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Isothiocyanates in the Prevention of Neural Tube Defects in Chick Embryos

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Honors Thesis

ISOTHIOCYANATES IN THE PREVENTION OF NEURAL TUBE DEFECTS IN CHICK EMBRYOS

by
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ABSTRACT

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NEURAL TUBE DEFECTS IN CHICK EMBRYOS

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Bachelor of Science

Neural tube defects (NTDs), including anencephaly and spina bifida, contribute significantly to neonatal morbidity and mortality worldwide. Despite the widespread implementation of folic acid (FA) supplementation, its ineffectiveness in certain genetic populations, alongside the persistent prevalence of NTDs, underscores the need for alternative preventative strategies. This study investigates the potential of isopropyl isothiocyanate (IPI), a compound derived from *Moringa oleifera (M. oleifera)*, in mitigating NTDs through the modulation of oxidative stress pathways. Using the chick embryo model, we aimed to determine whether IPI significantly reduces the incidence of chemically-induced NTDs by activating the Nrf2 cofactor, a key regulator of cellular response to oxidative stress. Here we report data supporting this hypothesis, suggesting that IPI could offer a novel, naturally derived alternative to FA supplementation for NTD prevention. Further research is warranted to know whether IPI and *M. oleifera* are valid alternatives for FA, particularly in regions with limited access to medical resources and in populations with FA-resistant genetic variants. This study contributes to the broader field of developmental biology by highlighting the importance of oxidative stress regulation in embryonic development and the potential of plant-derived compounds in preventing congenital defects.

Keywords: Neural tube defects, ceramide, isothiocyanate, valproic acid, oxidative stress, Nrf2 activation, *Moringa oleifera*, folic acid supplementation

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Special thanks to my Abigail Réileen for showing me the humanity of anencephaly and driving me to explore the pathogenesis and potential for preventing neural tube defects like hers. I will always love you.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Introduction

Background

Neural tube defects (NTDs), birth defects that hinder nervous system development early in an embryo's life, comprise around one tenth of all neonatal deaths around the world each year (Berihu et al., 2018). NTDs primarily occur sporadically, and do not run in families. The specific pathogenesis that leads a fetus to develop a NTD remains unclear, but is generally understood to be multifactorial. The problem arises early in embryonic development, between the 17th and 28th days of gestation, and most women do not even know they are pregnant when the NTD develops (Avagliano et al., 2019). Some toxicants are known to cause NTDs. Those explored previously in this lab by Dr. Micah Ross in collaboration with Dr. Michael R. Stark include ceramide (C2), fumonisin B(1), and valproic acid (VPA). Other factors of maternal health, including metabolic conditions such as obesity and type 2 diabetes mellitus (T2DM), are correlated with a higher incidence of NTDs (Ross et al., 2019). These clues to the pathogenesis of NTDs support the basis of this study: exploring, using the chick embryo model, whether isopropyl isothiocyanate (IPI), which is known to be neuroprotective, anti-inflammatory, and antidiabetic, will reduce the incidence of NTDs.

NTDs manifest in a variety of ways, although the most prevalent are defined as anencephaly (Fig. 1C)—failure of the rostral neural tube to fuse, which would fully form the brain and central nervous system—and spina bifida (Fig. 1B)—failure of the caudal neural tube to fuse, which would fully complete the spinal cord and peripheral nervous system (Avagliano et al., 2019).

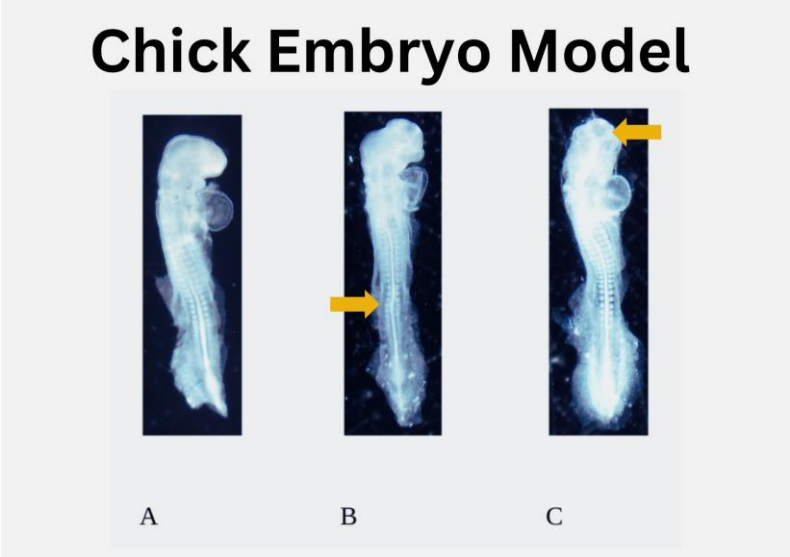

Figure 1

Examples of normal and disrupted development of the chick embryo model. A. Normal embryonic development; B. Posterior neural tube defect; C. Anterior neural tube defect

These failures occur during gastrulation and neurulation, the earliest beginnings of the entire human nervous system when a neural plate folds and fuses into a neural tube (Klein, 2013). The Sustainable Development Goals (SDG) of the United Nations include a call for both developed and developing nations to reduce NTDs incidence to .5 per 1000 births (Kancherla et al., 2019). This target highlights the high financial and human costs of NTDs. Bhide et al. (2013) performed a review of studies for known cases of NTDs throughout India, where 4.1 per 1000 births presented with NTDs nationwide, not taking into account stillbirths and elective abortions. A large disparity between regions emerges from the data, with a 2.4 per 1000 births NTD rate in the West and 7.3 per 1000 births rate in the North (Bhide et al., 2013, Table 2). The high incidence of NTDs in more rural areas of the North which lack hospital access suggests these regions face challenges of access. However, these figures may diverge merely because ultrasound screening and termination of fetuses with NTDs are more common practices where hospitals exist, obscuring the true incidence of these developmental defects. Even in nations with highly developed infrastructure, the incidence of NTDs does not meet the UN SDG of .5 per

1000 births. The United States and Canada are tied for an NTD rate of 0.7 per 1000 births, still above the target rate set by the United Nations (Allagh et al., 2015). Research on NTDs elucidates the etiology of these birth defects and explores evidence-based interventions to help developed and developing nations reach the target.

Existing Research

Previous research surrounding NTDs points to folate deficiency as a root cause. However, other nutrient deficiencies have also been found to result in higher incidence of NTDs. Selenium, for instance, is a dietary nutrient with known antioxidant properties. A deficit in selenium is associated with the occurrence of NTDs (Martín et al., 2004). The focus on antioxidants and oxidative stress in the pathogenesis of NTDs has been fruitful. The recent discovery that ceramides have a teratogenic effect related to neural tube defects (NTDs) clarifies a correlation between obesity/T2DM and the prevalence of NTDs—anencephaly and spina bifida—in affected maternal populations (Ross et al., 2019). It also confirms a previously defined role for oxidative stress in the pathogenesis of NTDs (Chang et al., 2003; Martín et al., 2004). Ceramides represent a category of sphingolipids known for their biological activity, functioning as critical components in cell membranes (Ross et al., 2019). However, when they are present at high levels in the circulating blood plasma, ceramides may cause or signal oxidative stress. Furthermore, in the maternal blood plasma, they significantly alter tissue redox environments, enough to affect the normal closure of the neural tube (Chang et al., 2003; Ross et al., 2019). A mouse study demonstrated that co-administering the amino acid methionine, known to help neutralize reactive oxygen species (ROS) and free radicals that can cause oxidative damage, with VPA can significantly reduce the incidence of the VPA-caused posterior neural tube defect spina bifida from 60% to 30% in a dose-dependent manner, without

impacting how valproic acid is metabolized (Ehlers et al., 1996). Despite this historical knowledge, addressing NTDs from the perspective of metabolic health and reducing oxidative stress has not been a key focus from a public health standpoint. This is likely due to the decades of focus on folic acid (FA) deficiency which has resulted in significant improvement of incidence of NTDs through FA supplementation of food staples like wheat and other cereals (Kancherla et al., 2019; Klein, 2013). Campaigns of FA supplementation have mostly succeeded in alleviating the contributing factor of poor nutrition to this state of oxidative stress, thereby reducing incidence of NTDs in countries with widespread FA supplementation.

Neural tube defects (NTDs), including conditions like anencephaly and spina bifida, remain a significant global health challenge, affecting a considerable number of newborns annually. Despite the benefits of folic acid (FA) supplementation in reducing the incidence of NTDs, a subset of these defects, termed folic acid-resistant NTDs, persists across various populations (Avagliano et al., 2019). This phenomenon underscores a critical need for alternative prevention strategies, especially for individuals carrying the MTHFR 677TT genotype. This genotype, associated with the methylenetetrahydrofolate reductase (MTHFR) enzyme, plays a pivotal role in folate metabolism and DNA methylation processes. Individuals with this genetic variant may require higher levels of B-vitamins due to reduced enzyme efficiency, which can impact the risk of developing NTDs (McGarel et al., 2014; Christensen et al., 2015). This is particularly relevant given the higher prevalence of the MTHFR 677TT genotype in certain populations, such as Hispanics, leading to an increased risk of NTDs in these groups (Lary & Edmonds, 1996; Guéant-Rodriguez et al., 2006; Huang et al., 2018).

In a cell culture study with lymphoblastoid cell lines, the homozygous and heterozygous type MTHFR C677T allele exhibited poor absorption of FA, but responded

well to a more bioavailable supplement, 5-methyltetrahydrofolate (5-Me-THF) (Vidmar Golja et al., 2020). A mouse study showed reduced MTHFR enzyme activity in the presence of high folic acid supplementation, a harmful outcome, especially for individuals who already have 30-60% reduced enzyme activity due to a genetic polymorphism (Christensen et al., 2015). Such reduced enzyme activity impacts the process of neural tube closure. Taken together, these studies suggest folate status and genetic polymorphism need to be considered when creating supplementation plans.

In light of the limitations associated with FA supplementation, especially among populations with FA-resistant genetic variants, the search for other effective alternatives has led to the exploration of the Moringa tree (*M. oleifera*). *M. oleifera* not only contains high levels of bioavailable folate but also isothiocyanates, compounds known for their anti-inflammatory and antidiabetic properties (Attakpa et al., 2017; Boon et al., 2013; Saini, et al., 2016). Specifically, IPI–derived from Moringa–shows promise in mitigating oxidative stress through the activation of the nuclear factor erythroid 2–related factor 2 (Nrf2) cofactor, a key regulator of cellular response to oxidative stress (Borgonovo et al., 2020; Cheng et al., 2019). Given the role of oxidative stress in the development of NTDs, IPI presents a novel avenue for prevention, especially in areas where access to conventional medical resources is limited. Since it is readily available, inexpensive, and commonly used in Asia and Africa, *M. oleifera* does not face the same hurdles as FA vitamin supplementation in areas with poor medical access, making it potentially an ideal avenue to achieve the United Nations SDG.

The significance of this research lies in its potential to offer an accessible, culturally accepted, and natural alternative to FA supplementation for preventing NTDs, particularly among populations with MTHFR C677T polymorphism. By leveraging the properties of *M. oleifera*, this approach could significantly impact global health, especially in regions

where nutritional and medical access is a challenge, thereby contributing to the achievement of the United Nations Sustainable Development Goals.

Methods

Determining the Optimal Dose

We conducted an initial MTT cell viability assay using P19 cells to determine the optimal dose of IPI for cell survival. These cultured cells were plated on a 96-well plate with various concentrations of αMEM media and IPI stock solution with a negative control of hydrogen peroxide, known to kill the cells and a positive control of only αMEM media, known to nourish the cells because it contains all 21 amino acids plus 5 additional vitamins.

After incubating for 24 hours, the 96-well plate was washed with 200µL serum-free media, and an MTT stock solution consisting of 5 mg MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) dissolved in 1 mL of PBS (phosphate buffer solution) was added to each well. Cells were then incubated at a temperature of 37 ℃ for three hours, before being washed again with PBS and treated with DMSO (100µL of dimethyl sulfoxide in each well). The 96-well plate was then wrapped in foil and placed in an orbital shaker for 15 minutes, after which a microplate reader set to read an absorbance of 570 nm analyzed the absorbance to determine cell viability at each of the concentrations in Table 1.

Column	1	2	3	4	5	6	7	8	9	10	11	12
Concentration	Cont-rol	0.1	2.5	5	10	20	50	100	500	1000	5000	H_2O_2
Stock solution	0	.08 mL	1mL	1mL	1mL	0.8mL	1mL	0.4 mL	1mL	0.4mL	5.33 µL	10µL
Media	2 mL	1.92 mL	1mL	1mL	1mL	1.2mL	1mL	1.6 mL	1mL	1.6mL	5000 µL	2µL

Table 1

These are the concentrations for the MTT Cell Viability Assay done in P19 cells according to a serial dilution beginning with 5000, which represents a molarity of 9.69 mM, or 5.33µL of 97% isopropyl isothiocyanate (IPI) stock solution in 5000 µL of cell culture media. The stock solution of IPI was mixed with alphaMEM, to determine the optimal dose of IPI for living P19 cells.

Treating Embryos with IPI vs. Teratogenic Controls

Eggs were acquired from a local supplier, then incubated at 37 °C for 30-36 hours to collect Stage HH8 embryos. Embryos were collected from eggs using the EC method described by Chapman et. al., in *Developmental Dynamics* (2001). The method involves placing a filter paper disc around the embryo, cutting it out of the yolk, and then lifting it so it can be plated flat on agar gel (Fig. 2).

Sample embryos were plated on agar gels in 6-well plates with IPI along with a teratogenic chemical—known to induce NTDs—ceramide (C2) or valproic acid (VPA). To study the effects of the long-chain type of ceramide most abundant in neural tissue and commonly elevated in serum under oxidative stress, we focused on C18:0 via a short chain analog tagged C2 (Ross et al., 2019). Control embryos were plated only with the teratogen. Embryos were then incubated with the chemical treatment or control for 24 hours. Embryos were then collected, fixed in 3.7% formaldehyde for preservation, dissected, and imaged to be scored for presence or absence of NTDs.

C2 and VPA have been studied previously in this lab, and their effects in causing developmental defects are well-established (Asan et al., 2019; Jentink et al., 2010; Ross et al., 2019; U.S. Food & Drug Administration, 2018; Wyszynski et al., 2005). C2 is a

sphingolipid with a long fatty acid tail, essential to many processes in the body. However, when found in high concentrations in the blood plasma of patients, it has a disruptive effect (Ross et al., 2019). High concentrations in maternal blood plasma is correlated with conditions including T2DM and obesity. Its presence in high amounts is also correlated with oxidative stress.

VPA is an active ingredient in some widely used seizure medication, which is also used for migraine relief. It is known to cause birth defects, but because the symptoms of these maladies are severe and the risk is relatively small, valproate was historically not discontinued during a pregnancy. Today, the FDA recommends stopping use of valproate in pregnancy only after consulting with a healthcare professional, since sudden cessation is dangerous, as well (U.S. Food & Drug Administration, 2018). Though newer epilepsy drugs are largely replacing sodium valproate, its widespread use makes it particularly relevant in a study like this, exploring potential for embryonic rescue from NTDs.

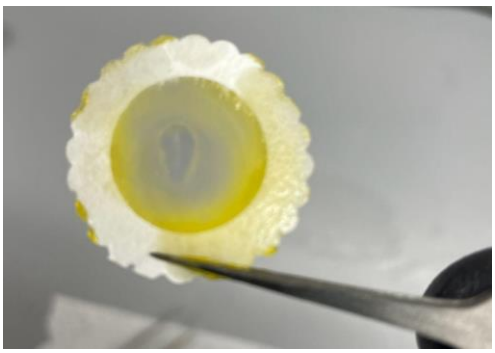

Figure 2

An embryo is captured and plated using the EC method, then treated with both VPA & IPI.

Finally, data on IPI-treated embryos was measured against control embryos which were plated with the teratogen (C2 or VPA) only. NTD rates for each teratogen were compared with established literature.

Measuring Nrf2 Targets in IPI-treated HepG2 Cells via Luciferase assay

As *M. oleifera* has been found in previous studies to increase Nrf2 activity, a luciferase assay was prepared using HepG2 Cells, a human liver cell line known for high proliferation rates and one that is ideal for studying this pathway. These cells were modified with a reporter for Nrf2 activity. The cells were allowed to grow overnight on a 96-well plate in their favored cell culture medium, Eagle's Minimum Essential Medium (EMEM). Then a concentration gradient of IPI was applied in duplicate with two columns treated with 77.52 µM IPI, two columns with 193.8 µM IPI, and two columns with 484.5 µM IPI. Additionally, two negative control columns received no treatment and two positive control columns contained t-Butylhydroquinone (TBHQ) dissolved in dimethyl sulfoxide (DMSO), diluted in a 50µM solution with EMEM.

After sitting in an incubator with the IPI gradient and control treatments for 24 hours, the HepG2 cells were treated with luciferase reagent buffer, added on top of the cells. The reagent was in contact with the HepG2 cells for at least 5 minutes before measuring luminescence, as this time allows for complete lysis of the cells and ensures the luminescence measurement accurately reflects the activity of the luciferase enzyme (BPS, 2024). Cell lysis-induced luminescence was read on a SpectraMax iD3 spectrometer (Fig. 7).

Results

MTT Assay Results

Using the in vitro MTT cell viability assay in P19 cells, the optimal concentration of 97% IPI was determined to be a concentration of 1:500,000 or 193.8 µM IPI, which resulted in consistent survival among the cultured, undifferentiated P19 cells (Fig. 3).

Here the optimal concentration is labeled 100, a dilution from the original stock solution of 5.33 µL of IPI in 5000 µL of media (9.69mM). A negative control is H_2O_2 (hydrogen peroxide), known to kill the cells, and a positive control is alphaMEM media, known to nourish the cells. We assumed 193.8 µM IPI as the optimal dose based on the MTT Cell Viability Assay.

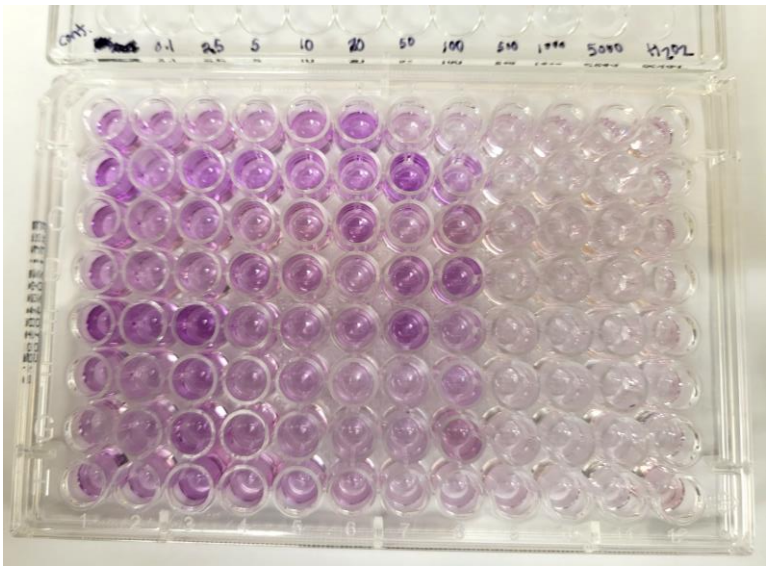

Figure 3

In this MTT Assay, the purple formazan crystals from (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT are signaling metabolically active P19 cells.

IPI Embryo Rescue Results

To measure effects of IPI in rescue of embryos from VPA-induced NTD, 36 embryos were plated, 20 with VPA + IPI and 16 with VPA alone. Initial results of the VPA/IPI experiment suggest IPI rescues embryos from neural tube closure defects (See Fig. 4). Out of 20 with VPA + IPI, only 1 presented with an anterior defect, while 19 were normally developing. Of the 16 with VPA alone, 11 were normal and 5 presented with defects: 1 anterior defect, 3 posterior defects, and 1 embryo with both anterior and posterior defects. We conducted independent samples t-tests to compare NTDs across the IPI Rescue and VPA Control groups (Fig. 4). As we hypothesized, there were significantly more NTDs in the VPA Control group [$t(34) = 2.179$, $p = .018$ one-sided]

indicating that the IPI rescue treatment significantly reduced the incidence of NTDs. Interestingly, among the specific types of NTDs, only the posterior defects were significantly increased in the VPA Control vs. the IPI Rescue group [t(34) = 2.088, p = .022 one-sided]. There may be something specific to the mechanism of VPA that favors posterior defects and drives the significance in total defects vs. IPI Rescue. Alternatively, the timing of treatment could play a role, as treatment must occur early in order to impact anterior neural tube development. A meta-analysis of eight published cohort studies representing 1565 pregnancies with first-trimester exposure to valproic acid, showed an increased incidence of spina bifida, the posterior defect of the neural tube, over other types of birth defects (Jentik et al., 2010). However, the authors excluded data on anencephaly from their analysis, so no direct comparison can be drawn between the two. Concerning timing of development, one study found that discontinuing use of valproate in the first trimester resulted in a decrease in major birth defects, including spina bifida (a posterior defect) and microcephaly (an anterior defect) (Fietz et al., 2024). The consequence of not discontinuing valproate use at that time resulted in a threefold increase in birth defects compared to the control group, suggesting that the timing of insult is significant.

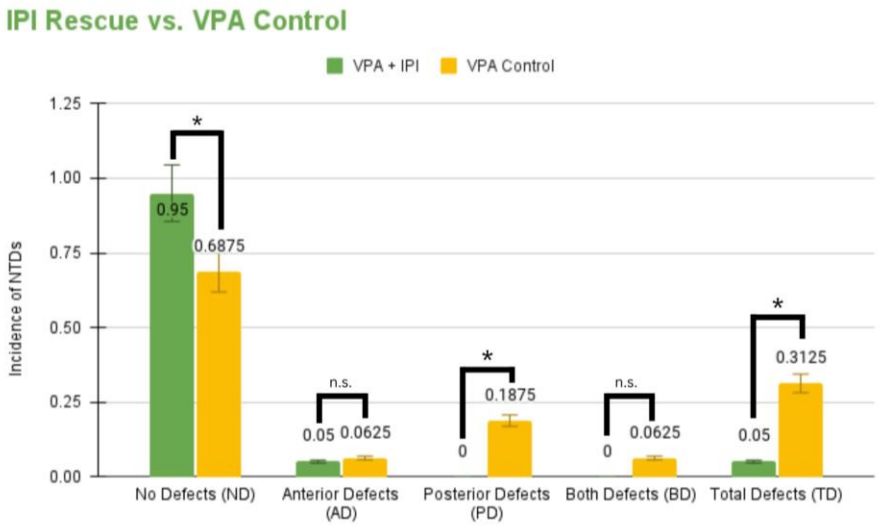

Figure 4

Results show IPI prevented neural tube defects in chick embryos in the presence of VPA, a known teratogen; and that embryos plated with VPA alone exhibited a higher percentage of defects. * denotes $p<.05$; n.s. denotes not significant.

To measure the effects of IPI in rescue of embryos from C2-induced NTDs, 19 embryos were plated, 11 with C2 + IPI, and 8 with C2 alone. Results from C2/IPI experiments show that, with a sample size of 11, only 2 embryos presented with NTDs (both posterior defects) in the C2 + IPI group for an overall 18.2% NTD rate in this group. Conversely, in the C2 Control group, in a sample size of 8, 4 embryos presented with NTDs (3 anterior defects, 1 posterior defect) for an overall 50% NTD rate in this group. This is consistent with the 45% NTD rate observed in C2 treated embryos in previous work (Ross et al., 2019). The much lower NTD rate in the IPI treatment group suggests a rescue effect by IPI in the presence of C2 (Fig. 5). To test the effect of IPI treatment on NTDs rescue we conducted independent samples t-tests to compare NTDs across the IPI Rescue and C2 Control groups (Fig. 5). Though there were more NTDs in the C2 Control group, the difference was not significant [$t(17) = 1.481$, $p = .079$ one-sided] indicating that the IPI rescue treatment trended toward reducing the incidence of NTDs and that further dose adjustment in embryo trials may be useful. Only the difference in anterior defects was significant [$t(17) = 2.430$, $p = .013$ one-sided] suggesting a need for further investigation and a larger sample size to determine if this is an artifact of the data or true significance.

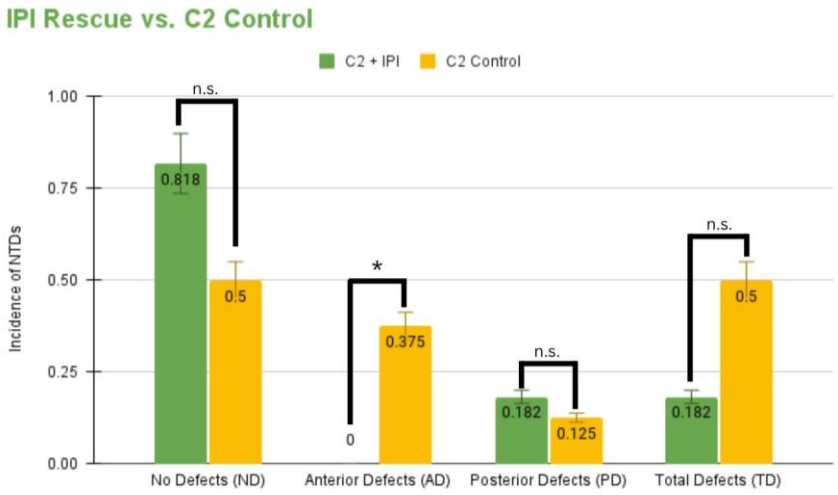

Figure 5

Results trend toward a rescue effect by IPI in the presence of C2, a known teratogen, but largely do not reach significance. * denotes $p < .05$; n.s. denotes not significant.

Nrf2 Target Gene Activity After IPI Exposure

As we see a rescue of NTDs in embryos against both teratogens to different degrees of significance, it's important to consider the mechanism at work that is reducing the incidence of NTDs in vivo. The assumption can be made, referencing previous studies that show Nrf2 activity increases in the presence of IPI, that this anti-inflammatory mechanism is an important component in the observed NTD reduction. To determine to what extent this rescue effect is a result of Nrf2 activity relieving oxidative stress in the embryos, an analysis of Nrf2 targets is necessary. An increase in Nrf2 target genes would be expected in this case (Fig. 6 & Fig. 7).

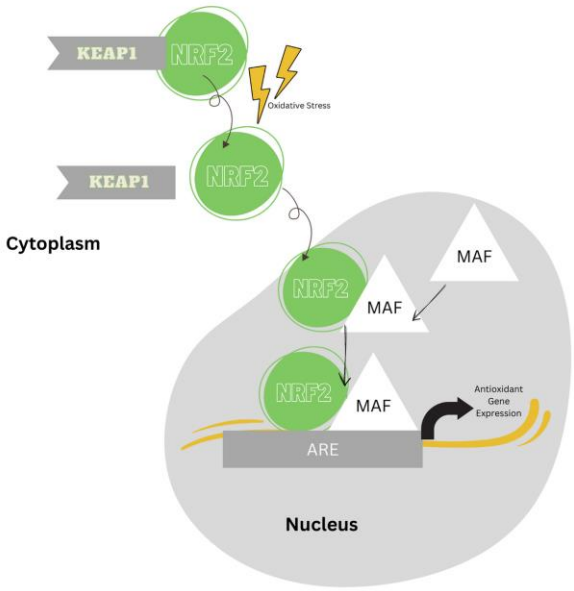

Figure 6

Nrf2 is a transcription factor sequestered in the cytoplasm of cells by Keap1. In cases of oxidative stress, Keap1 releases Nrf2, which enters the nucleus and binds with small Maf proteins. This dimer binds at the cis-acting enhancer (ARE) antioxidant response element to trigger the transcription of antioxidant target genes (Li et al., 2008).

A follow-up experiment was done involving culture of HepG2 cells, a human liver cancer cell line, and application of an IPI gradient (77.52 µM IPI; 193.8 µM IPI; and 484.5 µM IPI) for 24 hours. A luciferase assay was then performed to determine the prevalence of Nrf2 target genes in the experimental set vs. both a positive control—50µM of t-Butylhydroquinone (TBHQ) dissolved in dimethyl sulfoxide (DMSO) at 8.3mg TBHQ per mL of DMSO, where TBHQ is known to upregulate the Nrf2 pathway—and a negative control—only EMEM cell culture media without any drug.

To test the effect of IPI dosage on Nrf2 activity we conducted a one-way ANOVA (Fig. 7). There was a significant dose-dependent effect of IPI on Nrf2 activity [$F(4, 75) = 6.47$, $p = .00016$]. Follow-up pairwise comparisons using Tukey's test showed a statistically significant difference between the negative control and the highest IPI concentration of 484.5 µM (mean difference = 6.56, $p = .036$) indicating that IPI is an effective activator of the Nrf2 pathway in human cells. Critically, there was no significant

difference between the positive control and the higher concentration of IPI (mean difference = 3.75, $p > .40$) suggesting that the optimal dose of IPI works just as effectively as TBHQ in upregulating the Nrf2 pathway.

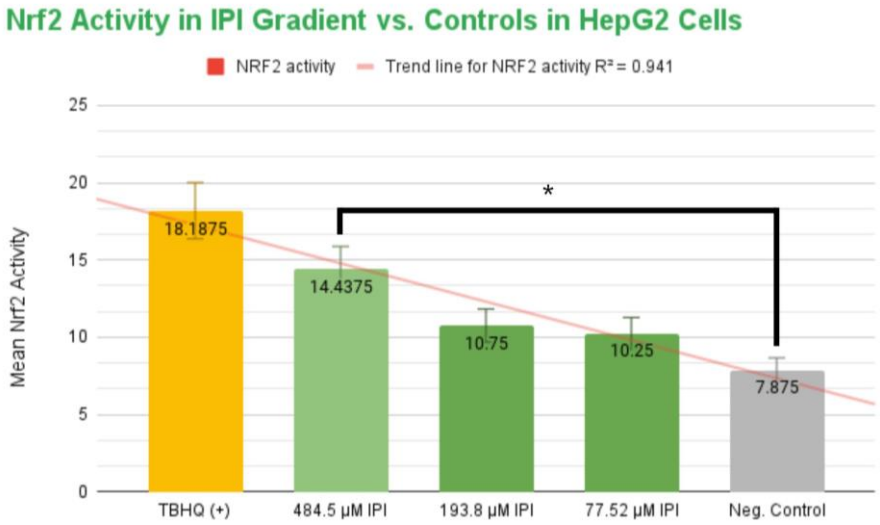

Figure 7

A luciferase assay on a 96-well plate of HepG2 cells with a reporter for Nrf2—where Nrf2 pathway activation causes luminescence—was performed with an IPI gradient: negative control (no IPI, only cell culture media EMEM); concentration gradient of 77.52 µM IPI, 193.8 µM IPI, & 484.5 µM IPI; positive control of TBHQ (known to upregulate the Nrf2 pathway). Cell lysis-induced luminescence was read on a SpectraMax iD3 spectrometer. Nrf2 is upregulated in both the positive control and the highest IPI concentration (484.5 µM). At the 484.5 µM IPI concentration, Nrf2 activity is significantly increased from the negative control. * denotes $p<.05$.

Interestingly, there was no statistically significant difference between the negative control and the 193.8 µM IPI dose which we originally used as the optimal dose in the chick embryo teratogen rescue experiments. This may explain why rescue by IPI was not more significant in those experiments. Future experiments should take this optimal dose into consideration when treating living embryos.

Limitations to our experiments included small sample sizes of chick embryos, limited undergraduate student hours in the lab, and a high failure rate in the embryo

collection process. Further research could include evaluating redox states, and performing a Nrf2 target genes expression analysis by quantitative PCR.

Discussion

Limitations and Consensus

Despite the limitations of this study, the aggregate of studies on Moringa-derived isothiocyanate depict a natural compound with potential public health impact in the area of maternal health and the prevention of birth defects. Ross et al. (2019) found that oxidative stress, not merely folic acid deficiency, is a likely instigator of developmental insults to an early-stage embryo. Other scientists discovered that IPI has antidiabetic and anti-inflammatory capacities (Borgonovo et al., 2020; Waterman et al., 2014). Where redox imbalance (i.e. oxidative stress) contributes to the development of neural tube defects, IPI contains the necessary qualities to mitigate and minimize this effect. It was posited that it does this by activating the Nrf2 cofactor (Cheng et al., 2019). If this is the case, activating Nrf2 cofactor will reduce chemically-induced oxidative stress, thereby preventing the development of NTDs. Our results support this model. Whether in the presence of VPA or C2, IPI demonstrated the ability to reduce the number of neural tube defects as a percentage of the total sample, though with varying degrees of significance. Additional research is needed to improve statistical power and to validate these results.

The question of optimal dose of IPI is one this study only began to explore. The form of isothiocyanate, suspended in an alcohol solution, that was used in this study is highly concentrated and had to be diluted. Ultimately, the concentrations that were used were remarkably small: 77.52 µM IPI; 193.8 µM IPI; 969 µM IPI. When taken dietarily, *M. oleifera* is recommended in doses no more than 1 Tablespoon of the powdered leaves and seeds, due to the potency of the nutrients *M. oleifera* contains. A different

isothiocyanate was isolated in the study by Borgonovo et al. (2020), the isothiocyanate compound moringin (4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate), which is present in *M. oleifera* seeds and leaves. They determined the moringin content in *M. oleifera* seed flour to be 0.344 ± 0.018 mg/g (or 344 ± 18 ppm) while in *M. oleifera* leaf powder, the moringin content was 0.074 ± 0.004 mg/g (or 74 ± 4 ppm). Based on their analysis, we can estimate that 1 tablespoon (7-8 grams) of ground *M. oleifera* leaf powder would contain approximately 0.52 - 0.59 mg of the isothiocyanate compound moringin. While this isothiocyanate content is not as high as that found in mustard powder, it does appear to be a more stable form, with positive implications for the shelf-life of *M. oleifera* powders (Chen et al., 2019; Faizi et al., 1994).

In animal studies and human trials, very small concentrations of IPI are used (100-300 mg/kg), and show desired effects, such as a 51.2% decrease in blood glucose levels in severely diabetic rats (Jaiswal et al., 2009) or to protect tissues against chemical and radiation damage (a review by Stohs & Hartman, 2015). In our luciferase assay, we found a significant increase in Nrf2 activity, similar to the positive control, when a dose of 484.5 µM IPI was used (Fig. 7). This is not the concentration that was used in the chick embryo model vs. known teratogens C2 and VPA; that concentration was 193.8 µM IPI. It is possible that future experiments using 484.5 µM IPI as an optimal concentration will see greater statistical significance than this study presented.

As studies using *M. oleifera* in various forms continue to grow in number, more available evidence demonstrates its ability to reduce oxidative stress. A study using an ethanol extract in cultured neurons showed significant increase in dendrite and axon growth, in both the length and number of these branches, and researchers believe it may be neuroprotective via oxidative stress reduction (Hannan et al., 2014). Cell proliferation also increased in a study exposing cultured cells to an ethanol extract of *M. oleifera*

(Stohs & Hartman, 2015). Each of these effects is potentially, and likely, based on the effect of IPI on oxidative stress.

Next Steps

Future research directions include validating these results in mammalian models and exploring the applicability to human populations, especially in areas with high NTD prevalence and those affected by genetic factors influencing folate metabolism. Epidemiological studies will also inform future research, showing how different populations respond to *M. oleifera* vs. FA by country of origin or genetic polymorphism.

In addition to evaluating the upregulation of Nrf2 target genes in the presence of IPI, further research should focus specifically on reducing neural tube defects in populations with MTHFR C677T polymorphism. The knowledge of gene mutations aids genetic counselors in advising prospective parents on maternal nutrition needs and risk reduction. However, this benefit is not accessible to the general childbearing population. This highlights the need for personalized medicine to mitigate potential harm from generalized public health practices. As research progresses, public campaigns on reducing neural tube defects (NTDs) through nutritional supplementation are crucial to bridge the research-communication gap and provide potentially life-saving information to the broader population.

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