



OPEN Lethal toxicity of metformin on zebrafish during early embryonic development by multi-omics analysis

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Metformin is an antidiabetic drug used in type 2 diabetes as well as indicators in polycystic ovary syndrome (PCOS) and cancer. Due to their increase in popularity, high amounts of metformin are being released into aquatic environments. However, the toxic effect of metformin on embryonic development in aquatic organisms remains limited. Therefore, this study aimed to elucidate the lethal embryotoxicity of metformin and determine the underlying molecular pathways influencing embryonic development using a zebrafish model through multi-omics analysis. Metformin was microinjected into zebrafish embryos at the 1-cell stage with varying concentrations (50 mM, 100 mM, 200 mM, 400 mM, and 800 mM). From the results, hatching rates decreased in a dose dependent manner. Fetal malformation and mortality ($LC_{50} = 339.8$ mM) increased in a dose dependent manner. In situ hybridization of whole-embryo assays demonstrated that metformin exerts a significant impact on the initial stages of embryonic development, leading to aberrant differentiation of the germ layers, perturbed organogenesis, and delayed development. Furthermore, transcriptomics, metabolomics, and lipidomics were used to study the molecular mechanisms of embryonic toxicity. The results showed that the cell cycle, dorsoventral axis formation, and collecting duct acid secretion pathways were significantly altered in treated embryos. In brief, these results provide useful information on the lethal toxicity mechanism of metformin overdose and provide clues for further studies in humans.

Metformin, an antidiabetic drug, is the preferred treatment option for type 2 diabetes over the last six decades¹. Additionally, it has demonstrated its usefulness in the treatment of polycystic ovary syndrome (PCOS) as it prevents and delays metabolic disorders^{2,3}. Moreover, its application in obstetrics and gynecology has also been suggested as a treatment regime for gestational diabetes mellitus (GDM)⁴. Currently, metformin is being investigated as a potential therapeutic agent for diseases associated with inflammation owing to its antioxidant properties⁵. Furthermore, a multidimensional life omics and tissue pathology study on 79 types of organs and tissues across 11 systems revealed that metformin preserves prefrontal cortex thickness, improves cognitive performance, and reduces aging effects in elderly primates, establishing a novel research paradigm for aging mechanisms and interventions⁶.

Recently, it has been determined that there are high amounts of metformin being release into aquatic systems due to an increase in usage. This release is a result of incomplete metabolism⁷. There are concerns regarding potential embryonic toxicity due to metformin exposure in aquatic organisms. Currently, animal models have been employed to study the effects of metformin on embryonic development, with some evidence indicating an increased risk of fetal malformation and mortality, as well as decreased hatching rates in mice⁸. However, the molecular mechanisms underlying these toxic effects require further investigation.

To fill in the current knowledge gaps about the embryotoxicity of metformin, this study aims to investigate the potential toxicity of metformin during early embryonic development in a zebrafish model. A zebrafish model was selected as it may best represent the effects of metformin in an aquatic species. Moreover, this model has a variety of benefits over mice or other animals, such as embryo transparency, ease of observation, cost-effectiveness, and

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the ability to survive without active circulation during early developmental stages. Most importantly, zebrafish serve as an excellent model for investigating compounds with toxicological significance and can be utilized as an alternative developmental toxicity model to predict their effects on mammals⁹. Elizalde-Velázquez et al. aimed to explore the potential embryotoxic effects of metformin on zebrafish (*Danio rerio*) embryos, focusing on developmental and redox balance changes. Their results indicated that metformin exposure significantly impacted embryonic development, leading to increased mortality, morphological abnormalities, and oxidative damage¹⁰.

Although previous studies have also explored the lethal effect of metformin on zebrafish embryos¹⁰, the molecular mechanism of metformin-induced embryonic death has not been explored using multi-omics. Therefore, this study attempts to determine the concentration at which lethal toxicity occurs in embryos of zebrafish model. More importantly, we used transcriptome, metabolome and lipid metabolome to analyze the mechanism of metformin-induced embryonic lethality in zebrafish.

Material and methods

Ethical approval

All experiments in this study were conducted in accordance with the “Guide for the Care and Use of Laboratory Animals” (Eighth Edition, 2011. ILARCLS, National Research Council, Washington, D.C.) and were approved by the Animal Care and Use Committee of West China Second University Hospital, Sichuan University (Approval ID: HXDEYY20131021). All methods were performed in accordance with the ARRIVE guidelines (<https://arriveguidelines.org>). Euthanasia of live fish was performed by submersion in ice water (5 parts ice/1 part water, 0–4 °C) for at least 10 min until cessation of opercular movement.

Chemicals

Metformin hydrochloride (Purity: 99.92%) was purchased from MEDChem Express (Cat. No.: HY-17471A; CAS number: 1115–70-4; NJ, USA). The metformin stock solutions were prepared in ultrapure water.

Breeding, embryo handling, and microinjection of zebrafish

Zebrafish wildtype AB strain was obtained from China Zebrafish Resource Center (CZRC) and maintained by our laboratory. Adult zebrafish were reared under a 14-h light/ 10-h dark cycle at a temperature of 28 ± 5° C in an indoor facility. The adult zebrafish were fed hatched shrimp three times a day. Zebrafish eggs were spawned through natural mating. The eggs were collected, washed, and transferred into freshwater containing methylene blue for culturing. After culturing for specific period, the embryos were collected and fixed with 4% PFA for assays. Embryo microinjections were performed as described previously¹¹. During the one-cell stage, zebrafish embryos were injected with 2 nl of a solution containing certain concentration of metformin or water (control).

Mortality, hatching, and malformation rates

To assess the potential embryotoxicity of metformin in zebrafish development, the embryos were divided into six groups, each of which was injected with a concentration of metformin of either with 50, 100, 200, 400 or 800 mM metformin, or water (control). The lethal concentrations studied in this work are independent of the water environment concentration. Serial gradients in concentration selection were chosen using integer concentrations. Embryonic malformations, mortality, or hatchings of zebrafish were recorded at 24, 48, and 72 h post fertilization (hpf). Phenotypic changes were also recorded. Embryonic malformations that were taken into account include delayed development, curved body axis, small head, short tail and pericardial edema. Whole experiment was conducted in triplicate, and the number of embryos in each group is around 100.

Whole embryo in situ hybridization (WISH)

The 50% epiboly stage (5 hpf) marks the initiation of zebrafish gastrulation, which gives rise to mesoderm and hematopoietic progenitors, and serves as a critical time point for inducing embryonic organizer formation¹². In situ hybridization was used to explore the effects of metformin on zebrafish embryonic tissue development. Zebrafish embryos were collected and placed in 4% PFA. Subsequently, the egg membranes were removed from the embryos and placed in methanol for WISH performance¹¹. The steps of WISH followed those of a previous study¹³. The embryos were placed into two groups, the first group was hybridized with the dorsal organizer marker, *gsc*, and ventral marker, *eve1*, at 5 hpf; the second group was hybridized with the endodermal marker, *sox17*, and neural precursor marker, *sox2*, at 7 hpf.

Transcriptomics, metabolomics, and lipidomics

To elucidate the alterations in metabolic pathways during embryonic development, embryonic metabolites were analyzed using both positive ion mode (POS) and negative ion mode (NEG) after metformin exposure.

Embryos injected with metformin solution were cultured in egg water for 5 hpf. The control and treatment groups contained 200 embryos each. The collected embryos were sent to Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China) for multi-omics analyses. Detailed methodologies and instruments for m6A-seq, metabolomics, and transcriptomics are provided in the Supplementary Methods.

Statistics and analysis

All assays were performed by two blinded researchers. Statistical analyses were performed using GraphPad Prism 8.0 software. Data are expressed as mean ± standard error of the mean (SEM). Differences between groups were assessed using Dunnett’s multiple comparison test and one-way ANOVA for malformation rates (Fig. 1B) and Survival rate (Fig. 1C). Two-tailed unpaired Student’s t-tests were used to compare hatching rate between two groups (Fig. 1E). Statistical significance was set as $P < 0.05$ (***) $P < 0.001$, (**) $P < 0.01$, (*) $P < 0.05$.

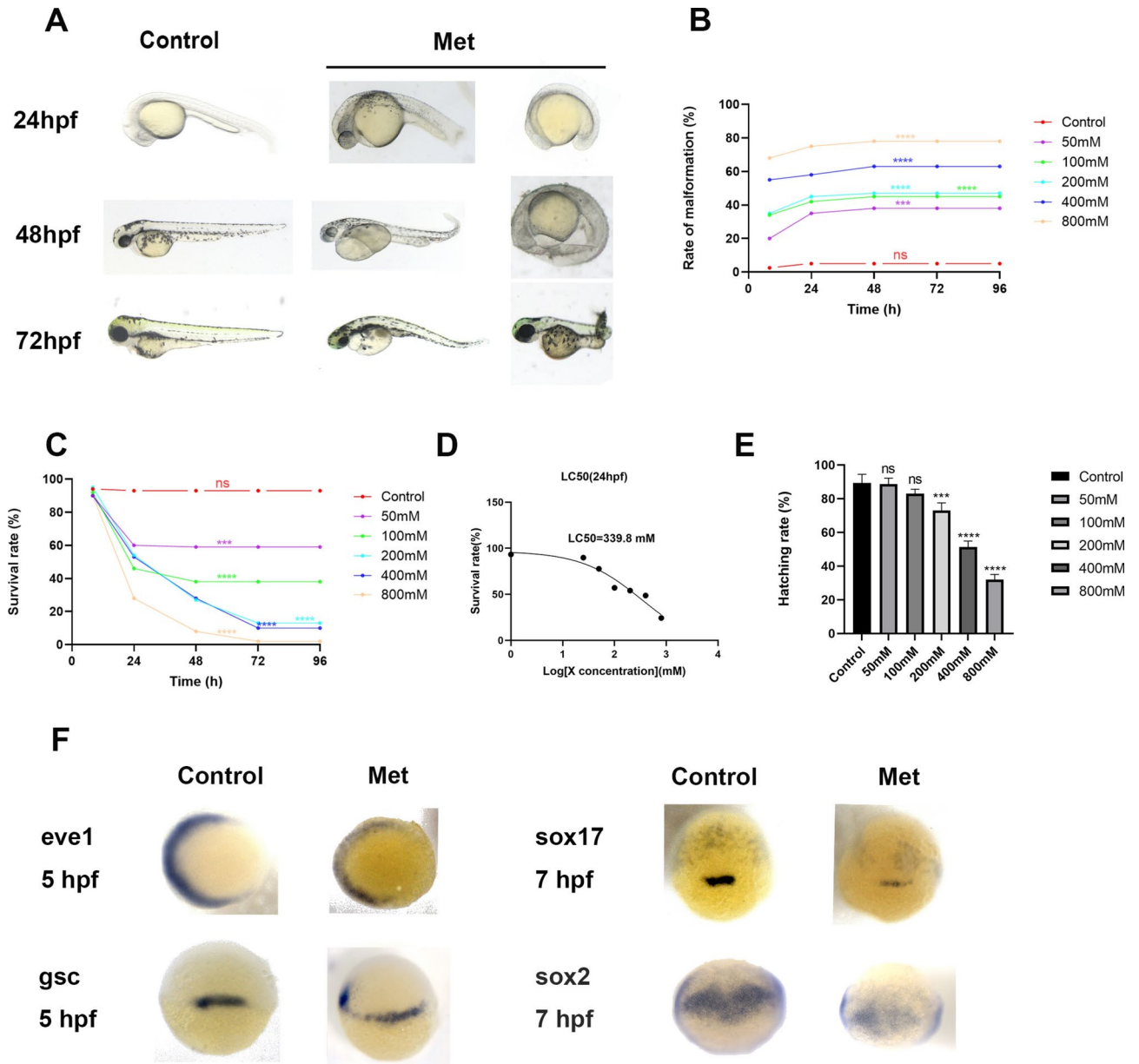


Fig. 1. (A) Phenotype of zebrafish embryos at 24, 48, 72 hpf in the control and 800 mM treatment group. (B) Deformity rate curves of zebrafish exposed to different concentrations of metformin (0–96 hpf). Unpaired t-test was used to test for statistical significance * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$, the same as below. (C) Survival rate curves of zebrafish exposed to different metformin concentrations (0–96 hpf). (D) The 24 hpf LC50 value of metformin to zebrafish embryos. (E) Hatching rate of embryos on the third day. (F) Embryonic organizer and germ layer marker expression detected by WISH during gastrulation.

Cumulative distribution analysis of the log2 fold changes of RNA levels of metformin exposure versus control embryos in maternal-early, maternal-late, zygotic-early, zygotic-late, semi-stable-1 and semi-stable-2 groups at 5 hpf was according to Zhao et al.'s description¹⁴. Lethality data were transformed as log (concentration), followed by nonlinear regression (variable slope–four parameters) to calculate Lethal Concentration 50% (LC₅₀) values using Graphpad Prism 8.0 following the guide of software (<https://www.graphpad.com/support/faq/how-to-determine-an-icsub50sub/>).

Results

Malformation, mortality, and hatching rates

The appearance of different degrees of deformities in embryonic morphology, such as deformities of the tail and curvature of the spine, yolk deformation, and delayed hatching, is shown in (Fig. 1A). The highest malformation rate (78%) was observed at 24 hpf at a metformin concentration of 800 mM compared with that of the other concentrations (Fig. 1B).

High embryonic mortality was consistently observed within the 24 hpf timeframe across all experimental groups. Notably, when the embryos reached 48 hpf, the survival rate dropped below 50% in all experimental groups except for the 50 mM group (Fig. 1C). After 72 hpf, the survival rates of the groups treated with 200 mM and 400 mM decreased to 12% and 10%, respectively, whereas all embryos in the group exposed to 800 mM metformin decreased to 1%. The LC_{50} of metformin in the zebrafish embryos at 24 hpf was 339.8 mM (Fig. 1D). Therefore, a dose of 339.8 mM was used for subsequent assays, such as WISH and multi-omics analysis.

The hatching rate of the embryos demonstrated a significant decrease at 72 hpf. At 3 dpf, the hatching rates for the control, 50 mM, and 100 mM groups were 88%, 87%, and 84%, respectively. However, the minimum hatching rates in the 400 mM and 800 mM groups decreased to 50% and 33%, respectively, at 3 dpf (Fig. 1E).

Compared to those in the control group, the zebrafish embryos exhibited pronounced deformities, which progressively intensified over time. Malformation and mortality rates of zebrafish embryos injected with metformin increased to varying degrees over time. Furthermore, the hatching rate was significantly reduced. These findings suggested that high dosages of metformin impede the development of early embryos to varying degrees.

Whole embryo in situ hybridization

At 5 hpf, there was a significant increase in the dorsal organizer marker (*gsc*) and a decrease in the ventral mesoderm organizer marker (*eve1*) in embryos injected with 339.8 mM metformin. In these groups, metformin altered the fate of dorsal–ventral cells and enlarged the dorsal area, suggesting that metformin affects cell development on the dorsal–ventral side of zebrafish. Furthermore, in contrast to the dorsoventral mesoderm marker genes, the endodermal marker (*sox17*) and neural precursor marker (*sox2*) showed decreased expression levels at the 70% epiboly stage (Fig. 1F).

Transcriptomics

Treatment with metformin resulted in the differential expression of 2660 transcripts, of which 1625 were upregulated and 1035 were significantly downregulated compared to the control group (Fig. 2A). Kyoto Encyclopedia of Genes and Genome (KEGG) enrichment analysis^{15,16} (Fig. 2B and Supplementary Table 1) revealed that these differentially expressed genes (DEGs) were primarily involved in the Metabolic pathways, as well as pathways associated with the Necroptosis, Viral carcinogenesis, Fanconi anemia, and ABC transporters. Volcano plot analysis was performed based on the significantly distinct genes in each comparison group. The volcano plot visually represented the differential gene expression between comparison groups, with genes positioned closer to either end indicating a higher degree of disparity (Fig. 2C).

Gene Ontology (GO) enrichment analysis revealed that the expression of genes related to 24 biological processes, 15 cellular components, and 12 molecular functions were significantly altered (Fig. 2D). Through enrichment analysis of cell components, DEGs were primarily associated with nucleosomes, intracellular membrane-bound organelles, and membrane-bound structures (Fig. 2E). Moreover, the molecular functions of DEGs were involved in protein heterodimerization activity, nucleic acid binding, organic cyclic compound binding, and heterocyclic compound binding (Fig. 2F). They were also related to chromatin silencing, DNA packaging, chromatin assembly or disassembly, and negative regulation of RNA biosynthesis when considering biological processors (Fig. 2G).

According to the transcriptome results, DEGs were observed in various biological systems, including the nervous (Fig. 3A), hematopoietic (Fig. 3B), axon (Fig. 3C), cardiovascular (Fig. 3D), and optic nerve development (Fig. 3E) systems.

Metabolomics and lipidomics

Metformin treatment resulted in 87 differentially expressed metabolites compared with the controls, of which 34 were upregulated and 53 were significantly downregulated (Fig. 4A). NEG metformin treatment resulted in 90 differentially expressed metabolites compared with the controls, of which 48 were upregulated and 42 were significantly downregulated (Fig. 4B). Using KEGG enrichment analysis, the metabolic pathways associated with fatty acid biosynthesis as well as the β -alanine and D-arginine metabolic pathways were identified (Fig. 4C).

Furthermore, the lipidome analysis (KEGG enrichment) results showed that the lipidome was mainly involved in choline metabolism in cancer, biosynthesis of secondary metabolites, and retrograde endocannabinoid signaling (Fig. 4D). According to the lipid cluster heat map analysis, the expression of lipids in all samples was visually displayed. The redder the color, the higher the abundance of lipids, and the bluer the color the lower the abundance of lipids (Fig. 4E and F, Supplementary Table 2 and 3). By clustering the lipids and samples separately according to their abundance, the samples or lipid expression patterns under the same branch were more similar.

The impact of metformin on the activation of the zygotic genome

Zygotic genome activation (ZGA) is a pivotal concept in the field of developmental biology, denoting the process by which a zygote's endogenous genome is activated following fertilization, representing an initial transcriptional event in an organism's life¹⁷. The timing and mechanism of ZGA vary in different species. In mammals, the ZGA process is complex and involves a multitude of transcription factors and regulatory mechanisms. In mice, ZGA is a dynamic process characterized by progressive binding of RNA polymerase II (Pol II) and gradual augmentation of transcriptional activity¹⁸.

Maternal-to-zygotic transition (MZT) is a pivotal stage in animal embryo development that marks the transfer of control over embryonic development from maternal factors to the zygote genome. This process encompasses two closely linked events: degradation of maternal mRNA and ZGA^{19,20}. During the MZT, early embryos rely

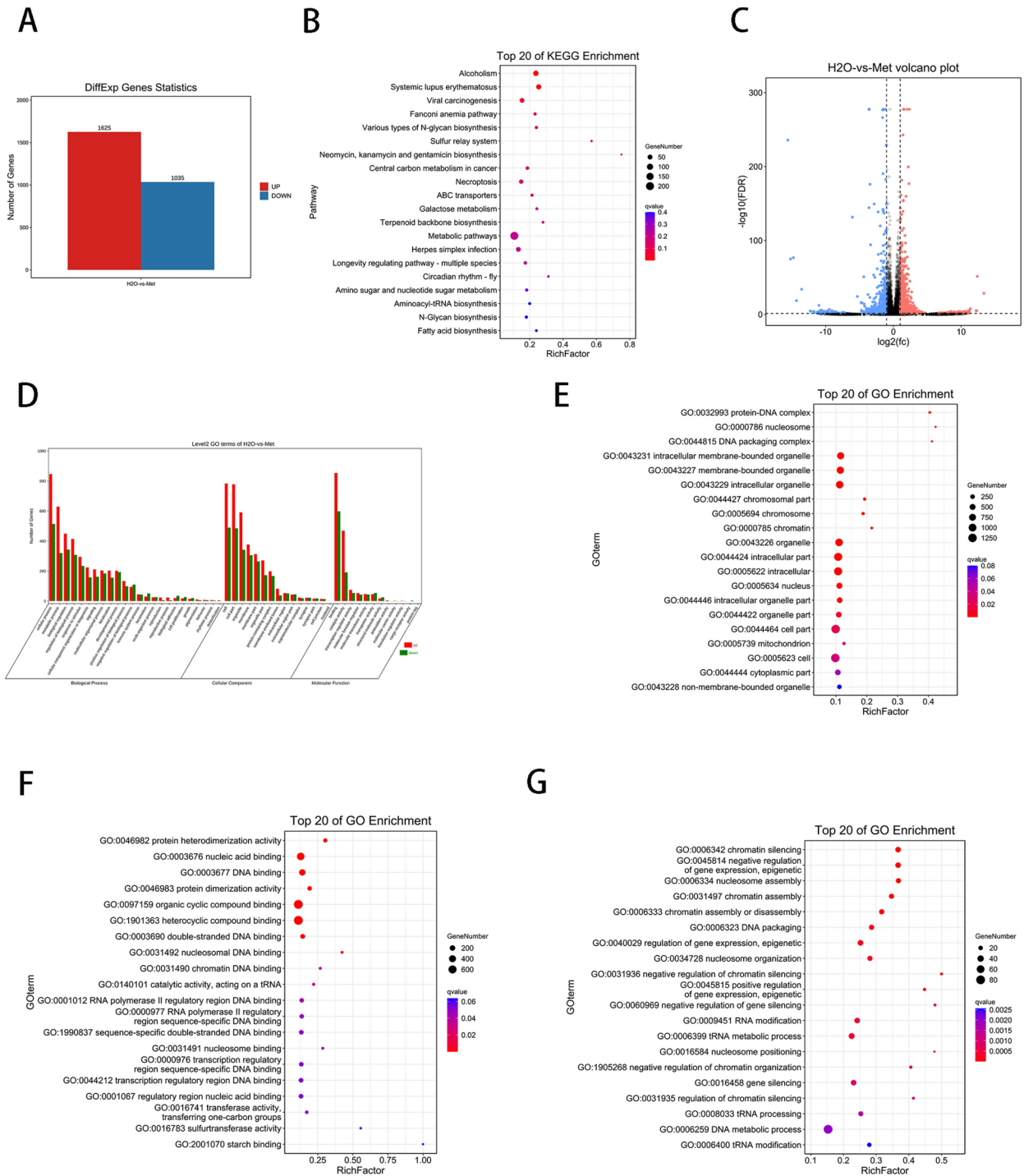


Fig. 2. (A) All statistical maps of differential genes. (B) The top 20 of KEGG enrichment results of RNA-seq. KEGG pathways were identified and visualized using the KEGG database (Kanehisa Laboratories, www.kegg.jp/kegg/kegg1.html). Permission to use KEGG pathways was obtained from Kanehisa Laboratories. (C) Volcano plot of differential genes. (D) The number of significantly differentially expressed genes for biological processes, cellular components, and molecular functions. (E–G) The top 20 of GO enrichment of common DEGs in RNA-seq. (E) cellular component. (F) molecular function. (G) biological process.

on RNA and proteins stored in the oocyte for initial development, followed by activation of the zygote genome, initiating autonomous transcription in the embryo²¹. Distinct alterations in RNA levels across six clusters of zygotic genome activation was observed, these were pronounced during the maternal-early and -late phases, with increases of 94% and 91%, respectively (Fig. 5A and B). There was a 90% and 80% decrease in the early and late stages of zygote growth, respectively (Fig. 5C

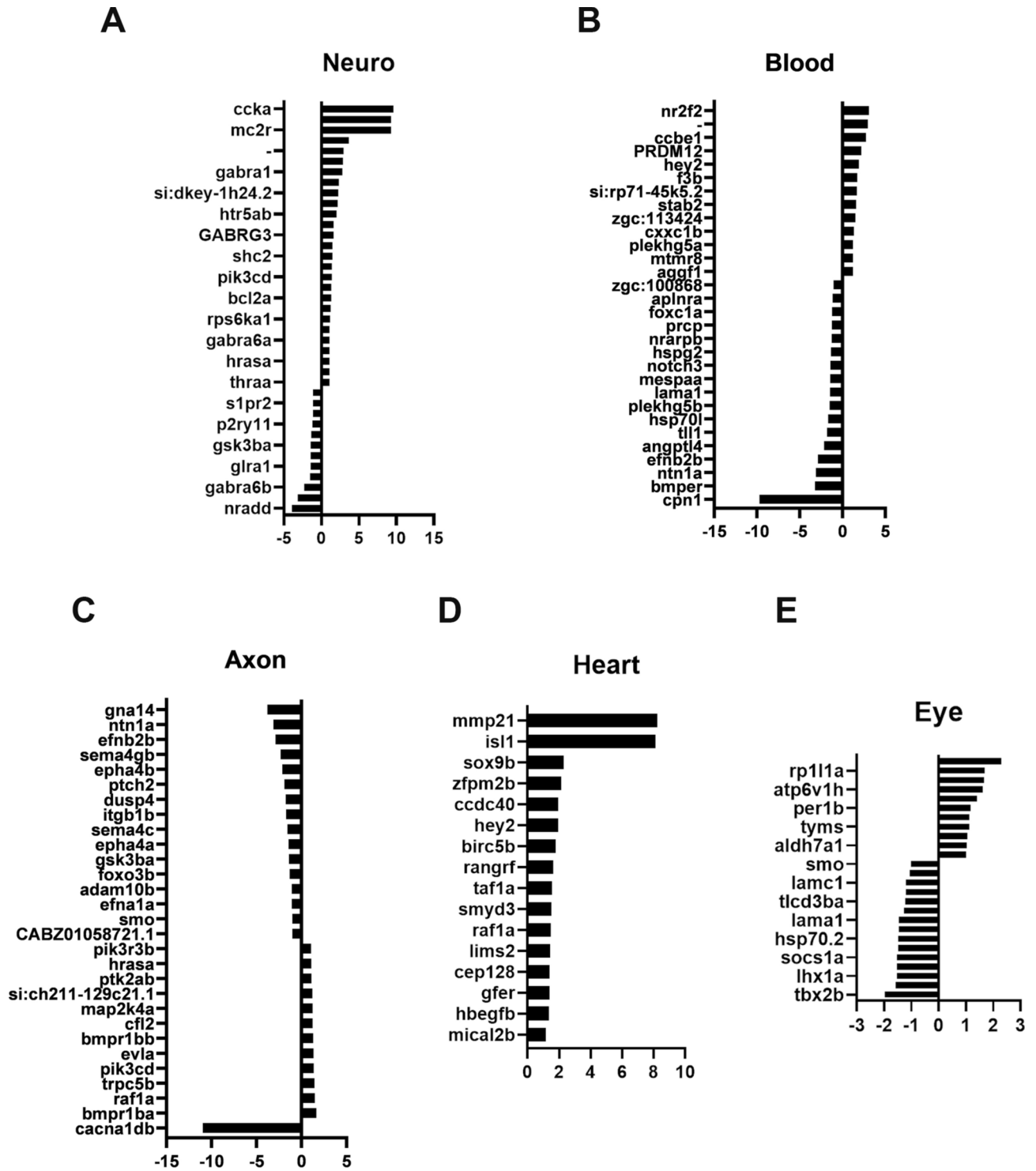


Fig. 3. Transcriptomic analysis of differentially expressed genes in the (A) nervous, (B) blood, (C) axon, (D) heart, and (E) eye systems.

and D). In addition, two clusters of semi-stable genes changed significantly (Fig. 5E and F). In brief, these results indicated that high dosages of metformin destroyed ZGA in early embryos.

Discussion

Metformin is a well-known antidiabetic drug used in a variety of emerging applications, including cancer therapy. These drugs have excellent safety and tolerability profiles²². In this study, a zebrafish model was used to determine the toxic effects of excessive metformin on the phenotype, mortality, and hatchability of zebrafish embryos at 24,

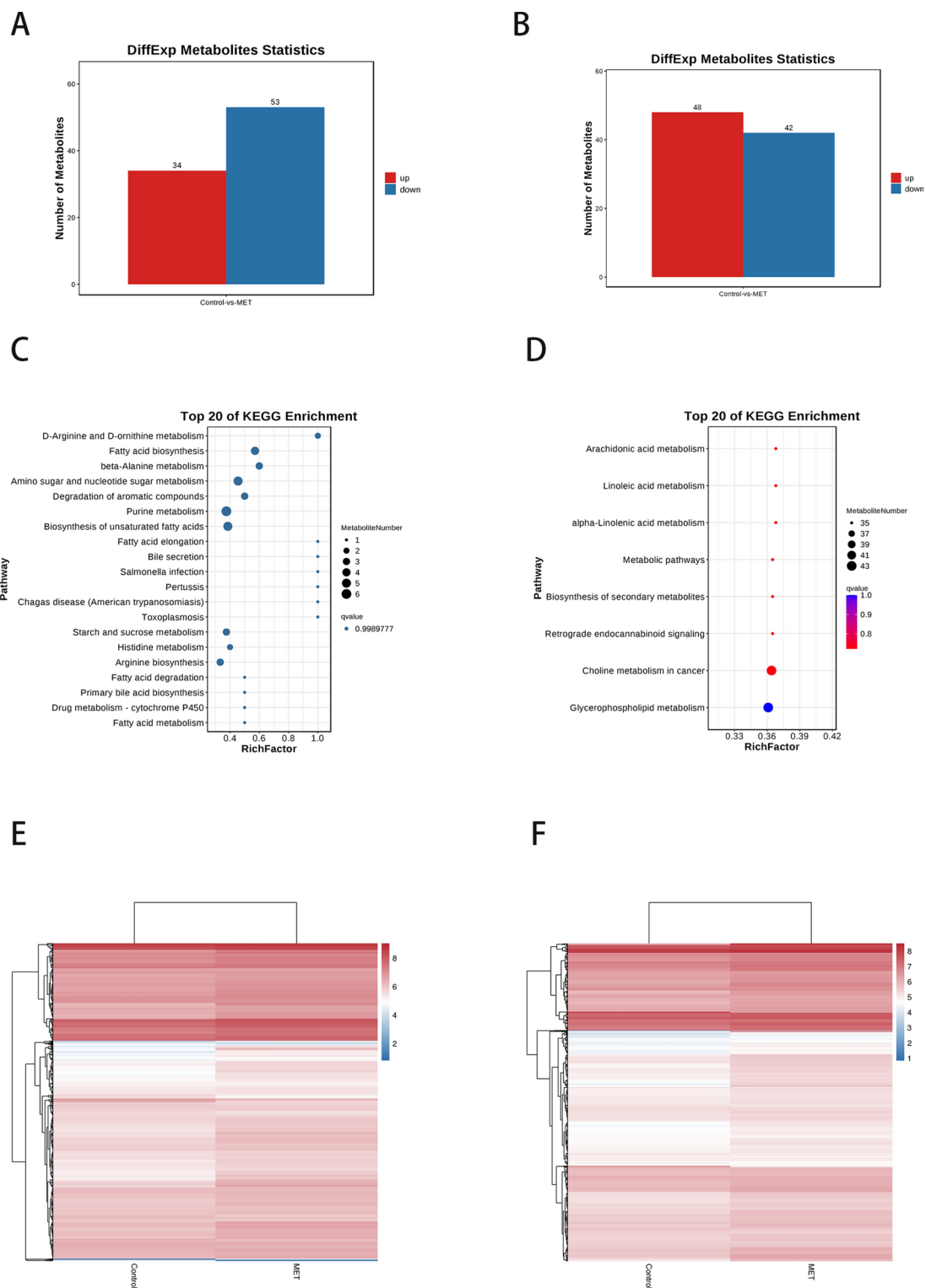


Fig. 4. All statistical maps of differential genes of metabolomics in (A) POS and (B) NEG. The top 20 of KEGG enrichment results of (C) metabolism (D) lipidome sequence. KEGG pathways were identified and visualized using the KEGG database (Kanehisa Laboratories, www.kegg.jp/kegg/kegg1.html). Permission to use KEGG pathways was obtained from Kanehisa Laboratories. Metabolite cluster heatmap analysis in (E) POS and (F) NEG.

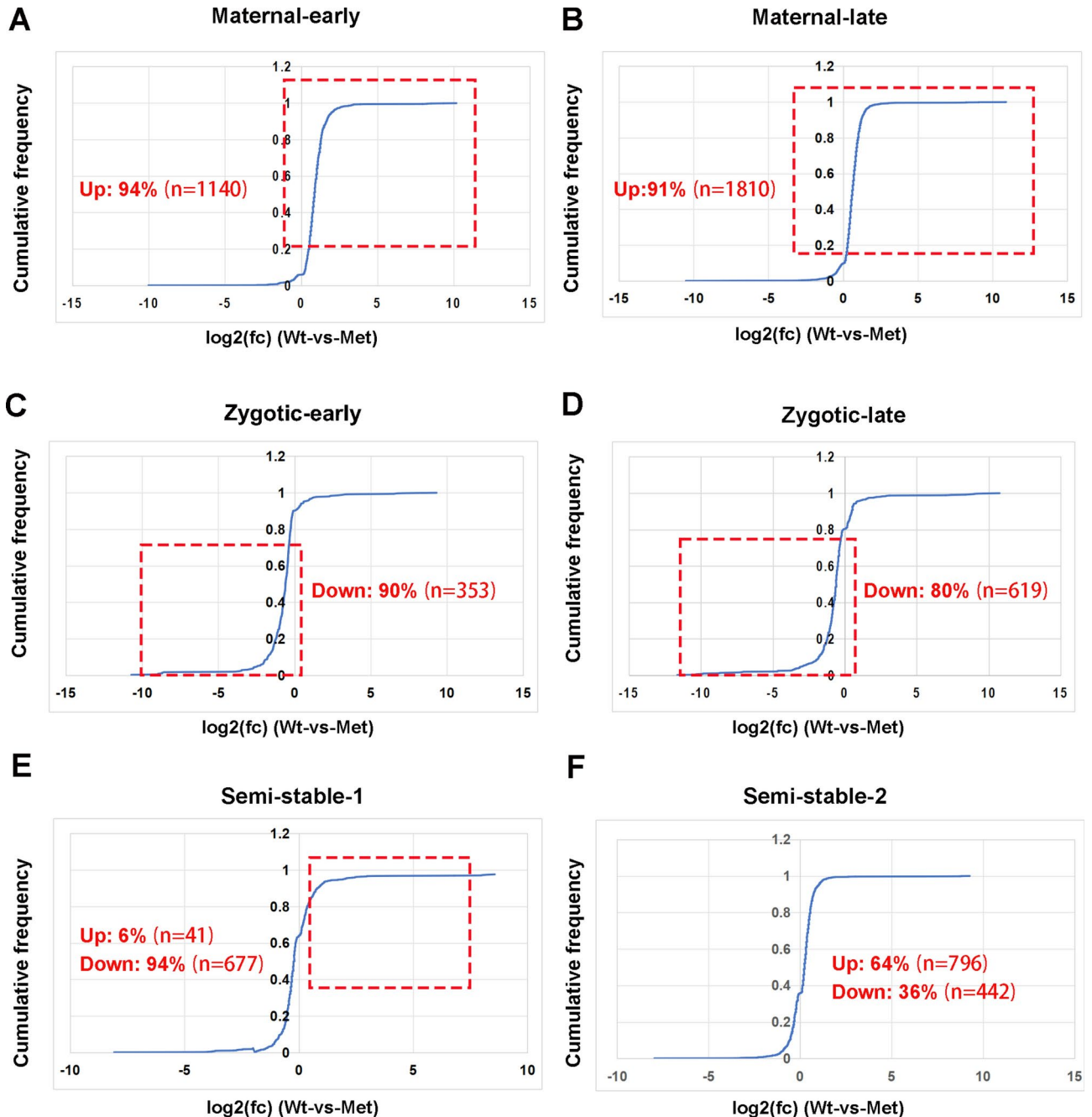


Fig. 5. Zygotic genome activation in (A) maternal-early and (B) maternal-late of metformin. Zygotic genome activation in (C) zygotic-early and (D) zygotic-late of metformin. Zygotic genome activation in (E) semi-stable-1 (F) semi-stable-2 of metformin.

48, and 72 hpf. In situ hybridization and multi-omics analyses (transcriptomics, metabolomics, and lipidomics) were performed to identify key factors involved in embryonic differentiation and tissue formation.

Our results showed that high doses of metformin-treated embryos exhibited altered developmental patterns and morphological features compared to those of the control group. The metformin-treated embryos exhibited deformities, particularly in the eye, optic tectum, and yolk sacs. The deformities were also dose-dependent, with higher concentrations having more adverse effects on the embryos. In situ hybridization showed that the dorsal organizer marker (*gsc*) and ventral mesoderm organizer marker (*eve1*) were significantly altered, and the endodermal marker (*sox17*) and neural precursor marker (*sox2*) showed decreased expression levels at the 70% epiboly stage. WISH revealed aberrant differentiation of the embryonic germ layers and abnormal formation of vital organs, implicating a crucial role for metformin in early embryonic development. Hatching is a critical period of embryogenesis and is, therefore, a pivotal point in the life cycle of fish, with hatching time commonly

employed as an endpoint in developmental toxicity studies²³. In this study, the treated embryos displayed a delayed hatch time compared to the control group.

The molecular mechanism underlying the impact of high does metformin on RNA levels in zebrafish embryos was investigated through transcriptomic analysis in this study. Treatment with metformin resulted in the differential expression of 2660 transcripts, of which 1625 were upregulated and 1035 were significantly downregulated compared to those in the control group, including genes related to systemic lupus erythematosus and alcoholism. Furthermore, there was a significant association between DEGs and cancer, suggesting a potential link between the toxic effects of metformin and carcinogenesis. It was determined that the CACNA gene family is associated with the nervous system, which has significant implications in the pathogenesis of migraine, epilepsy, cerebellar ataxia, dystonia, and cerebellar atrophy, among other diseases²⁴. The protein RP1L1, which is distinct in the visual nervous system, is a component of cilia found in photoreceptor cells. Defects in RP1L1 may lead to photoreceptor diseases, indicating that RP1L1 plays a pivotal role in the biology of photoreceptor cells²⁵. The DEG MMP21 within the cardiovascular system may serve as an independent prognostic biomarker for invasion and metastasis in gastric cancer²⁶. Moreover, high does metformin was also found to affect the metabolic and signal transduction pathways of metabolites in zebrafish embryos, as revealed by metabolomics analysis. Based on omics analysis, the molecular mechanisms underlying these toxic effects include alterations in cell cycle regulation, dorsoventral axis formation, and collecting duct acid secretion pathways.

Through KEGG analysis of the lipidomics, we found that choline metabolism in cancer had an impact on embryonic lethality, mainly reflected in disruption of choline metabolism pathways, cancer-related alterations in choline metabolism, nutritional and metabolic interactions. The dysregulation of choline metabolism in cancer can exert profound effects on embryonic development, with the potential to induce lethality. This is largely attributed to disruptions in critical metabolic pathways and enzymes involved in phosphatidylcholine synthesis^{27,28}. Further research is essential to elucidate the underlying mechanisms and to identify potential therapeutic strategies that could mitigate the adverse impact of cancer-related metabolic alterations on embryonic health²⁸. Through KEGG analysis of the metabolomics, Purine metabolism is intricately linked to embryonic development, and its dysregulation can have profound effects on embryonic viability and normal development. The spatiotemporal regulation of purine synthesis pathways, particularly the de novo and salvage pathways, is essential for supporting the high metabolic demands of neural stem/progenitor cells (NSPCs) during embryonic brain development²⁹.

A previous study examined the effects of metformin on zebrafish embryonic development and oxidative equilibrium, revealing that environmental concentrations of metformin can disrupt embryos, accelerate hatching, and cause morphological changes leading to mortality. It also showed that metformin increases antioxidant enzyme activity and oxidative damage biomarkers, suggesting that oxidative stress contributes to embryotoxicity¹⁰. In contrast, our study found that specific metformin concentrations increased mortality and malformation rates, and affected hatching. We assessed toxicity at multiple time points (24, 48, and 72 hpf), whereas the previous study focused only on oxidative stress mechanisms. Our study further explored molecular mechanisms using transcriptomic, metabolomic, and lipidomic analyses and tested higher concentrations to determine the median lethal concentration and mortality rate.

In summary, the presence of metformin in global waters and its biotic and abiotic effects are active areas of research, with studies aimed at determining the extent of metformin contamination and its implications in aquatic environments. This research demonstrates that lethal toxicity of metformin using zebrafish embryo model, thereby providing valuable insights for further investigation into the underlying toxic mechanisms associated with excessive metformin exposure.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Author contributions

CRedit authorship contribution statement: Ziyuan Lin: Writing-original draft, Data curation, Software, Formal analysis, Investigation, Methodology. Mingfeng Liu: Investigation, Formal analysis, Methodology. Feng Chen: Data curation, Software. Chanjuan Zhao: Data curation, Software. Yanyan Liu: Conceptualization, Formal analysis, Investigation, Supervision, Visualization. Huaqin Sun: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing-review & editing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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