



OPEN Esketamine induces embryonic and cardiac malformation through regulating the *nkx2.5* and *gata4* in zebrafish

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Esketamine (EK) has been widely used in the treatment of depression, but the effects of EK prenatal treatment on embryonic heart development have been rarely reported. This study assesses the effects of varying concentrations of EK on embryonic development and cardiogenesis to determine the teratogenic concentration in the zebrafish model, centering on the interaction between the genes *nkx2.5* and *gata4* to elucidate the mechanisms underlying cardiac morphogenesis. Zebrafish embryos were classified into six distinct groups and exposed to either a vehicle or EK to ascertain the median lethal concentration (LC₅₀) at 48 and 72 h post-fertilization (hpf) analyzing mortality rate data. Embryonic and cardiac morphologies were assessed utilizing live embryo imaging techniques and stereo microscopy. *Nkx2.5* and *gata4* were identified via whole-mount in situ hybridization (WISH) and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Exposure to EK leads to significant teratogenic effects on zebrafish embryos, which are both concentration- and time-dependent. The 48 h- and 72 h-LC₅₀ of EK for zebrafish embryos were 1.30 (95% CI 0.92, 1.60) millimolar (mM) and 0.71 (95% CI 0.46, 1.01) mM, respectively. A significant reduction in heart rates and body length were observed and the distance between the sinus venosus and bulbar artery (SV-BA) was found expanded, the pericardial edema area showed significant swelling, and the body axis curvature was more pronounced in the EK exposure groups. Both WISH and RT-qPCR analysis showed *nkx2.5* staining intensity and expression significantly decreased, while *gata4* assay results were in the opposite direction. Our findings indicate that exposure of zebrafish embryos to EK results in embryonic and cardiac malformations, primarily due to the down-regulation of *nkx2.5* and the over-expression of *gata4*. Equilibrium maintenance and compensatory mechanisms are crucial in spatiotemporal gene regulation.

Perinatal depression (PND) is a public health problem¹, which has received much attention in recent years due to its severe social dysfunction. The formation and development of PND have introduced numerous complex challenges to society, frequently resulting in maternal suicidal tendencies. PND refers to severe or mild depressive episodes that occur in a special period: during pregnancy or within one year after delivery, that is, including pregnancy depression and postpartum depression². Globally, anxiety and depression affect 15–20% of pregnant women today^{3,4}. Pregnancy's physiological shifts, postpartum recovery, and the psychological toll on new mothers collectively fuel PND. PND, alongside prenatal drug exposure, imperils fetal growth, giving rise to developmental complications and birth defects. These defects, born of inadequate intrauterine progress, stand as the chief culprits behind neonatal disabilities and fatalities. Consequently, they impose substantial economic and psychological burdens on society and families, adversely impacting population health and social progress. However, as the population of pregnant women with PND grows, the challenge of effectively and safely intervening in this condition during pregnancy without adversely affecting fetal development remains a significant and complex issue.

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As the classic representative, selective serotonin reuptake inhibitors (SSRIs) have been widely used in the treatment of PND⁵, but their use in the first trimester may be associated with fetal congenital heart defects (CHD)^{6–8}, and even their effectiveness remains unclear⁹. Being the S-enantiomer of ketamine, EK is an emerging antidepressant drug, which can rapidly and effectively relieve the symptoms of major depressive disorder (MDD) through inhibiting the N-methyl-D-aspartate (NMDA) receptor^{10,11}. Recently, Wang and his colleagues have reported on BMJ that a single low dose of EK after delivery reduced major depressive episodes by about three-quarters at 42 days postpartum¹². However, it remains to be verified whether the use of EK for the treatment of PND, particularly in the first trimester, may adversely affect fetal heart development.

In order to address this issue, the present study will systematically examine the toxicity of EK exposure on the development of embryos and cardiac function in zebrafish embryos. The use of zebrafish as a model organism is advantageous due to its high genetic homology with humans, coupled with the ability to perform in vitro fertilization and observe embryonic development externally, facilitated by the transparency of the zebrafish body¹³. Therefore, zebrafish embryo has become an excellent model for developmental biology, basic medicine, neurological and toxicology research during the last decades^{14,15}. The heart, which has a simplified structure consisting of only one atrium and one ventricle¹⁶, is the first organ to form and function during zebrafish embryogenesis. The zebrafish heart, despite its anatomical divergence from the human heart, possesses analogous characteristics that encompass atria, ventricles, heart valves, and a conduction system^{17,18}. The critical window for cardiac development in humans spans weeks 2 through 7 of gestation, a phase during which the heart undergoes significant morphogenesis. In contrast, the zebrafish heart achieves full development within approximately 48 h, highlighting species-specific differences in developmental timelines, as documented in Lohr's study¹⁹. Embryonic development represents a highly orchestrated and finely regulated spatiotemporal dynamic process. In the course of development, embryos are required to sustain this dynamic process while simultaneously safeguarding it from disruption by external, non-endogenous factors. Exploring and avoiding the toxic effects of EK exposure so that provide scientific theoretical support for PND women is meaningful treatment protocols, which also be of great significance to improve the health of the population. Therefore, we have conducted a methodical examination of the toxicity of EK exposure on both embryonic and cardiac development by zebrafish model to broaden the scope of EK prescription.

Given the aforementioned considerations, this study aims to elucidate the effects of EK on zebrafish embryonic and cardiac morphology and function, with an initial investigation into the underlying mechanisms by examining the expression patterns of the cardiac development-associated genes *nkx2.5* and *gata4* during the progression of cardiac dysplasia.

Materials and methods

Ethics

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the South China University of Technology (Guangzhou, Guangdong, P.R China, approval number SCUT-2022-096) and were carried out in accordance with relevant guidelines and regulations. All the methods are performed in accordance with ARRIVE guidelines.

Zebrafish husbandry and embryo collection

Four-month-old healthy wild-type zebrafish *Tuebingen* (TU) strain in this study are all from the Chinese Academy of Sciences Institute of Aquatic Organisms (Beijing, China). All the adult zebrafish were regularly fed brine shrimp twice a day. Water temperature maintained at $28.5 \pm 1^\circ\text{C}$ in the incubator. The salinity of the water was regulated by NaCl and maintained solution conductivity between 450 and 500 $\mu\text{S cm}^{-1}$. A 14-h light:10-h dark cycle was maintained to mimic natural day-night patterns. The day before the experiment, adult male and female zebrafish were placed in the breeding box at a 1:1 ratio and kept overnight under dark conditions. The next morning at 6:30 am, male and female zebrafish were stimulated to mate and lay eggs. Zebrafish eggs were collected for subsequent experiments.

Esketamine exposure and experimental design

In order to study the EK (Hengrui Medicine, Lianyungang, China) on zebrafish embryonic development and the influence of cardiac toxicity, the egg shells were removed using demembranase (contains chain protease) at a 1:10 dilution on the first day of the experiment, then the collected fertilized eggs at 24 hpf were incubated in six-well plate 24 h with 30 embryos each well, six groups randomly incubated in a range of EK concentrations (0, 0.21, 0.42, 0.84, 1.68 and 3.36 mM, diluted with E3 embryo medium: 5 mM NaCl, 0.67 mM KCl, 2.4 mM NaHCO_3 and 0.9 mM CaCl_2) through 48 h, exposure solution was changed every 24 h, according to the drug screening protocol²⁰. Each group was set at least three repeated experiments. We utilized a Nikon SMZ18 stereo microscope (Japan) to observe and record the hatching condition and mortalities at 24-h intervals. EK stock solutions were prepared in E3 medium and then diluted to attain the requisite concentrations for the purposes of this study. Zebrafish embryos were treated with the above protocol to determine its toxic effects. Through counting the mortality, heart rate and body length in designed timepoints until 72 hpf, the LC_{50} values were determined by Graph-pad Prism 10.0 software. The hatching rate of the zebrafish embryos without dechorionating was observed at designed timepoints during the EK exposure in other groups. For calculating the larval hatch rate, successful hatching was defined as a larvae's head or tail breaking out of the chorion, with the rate expressed as a percentage of living embryos hatched during the designed timepoints. Zebrafish embryos were placed in an incubator at $28.5 \pm 1^\circ\text{C}$ and cultured until the target time.

Determination of the optimal concentration exposure

Based on the mortality of zebrafish embryos that resulted from a series of different concentrations of EK exposure, we have calculated the LC_{50} of EK in zebrafish embryo model at 48 and 72 hpf. 0.21, 0.42 and 0.84 mM were selected as the low, median and maximum (optimal) concentrations to treat zebrafish embryo for further revealing the teratogenic effects on cardiogenesis and embryonic development. Solution in each group were changed every 24 h. EK or vehicle-treated zebrafish embryos were stored at 4 °C condition in refrigerator for subsequent experiments including observation of zebrafish embryo phenotypes under stereoscopic microscope, WISH test and RT-qPCR analyses. Combined with the mortality, hatching, and malformation phenotypes under a range concentrations of EK, we selected some optimal concentrations to further prove the mechanism of EK developmental cardiotoxicity through WISH and RT-qPCR tests.

Live embryos imaging and heartbeats counting

Selective concentrations of EK exposure were conducted until 72 hpf and solutions were refreshed per 24 h. The embryonic development and morphological changes of zebrafish embryos were recorded under stereo microscope. All survival zebrafish embryo were anesthetized with 0.03% tricaine (MS-222; Sigma-Aldrich, Germany) and mounted in 1% low-melting agarose with 0.03% tricaine, and their phenotypes were observed and measured by stereo microscope equipped with a digital camera (Olympus ZX) at 72 hpf. Quantifying the body length (μm), SV-BA distance (μm), and pericardial area (μm^2) of zebrafish three times in each group and the average was analyzed through Image J software. Zebrafish were exposed to 30 g/L methylcellulose, and the heartbeats of 48 and 72 hpf in 1 min were counted under anatomic microscope. The assessment of cardiac morphology and function according to the treatment protocol published by Hoage and Glickman^{21,22}. Any anomaly of cardiac looping may cause disruption of its function. Thirty fish were taken from each group, and each experiment was repeated at least three times.

WISH analysis of the CHD susceptible genes

Congenital heart disease susceptible genes *nkx2.5* and *gata4* were selected as the indexes for the cardiotoxicity of esketamine²³. WISH using digoxigenin (DIG)-labeled antisense RNA probes for *nkx2.5*, *gata4* were performed based on previous protocols²⁴. Subsequently, the corresponding probes (500 ng/mL) were added to the mixture, which was then incubated overnight at 70 °C in a water bath. Combined with the above mortality, hatching, and malformation phenotypes, the optimum concentrations were chosen to further prove the mechanism of EK developmental cardiotoxicity through WISH tests.

Plasmid templates (*nkx2.5*, *gata4*) for the preparation of in situ hybridization RNA probes were presented by Wang Qiang Laboratory of South China University of Technology. After that, procedures In Ambion's MAXIScript In Vitro Transcription Kit were followed. Full RNAase free operation prevents degradation of the probe. After treatment in different concentrations according to experimental requirements embryos were collected for WISH analyses at 72 hpf, with 15 embryos per tube, three tubes for each group. *Nkx2.5* and *gata4* probes specifically expressed in zebrafish hearts were selected for hybridization. The specific operation process is carried out according to the standard experimental guideline published by Institute of Zoology, Chinese Academy of Sciences. At the end of color development, the embryos were completely immersed in te color development solution and placed in a 28.5 °C incubator for 5~8 h, washed with buffer solution after color development, and 4% paraformaldehyde was fixed for reserve use. When photographs are needed, the embryos are transferred to 90% glycerol, photographed under a Leica stereo microscope, and the images are taken.

RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Gene transcription of *nkx2.5* and *gata4* were detected at 72 hpf in zebrafish embryos exposed to various concentrations of esketamine (0, 0.21, 0.42 and 0.84 mM). Thirty zebrafish embryos were collected in each group, and the total RNA was extracted by Trizol according to the manufacturer's instructions. The total RNA concentration was detected by a NanoDrop spectrophotometer with a target of OD260: OD280 \approx 2.0. Then, the cDNA synthesis was conducted employing the PrimeScript RT Reagent Kit of Takara Company. RT-qPCR was performed in triplicate using tenfold diluted cDNA and a TB Green Premix Ex Taq II kit on a CFX Connect™ Real-Time System (Bio-Rad, Hercules, CA, USA).

The following primers were used for RT-qPCR:

NKX2.5: forward 5'-CGG ATC CTC TCT CTT CAGCG -3',

reverse 5'-TGA CAA CAG CCG ATG TCT TTTT-3';

GATA4: forward 5'-CTA CAG GCA CCC CAG CAGA-3',

reverse 5'-AGA GCC CGA GAC CCG AAAT-3'.

β -actin: forward 5'- AGC ACG GTA TTG TGA CTA ACTG -3',

reverse 5'-TCG AAC ATG ATC TGT GTC ATC-3'.

The PCR program included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. β -actin was used as the internal reference gene, and all data was analysis using the $2^{-\Delta\Delta CT}$ method. Each experiment was repeated for at least three times.

Statistical analyses

All experiments were conducted in triplicate to ensure the reliability and validity of the experimental results. Statistically differences were analyzed by one way ANOVA and the Dunnett's multiple comparison (mean \pm SEM). All statistical analysis using GraphPad Prism software (version 10.0) and SPSS software (version 25.0). $P < 0.05$ was considered statistically significant.

Results

Determination of the optimal concentration

Esketamine exposure was started from 24 to 72 hpf, the medication was changed twice during the period with an interval of 24 h. We observed the mortality at multiple designed timepoints, including 3, 6, 12, 24 and 48 h post-exposure (hpe) to determine its toxicity effect on embryos, respectively. Embryos with no heartbeat were counted and the numbers were marked in each group. As the Fig. 1A shown, consistent with control group, zebrafish embryo survival rate was 100% at the both concentration of 0.21 and 0.42 mM following a long duration of EK exposure from 24 to 72 hpf. In the 0.84, 1.68 and 3.36 mM groups (Fig. 1A), all the survival rate decreased slowly with the exposure duration extended. However, in 0.84 and 1.68 mM groups, when EK exposure time reached to 48 h, the survival rate decreased to 37% and 0%, respectively. In 3.36 mM group, all the zebrafish embryos died at the beginning of the second phase of the study (24hpe). Therefore, concentrations of 1.68 mM and 3.36 mM are unsuitable for further studies on the toxicity effects on zebrafish embryonic development due to the high mortality observed. Based on the results of the above studies, we speculate that the 24 hpe-LC₅₀ value of EK was 1.30 mM, with a 95% confidence interval between 0.92 and 1.60 mM, and the 48 hpe-LC₅₀ value of EK was 0.71 mM, with a 95% confidence interval between 0.46 and 1.01 mM.

The number of dead zebrafish embryos were calculated at the designed timepoints in each group. According to the established toxicity grading criteria, EK is identified as having a median toxic effect on zebrafish embryos. Furthermore, a concentration of 0.84 mM has been determined to exhibit both low mortality and a high degree of teratogenicity, representing the optimal concentration for zebrafish embryos. This concentration is notably consistent with the therapeutic range reported for ketamine in zebrafish toxicity studies^{25,26}. A range of three concentrations: 0.21, 0.42 and 0.84 mM were chosen to determine the teratogenicity of EK on zebrafish embryos and the cardiac development defects. Zebrafish embryos were exposed to EK 24 hpf, the mortality, hatching rate and malformation rate showed concentration- and time-effect dependent manner. The above results are based on the pharmacodynamic ratio of EK and ketamine. Each treatment was repeated three times for analysis.

Effects of esketamine on embryonic and cardiac morphology in zebrafish

Assessment of heart phenotype *via* stereomicroscope (Fig. 2). The pericardial area (μm^2) under different concentrations of EK exposure and control group. At 72 hpf, mild pericardial edema was observed in the 0.42 mM group, and the degree of pericardial edema became more severe with the increase of concentration. The phenotype of pericardial edema was in a concentration-dependent manner. Different concentrations of EK exposure result in expanded SV-BA distance (μm). At 72 hpf, mild lengthened SV-BA distance was observed in the 0.42 mM group, and the SV-BA distance became longer with the increase of concentration. The phenotype of SV-BA distance was also in concentration-dependent manner.

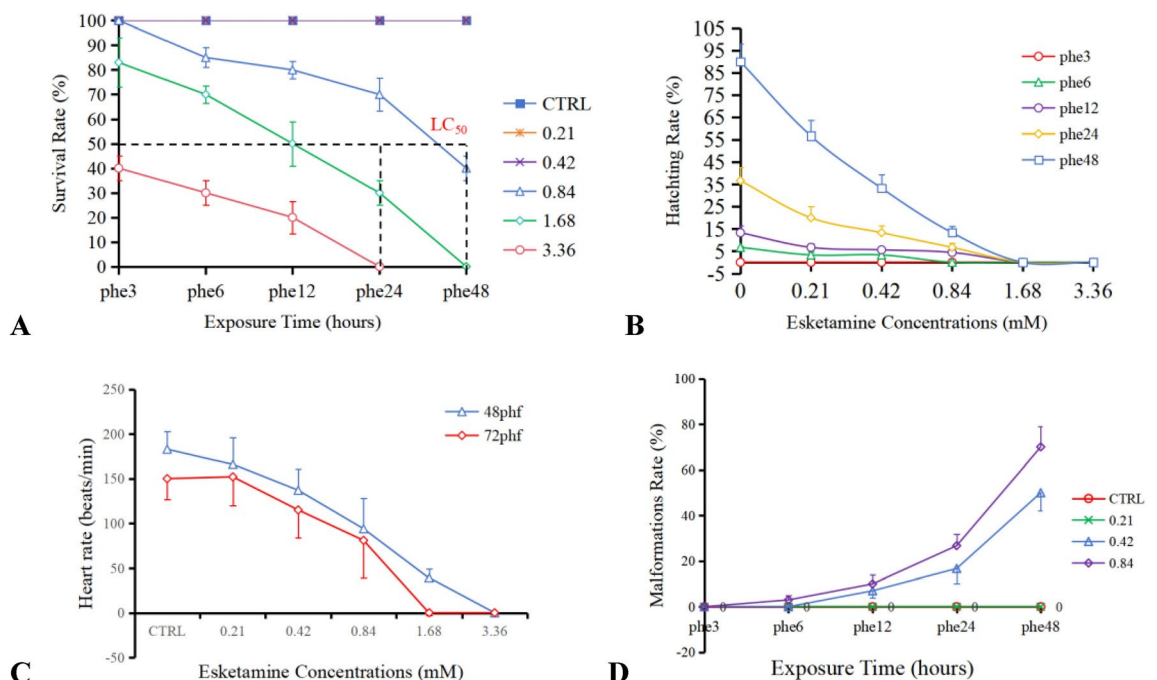


Fig. 1. Effects of EK exposure on zebrafish embryos during different post-fertilization periods. **(A)** (Table 1 suppl.), Survival rates in different concentrations and the determination of LC₅₀ at 48 and 72 hpf. **(B)** (Table 2 suppl.), Hatching rate of zebrafish embryos exposed to vehicle or EK. **(C)** (Table 3 suppl.), Effects of different exposure concentrations on heart rate in both timepoints. **(D)** (Table 4 suppl.), Malformation rate of zebrafish embryos exposed to different concentrations and exposure time. The data represent as mean \pm SD ($P < 0.05$).

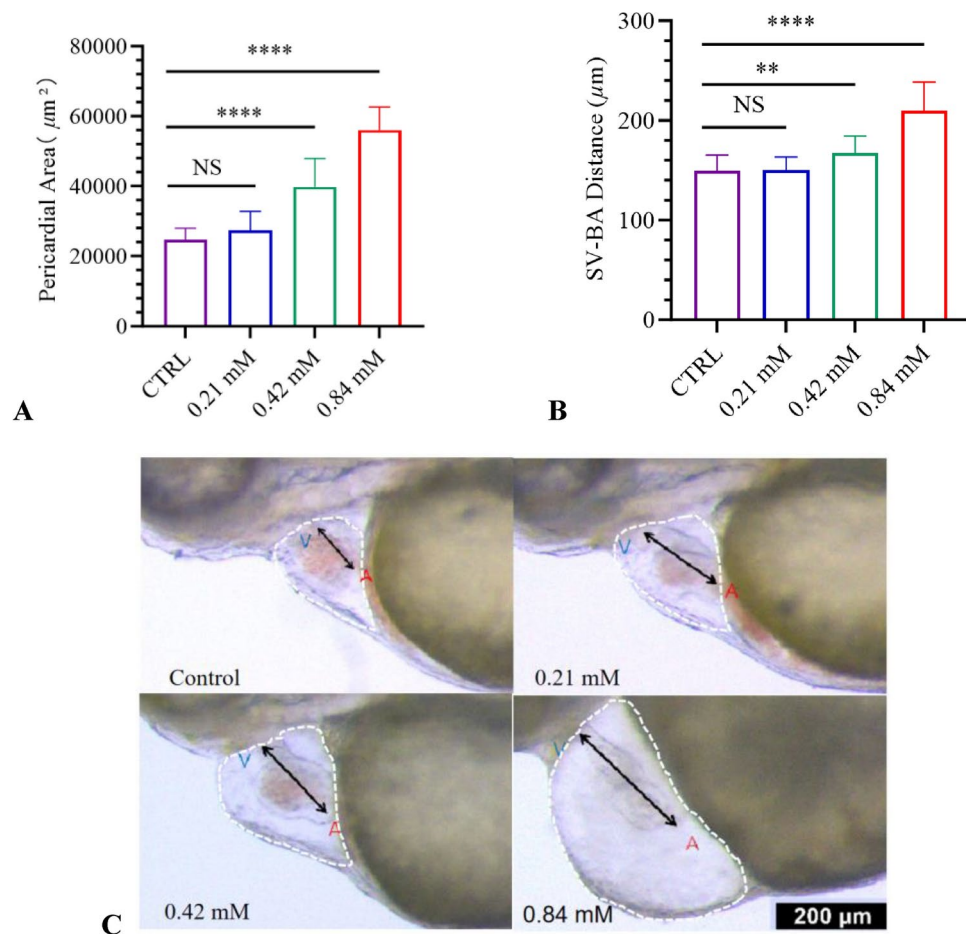


Fig. 2. EK exposure (0.21, 0.42 and 0.84 mM) induces embryonic dysplasia with pericardial and SV-BA distance (Fig. 2C). (A) (Table 5 suppl.), Pericardial area at 72 hpf of four randomly chosen zebrafish embryos, there was no pericardium swelling in the control and 0.21 mM groups ($P > 0.05$), the embryos both in 0.42 and 0.84 mM groups were pericardium swelling (rate = 100%, $P < 0.01$). (B) (Table 6 suppl.), SV-BA distance of four randomly chosen zebrafish embryos at 72 hpf. C, Images of zebrafish at 72 hpf under a stereo microscope; scale bar: 200 μm; magnification: $\times 6.25$. Each group (except 0.84 mM) contains 30 zebrafish embryos and all reperformed for three independent experiments. All data were analyzed using one-way ANOVA with Bonferroni correction. A, bulbar artery; V, sinus venous. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Phenotypes of zebrafish embryo exposed to different concentrations of EK

Exposure to esketamine induced dysplasia within the chevron-shaped somites, where the angle of dashes in the 0.84 mM group was significantly enlarged. The impact of varying concentrations of EK on the body axis length across different groups was examined, a gradual reduction in body axis length was observed, as the concentration increased. These morphogenesis presents a pronounced time- and concentration- dependent manner.

The expression of *nkx2.5* and *gata4* by WISH analysis

Exposure to EK markedly decreased the expression of *nkx2.5* (Fig. 4A), *gata4* otherwise was opposite (Fig. 4C). We have analyzed 15 zebrafish embryos in each group against *nkx2.5* and *gata4* by WISH assay, *nkx2.5* staining gradually became lighter with the increase of EK concentration. The ratio of deregulation for *nkx2.5* and *gata4* is nearly 80% in the 0.42 mM group, but in the 0.84 mM groups the ratio decreased to nearly 40% due to the high mortality.

The zoom images of Fig. 4A showed the cardiac looping defects under 0.21, 0.42 and 0.84 mM exposure. Different types of heart looping at 72 hpf following EK exposure of different concentrations were visualized by *nkx2.5* expression (Fig. 4A). The zebrafish embryos showed a “no-looping” heart under the EK exposure, which is demonstrated in the zoom images. Figure 4A,B presents the outcomes derived from the application of WISH.

EK down-regulate *nkx2.5* and up-regulate *gata4* through Real-Time Quantitative PCR (RT-qPCR) test

Nkx2.5 and *gata4* expression levels were quantified using qPCR, which corroborated the trends and findings observed in the WISH assays. (Fig. 5A Table 9 and B Table10 suppl.). The trends identified were consistent with those obtained through WISH results.

Discussion

Previous studies have conclusively demonstrated the neurotoxicity effects of EK exposure on pregnant rats and their offspring. Zhang²⁷ has reported that exposure to EK in the maternal generation results in memory impairments in offspring rats. This phenomenon could potentially result from EK crossing the placental barrier, thereby inducing developmental impairments in the offspring mice. A multitude of epidemiological investigations have examined the potential for first trimester use of SSRIs to cause CHD²⁸. However, whether EK exposure during this special period has any effect on fetal cardiac development remains unascertained and needs in-depth discussion and further study.

In recent years, zebrafish embryo, which is closely parallels mammalian fetal development, has been widely used in various fields as a model for studying drug cardiotoxicity²⁹. In this current research endeavor, we have conducted a thorough investigation into the toxicological implications of EK exposure on zebrafish embryos, meticulously observing alterations in their developmental morphology and functionality. Our study reveals that EK exposure not only affects the molecular mechanisms of zebrafish embryos but also causes damage to cardiac function, such as heart rate. Figures 2 and 3 demonstrate the detrimental and cardiac teratogenic impacts of EK on zebrafish embryos and cardiogenesis, with these effects manifesting in a concentration- and time- dependent fashion.

A specific range concentrations of EK modulates the morphology and functionality of developmental heart, which is consistent with our research hypothesis. The concentrations that we identified as optimal through the 48- and 72-h LC₅₀ assessments exhibit high teratogenic potential with low mortality rates (Fig. 1A,D), which have notably altered the gross morphological development of the organisms, specifically impacting the pericardium (Fig. 2A,C), SV-BA distance (Fig. 2B,C), body length (Fig. 3A,B), and chevron-shaped somites angles of zebrafish (Fig. 3C,D). The exposure to EK also led to a reduction in heart rates (Fig. 1C), and a positive correlation between these two factors was observed, which is in line with the research conducted by Yuan²⁵.

As depicted in Fig. 1A, the exposure to EK exhibits a time- and concentration- dependent effect, the significant differences were observed among the three groups at a same timepoint. When the exposure time reached 24 h, all embryos in the 3.36 mM group succumbed to death, mirroring the fate of embryos in the 1.68 mM group that

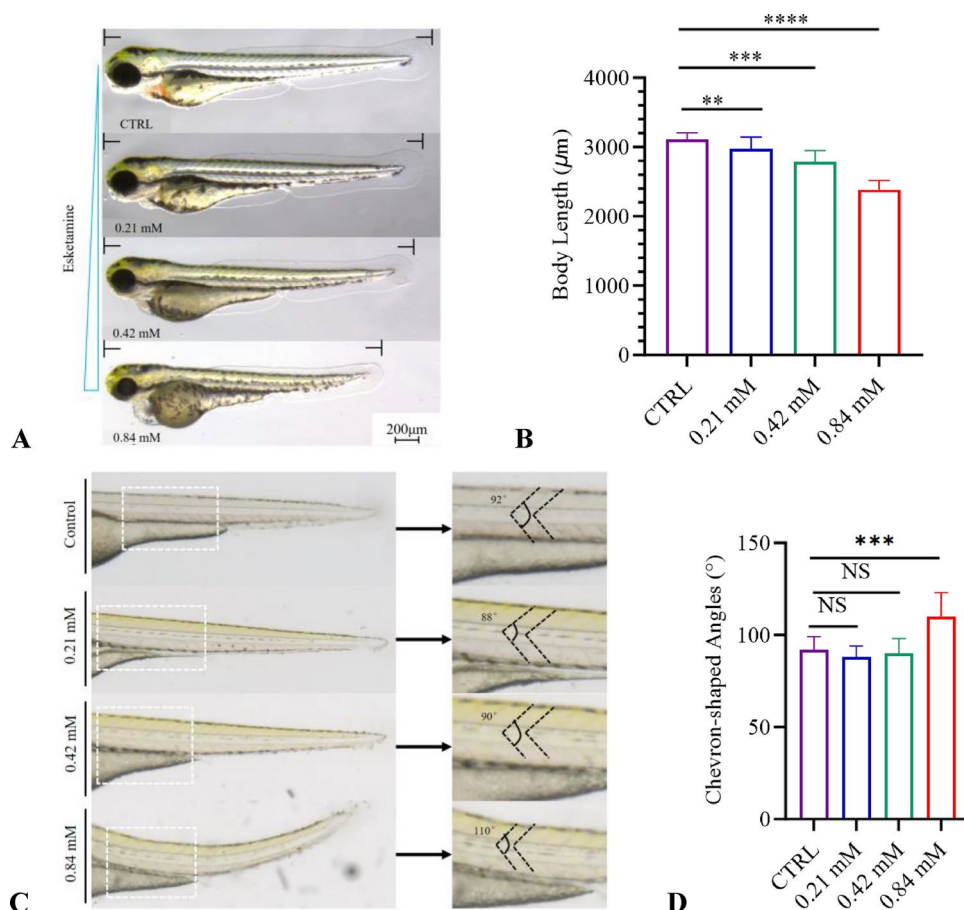


Fig. 3. (A,B) (Table 7 suppl.), Comparison of the zebrafish body length at 72 hpf; length of four embryos were randomly selected to be measured. (C,D) (Table 8 suppl.), chevron-shaped somites angles (small dashes) which induced by malformation of zebrafish at 72 hpf was expressed in (C) and zoomed images, the angles were measured randomly through the survival zebrafish embryos in each group. All data were analyzed using one-way ANOVA with Bonferroni correction. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

perished after 48 h of exposure. Therefore, we excluded the aforementioned two concentration groups, 1.68 and 3.36 mM, from further investigation.

In the CTRL and 0.21 mM groups, no instances of malformation were observed. At the identical timepoint, the incidence of deformity in the 0.84 mM group was notably elevated compared to the 0.42 mM group, exhibiting a clear time- dependent trend. In this research endeavor, the teratogenic concentration of EK exposure that was selected aligns precisely with the ketamine concentrations reported by Guo, based on the similarity in their pharmacokinetic profiles³⁰. The 72 h-LC₅₀ of EK was approximately 0.71 mM, as determined through a rigorous analysis of survival rate (Fig. 1A). Consequently, for the teratogenic analysis of zebrafish embryos, three specific concentrations of 0.21, 0.42, and 0.84 mM were carefully selected.

To meticulously investigate the developmental toxicity of drugs on zebrafish embryos, we have selected the hatching rate, heart rate, pericardial area, body length, and SV-BA distance as the key characterized parameters for analysis. Our research concentrated on the initial stages of cardiac development in zebrafish, which serves as a model for the early fetal development process in human. Notably, the aforementioned parameters were most optimally observed during this particular phase. Except for the two elevated concentrations of EK, specifically 1.68 mM and 3.36 mM, which significantly induced pericardial edema and mortality at extremely high levels, we also discerned a consistent trend with Luis M Félix's reports³¹, observing a marked slowing of heart rate as the concentration of EK increased within a particular range. Zebrafish embryos exposed to that range of EK concentrations at the early stages of cardiac development showed enlarged pericardial area and longer SV-BA distance, all of these results were consistent with previous studies^{32,33}. Based on the outcomes obtained, concentrations less than 0.84 mM were deemed as the optimal selection for the investigation and analysis of teratological effects, specifically focusing on the examination of two pivotal genes related to CHD formation utilizing the WISH technique (Fig. 4A,C).

Given the high teratogenic rate of cardiac phenotypes in morphant embryos, we utilized *nkx2.5* and *gata4* markers to confirm disturbances in cardiac development, which have been established as critical factors in CHD during fetal development³⁴. Numerous studies^{35,36} have conclusively shown that *nkx2.5* is localized within heart precursors, serving a crucial regulatory role in cardiac morphogenesis. The mutation or abnormal expression of *nkx2.5* leads to impairments in cardiac development, encompassing morphological abnormalities in both the ventricle and atrium. These abnormalities manifest as cardiac looping disorders, characterized by median cardiac morphology, and can ultimately culminate into CHD. The insights derived from our extensive evaluation of cardiac dysplasia are markedly apparent, likely attributable to the diminished expression of *nkx2.5* subsequent to EK exposure, which demonstrated that *nkx2.5* staining intensity diminished at WISH, and its expression levels were significantly reduced at q-PCR. The influence of EK exposure on *nkx2.5* in our research was found to be in alignment with Martin's, which also has revealed that the exposure to external stimuli, such as drugs, has been observed to elicit a response within 24 h, specifically through affecting the expression of *nkx2.5*³⁷ during embryonic development. Although Harrington³⁸ has discovered that the *nkx2.5* loss-of-function zebrafish demonstrates an elevated heart rate, exhibiting a contrasting trend in comparison to ours. This discrepancy may stem from differing drug cardiotoxicities, alternative factors, or genetic compensatory mechanisms.

Gata4 is another factor plays a pivotal role in heart development, and the occurrence of mutations within the *gata4* gene also can potentially result in heart dysmorphology, encompassing a range of congenital defects that affect its shape, size, and function³⁹. In the absence of developmental anomalies, the expression of *gata4* is gradually increased throughout the whole heart development process⁴⁰. Vikas Gupta' study⁴¹ has revealed that the zebrafish heart's responses to various stressors are interconnected through a shared mechanism involving the activation of *gata4*. The *gata4*-mediated stress response pathway is consistently employed by zebrafish during growth to confront external stimuli⁴². Furthermore, *gata4* regulation is pivotal for ventricle formation during both zebrafish juvenile morphogenesis and adult heart regeneration⁴³. An additional study⁴⁴ has likewise demonstrated a correlation between atrial septal defect (ASD) and *gata4* mutation. Our study conclusively demonstrated a positive correlation between increasing EK concentrations and heightened *gata4* staining intensity via WISH, alongside a statistically significant rise in *gata4* gene expression as shown by qPCR analyses.

The aforementioned phenomenon unveils an intriguing pattern: an increase in EK concentration correlates with down-regulation of *nkx2.5* and up-regulation of *gata4* gene expression. *Gata4* has the ability to interact with the C-terminal auto-inhibitory region, thereby releasing the active domain of *nkx2.5*²³. This process could potentially serve as a compensatory mechanism in response to the down-regulation of *nkx2.5* expression induced by EK exposure⁴⁵. Tong's research⁴⁶ demonstrated that *nkx2.5* engages in an interaction with *gata4*, and any imbalance in this interaction can lead to the development of severe cardiac abnormalities. It is noteworthy that mutations in *gata4* may give rise to defective interactions with *nkx2.5*, ultimately resulting in the development of CHD⁴⁷. Another investigation has revealed that the *nkx2.5* mutation could potentially be linked to Tetralogy of Fallot⁴², which may also be influenced by *gata4* mutations⁴⁸. *Gata4* and *nkx2.5* are key synergistic regulators of atrial natriuretic factor and B-type natriuretic peptide gene, which play a critical role in repairing and remodeling cardiogenesis^{49,50}. Carl O. Brown also has disclosed that the incorporation of GATA transcription factors can potentially trigger the early expression of *nkx2.5*, thereby playing a crucial role in the development of cardiac progenitors⁴³. Daniel Durocher' research has reported that the *nkx2.5* and *gata4* provide cooperative crosstalk, which may present a paradigm for transcription factor interaction during cardiogenesis⁵¹. *Nkx2.5* serves as a pivotal co-factor for *gata4*, both of which are indispensable for the development of the heart, and their mutations are intricately linked to CHD. Wang and colleagues⁵² speculated that the *nkx2.5* lies in the upstream of *gata4*, and also prior expression to *gata4* in the cardiogenic area at embryonic period. The functional cooperation of *gata4* and *nkx2.5* plays crucial role in the cardiogenetic process⁵³. *Nkx2.5*, as also a cofactor of *gata4*, has been shown to be able to recruit *gata4* to cardiac gene promoters⁵⁴, but our findings through WISH (Fig. 4A,C) and RT-qPCR (Fig. 5A,B) analyses indicate that over-expression of *gata4* does not produce identical results to those of *nkx2.5*.

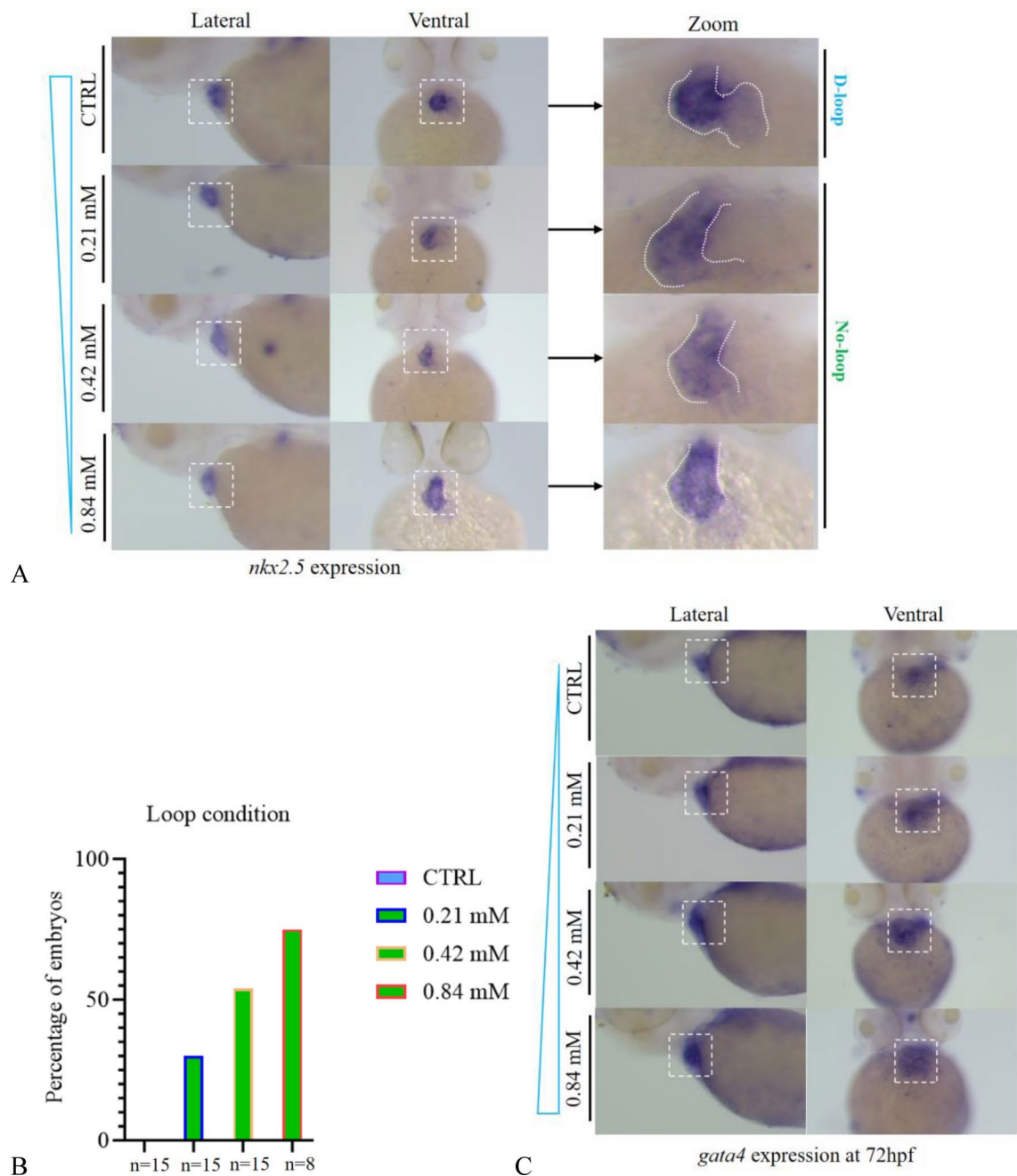


Fig. 4. The expression of heart-specific genes examined through WISH against *nkx2.5* and *gata4* at 72 hpf (A,C), and the cardiac looping is shown in (B). CTRL, control.

Maybe prolonging the EK exposure time under lower concentrations can induce more significant compensatory effect between the two variables.

We furthermore exhibited that as the concentration increased, zebrafish embryos gradually manifested cardiac looping disorder, looks like median cardiac morphology phenotype through WISH from the ventral view. Gradual reduction in atrioventricular overlap and a tendency of distributing along a central axis were observed under the stereo microscope. The initiation of cardiac looping and the process of tube formation may potentially be impeded by exposure to EK, particularly in instances where an imbalance arises between the expression levels of *nkx2.5* and *gata4* (Fig. 4A, zoom images). This imbalance disrupts the delicate equilibrium necessary for proper heart development, leading to potential CHD.

Conclusions

In conclusion, our research confirms the 48 and 72 h-LC₅₀ of EK and its teratogenic effects on embryonic and cardiac development in time- and concentration- dependent manner. Selective EK exposure leads to compromised cardiac morphology and function in zebrafish embryos, potentially due to an imbalance in the expression of

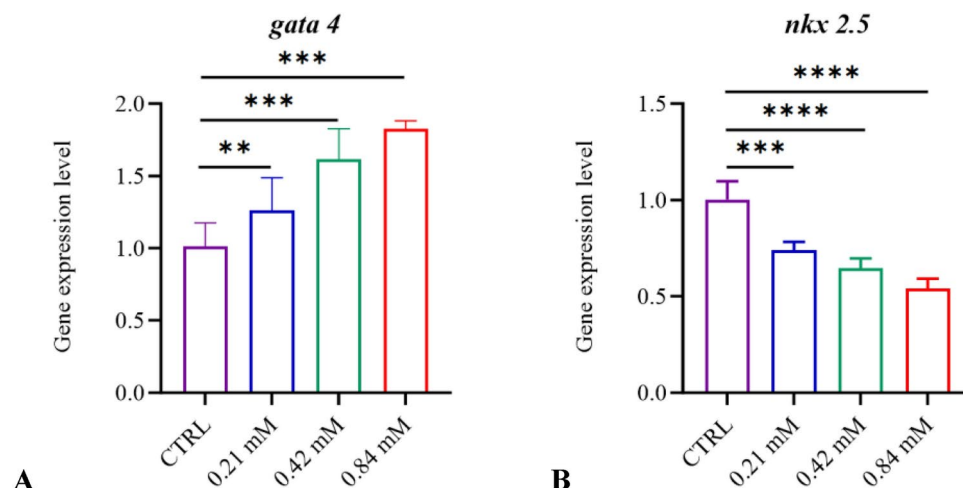


Fig. 5. The expression of cardiac development related genes in zebrafish embryos exposed to EK. The relative expression level of *gata4* showed an upward exposed to EK (A, Table9 suppl.), and the levels of *nkx2.5* decreased significantly (B, Table10 suppl.). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

cardiogenesis-susceptible genes such as *gata4* and *nkx2.5*, along with disruptions in their regulatory networks. Our findings suggesting that caution is warranted when considering its use to treat antepartum depression.

Data availability

All of the data presented in this study could be obtained from corresponding author.

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Author contributions

J.T., S.L., X.L. and R.Z. collaboratively designed and executed the majority of the experiments, with invaluable assistance from Q.O. contributed to the execution of the zebrafish behavioral experiments. J.T. and S.L. were responsible for devising the experimental protocols, interpreting the data obtained, and drafting the manuscript. Additionally, R.Z. and T.J. dissected the relevant tissues and conducted the WISH procedure. Throughout the process, H.H. offered their insights and provided crucial guidance in discussing the results.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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