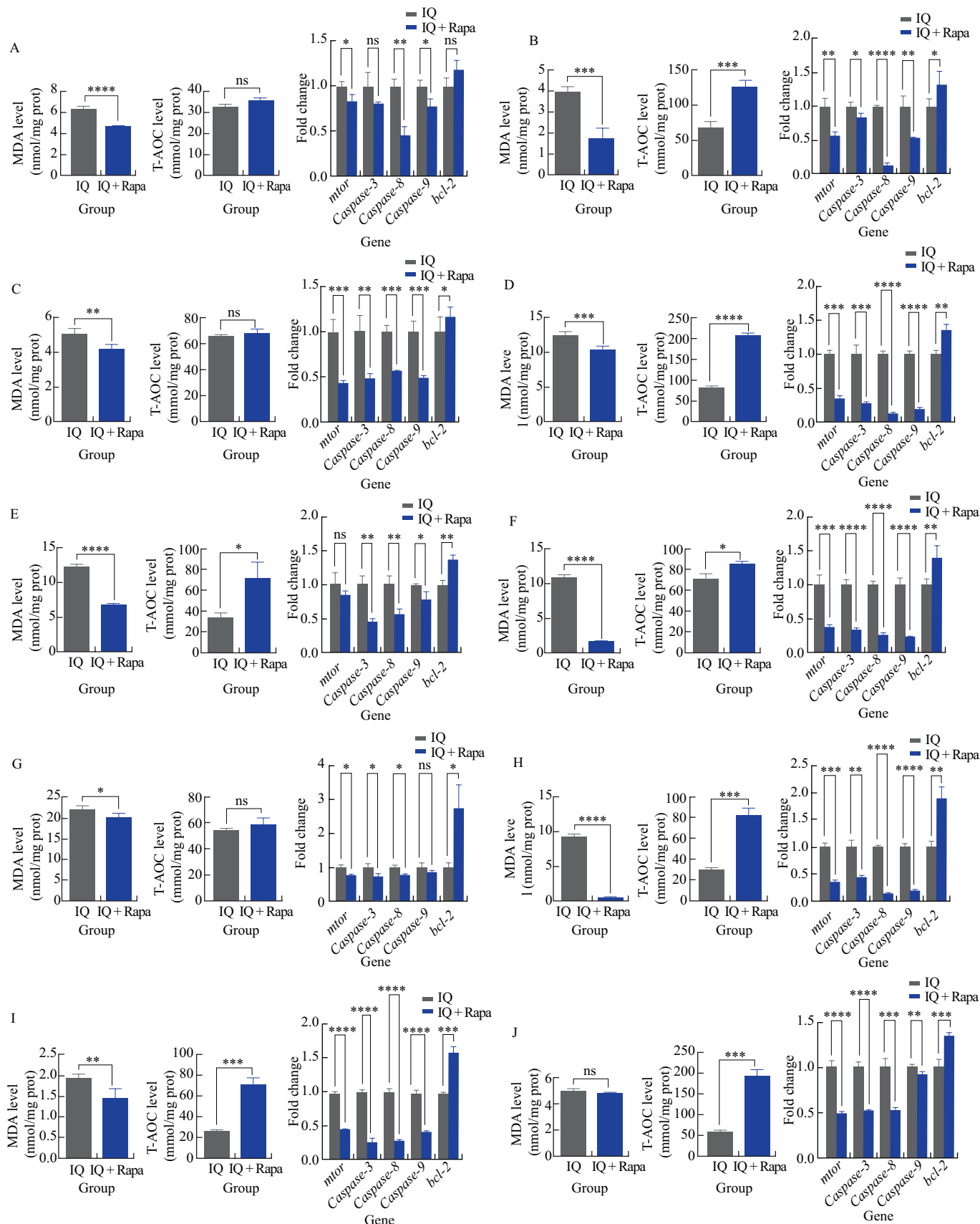


Fig. 9 Effects of mTOR inhibition on oxidative stress and apoptosis markers in multiple tissues of IQ-exposed zebrafish. Levels of MDA and T-AOC, and the gene expressions of *mtor*, *Caspase-3*, *Caspase-8*, *Caspase-9*, and *bcl-2* in (A) eye, (B) spleen, (C) gonad (ovary), (D) gonad (testis), (E) heart, (F) intestine, (G) kidney, (H) muscle, (I) liver, and (J) brain of IQ-exposed zebrafish with or without rapamycin treatment. Expression of target gene through qPCR analysis using gene-specific primers; β -actin served as the internal loading control for transcript abundance analysis. Data are presented as mean $\pm$ SD ($n = 3$), analyzed by Student's t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

4. Discussion

Schut et al.^[31] studied the accumulation of DNA adducts of IQ in tissues and white blood cells (WBCs) of the Fischer-344 rats using ³²P-postlabeling assays. Results showed that accumulation of adducts occurred in all organs and in WBCs in a dose- and time-dependent manner, and adduct formation was highest in the liver, followed by lung, kidney, stomach, large intestine, WBC and small intestine. The accumulation of IQ in different tissues has not been reported previously. Previous study has reported HCAs (IQ, MeIQ, MeIQx and PhIP) were rapidly and almost completely absorbed in the *in vitro* gastrointestinal model (TIM), more than 90% was removed from the jejunal and ileal segments after 4 h, while nearly 10% was delivered at the end of the intestinal compartment^[32]. In addition, a study in rats provided direct empirical support for the role of enterohepatic cycling in the genotoxicity of IQ^[33]. Our results also showed that IQ is still accumulated at higher levels in the liver and intestine. IQ has been detected in various biological samples of zebrafish after exposure, including eye, spleen, gonad (ovary or testis), heart, intestine, kidney, muscle, liver, and brain. Muscle contained the lowest level of IQ, and eye had the highest level. Accumulation in the eye might originate from outer epidermal exposure. It was discovered in brain, which indicated that IQ could cross the blood-brain barrier. High levels in gonad indicated that these accumulated IQ were potentially stored in gonad and could be transferred to the next generation. The current study provides strong evidence that IQ could be taken up by developing zebrafish, and accumulate in multiple tissues, especially across the blood-brain barrier, accumulate in the brain, and potentially exert toxic effects.

Oxidative stress is the first response of an organism when exposed to any exogenous contaminant. Since antioxidants are primarily responsible for scavenging ROS, excessive ROS is one of the indispensable reasons for the occurrence of oxidative stress and the depletion of antioxidant defenses. Higher levels of ROS and their long-time exposure can incur damage to cellular biomacromolecules such as DNA. In addition, increased oxidative stress can mediate abnormal development in the eye, heart, blood vessels, and liver^[34]. IQ exposure has been shown to increase ROS levels and inhibit antioxidant systems *in vitro*^[35]. We also reported that the liver and brain in zebrafish exhibit antioxidant levels reduction after IQ exposure^[19,25]. In this study, a significant reduction in antioxidant activity levels (CAT, GSH, GSH-Px, and SOD) and in expression levels of antioxidative genes (*cat*, *gpx1a*, *sod1*, *sod2*, *gstp1*, and *hmox1a*) in several tissues of IQ-exposed zebrafish. Moreover, IQ can activate aromatic hydrocarbon receptors (AHR)^[36], leading to substantial ROS production^[37]. Consistently, the change of ROS level in all the tissues was contrary to the change of oxidative enzyme activities in this study. Lipid peroxidation is generally thought to be associated with oxidative stress^[38]. We speculate that IQ-induced oxidative stress leads to lipid peroxidation in multiple tissues. According to the results of this study, we found that the MDA content increased significantly after IQ exposure. A recent study reported that AHR acts as a mediator of extractable organic-induced oxidative stress, leading to apoptosis and DNA damage^[37], which are common contributors to hepatotoxicity and nephrotoxicity^[39], brain and spleen toxicity^[40], testicular toxicity^[41], cardiotoxicity^[37], and aging^[28]. In this study, we found that IQ exposure induced oxidative damage, upregulated DNA damage and repair-related gene expression,

and increased the rate of TUNEL-positive cells in all of the IQ-accumulated tissues. Hence, pathological damage of multiple organs and tissues might be associated with oxidative stress, DNA damage, and apoptosis. The decreased antioxidant capacity of multiple organs in IQ-exposed zebrafish is responsible for oxidative stress. We have detected the accumulation of fluorescence signal of γ -H2AX, which is one of the products of DNA damage, suggesting that IQ indeed induced DNA damage. Our results are similar to previous studies in which IQ exposure can cause DNA damage^[7,10].

Oxidative stress stimulation was identified to induce cell death evidenced by increasing the rate of TUNEL-positive cells in different cellular systems. However, the exact molecular mechanism remains elusive. Zhang et al.^[42] demonstrated that co-exposure activated the mTOR signaling pathway and subsequently promoted apoptosis in zebrafish by potentiating oxidative stress, presenting synergistic toxicity. Therefore, we hypothesized that IQ exposure induced oxidative damage through an mTOR-dependent mechanism.

In zebrafish, mTOR is analogous to its role in mammals and regulates physiological adaptation to stressful events in a relatively similar manner during development^[43]. We previously found that IQ-induced liver damage was associated with autophagy inhibition^[15]. In this study, the gene expression levels of *mtor* were significantly upregulated in multi-organ of zebrafish after IQ exposure. Such results confirmed that inappropriate *mtor* expression during development was a probable cause of various organ malformations in zebrafish identified in the IQ-exposed group. Evidence demonstrated that because mTOR signaling has emerged as an important player in the pathogenesis of psoriasis, the authors found that the mTOR inhibitor rapamycin alleviates 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced aggravated dermatitis in mice with imiquimod-induced psoriasis-like dermatitis by increasing the expression of AHR and decreasing the expression of autophagy-related factors^[44]. Thus, changes in *mtor* gene expression after IQ exposure might indicate alterations in the mTOR signaling.

In the present study, the protein phosphorylation levels of mTOR were also significantly increased, evidenced by immunohistochemistry results, which were consistent with the oxidative stress response. mTOR signaling is one of mechanisms closely related to oxidative stress and a key regulatory factor of autophagy^[43,45]. The activation of mTOR promotes mitochondrial biogenesis and oxidative metabolism. It inhibits autophagy by phosphorylating downstream complexes and regulating lysosomal function, a central mechanism for degrading and recycling damaged organelles in cells^[46]. Sustained hyperactive mTOR signaling plays a crucial role in connecting metabolic diseases, cancer and aging through stimulation of oxidative stress^[46-48]. Thus, the multiple organ damage observed in IQ-exposed zebrafish might be due to downstream events mediated by mTOR.

Autophagy, generally mediated by mTOR, is a multistage biological mechanism that is essential for the development and survival of organisms, and plays a pivotal role in organogenesis and maturation^[48]. We previously found that IQ-induced liver damage in zebrafish was associated with autophagy inhibition, including inhibition of autophagy-related gene expression^[15]. In this study, IQ exposure inhibited autophagy also by decreasing the expression of autophagy-related genes. Our results showed that IQ exposure caused histopathological alterations in multiple organs. These alterations may be linked to the autophagy mechanism. Although there are different

mechanisms for controlling autophagy and causing disease during development in multiple organisms, abnormal regulation of mTOR-dependent autophagy may be sufficient to cause developmental abnormalities. The mTOR-dependent autophagy defect in IQ-exposed zebrafish is noteworthy because lysosomal diseases such as Danon disease, Pompe disease, and Gaucher disease have autophagic vacuoles accumulation, as well as in old tissues^[49]. Upregulation of mTOR levels has been shown or is suggested to be involved in aging in model organisms or humans^[50]. It may be an important finding that IQ intake poses a health risk to multi-organ by altering mTOR-dependent autophagy.

During stress conditions, mTOR contributes towards autophagy inhibition and apoptosis^[26]. In the present study, by using rapamycin, a potent and specific mTOR inhibitor, we conducted an experimental intervention to validate mTOR activation as a core mechanism underlying the toxic effects of IQ. To better understand the autophagic interference of mTOR and its association with oxidative stress and apoptosis, it was essential to investigate related markers. Bcl-2, which inhibits Caspase activity, is well known to inhibit apoptosis. The gene expression of *mtor* was downregulated in the IQ + Rapa group in comparison to IQ group, indicating that mTOR is inhibited. Rapamycin treatment decreased MDA contents and increased T-AOC capacities in comparison to IQ group in multi-organ tissues, suggesting that inhibition of mTOR could attenuate the observed oxidative stress. Interestingly, the expression of apoptotic markers (*Caspase-3*, *Caspase-8*, and *Caspase-9*) was reduced, and *bcl-2* was upregulated in the rapamycin-treated group in comparison to IQ group in multi-organ tissues of zebrafish probably *via* mTOR inhibition. These results more fully illustrated that IQ could affect apoptosis-related regulators by activating mTOR, leading to apoptosis. Previous study showed that excessive oxidative stress markers triggered stress in the heart; Treatment with rapamycin attenuated the cardiac oxidative stress marker supporting its protective effect against seizures-associated cardiac damage^[26]. Thus, the present study showed that mTOR activation is a direct consequence of IQ-induced oxidative stress, and inhibition of mTOR can improve IQ-induced oxidative stress and tissue damage-related markers in zebrafish.

Herein, we demonstrated that IQ can be accumulated in multiple organs of zebrafish after exposure. The present results suggest that long-term IQ exposure may induce systematic tissue damage and mTOR activation. Loss of tissue integrity is a mark of aging, and cancer is one of the classic diseases of aging. In the following studies, we will explore the influence of IQ on the upstream and downstream of mTOR.

5. Conclusion

Overall, this study represents the first report using zebrafish to evaluate IQ accumulation and its associated tissue damage across multiple organs (eye, spleen, gonad (ovary or testis), heart, intestine, kidney, muscle, liver, and brain) following a 35-day exposure period. Our findings revealed that exposure to IQ could be taken up by developing zebrafish, and accumulate in multiple tissues, especially across the blood-brain barrier, accumulate in the brain, subsequently causing systemic tissue damage. Significant histopathological changes were detected in tissues where IQ was shown to accumulate. It also leads to oxidative stress, apoptosis, DNA damage, and mTOR over activation. Additionally, IQ accumulation was higher in internal

organs (spleen, kidney, liver, intestine) after IQ exposure. mTOR inhibition attenuated oxidative stress and apoptosis in IQ-induced zebrafish multiple tissues. Our results provide evidence for the risk and mechanisms of IQ-induced multi-organ tissue damage, indicating the potential long-term negative effects of IQ exposure on the systemic health of organisms, further emphasizing the necessity of controlling or intervening in IQ intake.

Conflict of interest

Shuo Wang is an editorial board member for *Food Science and Human Wellness* and was not involved in the editorial review or the decision to publish this article. The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://doi.org/10.26599/FSHW.2025.9250486>.

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