Long-Term Exposure of Binary Mixture of Cadmium And Mercury Through Alteration of Nrf2 Signalling Pathway Damages The Developed Ovary of Zebrafish

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Abstract

The toxicity of the binary mixture of cadmium (Cd) and mercury (Hg) on the ovary of adult zebrafish was evaluated in the present study. Adult female zebrafish were exposed to cadmium chloride (1 mg/L), mercury chloride (30 µg/L) and a binary mixture of both metals for 21 days. The toxic effects of both metals on the ovary were investigated by evaluating the oxidative stress markers and related gene expression in ovarian tissue along with the histopathological examination. The significantly decreased level of GSH and increased level of MDA in ovarian tissue of adult female zebrafish exposed to Cd + Hg indicated that the exposure of binary mixture of Cd and Hg caused more lipid peroxidation in the ovary. The decreased expression of mRNA of catalase (CAT) and Nuclear factor erythroid 2 (NF-E2)-related factor 2 (Nrf2) were observed in the ovary of zebrafish exposed to binary mixture. The down regulation of Nrf2 might be responsible for suppression of the antioxidant system which resulted in cellular alterations in the ovary. Upon histological evaluation, a decreased number of full-growth (mature) oocytes along with degenerative changes due to Cd exposure were noticed. While, ovary of zebrafish of Hg-exposed group had shown decreased number of pre- and early-vitellogenic oocytes along with atretic pre-vitellogenic oocytes compared to the control group. The ovary of zebrafish of Cd + Hg-exposed group had shown decreased number of pre-vitellogenic oocytes with marked pathological changes in mature oocytes. Present findings elucidate that simultaneous long-term exposure of Cd and Hg may significantly affect the ovary through alteration of Nrf2 mediated cascade in adult zebrafish.

Introduction

The presence of pollutants in air and water sources because of industrial plants nearby residential areas is a critical situation for the health of humans and animals. Diverse pollutants are being discharged mainly into air and water resources, which also affects the health of aquatic animals. Amongst all pollutants, heavy metals cause intense harmful consequences on aquatic animals due to their high toxicity potential with environmental persistence (Srikanth et al. 2013). Cadmium (Cd) and mercury (Hg) are placed in upper position in the list of toxic metals and the levels of them in the environment are increasing. The metals like Cd and Hg can greatly accumulate in the brain, liver and kidneys of humans and animals after continuous exposure (Liao et al. 2006; Mieiro et al. 2011[a,b]). Nevertheless, the both metals can also accumulate in other organs such as gonads (ovary and testis), which may affect reproductive efficiency (Liao et al. 2006).

Cadmium remains in the environment without degradation and causes harmful effects to humans and animals (Van Dyk et al. 2007). Mercury is also found in the aquatic environment with the ability to cause bioaccumulation in the aquatic species (Gworek et al. 2016). After consumption of such fish and other aquatic food animals, Hg enters in the body of humans and causes severe pathological alterations. Individually, Cd and Hg has been reported to alter the antioxidant system. Nuclear factor-erythroid 2 - related factor 2 (Nrf2) has an important role to stimulate the antioxidant system (Lungu-Mitea et al. 2014). Significant down-regulation of Nrf2 has been observed in the brain of zebrafish following

exposure to binary mixture of Cd and Hg (Patel et al. 2021). However, exposure of zebrafish to binary mixture of Cd and Hg didn't produce the significant additive or synergistic toxic effect on brain.

Higher level of cadmium as well as mercury has been reported in water resources (Patel and Vediya, 2012) as a result of anthropogenic activity. The Cd exposure at higher level causes the damage to multiple organs. Even in zebrafish the exposure of Cd at 1.05 mg/L for 21 days has been reported to affect the liver and ovary of zebrafish (Sunaina and Ansari, 2015). Similarly, marked pathological lesions in brain of zebrafish have been reported following exposure to Cd at 1.0 mg/L concentration for 16 days (Favorito et al. 2011). Likewise, severe oxidative stress-mediated changes in hypothalamic-pituitary-gonadal axis of zebrafish have been reported following long-term exposure of mercury chloride (Zhang et al. 2016[a]). Severe oxidative stress in gonads of zebrafish has been reported when fish exposed to Hg at 30 µg/L for 30 days (Zhang et al. 2016[a]). Additionally, binary mixture of the Hg and Cd has been reported to alter the embryonic development in pregnant golden hamsters (Gale, 1973). However, Cd has been reported to counteract the biochemical and histological alterations in organs of Snakehead fish (Arya and Sharma, 2015). The lipid peroxidation is caused in ovary of zebrafish following individual exposure to Cd and Hg (Jin et al. 2015; Zheng et al. 2016). In fish, hypothalamus-pituitary-gonadal axis plays crucial role in regulation of reproductive functions (Ankley et al. 2010) through hormones secreted from them. Through disturbance of endocrine system, heavy metal-induced reproductive toxicity is well studied in fish (Ankley et al. 2010; Kwon et al. 2016; Su et al. 2016; Zhang et al. 2016[a,b]). The reports related to heavy metal-induced oxidative stress mediated reproductive toxicity are limited and little information is available on toxic effects of metals like Cd and Hg on ovary of aquatic animals following individual and simultaneous exposure.

The zebrafish (*Danio rerio*), a popular lower vertebrae animal model that is widely used in toxicity studies particularly in the field of reproductive and neurotoxicity. Toxicity potential of Cd and Hg following individual exposure in various species of animals have been studied well. However, in the real field condition, there is exposure of more than one metal in aquatic animals at a time. It is the need to evaluate the ovarian toxicity potential of a mixture of heavy metals which persist at high concentration in the environment. The evaluation of alterations of oxidative stress markers, expression of antioxidant genes and histological changes in the ovary of adult zebrafish would be useful to understand the toxicity potential of such heavy metals following simultaneous exposure. Thus, the present study was planned to evaluate the ovarian toxicity following single and combined exposure of Cd and Hg in adult zebrafish. As per our knowledge, this is the first report which explores the effect of co-exposure of Cd and Hg on the developed ovary of adult zebrafish in terms of status of antioxidant system, gene expression and histological changes.

Materials And Methods

Chemicals

Analytical grade cadmium chloride ($CdCl_2$) and mercury chloride ($HgCl_2$) were purchased from Himedia, Mumbai (CAS number – 35658-65-2) and Merck, Mumbai (CAS number- 7487-94-7), respectively. Other chemicals used in the analysis were of molecular or analytical grade.

Experimental animals and environment

Adult female zebrafish (Wild-type) of 5–6 months of age were procured from Vikrant aqua culture, Mumbai. Fish were kept in tanks having capacity of 20 L with fitting of aerators. Two weeks of acclimation period were monitored before starting the experiment. Temperature of the water in the tank was in the range of 25–28°C and light/dark cycle of 14:10 h was also maintained. The fish were fed with equal quantities of freeze-dried blood worms (Hallofeed®, Maharashtra Aquarium-Mumbai) and fish pellets (Tetra bits complete®, Tetra GmbH-Germany) (10 mg/fish twice a day freely available to each group). The filtered reverse osmosis (RO) water (pH of water: 6.8–7.4, Hardness: 200–250 mg/L, Electrical conductivity: 500–600 µs) was used during the experiment to maintain the fish.

Experimental design

In the study, the levels of exposure of both heavy metals were selected based on previous studies related to toxicity of cadmium (Favorito et al. 2011) and mercury (Zhang et al. 2016[a]). A total of 136 fish used for this study which were randomly divided into four groups (34 fish in each group). Fish of the control group were kept untreated. Second group of fish was exposed to cadmium chloride dissolved in water (1 mg/L). Third group was exposed to mercury chloride dissolved in water (30 µg/L). Fourth group was exposed to both heavy metals in combination at the above specified strength. The exposure of fish to heavy metal/s was continued for 21 days. The used strength of both metals in a binary mixture form in the study has created more practical or environmental realistic exposure in the fish. Both metals were accurately weighted using precise analytical weighing balance (Model: MS 204S/A01, Mettler Toledo, US). The concentration of metal/s in each tank was maintained by changing the water daily with fresh water containing particular strength of metal/s. After an exposure period of 21 days, oxidative stress markers in the ovary were evaluated using 96 zebrafish (24 in each group; pooled samples and 6 replication). While, 20 fish were used for evaluation of expression of antioxidant genes in the ovarian tissue (5 from each group). Histological changes in the ovary were also observed microscopically using 20 zebrafish (5 from each group).

All fish were humanely sacrificed by the ice-cold method on 22nd day of the experiment. The ovary of each fish was dissected under Stereo Microscopes (Model CZM6, Labomed Inc., USA). The dissection of the ovary from zebrafish was completed approximately within a minute. Samples of ovary from 24 zebrafish were collected in Tris-EDTA buffer (8.5 pH to evaluate the activity of SOD (Samples from 6 fish/group). For evaluation of CAT activity and level of GSH, samples from 48 fish (12 fish/group; a pooled sample from 2 zebrafish) were collected phosphate buffer saline (7.4 pH; PBS). Samples of ovary from 24 zebrafish were collected in a butylated hydroxytoluene buffer to estimate the MDA level. For histopathological examination, 05 whole fish from each group were collected in 10 % Neutral Buffered Formalin (NBF). Few incisions on the body of each fish were made to facilitate the penetration of

buffered formalin in the body. In addition to this, the tissue samples of the ovary from 5 fish of each group (5 replications) were washed with PBS and collected in a nuclease-free storage vial containing RNAlater solution (Invitrogen, Bangalore, India) and processed for isolation of RNA and complementary DNA (cDNA) synthesis to study the mRNA expressions of *sod, cat*, and *nrf2* genes.

Evaluation of oxidative stress markers in ovarian tissue

The SOD activity in the tissue was assayed using the method of pyrogallol autoxidation by superoxide radicals (Marklund and Marklund, 1974). Catalase (CAT) activity was determined according to the method described earlier (Sinha, 1972). The GSH level in the ovarian tissue was estimated using a method described by Ellman (1959). The level of malondialdehyde (MDA) in the sample was measured using steps described in the product catalog of Sigma-Aldrich Co. LLC., USA (Catalog number MAK085). ELISA plate reader (Thermofisher Scientific, Model: Multiskan Go) was used to measure the absorbance.

Evaluation of expression of genes

Trizol reagent (Ambion Life Technologies, USA) was used to extract total RNA from ovarian tissue as per the standard procedures described by Sambrook and Russel (2001). Nano-Drop spectrophotometer (ND-2000, Thermo scientific, USA) was used to check the quantity and quality of total RNA isolated. The sample of RNA with 260/280 ratio between 1.8 and 2.0 were further processed to remove the residual genomic DNA contamination using DNA-free™ Kit (Ambion Life Technologies, USA). Complementary DNA (cDNA) was synthesized from total RNA using the first-strand cDNA synthesis kit (Genetix Biotech Asia Pvt. Ltd, New Delhi, India) as per the manufacturer's instructions. High-performance liquid chromatography (HPLC) purified primers (Eurofins Genomics India Pvt. Ltd., Bangalore, India) were used for amplification of genes viz. *sod*, *cat, nrf2*, and *β-actin* are mentioned in Table 1. The target and house-keeping genes were amplified in a real-time PCR system (LightCycler® 480 II, Roche, Switzerland), using Maxima™ SYBR Green/ROX qPCR Master Mix (Genetix Biotech Asia Pvt. Ltd, New Delhi, India). Amplifications for each mRNA were performed with the cDNA template and fold expression analysis was carried out $2^{-\Delta\Delta Ct}$ method by keeping β-actin as the endogenous control gene (Livak and Schmittgen, 2001).

Table 1
Sequences of primers of targeted genes used for real-time PCR amplification

Target gene	Primer sequence (5'-3')	Annealing temperature (°C)	Product size (bp)	Reference
sod	FP 5'-CAACACAAACGGCTGCATCA – 3' RP 5'-TTTGCAACACCACTGGCATC – 3'	60	132	(Sarkar et al., 2014)
cat	FP 5'-AGTTCCCTCTGATTCCTGTG – 3' RP 5'-ATGGCGATGTGTGTCTGG – 3'	60	173	(Jaramillo et al., 2017)
Nrf2	FP 5'-TGTTGGTTCGGAGGCTCTTAA – 3' RP 5'-AGGCCATGTCCACACGTACA – 3'	60	62	(Velasques et al., 2016)
β-actin	FP 5'-GCTGTTTTCCCCTCCATTGTT – 3' RP 5'-TCCCATGCCAACCATCACT – 3'	60	60	(Bautista et al., 2018)

SOD: superoxide dismutase, CAT: catalase, Nrf2: Nuclear factor erythroid 2 (NF-E2)-related factor 2

Histopathological examination

The formalin-fixed fish were placed in sodium EDTA solution (0.35 M, pH 7.8) for decalcification for 7 days. The volume of sodium EDTA solution was 20 times the fish volume. The decalcified fish were embedded in paraffin which was used to get sections of 5 µ thickness using a semi-automated rotary microtome (Leica Biosystems, Germany). Tissue sections were stained with hematoxylin and eosin (H & E) stains. The stained slides were observed for microscopic pathological changes in the ovary using an optical microscope (Zeiss primo star) attached with a microscopic camera (ZEISS Axiocam ERc 5) and digital histological photographs were captured with the help of Carl Zeiss ZEN 2 (Blue edition) software.

Statistical analysis

Statistical analyses of all data were carried out using GraphPad prism. Kolmogorov-Smirnov test was used to evaluate the normality of data along with Bartlett's test to confirm the equal variance. All data

were analyzed by parametric one way analysis of variance (ANOVA) followed by Tukey's HSD test as they were found with normal distribution and homogeneous variance. The value of $p < 0.05$ (*) was considered as statistically significant and $p < 0.01$ (**), $p < 0.005$ (***) and $p < 0.001$ (****) were considered for highly statistical significant differences.

Results

Oxidative stress markers evaluated in ovarian tissue of zebrafish of different groups are presented in the Fig. 1. The SOD activity in ovarian tissue was not significantly ($p > 0.05$) altered in zebrafish of all toxicity groups. While, CAT activity in the zebrafish exposed to Cd + Hg was non-significantly lower than those of other groups. The levels of GSH in the ovarian tissue of Cd- and Hg-exposed zebrafish were slightly lower (non-significant) ($p > 0.05$) than that of the control group. However, the ovarian tissue of zebrafish exposed to Cd + Hg had shown a significantly lower level of GSH ($p < 0.01$) as compared the control group ($p < 0.05$). The MDA level of the ovary was slightly ($p > 0.05$) higher in zebrafish exposed to Cd and Hg alone as compared to that of the control group. However, that of the Cd + Hg-exposed group was significantly ($p < 0.001$) high as compared to that of the control group.

The expression levels of mRNA of SOD, CAT, and Nrf2 of ovary of zebrafish from different groups are given in Table 2. PCR amplification of β-actin (BA), CAT, SOD, Nrf2 from the ovarian tissue of adult zebrafish is shown in Fig. 2. The expression levels of SOD mRNA in all toxicity groups were slightly increased (non-significantly) as compared to control group. The CAT mRNA expression level in the ovary of zebrafish exposed to Cd + Hg was 4.92 fold lower than that of control group ($p > 0.05$). Similarly, expression level of Nrf2 mRNA in ovary of zebrafish exposed to Cd + Hg was also 7.08 fold lower than that of control group ($p > 0.05$).

Table 2
SOD, CAT and Nrf2 mRNA expression in the ovary of adult zebrafish exposed to Cd, Hg and Cd + Hg for 21 days

mRNA expression	Toxicity group	ΔΔCt value	Fold changes ($2^{-\Delta\Delta Ct}$)	Change in expression
SOD	Cd	2.96 ± 1.18[a]	0.13	Increase
	Hg	5.01 ± 0.70 [a]	0.03	Increase
	Cd + Hg	3.08 ± 0.77 [a]	0.12	Increase
CAT	Cd	-1.99 ± 1.27 [a]	3.98	Decrease
	Hg	-1.52 ± 0.80 [a]	2.88	Decrease
	Cd + Hg	-2.03 ± 1.04 [a]	4.92	Decrease
Nrf2	Cd	-0.90 ± 1.52 [a]	1.87	Decrease
	Hg	-2.33 ± 0.86 [a]	5.02	Decrease
	Cd + Hg	-2.82 ± 0.56 [a]	7.08	Decrease

Cd: group exposed to cadmium chloride, Hg: group exposed to mercury chloride, Cd + Hg: group exposed to cadmium chloride along with mercury chloride. Data were analyzed one-way ANOVA followed by Tukey's HSD test. No significant difference between groups ($p > 0.05$)

Upon microscopic examination, the ovary of normal zebrafish revealed normal architecture of oocytes of various developmental stages (pre-vitellogenic oocyte (PVO), early-vitellogenic oocyte (EVO) and full-growth (Mature) oocyte (FGO) (Fig. 3A & B). Early-vitellogenic oocyte stage is also called the cortical alveoli stage. The ovary of cadmium-exposed zebrafish had shown decreased number of FGO, increased number of PVO and EVO with loss of contacts between the oocyte cell membrane and the follicular cell layer (Fig. 4A). Various alterations like accumulation of proteinaceous fluid in EVO and FGO, degenerated PVO, atretic EVO and atretic PVO were noticed (Fig. 4B, C & D). The ovary of mercury-exposed zebrafish had shown accumulation of proteinaceous fluid (Degenerative changes) in EVO, degenerated FGO and atretic PVO (Fig. 5A to D). Ovary of zebrafish exposed to cadmium along with mercury had shown disorganized structure of FGO, depletion of yolk granules in FGO, accumulation of proteinaceous fluid in EVO and FGO, and atretic PVO (Fig. 6A to D).

The values of no. of oocytes at different stages in the ovary of adult zebrafish of different groups are shown in Fig. 7. No. of atretic pre-vitellogenic and early-vitellogenic and mature oocytes with degenerative changes in the ovary of adult zebrafish of different groups are shown in Fig. 8. In the control group, the

number of PVO, EVO and FGO were 45.76, 24.80 and 29.44 %, respectively. The Cd-exposed zebrafish exhibited significant increase of PVO and decrease in FGO compared to control fish. However, no significant effect of Cd was noticed on the number of EVO. The fish exposed to either Hg or Cd + Hg showed the significant decrease of PVO and EVO compared to the control and the Cd group. However, there was no significant difference in the number of PVO, EVO and FGO between Hg and Cd + Hg exposed groups. Compared to other toxicity groups, Cd exposure resulted in the significantly higher no. of atretic PVO. Similarly, Cd + Hg exposure resulted in the significant higher no. of EVO with degenerative changes. Interestingly, no. of FGO with degenerative changes was significantly higher in the Cd- and Hg–exposed groups as compared to control group. However, almost all FGO of Cd + Hg group showed structural damage with depletion of yolk granules as compared to that observed following individual exposure of Cd or Hg.

Discussion

The data related to the reproductive toxicity potential of binary mixture of Cd and Hg in laboratory animals, especially in zebrafish, are scarce. As per our knowledge, this is the first report which includes the findings related to effect of binary mixture of Cd and Hg on developed ovary of adult zebrafish exposed for longer duration. We focused on evaluation of oxidative insult along with mRNA expression levels of related genes in the ovary of adult zebrafish. We also observed important histological changes in the ovarian distribution of various types of oocytes along with histological changes in the ovary of adult zebrafish exposed to both Cd and Hg individually or Cd + Hg for a long time. Thus, the findings of the study may be helpful to understand or explore the risk hazard when animals or humans are exposed continuously to both metals at a time.

In line of our observations related to antioxidant enzymes in the present study, in the ovary of zebrafish, a significantly decreased GPx activity and GSH content with little or no effect on the activity of CAT and SOD after 30 days of exposure of Hg has been reported previously (Zhang et al. 2016[a]). However, Cd exposure for 21 days at high dose in zebrafish has been reported to cause significant decrease of CAT, SOD, GPx activity along with higher level of MDA in the ovary (Banni et al. 2011, Sunaina and Ansari, 2015). Cadmium leads to increase the oxidative stress through production of the hydroxyl radical species which initiates the lipid peroxidation (Jurczuk et al. 2004; Dondero et al. 2005) and also alters the anti-oxidative stress enzymes (Bauer et al. 1980; Jihen et al. 2009). The less alteration in CAT activity by Cd and Hg in the ovary of zebrafish might be due to less accumulation of H_2O_2 and other cytotoxic radicals. The GSH specifically after binding with Cd and Hg forms a complex which prevents it’s binding to cellular proteins, and therefore plays a critical role in the cellular regulation of detoxification. On the other hand, the MDA is an indicator of lipid peroxidation (LPO) which is a major contributor to the disruption of cell function under oxidative stress (Storey, 1996). The enhanced LPO in the ovary of fish exposed to Cd + Hg might be due to inhibition of antioxidants enzymes, which are more concerned with defense against free radical induction.

Following entry of heavy metals including Cd and Hg, the antioxidant system at cellular level is altered (Regoli et al. 2002) through increasing the levels of reactive species. The heavy metals also alters the activities of antioxidant enzymes of aquatic animals through alterations in activity and levels of antioxidant enzymes, particularly SOD, CAT and GSH which provide the first line of cellular defense against the free radicals. The heavy metals causes cellular alterations following entry in the body and affects integrity of DNA (Druwe and Vaillancourt, 2010; Gonzalez et al. 2010; Bertin and Averbeck, 2006). The activation of Nrf2 is a master key process of the cellular antioxidant response. The ROS binds to antioxidant response element (ARE) sequence in the promoter region of antioxidant enzyme genes and activates gene expression (Osburn and Kensler, 2008). Nrf2 in its inactive form can binds to Kelch-like-ECH-associated protein 1 (Keap1) which is inhibitor of Nrf2 which leads to alter the transcriptional activity (Sporn and Liby, 2012). Upon initiation of oxidative stress, the Nrf2 is liberated from Keap1 and translocated into the nucleus where it regulates the transcription of antioxidant genes (Dai et al. 2007). Till date, little information is available regarding the role of Nrf2 in Cd and Hg induced oxidative stress in the ovarian tissue of adult zebrafish except few studies on other metals (Jiang et al. 2014; Zheng et al. 2016; Mondal et al. 2019). We also observed slightly up-regulation of expression level of SOD mRNA in the ovary of zebrafish of toxicity groups. The down-regulation of mRNA expression of NrF2 was accompanied with down-regulation of CAT mRNA expression in all toxicity groups. Similar to our observations, the significant up-regulation of the SOD mRNA expression along with non-significant decrease of SOD activity was reported in the ovary of zebrafish exposed to Hg (30 µg/L) for 30 days. The CAT activity was also reported to be non-significantly affected (slightly lower) with the down-regulation of CAT mRNA in zebrafish exposed to Hg (30 µg/L) (Zhang et al. 2016[a]). The significant lower activities of SOD and CAT have been reported along with significant up-regulation of their relative genes in ovary of zebrafish following Cd exposure to 0.4 mg/L for 21 days (Banni et al. 2011). The up-regulated mRNA level of Nrf2 due to Cd exposure for 96 h has been reported with the up-regulation of Cu/Zn-SOD and CAT activity in the ovary of zebrafish (Zheng et al. 2016). The relatively stable activity of SOD and CAT along with expression of CAT mRNA in the ovary of zebrafish has been reported even though having an up-regulated expression of SOD1 (Zhang et al. 2016[a]). Fish can combat the increased level of ROS in the body by producing scavenging enzyme such as SOD which converts superoxide anions into H_2O_2. It might be possible that an increase in the transcription of this gene would contribute to the elimination of Cd and Hg-induced ROS from the cells. Continuous production of H_2O_2 in ovarian tissue may decrease the CAT activity and its relative mRNA expression level.

In line with our observations related to histological changes in the ovary, Cd exposure (0.4 mg/L for 21 days) has been reported to cause the histological changes in the ovary of zebrafish. Such effects might be due to the down regulation of Zn transporter 1 (ZnT1) Zrt- and over-expression of Irt-related protein 10 (ZIP10) in zebrafish ovary (Chouchene et al. 2011). The ZIPs and ZnTs are involved in cytokine- and growth factor-mediated signalling, and the regulation of enzymes, receptors, and transcription factors of cellular signalling pathways. The Hg exposure has been reported to cause oocyte atresia, loss of contacts between the oocyte cell membranes and the follicular cell layer at different exposure levels (15 and 30 µg/L for 30 days) in zebrafish (Zhang et al. 2016[a]). The Cd co-exposed with Hg in the present study had

shown significant down regulation of Nrf2 gene along with significant decrease of GSH content with increase of MDA level which indicates the possible toxicity potential of both metals following co-exposure as compared to individual exposure. This finding was also supported with histological changes in the ovary like degenerative changes in FGO along with atresia of PVO and EVO with significant decrease in the no. of PVO and EVO. Nuclear factor-erythroid 2 -related factor 2 (Nrf2) is the primary transcription factor which regulates cytoprotective genes, including the antioxidant glutathione (GSH) and protects the cells from oxidative stress (Harvey et al. 2009). Nrf2 has an important role in maintaining the GSH redox state through transcriptional regulation of glutathione reductase and protecting cells against oxidative stress.

Conclusions

The result of the present study showed that the cadmium exposure resulted in more number of atretic pre-vitellogenic oocytes along with reduced size of mature oocytes, while mercury exposure decreased the number of pre-vitellogenic oocytes without significant effect on size of mature oocytes. Such finding suggests that following long-term exposure of cadmium chloride alone may damage the mature and early-stage oocytes, while mercury chloride had shown ability to damage the oocytes at early stage in the ovary of adult zebrafish. Overall, from the results obtained in the study, it is inferred that waterborne exposure of cadmium along with the mercury causes significant oxidative stress-mediated histological damages through alterations of Nrf2 mediated signalling pathway in the ovary of zebrafish as compared to individual exposure, which may subsequently impair the reproduction capacity of fish.

Declarations

Ethics approval and consent to participate: Experimental procedure was approved by the Institutional Animal Ethics Committee of the college.

Consent for publication: All the authors approved the manuscript for publication.

Availability of data and materials: All data are made available in the manuscript and supplementary file.

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Authors' contributions:

Utsav N. Patel: investigation, validation, formal analysis, writing – original draft.

Urvesh D. Patel: conceptualization, writing – review and editing, supervision.

Rahul K. Vaja: investigation, data curation.

Aniket V. Khadayata: investigation, data curation.

Chirag M. Modi: methodology, editing.

Harshad B. Patel: investigation, validation, formal analysis.

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Figures

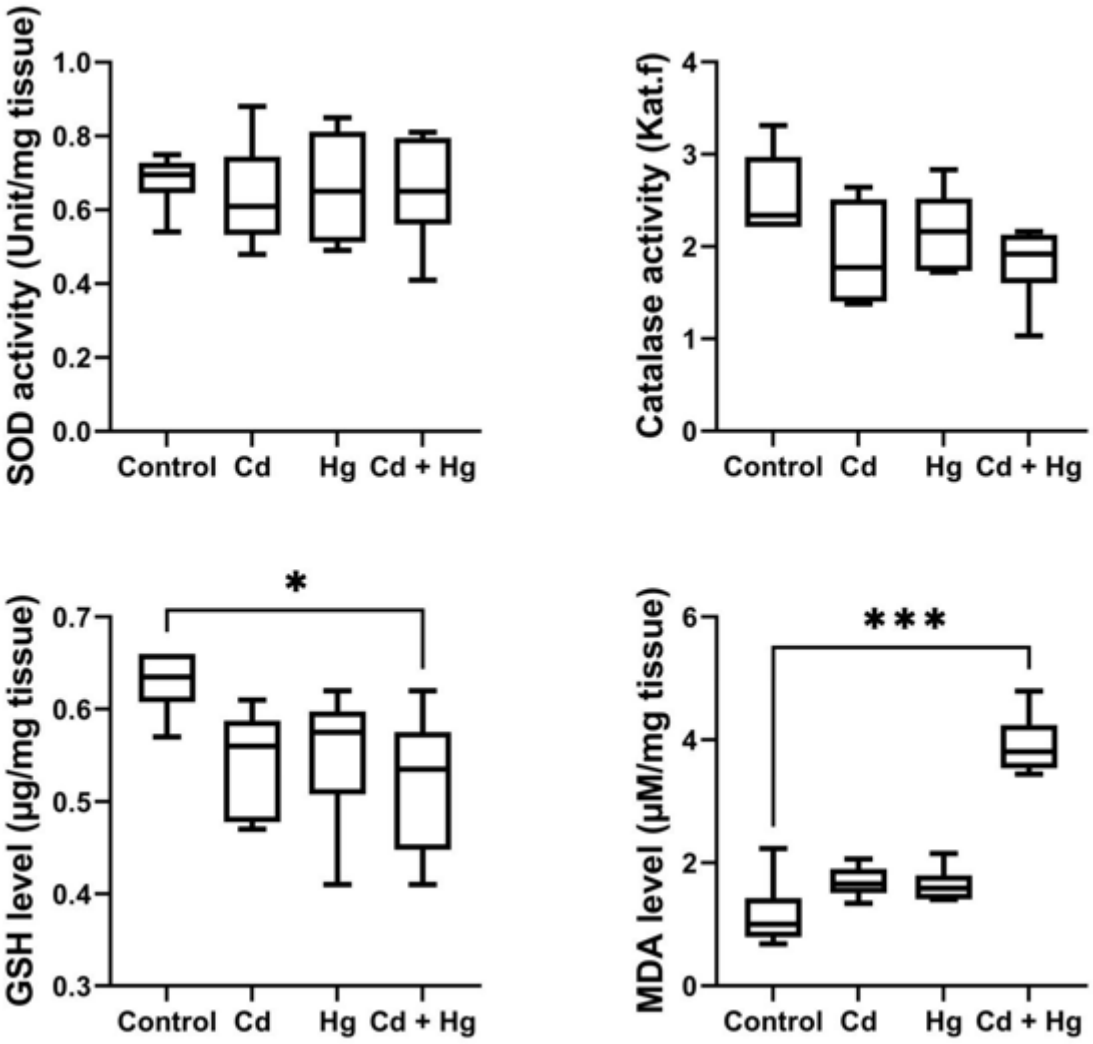

Figure 1

Oxidative stress markers in ovarian tissue of adult zebrafish exposed to Cd, Hg and Cd + Hg for 21 days. Cd: group exposed to cadmium chloride, Hg: group exposed to mercury chloride, Cd + Hg: Cd: group exposed to cadmium chloride along with mercury chloride. * Data were analyzed by one-way ANOVA followed by Tukey's HSD test, *Indicates significant differences between the groups. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

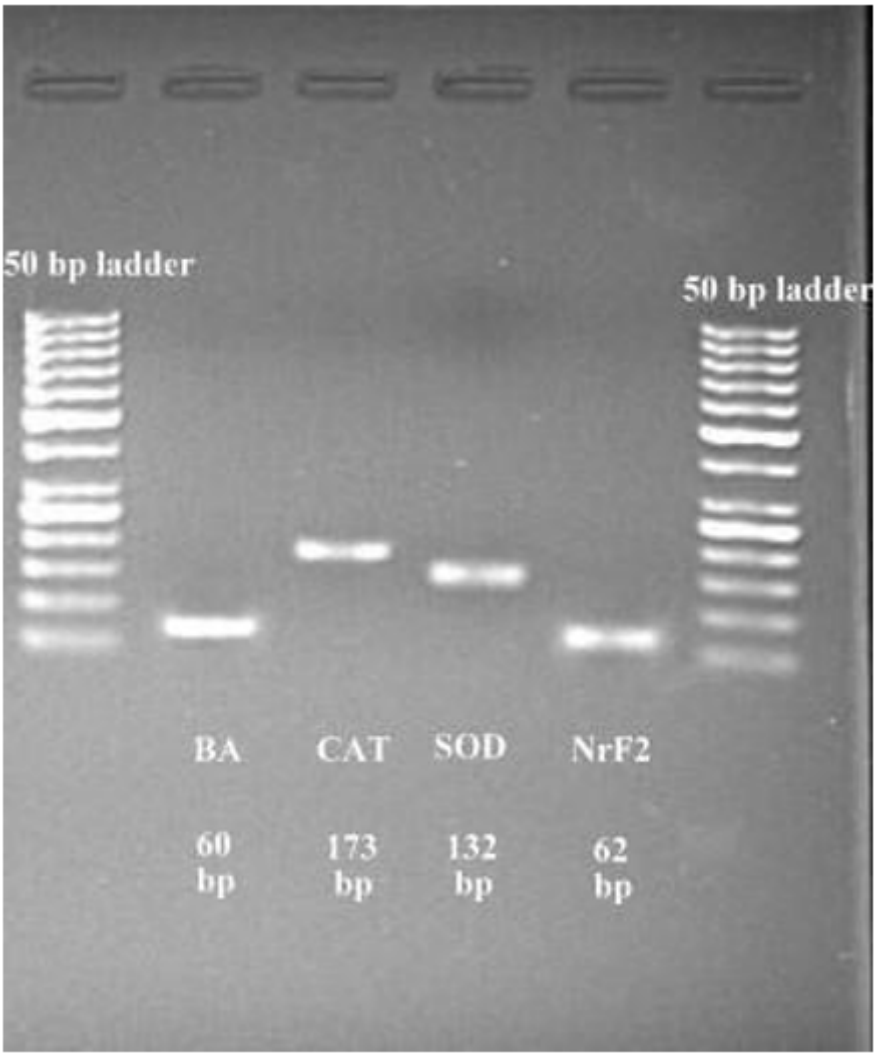

Figure 2

PCR amplification of β-actin (BA), CAT, SOD, Nrf2 from the ovarian tissue of adult zebrafish

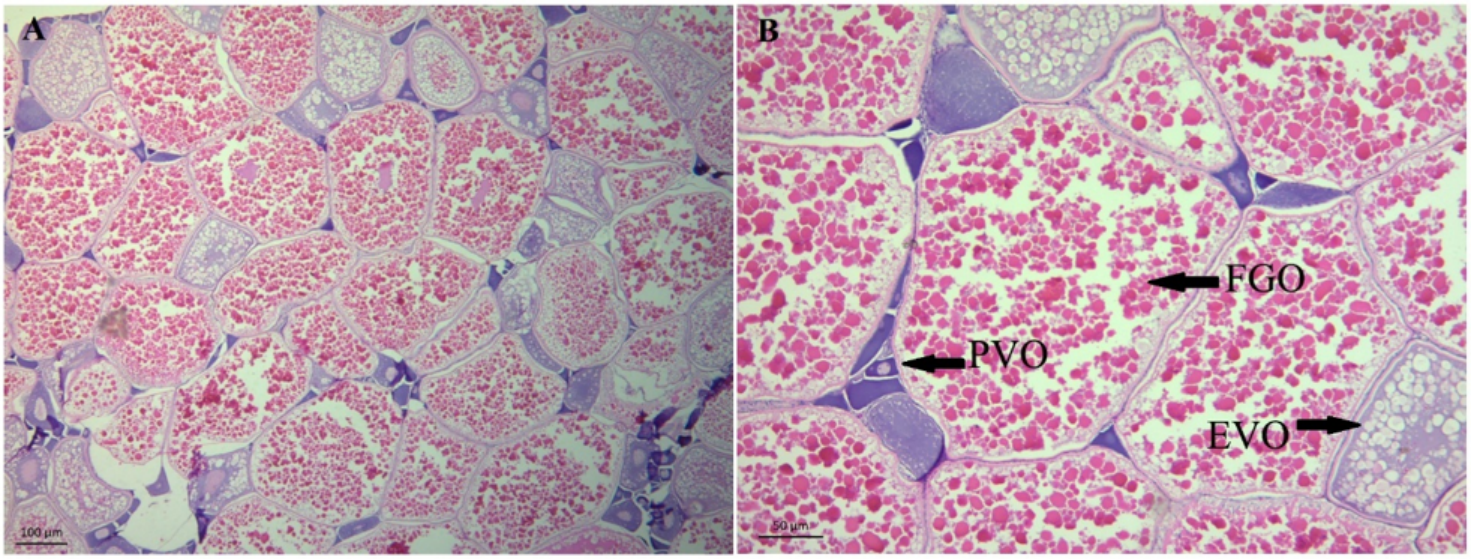

Figure 3

Microscopic view of ovary of zebrafish of control group showing various developmental stages of oocyte like pre-vitellogenic oocyte (PVO), early-vitellogenic stage (EVO) and full-growth (Mature) oocyte (FGO) (A: Scale of 100 µm; B, C, D: Scale of 50 µm).

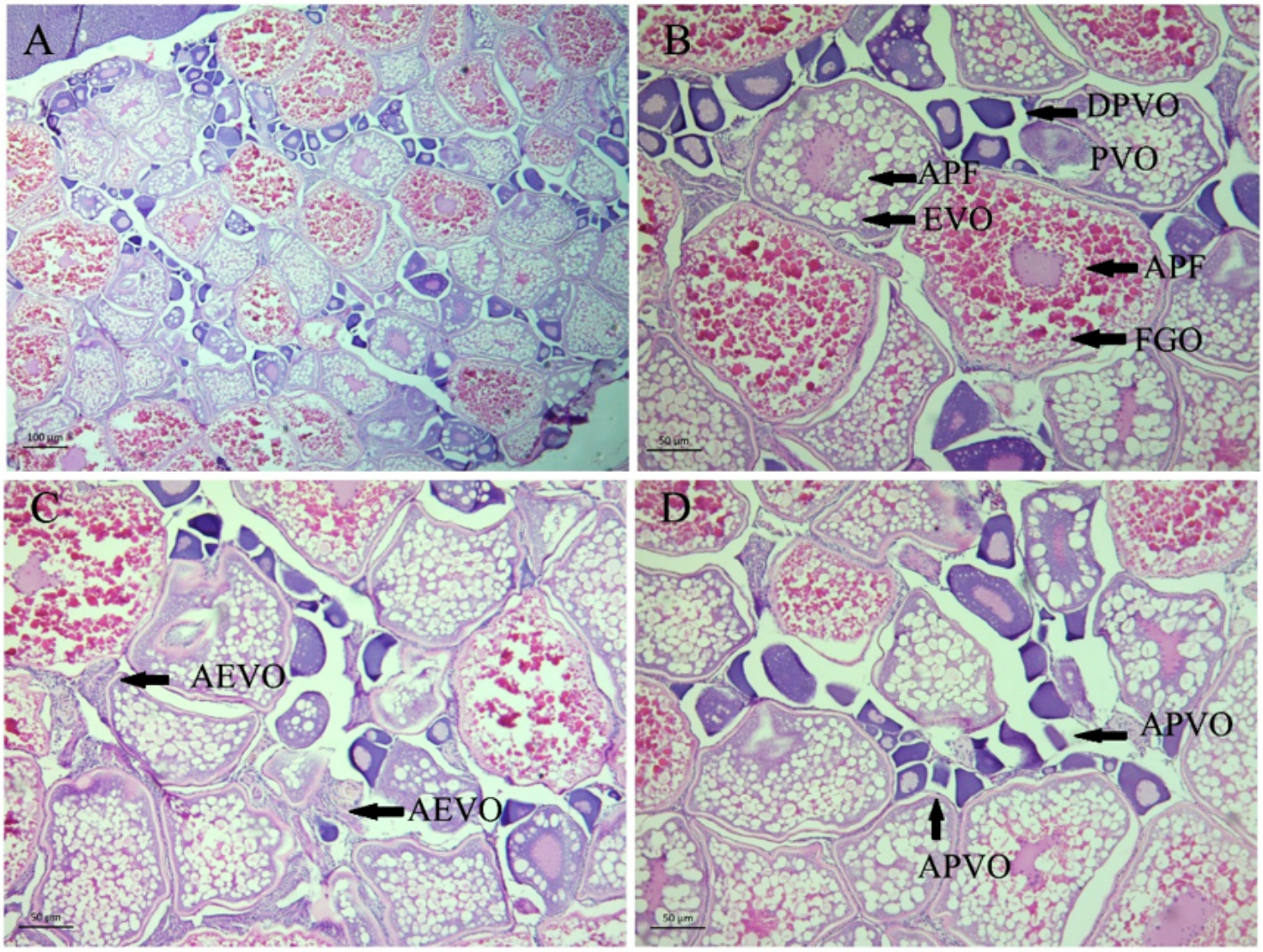

Figure 4

Microscopic view of ovary of cadmium-exposed zebrafish (A: 40 X and B, C, D: 100 X, H & E staining). A) Less number of full-growth (Mature) oocytes, increased number of pre-vitellogenic oocyte (PVO) and early-vitellogenic oocyte (EVO) with loss of contacts between the oocyte cell membranes and the follicular cell layer; B, C, D) accumulation of proteinaceous fluid (APF) in EVO and FGO, degenerated pre-vitellogenic oocyte (DPVO); atretic early-vitellogenic oocyte (AEVO); atretic pre-vitellogenic oocyte (APVO) (A: Scale of 100 µm; B, C, D: Scale of 50 µm).

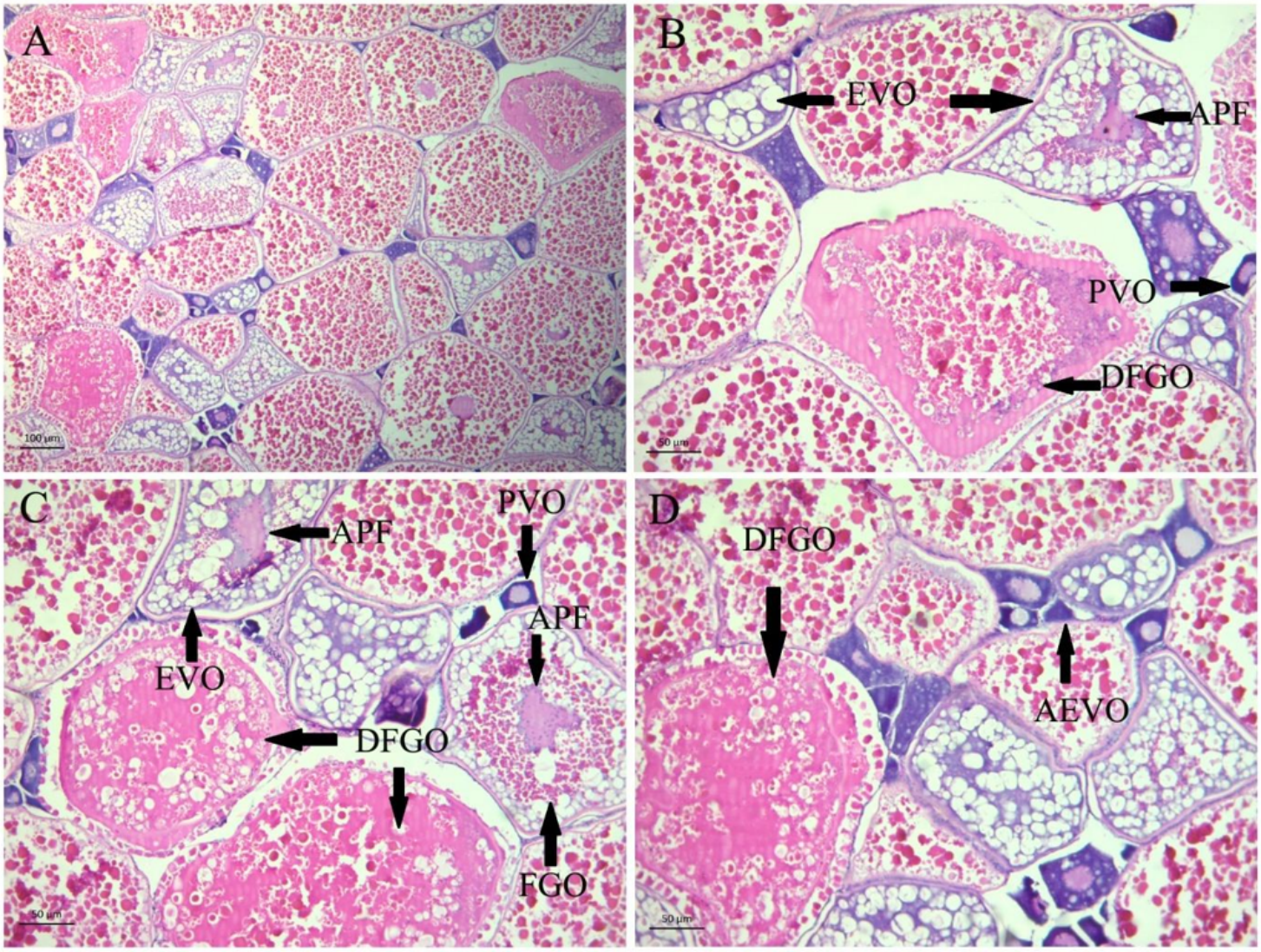

Figure 5

Microscopic view of ovary of mercury-exposed zebrafish. A) All types of oocytes (40 X); B, C, D) accumulation of proteinaceous fluid (APF) in EVO, degenerated full-growth (Mature) oocyte (DFGO) and atretic pre-vitellogenic oocyte (APVO) (A: Scale of 100 µm; B, C, D: Scale of 50 µm).

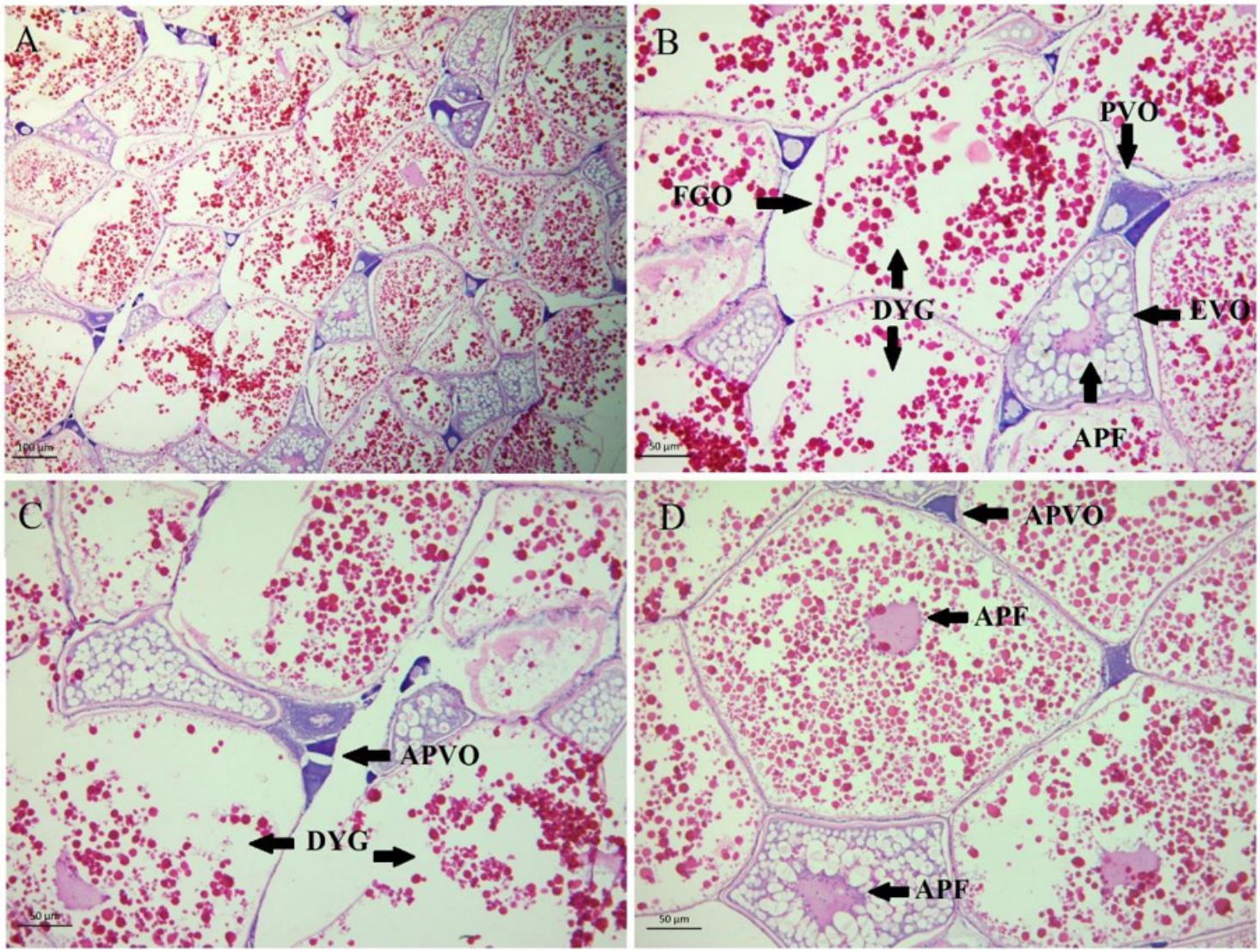

Figure 6

Microscopic view of ovary of Cadmium + mercury-exposed zebrafish. A) All types of oocytes with disorganized structure of full-growth (Mature) oocyte (40 X); B, C, D) depletion of yolk granules (DYG), accumulation of proteinaceous fluid (APF) in EVO and FGO, and atretic pre-vitellogenic oocyte (APVO) (A: Scale of 100 µm; B, C, D: Scale of 50 µm).

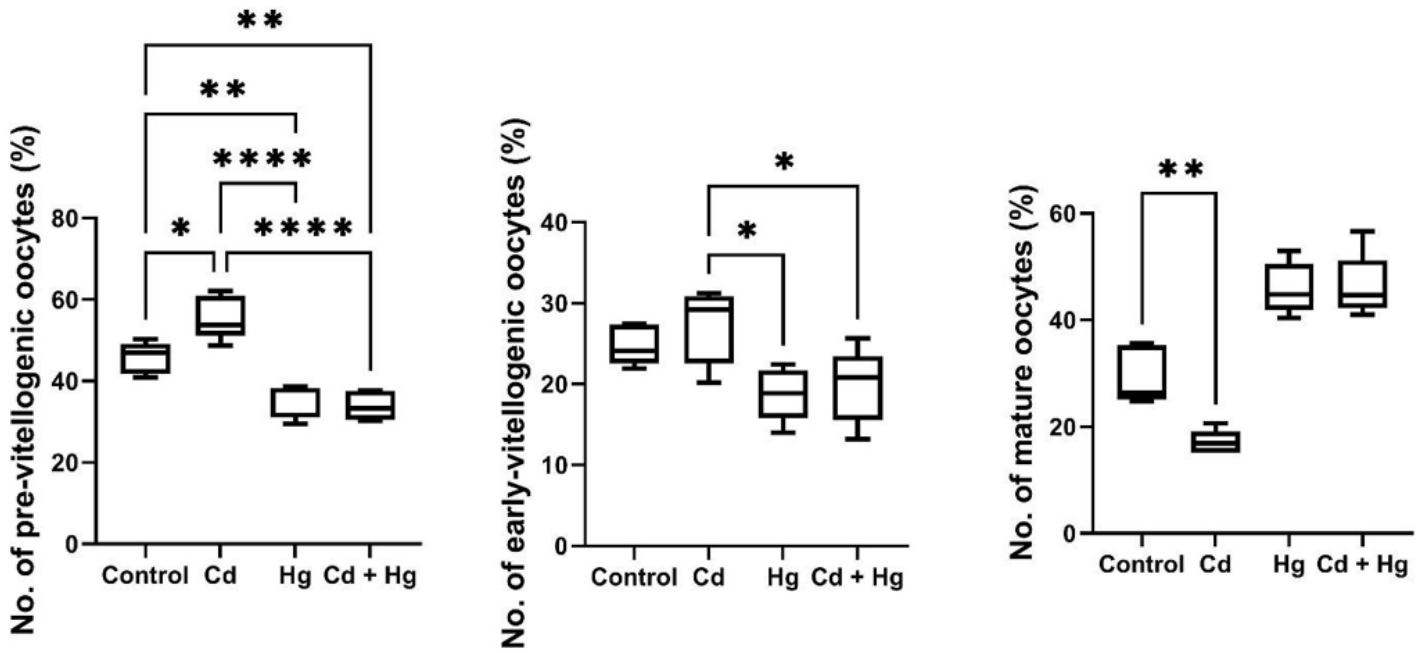

Figure 7

No. of oocytes at different stages in the ovary of adult zebrafish of different groups. Data were analyzed by one-way ANOVA followed by Tukey's HSD test, * Indicates significant differences between the groups (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$)

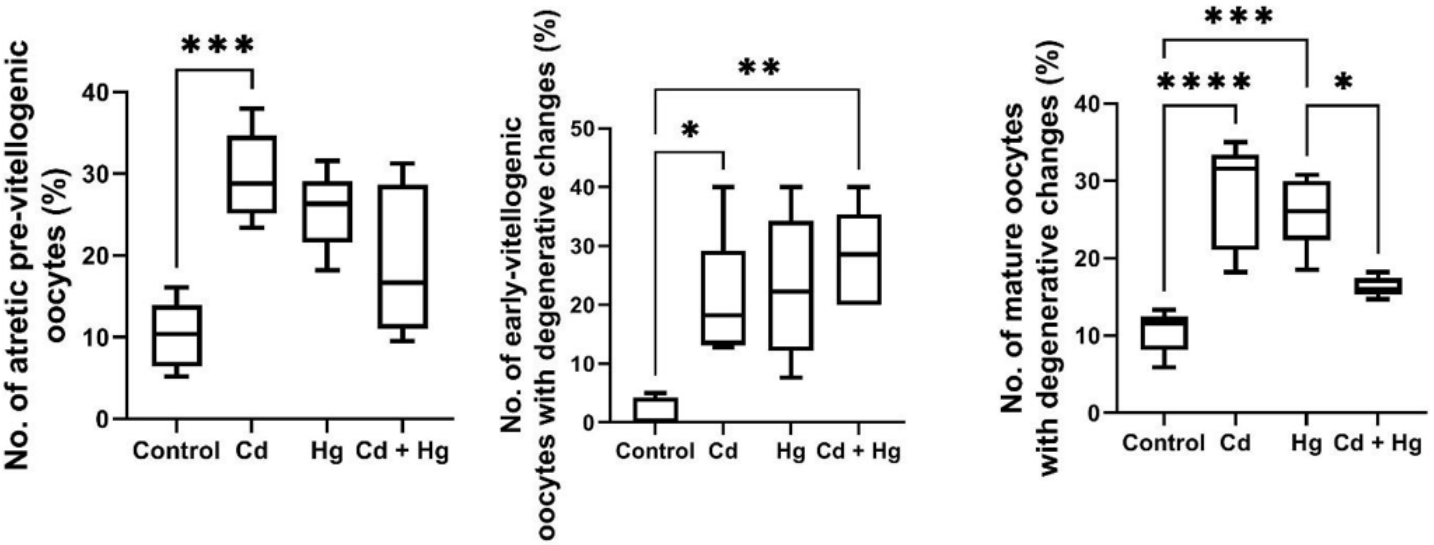

Figure 8

No. of atretic pre-vitellogenic and early-vitellogenic and mature oocytes with degenerative changes in the ovary of adult zebrafish of different groups. Data were analyzed by one-way ANOVA followed by Tukey's HSD test, * Indicates significant differences between the groups (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

Supplementary Files

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