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*Research*

# Anti-Teratogenic Potential of Exogenously Applied Over-The-Counter L-Glutathione Supplement on Ethanol-Induced Teratogenesis in Zebrafish (*Danio rerio*)

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**ABSTRACT**

Glutathione is the body's most abundant endogenous non-enzymatic antioxidant and is used as a substrate for free radical scavenging in the body, especially during ethanol metabolism. This study aims to shift the paradigm of using glutathione as a whitening agent into a potent antioxidant for therapy, particularly for ethanol-induced teratogenesis in the Philippines. Zebrafish embryos were treated with glutathione at various time points of ethanol exposure and concentration. Pre-treatments, co-treatments, and post-treatments with 100 µM glutathione solution were done to assess the most appropriate time point for glutathione intake upon exposure of the embryo to ethanol. Eye diameter and otic vesicle diameter were chosen as morphological parameters because dysmorphogenesis of these organs resembles mammalian fetal alcohol syndrome disorders. For eye diameter, alleviation of microphthalmia by glutathione was seen in pre-treatment (1% ethanol only) and post-treatment (1% & 1.5%) while co-treatment did not exhibit rescue for eye diameter reduction. For otic vesicle diameter, pre- and co-treatment with glutathione did not exhibit any changes in size but post-treatment showed abnormal enlargement suggesting possible teratogenic effect across all ethanol concentrations. The 2,2-diphenylpicryl-1-hydrazine (DPPH) assay was used as a confirmatory test for the free radical scavenging activity (FRSA) of treated tissues. Pre-treatment with GSH at 1% ethanol showed the highest FRSA while post-treatment showed FRSA insignificantly different to controls. This study suggests that glutathione can alleviate oxidative stress in embryo development which may lead to dysmorphogenesis and that supplementation before and after ethanol exposure may be a viable form of therapy for ethanol-induced teratogenesis.

**Key words:** Ethanol, teratogenicity, toxicity, Zebrafish

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## INTRODUCTION

Embryonic exposure to ethanol has been known to cause a wide variety of developmental defects linked to fetal alcohol syndrome disorder (FASD). FASD may manifest throughout childhood up until adulthood and can range from physical deformities to neurocognitive problems. Common deformities include facial abnormalities with a variety of different diagnosis methods used by physicians (Mukherjee *et al*. 2006).

The main proponent of ethanol teratogenicity leading to FASD is oxidative stress. It is known that redox reactions are prevalent in fetal development and the introduction of ethanol disrupts these reactions. Any aerobic organism produces reactive oxygen species (ROS) as a result of metabolic respiration, especially during embryonic development (Guérin *et al*. 2001). Ethanol is metabolized in most aerobic organisms with the enzymes, alcohol dehydrogenase 1 and cytochrome P450 2E1. The reactions of the enzymes produce reactive oxygen species (ROS). ROS alters the structure of proteins, lipids, and other components causing malformation of embryonic structures and disruptions in cell signaling (Ufer & Wang 2011).

To protect the embryo from the harmful effects of the ROS produced during embryo development, numerous enzymes work to sequester these free radicals and allow

normal redox reactions to occur. Reduced glutathione is a non-enzymatic ROS scavenger prevalent especially during pregnancy and functions to protect the developing embryo from oxidative stress (Guérin *et al*. 2001). Glutathione (GSH) has been shown to contribute to cell cycle regulation, proliferation, and differentiation specifically in the mechanism of DNA synthesis (Lu & Holmgren, 2014). Glutathione peroxidase (GPX) eliminates the threat of hydrogen peroxide poisoning and radicals brought about by lipid peroxidation. GPX uses glutathione as its main substrate so having an abundance within the cell is crucial for cell survival and protection against oxidative stress.

Given the toxicity of ethanol to embryos during fetal development, studies have been conducted to alleviate the disorders that characterize fetal alcohol syndrome. Antioxidant therapy is currently being studied as a way to counteract the negative effects of increased oxidative stress caused by embryonic ethanol exposure. Reimers *et al*. (2006) studied the effect of lipoic acid, ascorbic acid, and glutathione on the occurrence of pericardial edema. The researchers found that co-treatment with antioxidants reduced the frequency of pericardial edema by 20% in zebrafish. Marrs *et al*. (2011) observed that craniofacial cartilage in zebrafish specifically that of the ethmoid plate was reduced upon exposure to ethanol. However, co-treatment with retinoic acid attenuated ethanol-induced teratogenesis. A study by Arnold *et al*. (2016), also showed antioxidant rescue from selenomethionine, a toxic substance commonly found in agricultural run-offs. The antioxidant used by the researchers was n-acetylcysteine, a substance that increases glutathione levels in the body to scavenge free radicals. Upon pre-treatment with n-acetylcysteine, observed deformities were reduced after ethanol exposure. These anti-teratogenic studies showed that antioxidant activity can ameliorate defects induced by ethanol. However, it is not yet identified whether co-treatment or pre-treatment is the effective time point for anti-teratogenic exposure.

This study focuses on the antioxidant properties of glutathione in alleviating oxidative stress caused by ethanol metabolism in zebrafish embryos, specifically facial deformities resembling FASD caused by ethanol consumption during embryo development. Furthermore, this study aims to determine whether GSH can ameliorate the defects caused by ethanol at different time points, before, during, and after ethanol exposure. Zebrafish was used in this study due to the high reproducibility of the experiment is also possible due to the high fecundity of the organism per spawning event (Bilotta *et al*. 2002; Lieschke & Currie 2007). The parameters assessed in the study include otic vesicle length and eye diameter in zebrafish. Free radical scavenging activity using the 2,2-diphenylpicryl-1-hydrazyl (DPPH) assay was also checked in this study to confirm antioxidant activity in treated embryos.

## MATERIALS AND METHODOLOGY

### Zebrafish husbandry and maintenance

Wild-type zebrafish acquired from the Freshwater Aquaculture Center, Central Luzon State University, Science City of Muñoz was used for the study. A culture of brine shrimp was fed to the fish twice a day, once in the morning and once in the afternoon. 1 liter of dechlorinated, UV-treated water was used per fish in a tank to ensure sufficient volume for each fish. The methodology of Halili and Quilang (2011) from the University of the Philippines Diliman for zebrafish maintenance and spawning was used. The fish samples were reared in a glass tank using 4 females and 2 males for optimal spawning conditions. The spawning followed a 12-hr light and 12-hr dark cycle and was checked the following day. Embryo medium (EM) containing 2.65 mL $CaCl_2$, 500 µL 1 M $MgSO_4$, 771 µL 1 M $NaHCO_3$, and 77 µL 1 M KCl per liter was used as the vehicle for keeping the eggs during incubation and for solution preparation of ethanol and glutathione treatments. Zebrafish eggs were harvested after spawning from adult zebrafish and incubated up to 3 hr post fertilization (hpf) in petri dishes.

### Preparation of ethanol and glutathione

One percent, 1.5%, and 2.4% ethanol solutions were prepared from 95% ethanol mixed with autoclaved embryo medium. These concentrations are considered to be sublethal as stated in various studies also done using zebrafish teratogenicity assays (Bilotta *et al*. 2004; Soares *et al*. 2012). 100 µM glutathione solution was prepared using commercially available L-glutathione (GSH) capsules and dissolved in embryo medium. The concentration used for glutathione has been determined to be the maximum non-teratogenic concentration wherein pericardial edema is not observed (Reimers *et al*. 2006).

### Zebrafish toxicity assay and treatment with glutathione

Three variations of the toxicity assay were done to assess the ameliorative effect of glutathione at varying time points of exposure. Glutathione exposure was administered at different time points relative to ethanol exposure, namely pre-treatment (PrGlut + EtOH), co-treatment (CGlut + EtOH), and post-treatment (PoGlut + EtOH). PrGlut + EtOH set-up was conducted by pre-exposure of embryos to glutathione from 0-3 hpf before transferring to the ethanol concentrations (1.0%, 1.5%, & 2.4%) dissolved in embryo medium until 24 hpf. CGlut + EtOH set-up was done by exposing embryos to combined ethanol concentrations and glutathione from 3 hpf until 24 hpf. PoGlut + EtOH set-up was done by exposing the embryos to the ethanol concentrations from 3-12 hpf and then transferring them to

glutathione solution until 24 hpf. All the embryos from the set-ups were then placed in embryo medium after 24 hpf and were incubated until 3 days post fertilization (dpf) for morphological examination.

**Assessment of normal and teratogenic effects**

Surviving embryos were collected and examined to check for eye diameter and otic vesicle diameter. Normalization of measurements was done by dividing length measurements against the head-to-heart cavity length. Measuring of the eye diameter and otic vesicle was done using a light microscope at 400X and image calibration via Image J. Free radical scavenging activity (FRSA) was measured using a spectrophotometer and was calculated using Equation 1 (Damgaard *et al*., 2014):

$$\%\ FRSA = \frac{Absorbance\ of\ blank - absorbance\ of\ sample}{Absorbance\ of\ blank} \times 100$$

- Equation 1

**DPPH assay for free radical scavenging activity**

The 2,2-diphenyl picryl-1-hydrazyl assay (DPPH) was used to determine the antioxidant capacity of the tissues treated with glutathione after 24 hpf. After analysis of the most appropriate timepoint to apply glutathione, the DPPH assay was used to correlate the presence of free radicals to the degree of teratogenesis alleviated by glutathione. This test confirms the potency of exogenous glutathione as an anti-teratogenic agent against ethanol-induced teratogenesis. Larvae were anaesthetized in 1× MS-222 (tricaine methanesulfonate) and then transferred to a 1.5 µL Eppendorf tube where it was homogenized using a plastic pestle. The samples were then centrifuged for 2 min at 2500 r.p.m. and the supernatant of each solution was subjected to the assay. Five µL of sample was mixed with 95 µL DPPH solution in absolute ethanol and absorbance was read at 570 nanometers using a spectrophotometer (Damgaard *et al*. 2014).

**Statistical analysis**

Results were statistically evaluated using a two-way analysis of variance (ANOVA). Tukey's honestly significant difference (HSD) test was done for post-hoc testing to determine the relationship between treatment groups. All analyses were done using GraphPad Prism statistical software.

**RESULTS**

After exposure to treatments, larvae were anesthetized and subjected to morphological analysis using ImageJ. Normalization of values was done on head-heart cavity lengths to account for any whole embryo teratogenesis.

**Ethanol exposure caused a reduction in eye diameter and enlargement of otic vesicle diameter**

After exposure to ethanol concentrations of 1.0%, 1.5%, and 2.4% from 3-12 hpf and 3-24 hpf eye diameter showed a significant overall reduction. A linear trend was observed with the largest eye diameter reduction seen at 2.4% and the smallest reduction was seen in 1.0% ethanol (Figure 1A) regardless of the length of exposure. This suggests that the effect of the alcohol is dose-dependent which confirms the teratogenicity of alcohol in the experiment. However, it was evident that the decrease in eye diameter was severe in 3-24 hpf since the exposure length is 19 hr while the 3-12 hpf or 9 hr exposure followed the same dose-dependent teratogenic effect albeit less severe. Cardiac edema was also seen in larvae exposed to ethanol at 3-24 hpf (Figure 1C).

Enlargement of otic vesicles was seen in the groups exposed to 1.5% and 2.4% ethanol at 3-24 hours compared to negative control (Figure 1B). No enlargement nor reduction in otic vesicle diameter was seen in the group exposed at 3-12 hpf.

**Differential effect of pre-treatment and co-treatment with GSH during ethanol exposure (PrGlut + EtOH and CGlut + EtOH) on eye diameter and otic vesicle diameter of zebrafish**

For pre-and co-treatment with GSH, the larvae were exposed to ethanol for 3-24 hpf (19 hr). Hence, the experiments were conducted at the same time. Upon pre-treatment with GSH, the eye diameter of embryos exposed to 1.0% EtOH resembled that of the negative control. Those exposed with 1.5% and 2.4% EtOH still showed a significant decrease in overall eye diameter compared to the negative control. For Co-treatment of GSH and EtOH, the data did not show any rescue of the reduction of eye diameter across all concentrations. It was also seen that eye diameter reduction was even greater in embryos exposed to 2.4% EtOH co-treated with GSH (Figure 2A). This shows that GSH may be effective in ameliorating the teratogenic effects of low ethanol concentration but only when administered before EtOH and not together with EtOH.

Otic vesicle diameters of embryos in both PrGlut + EtOH and CGlut + EtOH treatments across all EtOH concentrations showed no significant difference with the controls (Figure 2B). This suggests that pre-treatment and co-treatment with GSH did not significantly reduce the teratogenic effect of EtOH on otic vesicle diameter.

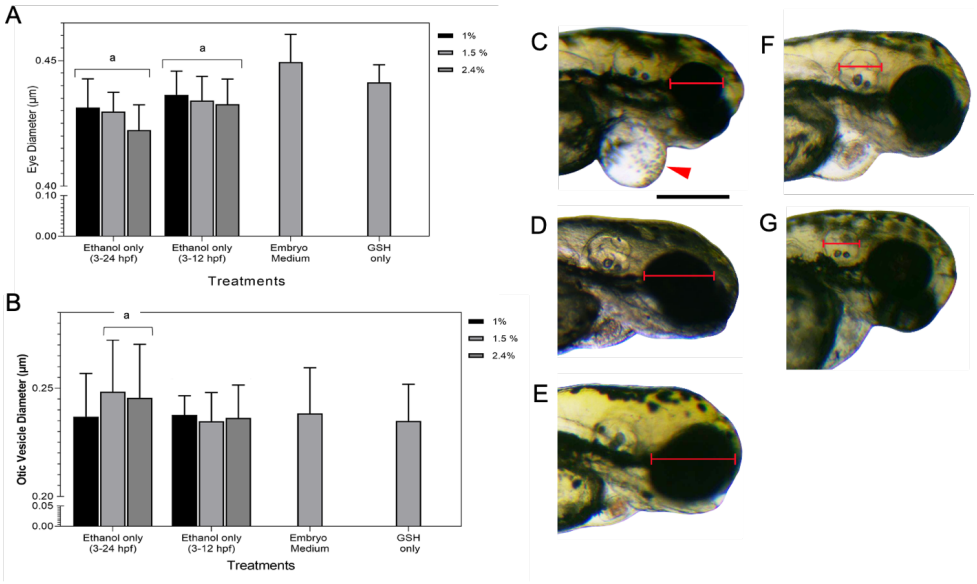


**Fig. 1.** Effect of different concentrations of ethanol on zebrafish (A) eye diameter and (B) otic vesicle diameter after exposure from 3-12 and 3-24 hr post fertilization. Treatments with lower-case letters indicate significant differences: a - statistically significant from negative control (embryo medium & GSH). Eye diameter of larvae exposed to ethanol at 3-24 hpf (C), 3-12 hpf (D), and embryo medium (E). Otic vesicle diameter of larvae exposed to ethanol at 3-24 hpf (F) and embryo medium (G). The red arrow indicates the presence of cardiac edema. The scale bar corresponds to 280 µm. Magnification is at 400x.

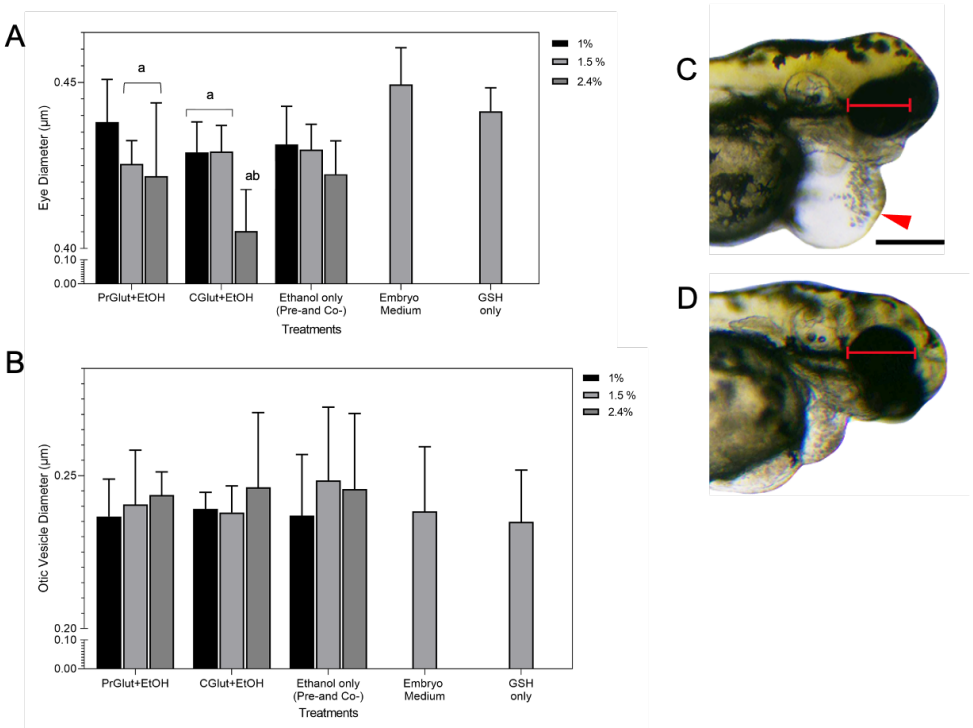


**Fig. 2.** Effect of pre-treatment with glutathione before ethanol exposure (PrGlut+EtOH) and co-treatment with glutathione during ethanol exposure (CGlut+EtOH) on 24-hpf zebrafish. (A) eye diameter of zebrafish exposed in different concentrations of pre-treatment and co-treatment set-ups; (B) otic vesicle diameter of zebrafish exposed in different concentrations of pre-treatment and co-treatment set-ups. Treatments with lower-case letters indicate significant differences ($p \leq 0.05$). a - statistically significant from negative control (embryo medium); b – statistically significant from positive control (EtOH only); ab - statistically significant from both positive and negative controls. Eye diameter of larvae exposed to 2.4% ethanol and GSH co-treatment (C) and corresponding positive control (D). The red arrow indicates the presence of cardiac edema. The scale bar corresponds to 280 µm. Magnification is at 400x.

**Post-treatment with GSH (PoGlut + EtOH) rescued the teratogenic effect of EtOH on eye diameter and induced otic vesicle diameter enlargement**

Post-treatment with GSH showed a consistent linear trend exhibiting significantly larger eye diameter compared to the positive control set-ups (Figure 3A). The greatest rescue was observed at 1.0% ethanol. It is important to note that post-treatments were exposed to ethanol for a shorter duration, 9 hr only. In terms of otic vesicle diameter, post-treatment with GSH caused further enlargement across all concentrations of ethanol (Figure 3B). This suggests that an ameliorative effect of GSH may be possible after EtOH treatment but only for eye teratogenic phenotype.

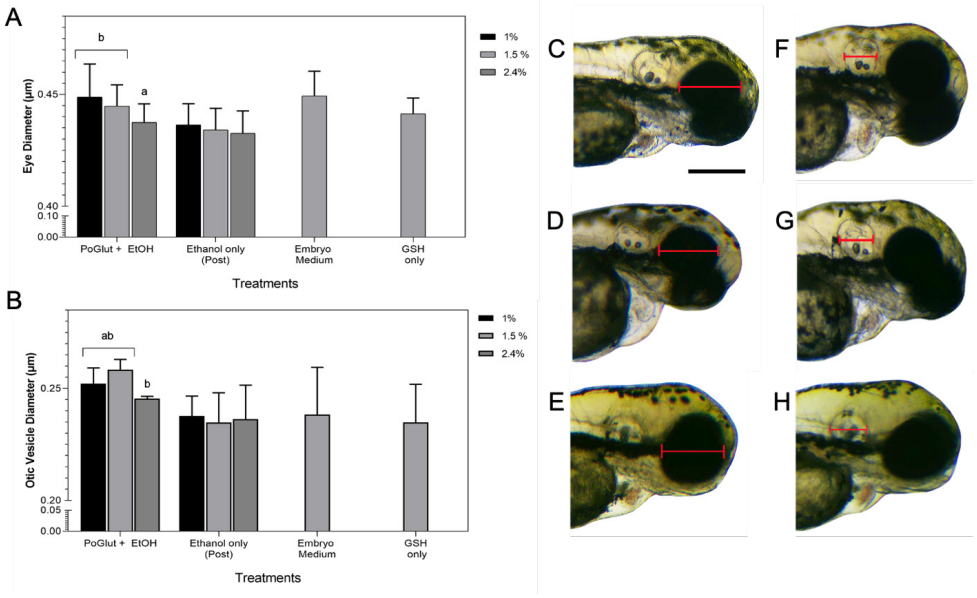


**Fig. 3.** Effect of post-treatment with glutathione after ethanol exposure (PoGlut+EtOH) on 24-hpf zebrafish. (A) eye diameter of zebrafish exposed in different concentrations of post-treatment set-ups; (B) otic vesicle diameter of zebrafish exposed in different concentrations of post-treatment set-ups. Treatments with lower-case letters indicate significant differences ($p \leq 0.05$). a - statistically significant from negative control (embryo medium); b – statistically significant from positive control (EtOH only); ab - statistically significant from both positive and negative controls. Eye diameter of larvae post-treated with GSH (C), positive control (D), and embryo medium (E). Otic vesicle diameter of larvae post-treated with GSH (F), positive control (G), and embryo medium (H). The scale bar corresponds to 280 µm. Magnification is at 400x.

**Antioxidant activity of GSH may reduce the teratogenic effects of EtOH**

Since the ameliorative effect of GSH was seen mostly in PrGlut and PoGlut set-ups, a DPPH assay was conducted to confirm whether the rescue of teratogenic phenotypes was due to the antioxidant activity of GSH. The DPPH assay for PrGlut + EtOH embryos shows that free radical scavenging activity (FRSA) was significantly evident in all concentrations with the highest % FRSA seen in 1.0% ethanol treatment. Percent FRSA was higher compared to the control set-up across all concentrations of ethanol treatments and higher than the negative control.

PoGlut + EtOH embryos, however, showed very minimal % FRSA with the highest seen at 1.0%. Differences between treatment groups were also not significant as the post-treated group resembled the control. The trend suggests free radical scavenging activity is highest at 1.0% ethanol (Figure 4).

## DISCUSSION

**Ethanol teratogenicity in zebrafish**

Zebrafish models have become a new paradigm in the modeling of fetal alcohol syndrome disorders (FASD) due to the ease of visualization and induction of teratogenesis in zebrafish embryos. It is known that manifestations of FASD in zebrafish models resemble that of FASD seen in vertebrates hence its emergence in toxicological studies (Loucks & Ahlgren, 2012). Previous literature recommends a range of below 3% for sublethal concentrations and enough ethanol to induce teratogenicity in developing embryos hence the use of concentrations 1.0%, 1.5%, and 2.4% in this study (Bilotta *et al*. 2002; Arenzana *et al*. 2006; Ali *et al*. 2011; Loucks & Ahlgren, 2012).

In zebrafish, embryonic ethanol exposure results in reduced eye size or microphthalmia. This condition is a consequence of reduced retinal cell differentiation, lens abnormalities, and overall developmental delay in zebrafish (Kashyap *et al*. 2007). Microphthalmia in zebrafish exposed to ethanol is immediately measurable and persistent even after 24 hr which makes zebrafish an ideal model for

studying the effects of embryonic ethanol exposure in humans. Also, the effect of ethanol on eye size was seen to be dose-dependent, in terms of concentration and timing of ethanol exposure (Bilotta *et al*. 2004). This reduction in eye diameter was consistently observed in our results, for both 3-12 hpf and 3-24 hpf exposure to ethanol treatments.

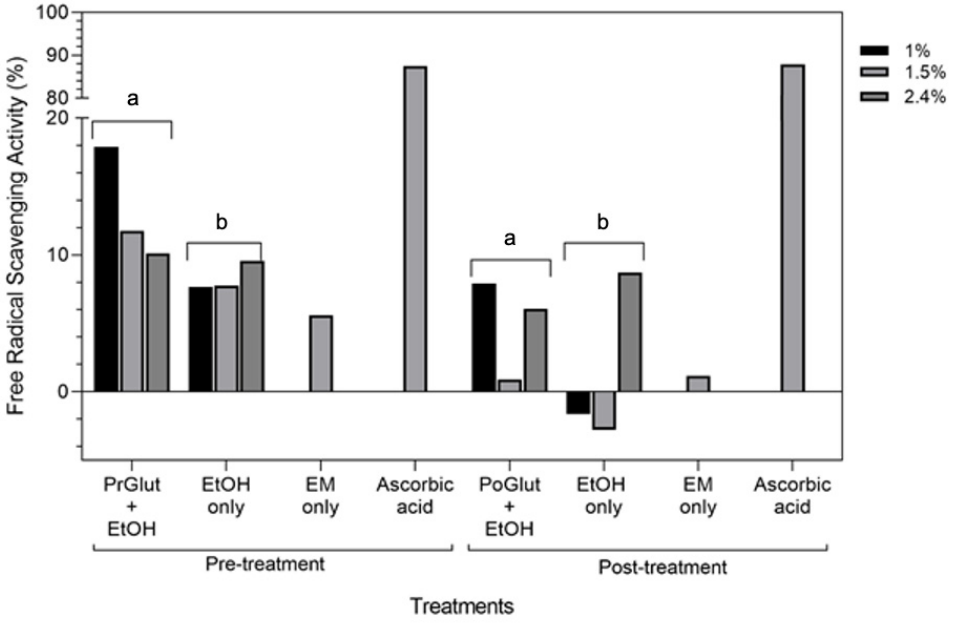


**Fig. 4.** Free radical scavenging activity (%) of pre- and post-treated larvae via DPPH assay on 24-hpf zebrafish. (A) Pre-treated zebrafish larvae against DPPH (B) Post-treated zebrafish larvae against DPPH. Values with asterisk indicate significant differences ($p \leq 0.05$). a - statistically significant from positive control (EtOH only) and negative control (embryo medium); b - not statistically significantly different from negative control.

Inner ear damage has also been observed in zebrafish exposed to ethanol. This may be characterized by morphological defects in the otoliths and otic vesicle size. Recent studies have shown that embryonic alcohol exposure altered the expression of genes involved in the induction and development of the otic vesicle in zebrafish. Zamora *et al*. (2013) revealed that alcohol exposure from 2-50 hpf (48 hr) led to early developmental defects in the octavolateral organs of zebrafish. Our results showed a slightly significant increase in otic vesicle diameter for embryos exposed to ethanol for 3-24 dpf (21 hr). This increase in otic vesicle size may be attributed to the exposure timing to ethanol which is critical during early octavolateral organ development. Zamora *et al*. (2017) tested different alcohol exposure timings within 24 hr and showed that otic vesicle size reduction is prominent in embryos at 12-17 hpf, which marks an alcohol-sensitive time window. There was no significant reduction in otic vesicle size observed in our set-up since the embryos were not exposed during the 12-17 hpf time window. However, our study is the first to show evidence that exposing embryos at a 3-24 hpf time window causes a significant increase in otic vesicle size.

**Pre-treatment and Post-treatment with glutathione partially alleviates microphthalmia in zebrafish**

Microphthalmia observed in the results resembles children affected by FASD upon embryonic exposure to ethanol. The mechanism surrounding microphthalmia is not specific to one structure but rather a collection of deformities of optic structures contributing to a reduction in eye diameter (Kashyap *et al*. 2007). These malformations include disruption of retinal cell differentiation, increased cell death, and delay in the development of the zebrafish embryonic eye. Lamination of neural retina is also shown to be delayed and pigmented epithelium was reduced in thickness after embryonic exposure to ethanol (Arenzana *et al*. 2006). Retinoic acid (RA) signaling, which is crucial in the differentiation of ocular structures in the embryo, is disrupted as ethanol competes with retinol (precursor of RA) in its metabolism into retinal via alcohol dehydrogenase (Han *et al*. 1998). Acetaldehyde, a byproduct of ethanol metabolism, may also compete with retinaldehyde for the reaction with retinaldehyde dehydrogenase causing inhibition of this pathway. Rescue of ethanol-induced teratogenesis was seen using retinoic acid and folic acid at varying time points upon exposure to ethanol (pre-, co-, & post-treatment) (Muralidharan *et al*. 2015). Co-treatments with RA were seen to be effective at ameliorating optic nerve hypoplasia and photoreceptor cell differentiation. In GSH treatments with ethanol, our data

shows rescue from microphthalmia phenotype in PrGlut + EtOH (1%) and PoGlut + EtOH (1% & 1.5%) treatments since no significant reduction in eye diameter was seen. However, microphthalmia was still present in co-treated groups. This may also imply that the amelioration of microphthalmia brought about by RA supplementation is mechanistically different from the antioxidant mechanism of glutathione. Co-treatment with glutathione then may presumably not be effective enough to ameliorate the damage caused by ethanol and its metabolism which may be ongoing simultaneously. It is important to note however that the structures checked in this study and in Muralidharan *et al*. (2015) are different which may indicate different mechanisms in each of the structures present in the embryonic eye.

**Ethanol-induced teratogenesis of otic vesicles may partially be rescued by glutathione pre- and co-treatment but may induce abnormal enlargement at post-treatment**

Upon exposure to ethanol, otic vesicles are commonly observed to be reduced in size according to previous literature. In a study by Zamora and Lu (2013), the otic vesicle area was seen to be significantly smaller than controls upon embryonic exposure to ethanol. The length of exposure in this study however did not coincide with the length of exposure of zebrafish to ethanol in the aforementioned study. This is important to note because of the possible variation of the effect of ethanol on octavolateral structures as seen with the enlarged otic vesicles in some of EtOH only groups. In addition, other studies have shown abnormal expansion of otic vesicles which may also be seen in Hedgehog (*Hh)*-inhibitor mutated zebrafish due to excessive *Hh* signaling (Hammond *et al*. 2010). This enlargement was seen only in embryos exposed to ethanol at 3-24 hpf. However, no enlargement was seen in embryos exposed to ethanol at 3-12 hpf since treatment with ethanol was stopped at 12 hpf. Zamora and Lu (2013) showed that growth for otic vesicles begins at 16 hpf so termination of ethanol exposure may not confer adequate teratogenic outcome to the otic vesicle.

PrGlut + EtOH and CGlut + EtOH showed no significant increase or decrease in otic vesicle diameter for all ethanol concentrations suggesting possible rescue. The glutathione intake may have conferred antioxidant protection from ethanol metabolism into free radicals, allowing for alleviation of dysmorphogenesis of the otic vesicle. PoGlut + EtOH, on the other hand, exhibited a larger otic vesicle diameter compared to positive control suggesting a teratogenic mechanism working with this kind of treatment set-up. No studies have been found as to why glutathione in this set-up may have conferred a teratogenic effect so further studies may be conducted to determine underlying molecular mechanisms and gene expression patterns resulting in this phenotype.

**Partial free radical scavenging activity is seen in glutathione-pre-treated larvae**

The results show minimal capacity for free radical scavenging in the tissue however it is seen that FRSA is still greater than the control set-up indicating a higher level of antioxidant rescue in these tissues across all ethanol concentrations. Studies have shown that increased free radical scavenging activity rescues in part some of the teratogenesis caused by ethanol metabolism in the developing embryo. The action of free radical scavengers such as superoxide dismutase is known to alleviate oxidative stress from increased superoxide anion generation and increased lipid peroxidation (Kotch *et al*. 1995). Studies by Wentzel and Eriksson (1997) on teratogenicity in rat models also explored the supplementation of superoxide dismutase, as well as, n-acetylcysteine which increases levels of endogenous glutathione in the body. They found that the antioxidant capacity of the embryos was increased and dysmorphogenesis was prevented. The results of the DPPH analysis of PrGlut + EtOH-treated larvae coincide partially with the aforementioned study showing increased free radical scavenging capacity upon pre-treatment with glutathione. Statistical difference was seen between PrGlut + EtOH and EtOH only signifying higher FRSA in pre-treated larvae. A significant difference was not seen between EtOH only and EM only and PrGlut + EtOH and EM only. Graphical trend, however, shows that EM has lower FRSA compared to EtOH implying a lack of scavenging activity, most likely due to the lack of free radicals since the negative control group was not treated with the teratogen. The trend in the graph also shows higher FRSA in PrGlut + EtOH treatments. PoGlut + EtOH showed that the highest FRSA was seen at 1.0% but is not statistically different from the control which may suggest that pre-treatment would be the more viable way of glutathione intake.

## CONCLUSION

The results shown above present evidence supporting the partial alleviation of teratogenesis caused by ethanol-induced oxidative stress via an antioxidant mechanism of glutathione. This may also suggest the viability of over-the-counter reduced L-glutathione supplements for antioxidant therapy and supplementation among pregnant women and not just as a whitening agent proposed heavily by the Philippine media. Furthermore, based on the given data, this study may add to the existing pool of literature on the efficacy of antioxidant therapy against fetal alcohol syndrome disorders. Based on the results presented above, pre-treatment with GSH seems to be the most viable treatment period for alleviation of dysmorphogenesis caused by ethanol exposure proven by morphological analysis and DPPH assay of treated tissues.

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**ETHICAL STATEMENT**
This study was approved by the University of the Philippines Diliman Institutional Animal Care and Use Committee (Protocol Review #: IB-2018-33-R).

**CONFLICT OF INTEREST**
The authors declare no conflict of interest.

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