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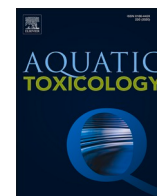
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Interference of gut-brain-gonad axis originating from triclocarban exposure to parent zebrafish induces offspring embryonic development abnormality by up-regulation of maternal circSGOL1

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ABSTRACT

Triclocarban (TCC) is a widely used antibacterial ingredient possessing acute toxicity effects; however, its chronic toxicity and underlying molecular mechanisms remain uncertain. Herein, we demonstrated that chronic TCC exposure affects the growth and development of adult zebrafish through inducing an intestinal flora disorder in the gut. The imbalance of intestinal flora caused functional barriers within the intestinal-brain-gonadal axis. This resulted in a series of anomalous nerve and motor behaviors, and reproductive toxicity as reflected in pathological damage to parental gonads and F₁-larval developmental malformations. Abnormal development of F₁ larvae was attributed to apoptosis induced by the up-regulation of circSGOL1. This up-regulation affected the activity and localization of the hnRNP A1 protein, which then promoted overexpression of pro-apoptotic related genes that ultimately lead to apoptosis during early embryonic development. Overall, these novel findings systematically elucidated the TCC toxicity mechanism in parent-offspring dyads, and provide important theoretical guidance for early risk warning and control of chronic TCC toxicity.

1. Introduction

Triclocarban (TCC) is a broad-spectrum lipophilic antibacterial agent that acts by preventing fatty acid synthesis and formation of microbial cell membranes (Hipwell et al., 2019; Kim et al., 2021). There was a sharp increase in the production and indiscriminate use of TCC following the outbreak of COVID-19 (Wang et al., 2022). With the COVID-19 pandemic, the use of disinfectants and hand soaps greatly increased around the world, which also elevated the TCC concentrations in domestic sewage and hospital sanitary effluents or sewage treatment plants (Li et al., 2020b). For example, the highest concentrations of TCC in the sludges of 21 provinces and cities in China are > 4870 ng/g (Chen et al., 2019). In Canada, the maximum concentration of TCC in biosolids from six wastewater treatment plants reached as high as 11,000 ng/g (Guerra et al., 2014). TCC was detected up to ~500 ng/L from 343 out of 365 surface water samples in China (Zhao et al., 2013). Due to its persistence, 84~114 µg/L of TCC was detected in the influent of U.S. municipal wastewater treatment facilities. In China, TCC is ubiquitously

detected in rivers and sediments along with organisms, such as fish and birds (Su et al., 2020; Zheng et al., 2021). TCC in pregnant women's serum, umbilical cord serum, urine and amniotic fluid is commonly found at detection rates and concentrations of > 76.9 % and >5 ng/L, respectively (Bai et al., 2020; Li et al., 2020a). Due to the potential for TCC bioaccumulation in the food chain, its environmental risk has attracted widespread public concern.

TCC exposure may trigger the risk of type 2 diabetes mellitus in women, enhance the occurrence of colitis and colon tumors, and interfere with the redox state of human liver cells (Dong et al., 2020; Zhang et al., 2020). Additionally, it can disrupt the maturation of mouse oocytes by affecting cell cycle progression, damaging autophagy of mouse neuronal cells and disturbing the signal transduction of estrogen receptors (Kajta et al., 2020). Moreover, our group found that TCC exposure to zebrafish caused high embryonic mortality, delayed hatching and yolk absorption, disrupted vascular development and significantly reduced the number of immune cells and their aggregation function (Xu et al., 2021). However, the underlying TCC toxicity mechanism is still

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ambiguous, especially regarding its reproductive toxicity to parent-offspring (F_0 - F_1).

The gut-brain axis is a multi-channel bidirectional regulatory network, which not only affects nerve and immune system functions, but also acts on the gonads through the endocrine pathway (Agirman et al., 2021; Hudry et al., 2019; Tysoe, 2021). The gut and brain can exchange signals through the efferent and afferent fibers of the vagus nerve, and also rely on immune factors in the blood to penetrate the blood-brain barrier and central nervous system (Agirman and Hsiao, 2021). Gut microbial metabolites and ghrelin drive sex-specific gene expression, regulate metabolism and organ rhythms, and convert inactive sex hormones into active states, thereby regulating sex hormones and fertility (Morais et al., 2021). Thus, a question arises as to whether chronic TCC exposure affects the gut-brain-gonad axis by interfering with the balance of intestinal flora in zebrafish.

The circRNAs are characterized by a closed continuous loop structure and widely expressed in vertebrate (Baumann, 2020; Bozzoni, 2021). Because circRNAs are highly stable and conserved in different species, they possess a wide range of molecular functions, including acting as miRNA sponges, regulating protein translation and degradation, and interaction with RNA binding proteins (RBP) (Chen, 2020). Notably, they have cell type-specific and developmental stage-specific expression characteristics (Kristensen et al., 2019), which may alter pregnancy-related diseases, apoptosis, gamete and embryonic development (Kristensen et al., 2022). As such, circRNA identification and functional analysis are important for estimating the quality of gametes and embryos, and for developing novel diagnostic and therapeutic targets for a variety of genetic diseases (Wu et al., 2022). Based on SRA shared database from NCBI (<https://trace.ncbi.nlm.nih.gov/Traces/sra/>), zebrafish sperm data were obtained with ID: GSM1085059 and ovum data ID: GSM1085060, and 2-cell zygote data were acquired with ID: ERR202500 from transcriptome sequencing. These data were employed to predict, screen and identify circRNAs inherited from parents to offspring. The intersection circRNAs were adopted from sperm, ovum and zygote, and circSGOL1 was screened out. A host gene of circSGOL1 was discovered to be Shugoshin-like 1 (SGOL1), which located on chromosome 19 of zebrafish, and contained 11 exons. The 7th exon of SGOL1 formed circSGOL1 (164 bp) through reverse splicing, and SGOL1 was a key protein to protect sister chromatids from premature separation during mitosis (Kahyo et al., 2011; Kim et al., 2013), which was associated with the occurrence, progression and prognosis of tumors (Pallai et al., 2015). Cancerous inhibitor of protein phosphatase 2A promoted cell cycle progression, premature chromosome segregation and aneuploidy, possibly through a novel interaction with SGOL1 (He et al., 2021). Sun's group reported that the knockout screen identified inhibition of SGOL1 expression as the most-effective genetic suppressor of sorafenib activity, and loss of SGOL1 was associated with sorafenib resistance (Tian et al., 2020). As a centromeric protein, SGOL1 played an important role in mitosis, and the levels of SGOL1 in hematological malignancies was abnormally expressed (Sun et al., 2018). However, so far there is a paucity of information regarding the functional research of circSGOL1.

Long-term TCC exposure to parents at environmental levels may greatly affect the development of their offspring, even if the offspring were not directly exposed to the pollutant. To address the many knowledge gaps concerning chronic TCC exposure, we herein employed zebrafish as a model organism to investigate the toxicity effects and underlying molecular mechanisms based on the gut-brain-gonad axis, and will explore the immunotoxic mechanism of TCC induced intergenerational transmission to F_1 from the perspective of maternal circSGOL1 regulation. These findings provide therapeutic targets and pathways for TCC-induced reproductive toxicity and enrich our understanding of their complex interactions at the molecular level.

2. Material and methods

A TCC-exposure concentration of 225 $\mu\text{g/L}$ was used to assess toxicity effects on F_0 zebrafish from 30-dpf (days-post-fertilization) larvae to 100-dpf sexually mature adults. Four groups were set: control, TCC-exposure, TCC-exposure +5 % sodium deoxycholate (SDA), and TCC-exposure +5 % vitamin C (VC). After exposure, the intestinal contents were aseptically dissected and collected for Illumina library construction, sequencing and bioinformatics analysis. Optical microscopy was used to assess histopathological injury of parents and whole-mount immunofluorescence staining of offspring was used to examine TCC-induced reproductive toxicity. Four behavioral assays were conducted on TCC-exposed 100-dpf adults: bottom dwelling test (BDT), conditioned place preference (CPP), social interaction test (SI) and T-maze trials.

Raw data for whole transcriptome sequencing of zebrafish sperm, oocyte and 2-cell stage were obtained from NCBI's public and open SRA databases. Screening and bioinformatics analysis of circRNAs were performed using OmicStudio tools at <https://www.omicstudio.cn/tool>. Cy3-labeled circSGOL1 probes were designed and synthesized by Gene-Pharma (Suzhou, China), and the hybridization signals of circSGOL1 were detected with fluorescent *in-situ* hybridization kits (Gene-Pharma). The circSGOL1 overexpression plasmid, siRNA and hnRNP A1 siRNA were synthesized by Gene-Pharma and injected into 1~2-cell-stage embryos at the yolk/cytoplasm boundary with 20 μM solution.

In the control group, ultrapure water containing 0.001 % acetone solvent was employed. Each treatment group included three biological replicates ($n = 3$), and each biological replicate was composed of three technical replicates. The sample size for each technical replicate was 24 larvae or adult zebrafish, and thus the total sample size for each biological replicate was 72 larvae or adult zebrafish. In order to remain the stable TCC-exposure concentration, the exposure solution was renewed per week for F_1 zebrafish. All experimental data were presented as the mean $\pm$ standard deviation (SD). Statistical analyses were conducted with the SPSS 18.0 software (Chicago, USA), and the significant levels were expressed at $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). To decrease the manuscript length to meet the requirements of this journal, all experimental protocols were rigorously described and placed in the Supplementary materials.

3. Results and discussion

3.1. Chronic TCC exposure induced developmental toxicity in adult zebrafish

A schematic representation of the overall TCC-exposure pathway is displayed in Fig. 1A. In order to investigate the gut-brain-gonad axis disorder caused by chronic TCC exposure through oxidative stress, four treatment groups (Control, TCC, TCC+SDA, TCC+VC) were set, and 5 % sodium deoxycholate (SDA) and 5 % vitamin C (VC) were added as antioxidant modulators to the TCC-exposure group, respectively. As a classic reduction substance, VC can eliminate excess ROS in the body and change the oxidative-stress state, and thus it was adopted as a rescue agent (Moslehi et al., 2023). Herein, VC partially rescued injury to the adult zebrafish gut-brain axis under TCC exposure. SDA is known to effectively disrupt many protein-protein interactions, and its injection into living fat can lyse adipocytes through mechanical cellular disruption to facilitate their absorption in the intestine (Muskat et al., 2022). Therefore, SDA was added to the zebrafish diet in an attempt to rescue the associated toxicity induced by chronic TCC exposure. SDA is beneficial for the development of nerves and intestinal flora (Xu et al., 2021), thereby exhibiting a restoration effect, to a certain degree, on the TCC-triggered toxicity. The result showed that no zebrafish death was observed from 30-dpf juvenile zebrafish to 100-dpf adults in the three TCC treatments or control group (Fig. 1B). Compared to the control group, there was no significant difference in body length across TCC

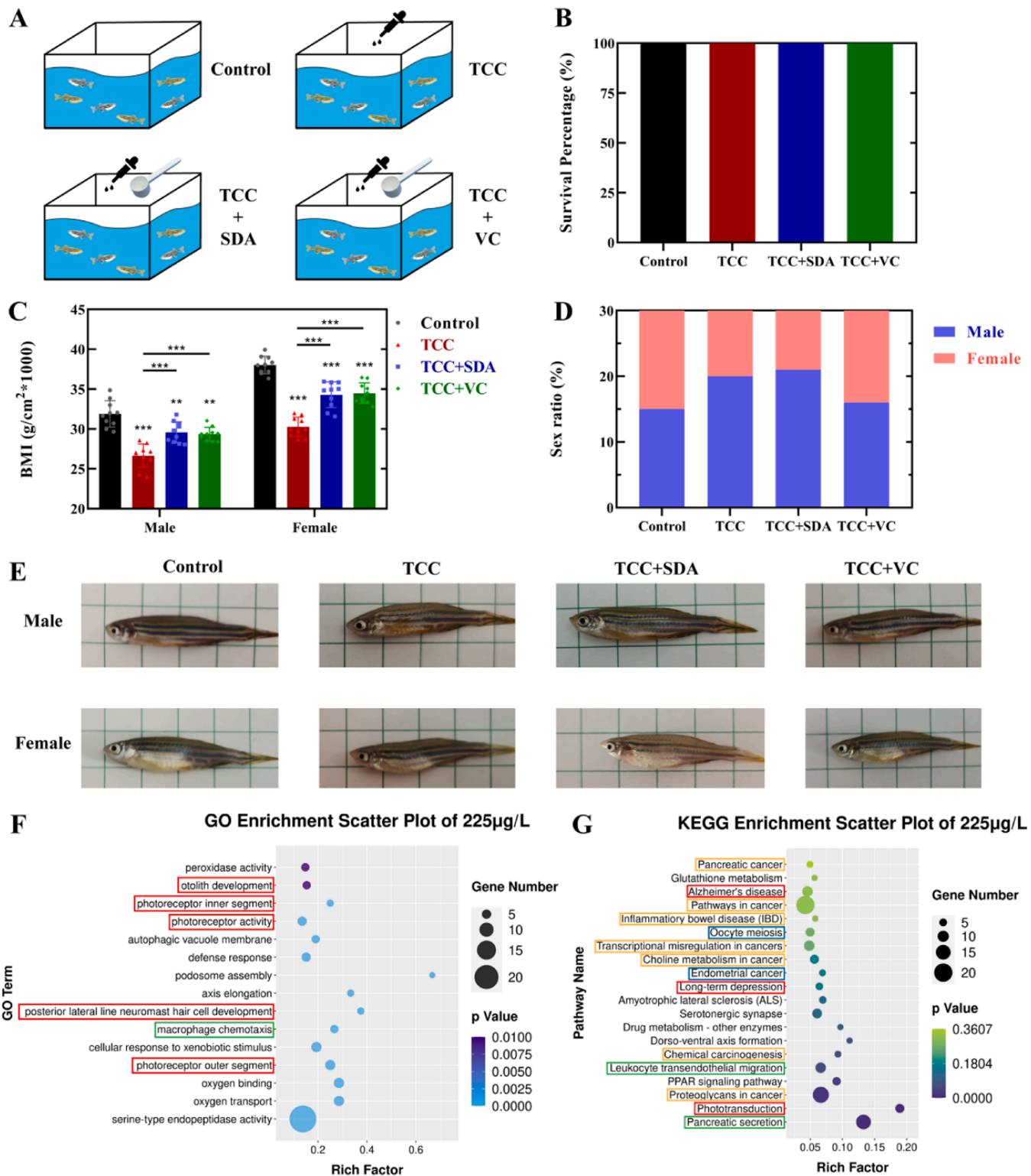


Fig. 1. Developmental toxicity in adult zebrafish exposed to TCC for 70 days

Notes: (1) A, Exposure strategy. (2) B, The survival rate of zebrafish in each group. (3) C, BMI index of female and male zebrafish in each group. (4) D, The sex ratio of zebrafish in each group. (5) E, The phenotypic morphology of 100-dpf zebrafish exposed to TCC. (6) F, GO enrichment scatter plot of 5-dpf zebrafish larvae exposed to 225 µg/L TCC. (7) G, KEGG enrichment scatter plot of 5-dpf zebrafish larvae exposed to 225 µg/L TCC. (8) Data are presented as the mean ± SD ($n = 3$; 10 adult zebrafish for each biological replicate in each treatment and control group). (9) Statistical analyses were carried out using the SPSS 18.0 software (Chicago, USA) at three significance levels: * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

treatments, whereas the body-mass index (BMI) significantly decreased for both female and male zebrafish in the three TCC-exposure treatments ($p < 0.001$; Fig. 1C). Notably, the average BMI values in the 225 $\mu\text{g/L}$ TCC+SDA and 225 $\mu\text{g/L}$ TCC+VC groups were significantly reduced by 9.2 % and 8.5 % for male and 9.8 % and 9.3 % for female; however, the addition of SDA and VC significantly attenuated the decreasing BMI trend ($p < 0.001$) induced by TCC. In the control group, the ratio of sexual differentiation was close to 1:1, whereas differentiation increased in the 225 $\mu\text{g/L}$ TCC and 225 $\mu\text{g/L}$ TCC+SDA treatments. As compared to the 225 $\mu\text{g/L}$ TCC treatment, the male-to-female ratio decreased in the 225 $\mu\text{g/L}$ TCC+VC group, but no significant difference was observed compared to the control group (Fig. 1D). The addition of VC significantly

reduced abnormal sexual differentiation induced by 225 $\mu\text{g/L}$ TCC, possibly owing to its antioxidant properties. The phenotypic morphologies of 100-dpf zebrafish showed normal physical structures, smooth body curves, and obviously differentiated gender characteristics between female and male adults (Fig. 1E). At 225 $\mu\text{g/L}$, TCC-exposed zebrafish displayed dysplasia, a thin body and spine curvature, however, these abnormal morphologies did not occur in the 225 $\mu\text{g/L}$ TCC+SDA and 225 $\mu\text{g/L}$ TCC+VC treatments. After zebrafish embryos exposure to the 225 $\mu\text{g/L}$ TCC, RNA-seq determined positively differential transcripts that were screened for GO functional and signaling pathway analyses. As detailed in Fig. 1F, the differentially-expressed genes were mainly related to redox processes, immunity,

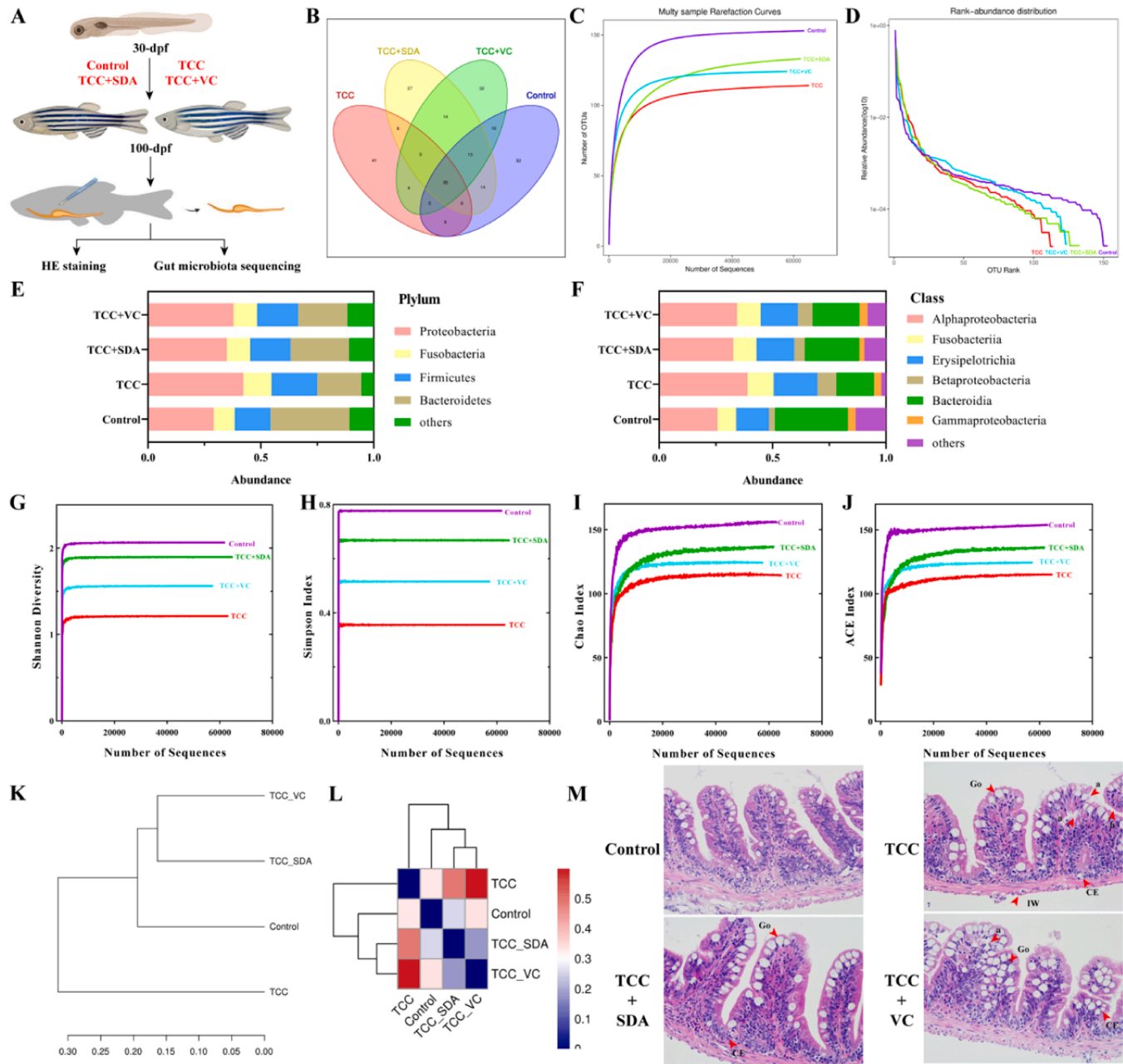


Fig. 2. Sequencing of intestinal flora and intestinal histopathological observation of adult zebrafish under chronic exposure to TCC

Notes: (1) A, The schematic diagram of the sequencing mechanism. (2) B, Cluster OUT analysis of sequencing data. (3) C, The Rarefaction-Curve. (4) The Rank-abundance graph. (5) E-F, Histogram of species distribution at phylum and class level. (6) G-J, Alpha diversity analysis, including Shannon index, Simpson index, Chao index, ACE index. (7) K-L, Beta diversity analysis, including the UPGMA cluster diagram and the Bray-Curtis multi-sample difference matrix heatmap. (8) M, HE staining of zebrafish intestinal tissue sections under chronic exposure to TCC. (9) "a" shows the rupture and ablation of intestinal cells; "b" shows the villous cells were arranged disorderly; CE, columnar epithelial cells; Go, goblet cells; IW, intestinal wall.

inflammation, nerve cell development, nerve organ function and serine-type endopeptidase activity. KEGG analyses determined that differentially-expressed genes were mainly enriched in the cancer-related pathways, such as pancreatic cancer, transcriptional misregulation, choline metabolism, chemical carcinogenesis and proteoglycans in cancer. Additionally, the related reproduction pathways included oocyte meiosis and endometrial cancer; and the neuro-behavioral pathways mainly contained Alzheimer's disease, long-term depression, amyotrophic lateral sclerosis, serotonergic synapse and dorso-ventral axis formation (Fig. 1G). Building upon the KEGG enrichment abundance analyses, we posit that TCC has a prominent endocrine disrupting effect. These findings informed further in-depth investigations of the TCC-triggered toxicity from the perspective of the gut-brain-gonadal axis in zebrafish.

3.2. Chronic TCC exposure induced gut dysbacteriosis and intestinal tissue damage in adults

After TCC continuously exposed to 70 days, the V3-V5 variable regions of 16S rRNA zebrafish gut contents were sequenced to determine changes in intestinal flora (Fig. 2A). Credible OTUs were obtained by clustering the sequencing data after quality control analysis (Fig. 2B). Curves for the four treatments at a 97 % similarity level were flat, suggesting that the amount of sequencing data was reasonable and abundant, and a larger amount of data would generate only a small number of new OTUs (Fig. 2C). Species were most abundant in the control group and the distribution was relatively uniform. Among the three treatment groups, the 225 µg/L TCC-exposure group showed a lower abundance and diversity of intestinal flora (Fig. 2D). The 225 µg/L TCC+SDA and 225 µg/L TCC+VC treatments had a greater abundance and uniformity of intestinal microbiota compared to the 225 µg/L TCC treatment (Fig. 2D). These observations offer strong evidence that chronic TCC exposure interfered with the structure and diversity of intestinal flora in adult zebrafish.

After annotating each OTU, the composition information for intestinal flora was determined at the phylum and class levels. The abundance of four phyla bacteria was significantly changed: *Proteobacteria*, *Fusobacteria*, *Firmicutes* and *Bacteroidetes* (Fig. 2E). After the 225 µg/L TCC exposure, the abundance of zebrafish-gut *Proteobacteria* was 1.4-fold higher than the control group, accounting for ~45.0 % of the overall abundance of intestinal flora. Similarly, the 225 µg/L TCC exposure led to an increase of 37.8 % and 27.7 % in the number of *Fusobacterium* and *Firmicutes*, respectively, but a decrease of 45.7 % for *Bacteroidetes* (Fig. 2E). At the class level, community structure showed a similarly changing trend as the phylum level, i.e., increased *Proteobacteria* and *Erysipelas* populations and a decreased *Bacteroidetes* population (Fig. 2F). In the 225 µg/L TCC+SDA and 225 µg/L TCC+VC treatments, the abundance of pathogenic microflora were reduced and probiotics increased to some extent, compared to the 225 µg/L TCC exposure group. Intestinal tract is an important immune and nutrient absorbing organ in zebrafish, and the decrease in diversity of intestinal flora may interfere with the gut-brain axis, which can explain the underlying mechanisms regarding neurobehavioral abnormalities induced by long-term TCC exposure to F₀ zebrafish. These findings infer that chronic TCC exposure has the potential risk of inducing immune and metabolic diseases. These inferences were further confirmed by analyses of Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolism-pathway enrichment for the transcriptome data. Consequently, we posit that intestinal flora disorder is closely related to abnormal BMI and male-to-female differentiation.

The alpha diversity metrics of Chao and ACE indices characterize community richness, while the Shannon and Simpson metrics assess community diversity. Zebrafish intestinal flora in the control group were most abundant and displayed the highest community richness and community uniformity (Fig. 2G–J). TCC exposure at 225 µg/L resulted in the lowest alpha diversity, implying that gut community diversity was

substantially reduced and more heterogeneous. The diversity was largely restored in the 225 µg/L TCC+SDA and 225 µg/L TCC+VC treatments compared to the 225 µg/L TCC-exposure treatment, which was consistent with the abundance rank curves (Fig. 2G–J). Notably, the UPGMA cluster diagram showed that the 225 µg/L TCC+SDA and 225 µg/L TCC+VC groups were clustered close to the control group and distinctly separated from the 225 µg/L TCC-exposure group, implying an effective attenuation of TCC toxicity by SDA and VC (Fig. 2K). A multi-sample differential matrix heatmap was determined from a Bray-Cutis analysis and the results were consistent with the cluster diagram (Fig. 2L).

H&E staining of control group zebrafish showed complete and clear intestinal structure, normal mucosal folds in outer muscle layer, intact intestinal villus, and neatly arranged villous cells (Fig. 2M). In sharp contrast, the 225 µg/L TCC exposure triggered cellular rupture and ablation, decreased the number of columnar epithelial cells, and increased and swollen goblet cells (Fig. 2M-red arrow). Meanwhile, the intestinal wall became thinner and villous cells displayed a disorderly arrangement. Notably, the 225 µg/L TCC+SDA and 225 µg/L TCC+VC treatments had alleviated intestinal injuries compared to the 225 µg/L TCC treatment. Overall, chronic TCC exposure induced intestinal flora disturbance and tissue damage in adult zebrafish that were largely attenuated by SDA and VC amendments.

3.3. TCC exposure triggered zebrafish neurotoxicity and abnormal motion behavior

To elucidate relationships within the gut-brain axis, whole-body immunofluorescence analysis was performed on 120-hpf (hours post-fertilization) larval zebrafish across a TCC concentration gradient. In the control group, the recently-developed neurons were clearly observed in the telencephalon, diencephalon, midbrain, cerebellum and oblongata, a "Y"-shaped neuronal cord was formed at the notochord-brain junction, and the neurons were abundant and densely/uniformly distributed (Fig. 3A). At the 25 and 125 µg/L TCC levels, the number of neurons in the brain decreased slightly ($p < 0.05$), and the formation of "Y"-shaped neuronal cords was inhibited and imperfect. At the 225 µg/L TCC, neurons were severely reduced and the "Y"-shaped neuronal cords were malformed ($p < 0.001$; Fig. 3A). Notochordal neurons showed obvious developmental defects, and no new neurons were observed in the body trunk and tail among all TCC-exposed treatments. These observations documented spinal curvature in adults after long-term TCC exposure, and demonstrated that TCC can affect development of the zebrafish central nervous system.

Bottom dwelling tests (BDT) indicated that zebrafish displayed higher locomotor activity in the absence of TCC, but the travel distance and speed were significantly decreased by 24.1 % and 20.6 % in the 225-µg/L TCC treatment, respectively ($p < 0.001$; Figs. 3B and 4A). Zebrafish in the control group displayed no preference for the upper or lower layer, and exhibited strong motion activity and daring to swim in the upper water layer. Under the 225 µg/L TCC exposure, zebrafish preferentially swam at the bottom and showed a significant decrease of time and frequency in the upper layer, as illustrated in the trajectory tracing heatmap (Figs. 2B and 4B). When the 225 µg/L TCC-exposed zebrafish occasionally swam into the upper area, they displayed excessive anxiety and restlessness, and then quickly moved to the lower layer ($p < 0.01$, $p < 0.001$; Fig. 4B). Compared to the 225 µg/L TCC treatment, zebrafish movement distance, speed, locomotor activity and habits were significantly improved in the 225 µg/L TCC+SDA and 225 µg/L TCC+VC treatments ($p < 0.05$, $p < 0.001$).

Zebrafish are social vertebrates that tend to gather together with their peers. The social intercourse test (SI) revealed that zebrafish swimming time and frequency were significantly higher in the social zone than in the non-social zone, demonstrating their preference for a zone with social interactions (Figs. 3B and 4C). Frequency and swimming time were significantly reduced in the social area for the 225 µg/L

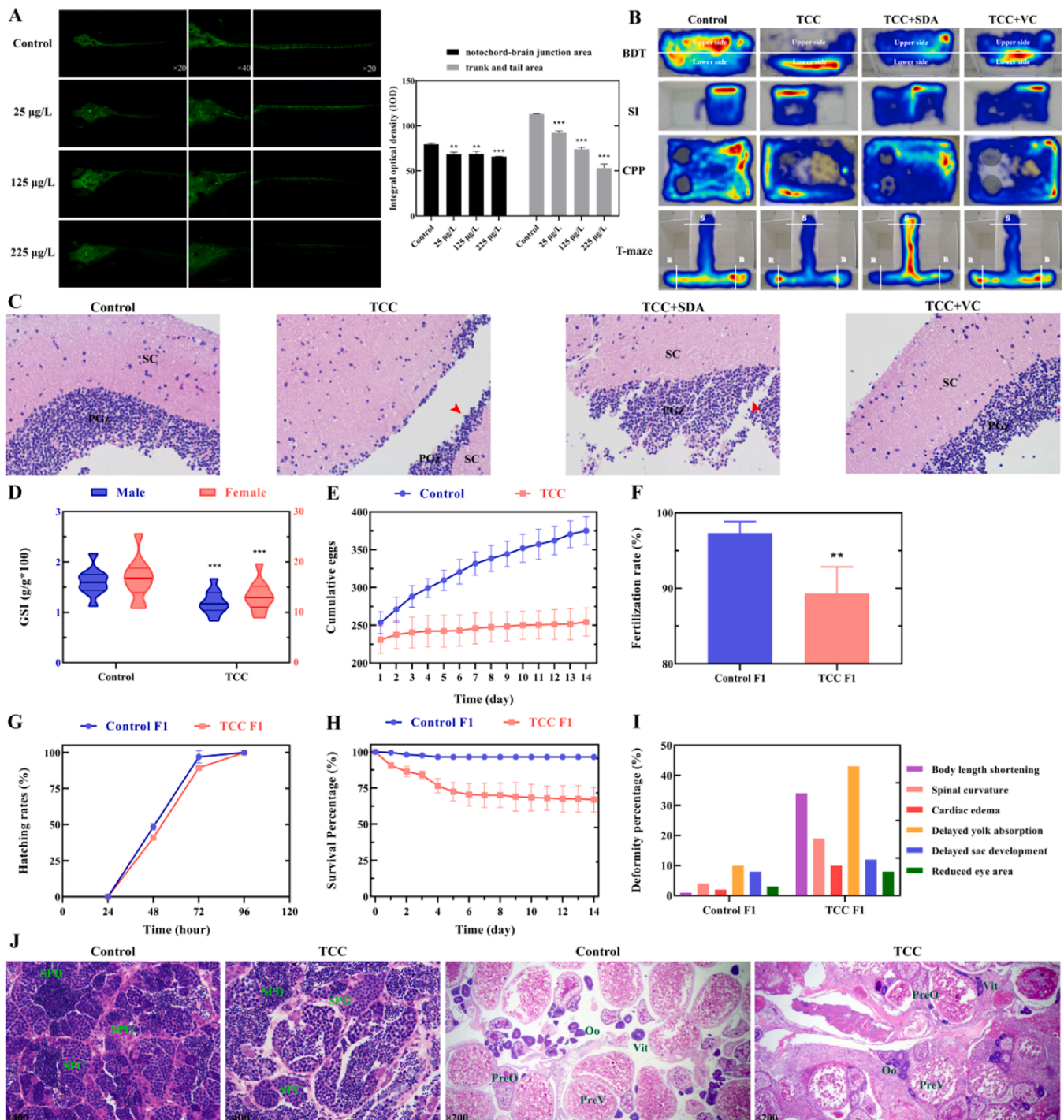


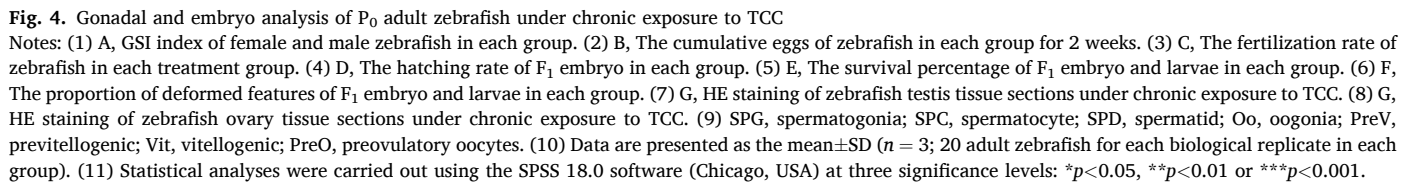
Fig. 3. Behavioral test and brain histopathological observation of adult zebrafish under chronic TCC exposure

Notes: (1) A, Whole-body immunofluorescence analysis of 120-hpf larval zebrafish exposed by TCC gradient concentrations and its quantification map. Green fluorescent dots represent newborn neurons. (2) B, Heatmap of behavioral tests such as bottom dwelling test (BDT), conditioned place preference (CPP), social interaction test (SI) and T-maze experiment. (3) S, start area; B, bait area; R, risk area. (4) C-D, BDT data analysis. (5) E-F, SI data analysis. (6) G-H, CPP data analysis. (7) I-J, T-maze data analysis. (8) K, HE staining of zebrafish brain tissue sections under chronic exposure to TCC. (9) PGz, periglomerular gray zone; SC, stratum centrale. (10) The Data are presented as the mean $\pm$ SD ($n = 3$; 20 adult zebrafish for each biological replicate in each treatment and control group). (11) Statistical analyses were carried out using the SPSS 18.0 software (Chicago, USA) at three significance levels: * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

TCC-exposed groups, while the frequency in the non-social area was 8.3-fold higher than the control group. Additionally, the swimming speed in the social area was very fast compared to the non-social area, indicating that the 225 µg/L TCC-exposed zebrafish were avoiding social situations and preferred quiet and isolated environments (Figs. 5B and 4D). Zebrafish abnormal motion behaviors substantially recovered in the

225 µg/L TCC+SDA and 225 µg/L TCC+VC treatments (Fig. 4C and D; $p < 0.5$, $p < 0.001$). These zebrafish behavioral characteristics indicate that TCC stress resulted in anxious, restless, unsociable and eccentric behaviors leading to lonely/depressed social interactions.

Conditioned place preference (CPP) trials evaluated the risk avoidance ability and vigilance behavior under adverse conditions. In the



After H&E staining, histopathological observations on diencephalon of control group zebrafish displayed a complete optic tectal region, abundant neuron cells of stratum centrale, and densely arranged neurons in the periglomerular gray zone (PGz; Fig. 3C). In the 225 $\mu\text{g/L}$ TCC-exposed group, neurons were sparsely distributed in PGz; the number of neurons was significantly reduced, and the PGz was

In terms of F₀ gonad, the spermatogonia (SPG), spermatocyte (SPC) and spermatid (SPD) in the control group were normally differentiated and matured in good shape and arrangement in testis (Fig. 3J). Under

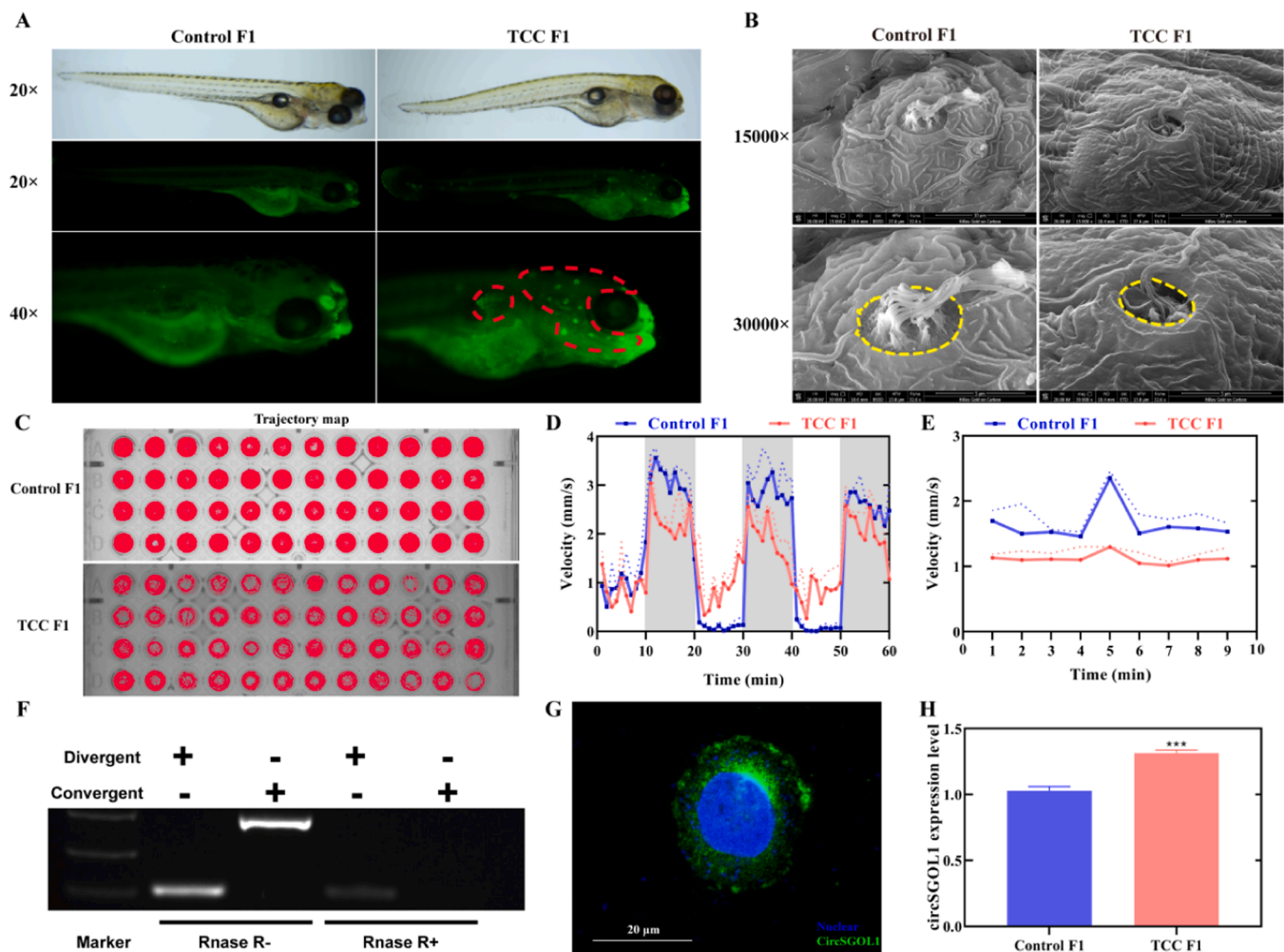


Fig. 5. Developmental toxicity and behavioral testing of F₁ larvae after chronic TCC exposure

Notes: (1) A, AO apoptosis staining of F₁ zebrafish in each group. (2) B, Observation of lateral line neuromast of F₁ zebrafish in each group by scanning electron microscopy. (3) C, Observation of spontaneous movement in F₁ zebrafish embryos under 24-hpf. (4) D, The motion trace map and heat-map of F₁ larval zebrafish movement under 120-hpf. (5) E, Distance traveled and swimming speed of F₁ larval zebrafish at 120-hpf. (6) F, Swimming speed of 120-hpf F₁ zebrafish larvae when subjected to a 40-min dark-to-light photoperiod. (7) G, Swimming speed of 120-hpf F₁ zebrafish larvae under the fifth-minute tap stimulation. (8) Data are presented as the mean ± SD ($n = 3$; 20 F₁ larvae for each biological replicate in each group). (9) Statistical analyses were carried out using the SPSS 18.0 software (Chicago, USA) at three significance levels: * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

the 225 μg/L TCC exposure, the number of SPG, SPC and SPD were greatly decreased, and some SPC cells experienced cytoplasmic vacuolization, seminal vesicle ablation and inflammatory infiltration, and partial formation of hollow fiber structure (Fig. 3J). Likewise, the number of oogonia (Oo), previtellogenic (PreV), vitellogenic (Vit) and preovulatory oocytes (PreO) from female zebrafish were significantly reduced, with Oo, Vit, and PreO decreasing by >80 % compared to the control group. This greatly contributed to decreased spawning in the F₀ generation. Moreover, the Oo and Vit cells did not develop completely, and became shriveled in shape with some necrosis.

3.5. TCC-induced developmental toxicity in F₁ larvae

To explore potential molecular mechanisms affecting F₁-larvae developmental toxicity from 225 μg/L-TCC-exposed F₀ parents, apoptosis staining was performed on F₁-generation larvae. At 225 μg/L TCC exposure, 5-dpf F₁ larvae experienced serious apoptosis in swimming sac, yolk sac, head and olfactory bulb (Fig. 5A), and prominent phenotypic malformations (shortened body length, curved spine and delayed yolk-sac absorption). SEM observation of F₁-larval development in the control group showed that hair cells and neuromast were in good

order, and hair cell bundles had many long stereocilia that were developmentally neat and dense. Conversely, the hair cell bundles in the 225 μg/L TCC-exposed F₁ larvae were short, sparse and malformed. The basal plate boundary of neuromast colliculus became blurred and irregularly textured, and the matrix of attached cilia was collapsed and smaller compared to the control-group F₁ larvae (Fig. 5B).

To assess the impacts of TCC-induced excessive apoptosis in brain and developmental defects of lateral neuromast in F₁ larvae, a behavioral test was employed. At 24-hpf, the average autonomic twitch frequency of F₁ embryos was 8 per minute in the control group. The twitch frequency decreased by 12.5 % ($p < 0.01$) in the 225 μg/L TCC-exposed group, indicating that TCC suppressed the early F₁-larval neuronal response (Fig. 4I). Further, at 120-hpf, F₁-larval movement distance in the 225 μg/L TCC-exposed group was decreased by 27.0 % compared to the control group, however, the average velocity showed no significant difference (Fig. 5C) as illustrated in the motor trajectory map and heatmap (Fig. 4J-K; $p < 0.001$). In the absence of TCC, F₁ larvae exhibited normal movement rhythms upon light-dark alternation, with low-speed movement in bright conditions and high-speed movement in dark conditions (Fig. 5D). 225 μg/L-TCC exposure resulted in higher and lower movements under light and dark conditions, respectively, and

these abnormal light-to-dark responses demonstrated that zebrafish were excessively frightened under illuminated conditions.

F₁-larval swimming speed showed a strong sensitivity to a 5-min sound stimulation, increasing by 60.9 % in the control group. In contrast, a prominent decrease in swimming speed of 225 µg/L-TCC-exposed F₁ larvae occurred under sound stimulation (Fig. 5E), owing to dysfunction in the zebrafish auditory or lateral neuromast, a key organ to sense temperature and sound signals (Xia et al., 2021).

3.6. Up-regulation of F₁-embryonic circSGOL1 inherited from parents

Because circSGOL1 contains specific binding sites for RNA binding proteins (RBPs), they form RNA-protein complexes and regulate the localization, transport and translation of RBPs. Therefore, circRNAs are emerging as effective markers of functional molecules in genotoxicity research for exploring underlying molecular mechanisms. Firstly, we analyzed and screened the raw data from the whole transcriptome sequencing of zebrafish sperm (data acquisition ID: GSM1085059), ovum (ID: GSM1085060) and 2-cell zygote (ID: ERR202500) using the Sequence Read Archive (SRA) database (Fig. 6A). In total, 364 circRNAs met the quality control requirements and were screened out, among which 146, 119 and 38 were distributed in the sperm, oocyte and 2-cell stage zygote, respectively; 14 were co-expressed in all three cell types

(Fig. 6B). The numbers of circRNAs with different lengths were elaborated as shown in Fig. S3-C. Due to the lack of RNA secondary structure, a steric hindrance exists between RNA strands with many bases and the reverse splicing enzymes, thereby making it difficult to form a loop. Thus, the predicted results were mainly focused on the < 1000 nt range. Then, we screened the circRNAs with lengths of 0–1000 nt that were co-expressed among sperm, egg and 2-cell stages, and finally identified a unique qualified circRNA designated as dre-circ-*chr19:49,970,754–49,970,590* (circSGOL1) derived from the *sgol1* gene. circSGOL1 (164 bp) is derived from its seventh exon by reverse splicing and cyclization as shown Fig. S3-D. Using Sanger sequencing, the nucleotide sequence of the seventh exon of *sgol1* was identified as "GGAA...ACAG". There was a reverse splicing site of "AGGG" in circSGOL1, confirming that circSGOL1 was a variant from the 7 exon of the *sgol1* gene formed by reverse alternative splicing. circSGOL1 was detected by PCR after reverse transcription with convergent and divergent primers, but was resistant to digestion by RNase R. These primers do not amplify any genomic DNA products, thus circSGOL1 did not result from a PCR artifact or genomic rearrangement. In contrast, convergent primers amplified the PCR products from linear *sgol1* mRNA, which disappeared after RNase-R digestion (Fig. 5F). Subsequently, fluorescence *in-situ* hybridization (FISH) was used to determine the cellular sub-localization of circSGOL1 in embryos. A large portion of

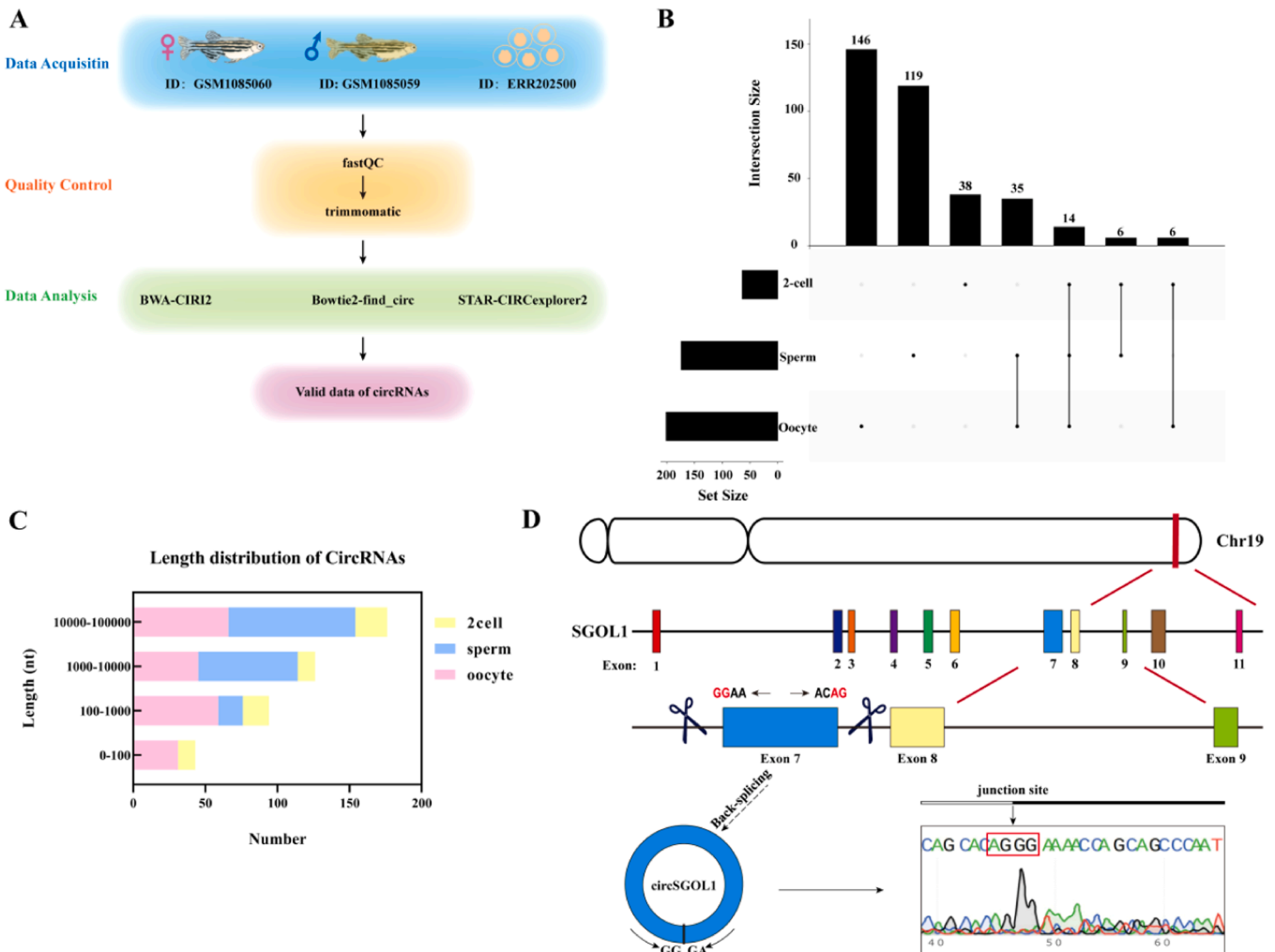


Fig. 6. Screening and prediction of parental circRNAs and characterization of circSGOL1.

Notes: (1) A, Flow chart of circRNA acquisition, analysis and screening. (2) B, The distribution of circRNAs in sperm, oocyte, and 2-cell stage zygote. (3) C, The length distribution of circRNAs. (4) D, Formation mechanism of circSGOL1 and sanger sequencing. (5) E, Total RNA from zebrafish with or without RNase R treatment were subjected to polymerase chain reaction (PCR). (6) F, Fluorescence in situ hybridization with junction-specific probes indicates the cellular localization of circSGOL1. (7) G, Expression levels of circSGOL1 in 120-hpf F₁ zebrafish larvae in each group.

circSGOL1 was distributed in the cytoplasm of the perinuclear region (Fig. 5G), concomitant with a large amount of endoplasmic reticulum. Sub-localization of circSGOL1 in cells supports its origination from reverse alternative splicing. By qRT-PCR, the F₁-adult expression of circSGOL1 was determined to be significantly up-regulated under TCC exposure (Fig. 5H; $p < 0.001$).

3.7. F₁-larval development disabilities and apoptosis associations with up-regulation of circSGOL1 under TCC exposure

To explore the effects of abnormal circSGOL1 expression on parental reproduction and offspring development, we artificially intervened the expression of circSGOL1 by microinjecting the over-expression plasmid (over-circSGOL1) and knock-down siRNA (si-circSGOL1) into 2-cell-stage embryos. At 48–96-hpf, larvae in the negative control group (NC, the synthetically unpaired or differential negative nucleic acid sequences for circSGOL1 and si-circSGOL1) displayed normal development and good phenotypic morphology similar to the blank control group (Fig. S1A). In contrast, the 48-hpf zebrafish in the over-circSGOL1 group showed a variety of malformations, including shortened body length, curved spine, microphthalmia, pericardial cyst and swimming-sac closure (Fig. S1B). These larvae developmental malformations at 72–96 hpf were obvious and severe. Conversely, the 48–96-hpf larvae in the si-circSGOL1 group showed only minor deformities (Fig. S1C and D). The phenotypic malformations caused by circSGOL1 over-expression were consistent with the main abnormal symptoms of F₁ larvae induced by TCC exposure. Thus, we posit that up-regulation of circSGOL1 in F₁ larvae is a plausible reason for the TCC-induced genotoxicity.

Apoptosis of larval zebrafish in the different TCC treatments was further detected by flow cytometry. In the NC group, apoptotic cells accounted for only ~0.61 %, which was in accordance with natural apoptosis levels for cell metabolism. Conversely, apoptotic cells in the over-circSGOL1 group were significantly enhanced during larval development, accounting for 0.94–2.12 % at 48–96 hpf (Fig. S1E–I).

3.8. CircSGOL1 binds hnRNP A1 protein to trigger over-expression of apoptosis-related genes and apoptosis in F₁ larvae

Based on the biological effects of circSGOL1 up-regulation, its downstream regulatory mechanism was further explored. Firstly, the RBPs integrated with circSGOL1 were predicted and analyzed on sequences from circSGOL1 by RegRNA 2.0 (an integrated web server for identifying functional RNA motifs and sites /index.html, Taiwan) and RBPmap (https://circinteractome.irc.nia.nih.gov/rna_binding_protein.html). Additionally, a miRNAs sponge was developed using the miRNAs target sites. A total of 9 recognition motifs were exactly matched as the 9 RBP hnRNP A1 (isomerization ribonucleoprotein A1) molecules in its 164 bp circular sequence through molecular docking (Fig. 7A). Because circSGOL1 does not effectively adsorb miRNAs (Fig. 7B), the functions of hnRNP A1 were analyzed in detail. Fluorescence co-localization experiments were carried out using a circSGOL1 probe integrated with hnRNP A1 antibody to demonstrate their combination in vivo. circSGOL1 was primarily observed in the perinuclear cytoplasm and a small amount in the nucleus as shown in Fig. 8C. Notably, the circSGOL1 presented as a green fluorescence that binds with hnRNP A1 protein in the purple region, which was confirmed by the co-immunoprecipitation localization in vivo (Fig. 7C).

Synthesizing the above information, we posit that the hnRNP A1 protein is a downstream factor of circSGOL1-induced apoptosis in F₁ larvae. In cytoplasm, hnRNP A1 is a negative regulator of an apoptosis inhibitory protein family via site-dependent translation into ribosome (Liu et al., 2020). It has a negative regulatory effect on the IRES activity of C-myc oncogene, heavy chain binding protein Bip, and vascular endothelial growth factor VEGF (Godet et al., 2019). hnRNP A1 can increase the mRNA stability of apoptosis inhibitory protein and prevent apoptosis, when ultraviolet radiation is applied to cells (Zeng et al.,

2021). To illustrate the effects of abnormal recruitment of hnRNP A1 by circSGOL1 on downstream apoptosis, sh-hnRNP A1 290 and sh-hnRNP A1 1231 were designed to inhibit the expression of hnRNP A1. They were micro-injected into the 2-cell-stage embryos to achieve the knock-down embryos of hnRNP A1. At 48–96 hpf, zebrafish in the NC group developed normal phenotypic morphology. At 48 hpf, all zebrafish micro-injected with sh-hnRNP A1 290 and sh-hnRNP A1 1231 developed a variety of malformations, including shortened body length, spine curvature, microphthalmos, pericardial cyst, cardiac hemorrhage and melanin inhibition. These developmental deformity became progressively more serious at 72 and 96 hpf (Fig. 7D–G). We conclude that the negative regulation of hnRNP A1 by increasing circSGOL1 seriously affected the development of larval zebrafish.

To disclose circSGOL1 binding with hnRNP A1 to induce apoptosis, we analyzed the hnRNP A1-targeting of downstream genes using the CLIP-seq database, thereby acquiring the RNA binding motif features and distribution location of the targeting RNAs (Fig. 7H). The hnRNP A1 mainly binds to the 3'-UTR or intergenic sequences of its target genes, whereas the binding motif sequences prefer "GGAGGAA" or "AUGGAG" (Fig. 7I), and AUG is the binding site of hnRNP A1 with the targeting RNA (Fig. 7J). Based on the above bioinformatics analysis, the target genes of hnRNP A1 with higher binding ability and lower binding free energy were screened out from the apoptosis-related genes, such as *Caspase3*, *Bcl2*, *Bax* and *TP53* (Fig. 7K). *Caspase3* is the most important terminal cleavage enzyme in the apoptosis process, and *Bcl2* is an inhibitor of apoptosis. Based on the Western blotting technique, the expressions of *Bax*, *Bcl2* and *Caspase3* were all significantly up-regulated in the 125 and 225-μg/L TCC treatments (Fig. 7L and M; $p < 0.01$, $p < 0.001$). Overall, these findings highlight a mechanism whereby abnormal recruitment of hnRNP A1 by circSGOL1 up-regulation triggered the overexpression of pro-apoptotic genes to further cause apoptosis during early embryonic development. A detailed discussion of the underlying results is presented in Supplementary materials.

4. Environmental toxicology implications

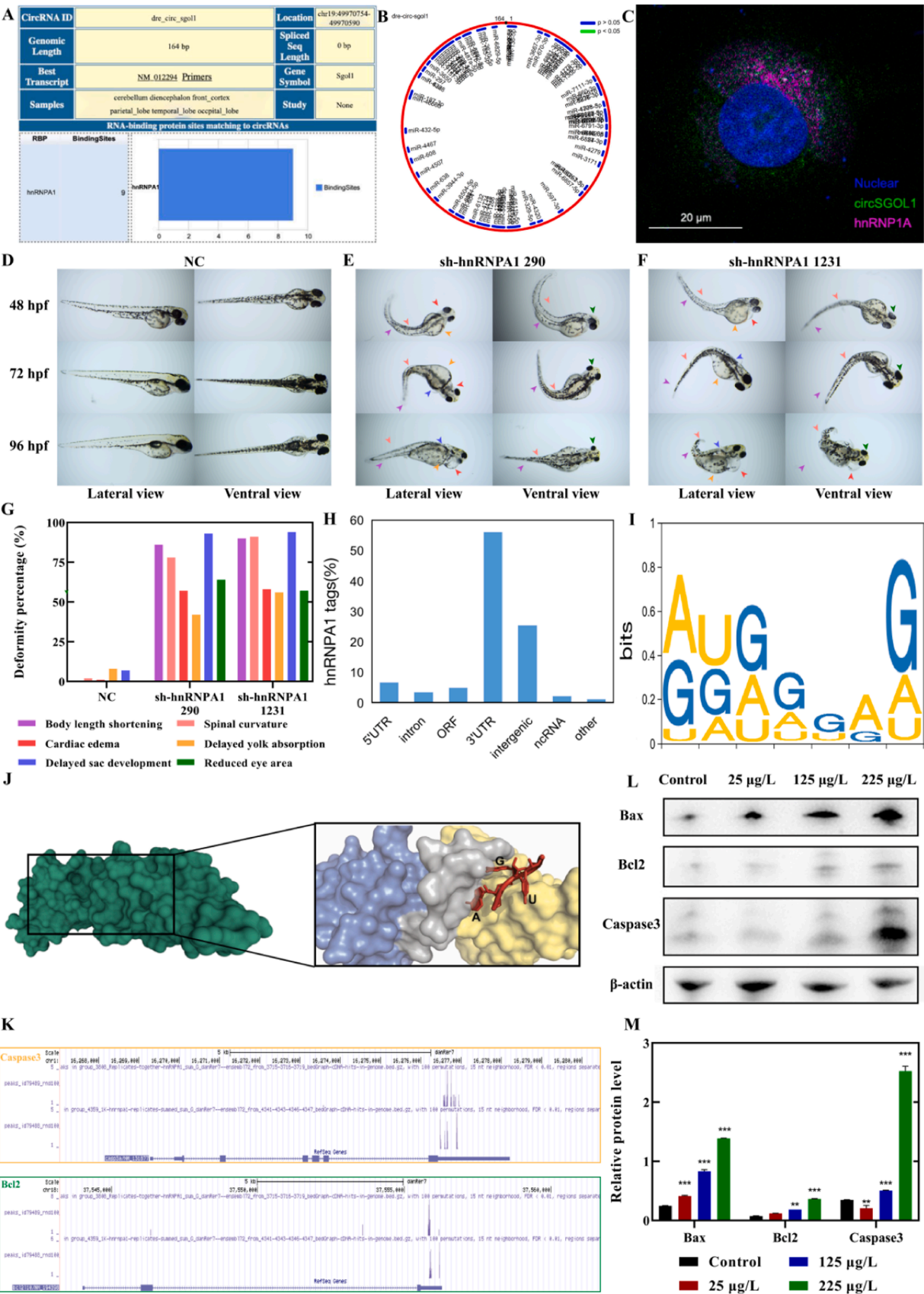
Long-term environmental exposure to TCC affects the growth and development of adult zebrafish through inducing disorders of intestinal flora in the gut. Alteration of intestinal flora triggered adverse impacts within the intestinal-brain-gonadal axis concerning signal transmission, nutrient circulation and energy flow. TCC induced a series of malformations in zebrafish nerve and motor behaviors, as well as reproductive toxicity. Mechanistic studies determined that F₁ larvae developmental disorders were due to apoptosis induced by the up-regulation of circSGOL1. This up-regulation affected the activity and localization of hnRNP A1, which in turn promoted the overexpression of pro-apoptotic related genes and ultimately apoptosis in early embryonic development (Fig. 8). Overall, these findings/data have practical significance for early TCC risk detection/control, and also provide therapeutic targets and pathways for reproductive toxicities and their complications.

CRedit authorship contribution statement

Huili Wang: Data curation, Formal analysis. **Xingcheng Liu:** Formal analysis, Methodology, Investigation. **Chenxi Zhao:** Conceptualization, Formal analysis, Investigation, Writing – original draft. **Jin Yan:** Writing – review & editing. **Zejun Wang:** Software, Data curation. **Randy A. Dahlgren:** Writing – review & editing. **Qiuhui Qian:** Conceptualization, Formal analysis. **Xuedong Wang:** Conceptualization, Supervision, Funding acquisition, Project administration.

Declaration of Competing Interest

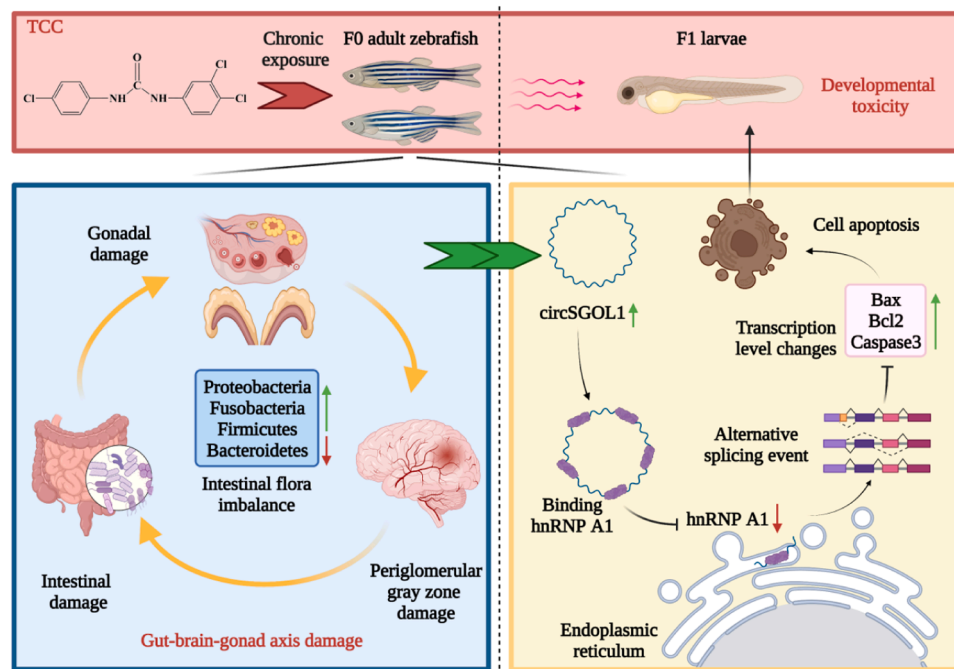
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



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Fig. 7. Exploration of the downstream pathway mechanism of circSGOL1-induced apoptosis in zebrafish

Notes: (1) A, Prediction of RNA-binding proteins that bind to circSGOL1. (2) B, Prediction of miRNAs binding to circSGOL1. (3) C, Fluorescence co-localization experiment of circSGOL1 and hnRNP A1. (4) D-F, The deformity observation of zebrafish larvae at 48, 72 96-hpf in negative control (NC), sh-hnRNP A1 290 and sh-hnRNP A1 1231 group, respectively. (5) G, The statistics of zebrafish deformity percentage in each group. (6) H, The RNA binding motif sequence distribution location in the targeting RNA. (7) I, The RNA binding motif sequence features. (8) J, Molecular docking analysis of the binding site of hnRNP A1 and target RNA. (9) K, Binding results of hnRNP A1 and target genes *TP53*, *Caspase3*, *Bcl2*. (10) L, Western blot results of *Bax*, *Caspase3*, *Bcl2* exposed to gradient concentrations of TCC. (11) M, Quantification results of L. (12) Data are presented as the mean $\pm$ SD ($n = 3$). (13) Statistical analyses were carried out using the SPSS 18.0 software (Chicago, USA) at three significance levels: * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

**Fig. 8.** Overall mechanistic illustration regarding on chronic TCC exposure disturbing the gut-brain-gonad axis of P₀ zebrafish and inducing the developmental toxicity of F₁ larvae by targeting the upregulation of maternal circSGOL1.

Data availability

The authors do not have permission to share data.

Acknowledgments

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.aquatox.2023.106782](https://doi.org/10.1016/j.aquatox.2023.106782).

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