

Section: Health, Nutrition and Food

Turmeric extract rescues ethanol-induced developmental defect in the zebrafish model for fetal alcohol spectrum disorder (FASD)

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## Abstract:

Prenatal ethanol exposure causes the most frequent preventable birth disorder, fetal alcohol spectrum disorder (FASD). The effect of turmeric extracts in rescuing an ethanol-induced developmental defect using zebrafish as a model was determined. Ethanol-induced oxidative stress is one of the major mechanisms underlying FASD. We hypothesize that antioxidant inducing properties of turmeric may alleviate ethanol-induced defects. Curcuminoid content of the turmeric powder extract (5 mg/mL turmeric in ethanol) was determined by UPLC and found to contain Curcumin ( $124.1 \pm 0.2 \mu\text{g/mL}$ ), Desmethoxycurcumin ( $43.4 \pm 0.1 \mu\text{g/mL}$ ), and Bisdemethoxycurcumin ( $36.6 \pm 0.1 \mu\text{g/mL}$ ). Zebrafish embryos were treated with 100 mM (0.6% v/v) ethanol during gastrulation through organogenesis (2-48 hours post fertilization; hpf) and supplemented with turmeric extract to obtain total curcuminoid concentrations of 0, 1.16, 1.72 or 2.32  $\mu\text{M}$ . Turmeric supplementation showed significant rescue of the body length at 72 hpf compared to ethanol-treated embryos. The mechanism underlying the rescue remains to be determined.

## Practical Application:

Fetal alcohol spectrum disorder is an umbrella term describing a range of defects caused by prenatal alcohol exposure. FASD prevalence ranges from 0.3 to 5% of the live born infants, and can be as high as 8.9% in low socioeconomic populations. These defects include mental retardation, learning and behavioral disabilities, and growth defects. If the Zebrafish model is applicable to humans, turmeric consumption during might ameliorate some of these alcohol-induced defects. Identifying the mechanism of the effect of turmeric could lead to potential preventative treatments.

**Keywords:** Fetal alcohol spectrum disorder, ethanol-induced defects, zebrafish, turmeric, nutritional supplement.

## Introduction:

Fetal alcohol spectrum disorder (FASD) is an umbrella term describing a range of birth defects caused by prenatal ethanol exposure. These defects include mental retardation, learning and behavioral disabilities, and growth deficits. FASD prevalence ranges from 0.3 to 5% of the live-born infants, and can be as high as 8.9% in low socioeconomic populations (May and others 2009). In addition to other factors, maternal nutritional status plays a critical role in the severity of the disorder. Zebrafish is an excellent model (Marrs and others 2010) for studying ethanol-induced developmental defects due to its rapid developmental time, and the ease of tightly controlling dosage. Several studies have explored the mechanisms underlying ethanol-induced birth defects (Muralidharan and others 2015; Sarmah and Marrs 2013; Eberhart and Parnell 2016; Lovely and others 2016). One of the pathways that contribute to ethanol-induced cytotoxicity is oxidative stress, which can lead to cell death. Increased oxidative stress caused by ethanol exposure without sufficient activation of detoxification activity led to the cell death in cell culture and mice (Brocardo and others 2011).

Turmeric, a spice from the rhizomes of *Curcuma longa*, has been widely used in food for flavor and color, and as an herbal medicine for its antimicrobial and anti-inflammatory properties. On average 3% of turmeric is comprised of yellow-pigmented biologically active curcuminoids, including curcumin (diferuloylmethane), desmethoxycurcumin, and bisdesmethoxycurcumin. Studies in animal models have shown the effect of dietary turmeric in cancers, cardiovascular diseases, Alzheimer's disease, and other clinical disorders (Kumar and others 2016; Wongcharoen and Phrommintikul 2009). Although the primary molecular target of curcumin has not been identified, its pharmacological properties have been attributed to interaction with

multiple pathways. One of the proposed mechanisms of curcumin action is scavenging reactive oxygen species (ROS) (Priyadarsini 1997).

Studies showed the protective roles of curcumin on the toxic effects of nicotine in rats and mice (Bandyopadhyaya and others 2008). Whether curcumin can protect deleterious effects of ethanol during development is not known. In this study, the effects of turmeric supplementation on ethanol-induced defects on zebrafish embryos were examined. This study was an extension of an undergraduate laboratory initiative at IUPUI to replace standard laboratory courses with Course Based Undergraduate Research Experiences (CUREs), discovery-based labs that provide undergraduate students with semester-long experiences aimed at developing laboratory expertise and critical thinking skills through integration of authentic biological research (Sarmah and others 2016).

#### Materials and Methods:

##### Turmeric Extracts:

Turmeric stock solution was made by extracting turmeric powder (McCormick, Sparks MD) with absolute ethanol (5 mg/ml), centrifuging to remove undissolved solids and filtering with a 0.45 $\mu$ m syringe filter.

##### Ultra Performance Liquid chromatography (UPLC):

Curcuminoids were separated and quantified by UPLC as previously described with minor modifications (Marczylo and others 2009). Briefly, analysis was conducted using a Waters Acquity UPLC (Waters, Milford MA) separation equipped with a photo-diode array detector.

Separation of the three main curcumin forms (Curcumin, Bisdemethoxycurcumin, and Demethoxycurcumin) was achieved using a BEH RP C18 (2.1 mm i.d. , 100 mm length, 1.7  $\mu$ m particle diameter) column thermostated at 30 °C using aqueous 2% acetic acid (A) and acetonitrile (B). The linear gradients were: 0 min 85:15 (A: B), 15 min 50:50 and 20 min 85:15. The content of individual curcumin forms was calculated based on a multilevel response curve constructed for curcumin at 425nm.

Zebrafish husbandry, embryo collection and experimental treatments:

Zebrafish (Hamilton TL strain) were raised and housed under standard laboratory conditions (Westerfield, 2000) in accordance with Indiana University Policy on Animal Care and Use. Embryos were treated with ethanol as previously described (Muralidharan and others 2015; Sarmah and Marrs 2013). Embryos were incubated in embryo medium, embryo medium containing ethanol (100 mM) or ethanol (100 mM) containing varying concentrations of turmeric extract from 2-48 hours post fertilization (hpf) at 28.5°C. Treatments using turmeric extract were adjusted so that the total ethanol concentration was 100 mM. The curcuminoid content of the turmeric treatments were 1.16  $\mu$ M, 1.74  $\mu$ M and 2.32  $\mu$ M.

Imaging:

Brightfield dissecting microscope images were acquired using a color Leica DFC450C camera mounted on a Leica MZ12 stereomicroscope.

Body length measurements:

Body length of the zebrafish embryos was measured using Image J software (NIH, Bethesda MD). The images were calibrated using a slide micrometer.

Statistics:

One-way ANOVA was used to test the group effect of each outcome, followed by LSD post hoc tests for individual comparisons. A 5% significance level was used for each test (Graphpad).

Results:

#### **Turmeric composition:**

The curcuminoid composition of the turmeric extract (5 mg/ml made in 100% ethanol) was determined using UPLC (Table1). The total curcuminoid concentration of the extract was 579  $\mu$ M.

#### **Ethanol-induced morphological defects**

Embryos treated with 100 mM ethanol from 2-48 hpf showed severe defects including pericardial edema, bent body structure, short body length, and microphthalmia (Fig 1B).

Inclusion of turmeric extracts in the ethanol treatments at the three tested concentrations showed rescue of these morphological defects (Fig. 1C-E).

#### **Ethanol-induced body length defects can be rescued by turmeric co-supplement:**

Ethanol treatment of zebrafish embryos caused severe body length defects in zebrafish embryos [F (4, 137) =5.87,  $p < 0.001$ ]. The ethanol treated embryos had a significant ( $p = 0.001$ ) reduction

in body length compared to controls (Fig 2). Inclusion of turmeric extracts reduced the effect of ethanol on the body lengths of the embryos. Embryos co-treated with 100 mM ethanol and 2.32  $\mu$ M curcuminoids from a turmeric extract showed a significant rescue of body length compared to the 100 mM ethanol treatment (100 mM ethanol vs 100 mM ethanol +2.32  $\mu$ M turmeric,  $p=0.001$ ). Body length of 100 mM ethanol +2.32  $\mu$ M turmeric was not different from the control embryos ( $p=0.899$ ). The body length of embryos co-treated with 100 mM ethanol and 1.74  $\mu$ M curcuminoids from a turmeric extract were not significantly different from controls ( $p=0.0428$ ), but significantly longer than 100 mM ethanol treated ( $p=0.016$ ) embryos. At the lowest level of curcuminoids supplementation (1.16  $\mu$ M), the body length of these embryos was not statistically different from the controls ( $p=0.303$ ) and significantly longer than ethanol treated embryos ( $p=0.029$ ).

These results indicate that turmeric supplementation rescues of some of the ethanol-induced developmental defects in our zebrafish FASD model.

#### Discussion:

FASD is one of the most frequent preventable birth disorder causing a wide range of birth defects which is a burden to society both socially and economically. Prevalence of FASD in low-socioeconomic populations is unusually high, and not surprisingly, these populations are at the greatest risk of poor diets. Previous studies on FASD patients characterize stunted growth as one of the FASD diagnosis criteria (Klug and others 2003; Landgraf and others 2013). Ninety percent FASD patients also report microphthalmia (Stromland and Pinazo-Duran 2002). Although mechanisms underlying ethanol-induced defects are not clear, some key pathways have been identified as severely affected. These include oxidative stress mediated cell death, epigenetics,



and gene expression regulation (Brocardo and others 2011; Muralidharan and others 2013; Zhou and others 2011). Studies showed that FASD phenotypes can be recapitulated in various animal models Ethanol exposure caused reduced eye diameter and body length in zebrafish embryos (Marrs and others 2010). Previous studies have shown the protective effects of nutritional supplements including vitamin A derivative (retinoic acid), folic acid, vitamin E, choline, among many others on ethanol-induced developmental defects (Muralidharan and others 2015; Sarmah and Marrs 2013; Heaton and others 2011; Mitchell and others 1999). Various compounds with antioxidant functions have been implicated in rescue of ethanol-induced cell death including superoxide dismutase (SOD), vitamin E, N-acetylcysteine, and lipoic acid (Peng and others 2004; Antonio and others 2011; Parnell and others 2010; Chung and others 2013). Studies on neural crest cell cultures and mouse models showed that treatment with Nrf-2 (Nuclear factor erythroid 2-related factor 2) inducers could reduce ethanol-induced ROS generation and cell death. Naturally occurring Nrf-2 activators, such as resveratrol, which is present in red grapes and blueberries, and other specific inducers such as tert-butylhydroquinone could alleviate ethanol-induced cell death (Kumar and others 2011; Yan and others 2010). This is the first study showing rescue of ethanol-induced defects on zebrafish using turmeric supplementation.

The primary active ingredients in turmeric are curcuminoids, primarily curcumin. Various studies have explored the roles of curcumin in relieving oxidative stress (Daverey and Agrawal 2016; Qin and others 2015). Studies on porcine granulosa cells showed a rescue of induced-oxidative stress by curcumin treatments (Qin and others 2015). In this study, oxidative stress lead to increased cell death due to ROS accumulation by decreasing antioxidative gene expression such as SOD, and catalase. Curcumin co- and pre-treatment restored gene expression, rescued cell death, and reduced ROS accumulation. Studies on animal models also showed that dietary

curcumin led to increased Nrf-2 nuclear translocation in liver and lungs, inducing phase II antioxidant gene expression (Farombi and others 2008; Garg and others 2008). Other mechanisms of curcumin action are still being explored including effects on miRNA function (Sreenivasan and others 2012). The mechanism underlying turmeric rescue in ethanol-treated embryos is unclear and may involve compounds other than curcuminoids that are present in the extract. Further examination of specific pathways including the oxidative stress pathway, particularly Nrf-2 gene targets, will provide insights into the response of ethanol-treated embryos to turmeric supplementation.

#### Conclusion:

Oxidative stress induced cell death is one of the major mechanism underlying ethanol-induced developmental defects. Many studies suggest role of curcumin in reducing oxidative stress. Curcumin co-treatment with ethanol rescues ethanol-induced decrease in body length providing insights in mechanism underlying FASD and potential approached for damage reversal.

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#### Contribution of Authors:

P. Muralidharan provided the embryos, supervised the students, and wrote the article. C. Connors and A. Mohammed conducted the laboratory experiments. S. Sarmah helped students in designing experiments. K. Marrs contributed to writing and editing of the manuscript. J. Marrs

provided resources, consulted on experiments and edited the manuscript, and G. Chism provided the idea and edited the manuscript.

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Table 1. Individual Curcuminoid Concentrations in the Turmeric Extract

Curcuminoid	Concentration (Mean±SEM) (µg/mL)	Concentration (µM)
Curcumin	124.1 ± 0.2	337
Desmethoxycurcumin	43.4 ± 0.1	128
Bisdemethoxycurcumin	36.6 ± 0.1	114

Figures:

Figure 1:

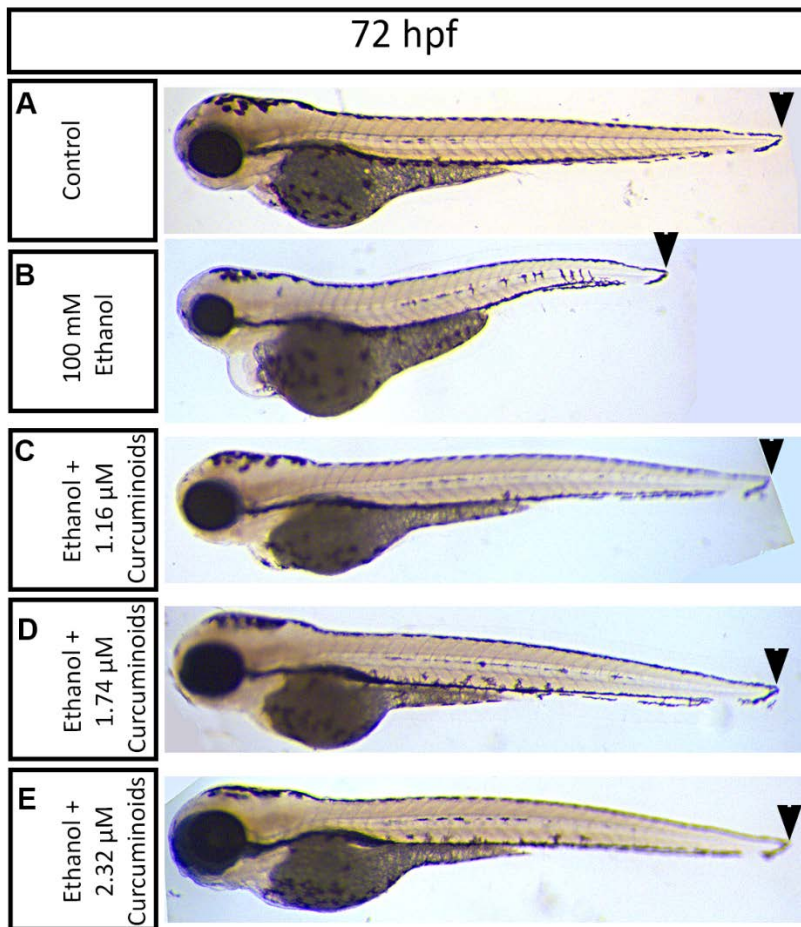


Figure 1: Ethanol-induced morphological defects are rescued by turmeric supplementation.

(A-E) Embryos were incubated from 2-48 hpf at 28.5°C in embryo medium (A), embryo medium containing ethanol (B) or ethanol containing 1.16  $\mu$ M, 1.74  $\mu$ M or 2.32  $\mu$ M curcuminoids (C-E) from a turmeric extract. Black arrowhead shows the end of the zebrafish pigmentation considered for body length measurements.



Figure 2:

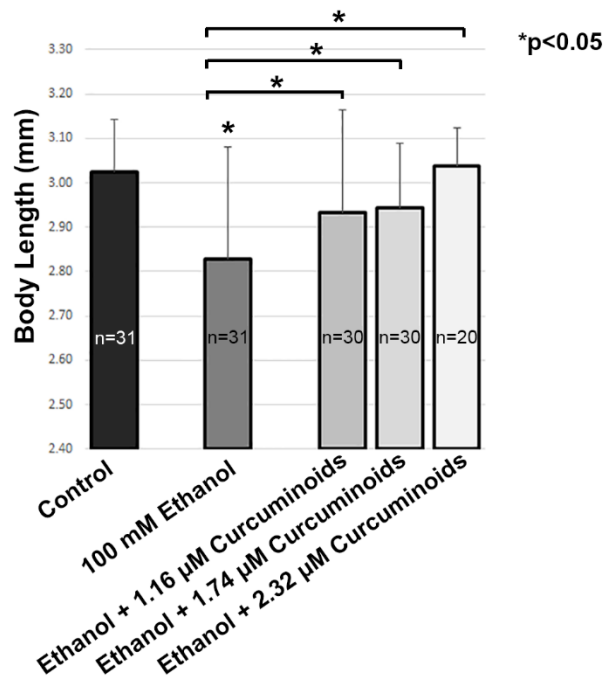


Figure 2: Effect of Turmeric Supplementation on body length of zebrafish embryos treated with ethanol. Embryos were incubated from 2-48 hpf at 28.5°C. in embryo medium, embryo medium containing ethanol or ethanol containing 1.16 µM, 1.74 µM or 2.32 µM curcuminoids from a turmeric extract. Lengths were determined using ImageJ from images taken using a Leica DFC450C camera mounted on a Leica MZ12 stereomicroscope.