The Effects of Curcumin and Epigallocatechin Gallate (EGCG) on Neurulation in *Xenopus laevis*

By:

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Abstract

Many studies have shown that curcumin, the yellow pigment in turmeric, and epigallocatechin gallate (EGCG) in green tea possess anti-inflammatory, anti-oxidative stress, tumor reduction, and neuroprotective properties against many neurodegenerative conditions. The hypothesis for this experiment is that the synergistic effects of EGCG and curcumin on the rate of neurulation in *Xenopus laevis* are greater than that of using factors alone. The effects of EGCG and curcumin are evaluated by incubating *Xenopus laevis* embryos in different concentrations of EGCG, curcumin, and a combination of both. Nieuwkoop and Faber development stage numbers of the embryos were recorded at various time intervals after the start of incubation. Time was measured starting with the neural plate stages, through the neural fold stages, and ending with the neural tube stages. The experimental groups are compared to the control group to observe any morphological changes. In addition, a literature review of the effects of dietary factor combinations on neurodegeneration disorders was done.

Table of Contents

Chapter 1: Introduction

1.1 Objectives

In these experiments, the combination of curcumin and EGCG on neurulation has been evaluated to see if the combination produces an effect different from or greater than the sum of their individual effects. Also, the rate of neurulation was measured after incubating *Xenopus* embryos in curcumin, EGCG, and a combination of both respectively. *Xenopus* embryo development was observed for any morphological changes. In addition to the lab component of this research, a literature review of the effects of dietary factor combinations on neurodegeneration disorders was done.

1.2 Xenopus laevis

Experimenting on *Xenopus* embryos is advantageous because these embryos are abundantly available, develop rapidly, and can be observed externally making them a valuable model to study embryonic development (7,8). In addition, the embryos have a fast development which can be seen externally, allowing quick analysis when observing the rate of neurulation. *Xenopus'* embryos have a fast development and within days of fertilization the embryos form a full set of differentiated tissues. Their development allows quick analysis of the effects of experimenting with embryonic gene expression. *Xenopus laevis* was used in this experiment; *laevis* has larger embryos that are better suited to microsurgery than *tropicalis* (7). Russell et al. observed embryogenesis and tracked cell movements and lineages in developing frog embryos. The results of Russell et al. experiment explains that during amphibian gastrulation, neurulation external ectodermal, and internal mesodermal tissues expand at different rates (9). The embryo's neural plate, folds, and tube are the changes observed during neurulation.

1.3 Neurulation

Neurulation is the development of the neural tube, the spinal cord, and the brain respectively. Researchers claimed that the pressure used by the ectoderm and mesoderm is important in neurulation (2). In amphibians neurulation occurs within the medullary plate and neural crest cells are created (1).

The process of neurulation begins with the formation of a neural plate. Alterations in cell shape and cell adhesion leads the edges of the plate to fold and rise, leading it to meet in the midline to form a tube. The cells at the top of the neural folds lie between the neural tube and the overlying epidermis. As a result, these cells become the neural crest cells in which the epidermis and neural plate give rise to neural crest cells. In addition, the notochord is necessary in order to prompt neuroplate formation. The notochord also regulates the location and formation of the neural tube (1). In amphibians, the morphogenetic movements of neurulation are associated with two cell shape changes (elongation and apical constriction) in the neural epithelium of the columnar neural plate cells (3). At the beginning of neurulation, the notoplate, a central posterior region of the neural plate, elongates through convergent extension. Anterior-posterior elongation of the entire neuraxis leads to the closure of the dorsal surface of the neural tube in amphibians (4). Neural tube defects are caused by unknown genetic trends along with critical environmental conditions (5). In Matteson et. al's study *Gpr 161* displays limited expression to the lateral neural folds, developing lens, retina, limb, and CNS (6). It is anticipated that a curcumin and EGCG environment will not harm the amphibians genes and influence a defect in the neural tube of the amphibians. During neurulation, the neural plate forms, then the neural folds, followed by neural tube development. According to the Nieuwkoop and Faber developmental stages, neurulation occurs from stage 13-21.

1.4 Curcumin

Curcumin is a main ingredient in the spice turmeric, which has many therapeutic properties. It is widely used as a spice, food preservative and coloring agent in India, China, and South East Asia. In addition, it has been used in traditional medicine as a remedy for diseases, such as, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism, and sinusitis (13). Studies have shown that curcumin has anti-inflammatory, anti-oxidative stress, and tumor reduction properties (13). Curcumin also has neuroprotection effects against a wide range of neurodegenerative conditions in animal models (14). Studies show that curcumin as an antioxidant, anti-inflammatory, and anti-amyloidal agent can improve cognitive function in patients with Alzheimer's disease. Curcumin can also benefit the brain through different mechanisms by providing protection against neurologic disorders (15). For example, in a population of elderly Asians, evaluation of cognitive function with a mental examination test showed people who consumed curry frequently performed significantly better as compared to those who almost never or rarely consumed curry (15); this suggests a possible ability of curcumin to affect brain function. Curcuim is insoluble in water and ligroin; it is soluble in alcohol, ether, and glacial acetic acid (16).

1.5 EGCG

Epigallocatechin gallate (EGCG) is the main active ingredient in green tea and possesses anti-cancer, anti-obesity, anti-atherosclerotic, anti-diabetic, anti-bacterial, and anti-viral properties. Many of the beneficial effects of green tea are due to activities of EGCG (17). Studies demonstrate that EGCG enhances adult hippocampal neurogenesis. Studies also show that EGCG reduced cerebral infarction and improved learning and memory deficits induced by cerebral ischemia. Long-term treatment with EGCG also increased malondialdehyde (MDA) levels, glutathione (GSH), and superoxide dismutase (SOD) activity in the cerebral cortex and hippocampus(17). This study demonstrated that green tea has a neuroprotective effect.

Another study demonstrated that green tea poly phenols may act as a potential neuroprotective agent against blood-brain barrier (BBB) damage at the early stage of focal cerebral ischemia through the regulation of TJ and PKCα signaling. Studies also show that EGCG remodels mature α-synuclein and amyloid-β fibrils and reduces cellular toxicity. For instance, studies show that EGCG converts large, mature α-synuclein and amyloid-β fibrils into smaller, amorphous protein aggregates that are nontoxic to cells (18). Studies demonstrate that EGCG compound binds directly to β-sheet- rich aggregates which lead to conformational changes without their disassembly into monomers or small Oligocene (18). These findings suggest that EGCG is a powerful transformational agent of mature amyloid fibrils. Effects of EGCG on neuro inflammation in LPS-induced BV-2 microglia cells were also examined (18).

1.6 Synergistic Effect of Dietary Factors

The literary research showed that studies have been done on the synergistic effect of dietary factors on cancer but not on neurulation. There have been studies done on the effect of combination of dietary factors, such as combination of curcumin and resveratrol as antioxidant agents. A study was done to compare antioxidant activities of curcumin and resveratrol, a polyphenol found in some non-dietary plants and dietary plants. In addition, combinations of curcumin and resveratrol were also study for potential synergism in a heme-enhanced oxidation reaction. This study showed that curcumin had antioxidant effects at all-time points (1-5 min; 10 microM), e.g., 30.5 +/- 11.9% (SEM) oxidation relative as compared to controls without phytochemicals ($p < 0.01$) at 3 min (19). On the other hand, resveratrol same concentration exhibited about half of curcumin's activity (19). However, when curcumin and resveratrol were combined together (5 microM each) the resulted synergistic antioxidant effect was 15.5 +/- 1.7% greater than an average of individual effect (19). Curcumin and resveratrol synergistic effect was significantly greater ($p < 0.05$; about 4-fold) than that of curcumin together with the

flavonolquercetin (19). This study demonstrated that curcumin is a powerful antioxidant in a reaction that may be relevant to in *vivo* toxicity. As compared to other well-known antioxidants, curcumin showed significantly greater synergism with resveratrol than with quercetin (19).
In the literature search, experiments that tested dietary combinations in regard to neurulation were not found. The results from Gang Xu et al. experiment showed that dietary curcumin, catechins, and combination administration significantly inhibited the total number of aberrant crypt fociper rat. The combination treatment displayed the most potent inhibitory effect, while there was no difference of inhibition between curcumin and catechins-treated groups. The incidence of colorectal cancer in the treated groups was significantly lower than that of positive control group. Findings suggest that the combination of curcumin and catechins may produce a synergistic colon cancer-preventative effect that would be more potent than each of the compounds alone (20). Two such naturally occurring agents, EGCG and curcumin, were noted to inhibit tumor growth by different mechanisms, a factor which may account for their demonstrable interactive synergistic effect (21). However, studies have not been done on the effects of the combination of curcumin and EGCG on neurulation. This research evaluates the effect of curcumin alone, EGCG alone, and the synergistic effects of curcumin and EGCG combined on neurulation in *Xenopus laevis*. The goal of the experiment is to evaluate if the combination of these two substances produces an effect different from or greater than the sum of their individual effects. In regards to the literature review component of this research, scientific research has shown that some combinations in dietary factors have positive effects on neurodegenerative disorders.

Polyphenolic dietary antioxidants, such as resveratrol, EGCG, and quercetin are effective neuroprotectants. Dietary intake of polyphenols is known to reduce oxidative stress and the risk

for related neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease. Some dietary factors may work synergistically to serve as a therapeutic function for individuals with neurodegenerative disorders by affecting the synthesis of brain membranes. Research investigates the benefits of such as carotenoids, folic acid, polyunsaturated fatty acids, and curcuminoids.

Chapter 2: Materials and Methods

2.1 Animal Care and Maintenance

Xenopus laevis embryos (100-150) unit were obtained from *Xenopus* Express. Upon arrival, using a plastic micropipette, four eggs for each concentration per group were each distributed into 2 inch Petri dishes for observation and photographed. The eggs from each Petri dish were transferred to 40mL of aged tap water in 50ml beakers with different curcumin and EGCG concentrations. The amphibians were fed a pinch of yeast daily after the post incubation time and photographed. *Xenopus laevis* were housed at approximately 70 degrees F in 24hr aged tap water. All experiments were carried out according to the guidelines of our institution's Animal Research and Care Committee.

2.2 Preparation of Solutions:

Curcumin:

Curcumin stock (Sigma Aldrich) was dissolved in anhydrous dimethyl sulfoxide (DMSO) as recommended by the supplier. The solution was stored in a glass test tube in a refrigerator at approximately -80 degrees Celsius. 1mL of DMSO was added into the beaker containing 11mg curcumin. Curcumin stock was then dissolved in DMSO completely by gentle swirl. The curcumin solution was stored in a glass test tube at -80 degrees C. Prior to use; the stock solution

was briefly placed in a 37 degrees C water bath. The two curcumin concentrations used in this experiment were: $4.36x10^{-4}$ m/L (2µL stock solution) and $8.72x10^{-4}$ m/L (4µL stock solution).

EGCG:
EGCG ≥80% (HPLC) stock, from green tea (Sigma-Aldrich) was stored at 2-8 degree Celsius. 50mg of EGCG stockwas dissolved in 250mL aged tap water. The two EGCG concentrations used in this experiment were: $1.75x10^{-4}$ m/L (5mL solution) and $3.50x10^{-4}$ m/L (10mL solution)

2.3 Incubation of embryos

The rate of neurulation in *Xenopus laevis* embryos was investigated based on their incubation treatment. The embryos were each housed in 40mL of aged tap water in 50mL beakers. Curcumin, EGCG, and a combination of both were then added respectively to incubate the embryos. The effects of EGCG and curcumin on neurulation were evaluated by incubating *Xenopus laevis* embryos in different concentrations of EGCG ($1.75x10^{-4}$ m/L and $3.50x10^{-4}$ m/L), curcumin ($4.36x10^{-4}$ m/L and $8.72x10^{-4}$ m/L), and combinations of both solutions. Two embryos for each concentration were used per trial. Embryos were divided into five groups: a control, DMSO control, curcumin, EGCG, and a combination of curcumin and EGCG. The embryos were transferred using a plastic pipette. The outer jelly layers of the embryos were removed for a better penetration of curcumin and EGCG using a Leica Zoom microscope, 2 inch Petri dishes, and #5 forceps. The embryos were monitored daily up to one week of incubation. In this analysis of neurulation, embryos of *Xenopus laevis* were examined by light microscopy at selected developmental stages.

2.4 Measurement of rate of neurulation

The photographed stages were compared with the Nieuwkoop and Faber developmental stages (Figure 1) to measure the rate of neurulation. According to the Nieuwkoope and Faber developmental stages,neurulation occurs from stage 13-21. During neurulation, the neural plate

forms, then the neural groove, followed by the neural tubes. The *Xenopus* embryos were photographed before incubation and approximately 2.5 hours after incubation.

2.5 Photography:

Using 319CU 3.2M CMOS camera connected to WF 10X/22 eyepiece stereomicroscope, each embryo was individually photographed to compare initial stages of development with the later stages of development after approximately 2.5 hours of incubation in different treatments. The Nieuwkoop and Faber developmental stages were retrieved from http://xenbase.org/anatomy/static/xenopustimetemp.jspand http://www.bio.davidson.edu/people/balom/stagingtable/xenopushome.html to determine the developmental stages that we will be observing.

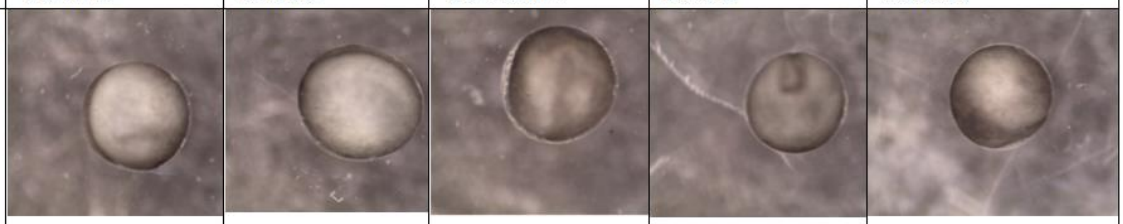
2.6 Figure 1: Nieuwkoop and Faber Developmental Stages

Nieuwkoop, Faber. Normal table of*Xenopuslaevis*http://www.xenbase.org/anatomy/alldev.do 13 March 2014

Chapter 3: Results

Figure 2 (a): *Xenopus laevis* Embryo Developmental Rate(1st concentration)

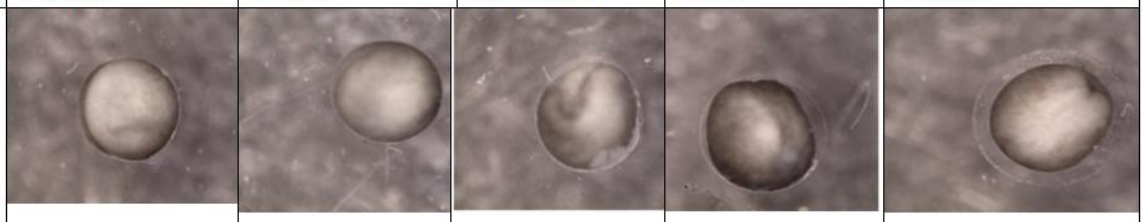
Curcumin: 4.36×10^{-4} m/L (2µL solution)
EGCG: 1.75×10^{-4} m/L (5mL solution)
Combination: Curcumin- 4.36×10^{-4} m/L (2µL solution) + EGCG- 1.75×10^{-4} m/L (5mL solution)

Treatment	Control	DMSO	Curcumin	ECGC	Combo
Photo before incubation					
Stage Before Incubation	15	15	15	15	15
Length of incubation time: ~147 minutes					
Stage After incubation	19	19	19	20	20

WF 10X/22 eyepiece stereomicroscope

Figure 2(b):*Xenopus laevis* Embryo Developmental Rate (2nd concentration)

Curcumin: 8.72×10^{-4} m/L (4µL solution)
EGCG: 3.50×10^{-4} m/L (10mL solution)
Combination: Curcumin- 8.72×10^{-4} m/L (4µL solution) + EGCG- 3.50×10^{-4} m/L (10mL solution)

Treatment	Control	DMSO	Curcumin	ECGC	Combo
Photo before incubation					
Stage Before Incubation	Stage:15	Stage: 15	Stage: 15	Stage: 15	Stage: 15
Length of incubation time: ~147 minutes					
After Incubation	Stage: 19	Stage: 19	Stage: 20	Stage: 20	Stage: 19

WF 10X/22 eyepiece stereomicroscope

Figure 3(a): *Xenopus laevis* Embryo Developmental Rate

Treatment	Embryo Stage Before Incubation	Embryo Stage After Incubation
Control	15	19
DMSO	15	19
Curcumin: $4.36x10^{-4}$ m/L (2µL solution)	15	19
EGCG: $1.75x10^{-4}$ m/L (5mL solution)	15	20
Combination: Curcumin- $4.36x10^{-4}$ m/L (2µL solution) + EGCG- $1.75x10^{-4}$ m/L (5mL solution)	15	20

Figure 3(a):The *Xenopus laevis* embryo incubated in a $4.36x10^{-4}$ m/L curcumin environment was at the same stage as the control after incubation (stage 19). Both embryos in$1.75x10^{-4}$ m/LEGCG and combination environment, respectively, show a stage increase (stage 20) compared to the control.

Figure 3(b): *Xenopus laevis* Embryo Developmental Rate

Treatment	Embryo Stage Before Incubation	Embryo Stage After Incubation
Control	15	19
DMSO	15	19
Curcumin: $8.72x10^{-4}$ m/L (4µL solution)	15	20
EGCG: $3.50x10^{-4}$ m/L (10mL solution)	15	20
Combination: Curcumin- $8.72x10^{-4}$ m/L (4µL solution) + EGCG- $3.50x10^{-4}$ m/L (10mL solution)	15	19

Figure 3(b): The *Xenopus laevis* embryos incubated in $8.72x10^{-4}$ m/L curcumin alone (stage 20) and $3.50x10^{-4}$m/LEGCG alone (stage 20) environment were both a stage after the control (stage 19). The embryo in the combination environment had the same stage as the control (stage 19).

After incubation, embryos in the higher concentration of curcumin treatment (C- $8.72x10^{-4}$ m/L) was at stage 20, which is a one stage increment compared to the control. Embryos in the cucumin treatment at its lower concentration (C- $4.36x10^{-4}$ m/L) was at stage 19, the same as the control. It is assumed that the higher the concentration of the solution, the greater the stage will be after a period of incubation. Both EGCG treatments ($1.75x10^{-4}$ m/L and $3.50x10^{-4}$ m/L) were

one stage incremented (stage 20) compared to the control after incubation. Embryos in the EGCG treatment regardless of the concentration was one stage more than the control. After incubation, embryos in the higher concentration combination treatment (C- $8.72x10^{-4}$ +E- $3.50x10^{-4}$ m/L) were at the same stage as the control (stage 19). While embryos in the lower concentration combination (C- $4.36x10^{-4}$ m/L + E- $1.75x10^{-4}$ m/L) were one stage incremented compared to the control. The hypothesis for this experiment is that the synergistic effects of EGCG and curcumin on the rate of neurulation in *Xenopus laevis* are greater than that of using factors alone was the case for embryos in the curcumin only treat at its lower concentration. Embryos in curcumin alone (C- $4.36x10^{-4}$ m/L) was at stage 19 after incubation while curcumin in combination with EGCG (C- $4.36x10^{-4}$ m/L + E- $1.75x10^{-4}$ m/L) was at stage 20. Embryos in the EGCG treatment at its higher concentration was at stage 20 after incubation but in combination the embryos were one stage less than when incubated alone. Further trials need to be done to support the hypothesis. Finally, Curcumin and EGCG did not disturb the embryonic development of *Xenopus laevis* and there were no morphological changes observed.

Literature Review: The Effects of Dietary Factor Combinations on Neurodegeneration Disorders

Combinations	Effects	Reference
Vitamin E & C	**Reduce prevalence and incidence of neurodegenerative disorder**	**Rahman K., 2007**
vitamin E and beta-carotene	**found to protect (rat) neurons against oxidative stress**	**Guyonnet S. et al., 2013**

vitamin C (120 mg), b-carotene(6 mg), vitamin E (30 mg), selenium (100 mg) and zinc (20 mg) in combination	**better episodic memory scores**	**P. Preziosi et al., 1998**
B vitamins plus omega-3 fatty acids	**less likely to have a decreased score on the temporal orientation task than those assigned to receive placebo**	**Guyonnet Set al., 2013**
berry fruit and fatty acids from walnuts and fish oils	**dramatically impact the aging brain, possibly leading to improved cognition and motor abilities**	**Joseph Jet al., 2009**
Curcumin synergized with fish oil	**reduced phosphorylated c-Jun N-terminal kinase, IRS-1, and tau and prevented the degradation of total IRS-1**	**MaQ.L et al., 2009**
association between fish consumption, monounsaturated fatty acids and polyunsaturated fatty acids (n-3 PUFA)	**Lowers risk reduction for neurodegenerative disease**	**Mattson M.P, 2004**
free radicals, antioxidants, and co-factors	**maintaining health, aging and age-related diseases**	**Rahman K, 2007**

nutritional flavonoids and polyphenols	**prevention of neurodegenerative diseases**	**RamassamyCet al., 2010**

Literature was scarce for the literature review of the effects of dietary factor combinations on neurodegeneration disorders. More literature works were found on the effects of single dietary factors on neurodegeneration. Researchers found that Vitamin E, Vitamin C, beta-carotene, omega-3 fatty acids, curcumin, and fish oil alone has helped to prevent neurodegenerative diseases. In limited literature supporting findings on the effects of dietary factor combinations on neurodegeneration disorders researchers have found that a combination of Vitamin E and C, berry fruit and fatty acids (walnuts and fish oils), and curcumin synergized with fish oil help to prevent neurodegeneration.

Chapter 4: Conclusions & Discussion

The data collected included three official trials. The hypothesis that the synergistic effects of EGCG and curcumin on the rate of neurulation in *Xenopus laevis* are greater than that of using factors alone was not supported by the data. More trials would need to be conducted for further support. There were no morphological changes determined from the collected data. Therefore, the concentrations of the solutions were reasonable to use as treatments for the *Xenopus* embryos.

Based on the results the developmental stage increments compared form the control varied based on the concentration and solutions used. The *Xenopus laevis* embryo incubated in an environment with a higher concentration of curcumin ($8.72x10^{-4}$ m/L) was one developmental

stage above the control after incubation. The embryo incubated in $4.36x10^{-4}$ m/L of curcumin, a lower concentration, was at the same stage as the control after incubation. The embryos in both EGCG environments (concentrations: $1.75x10^{-4}$ m/L and $3.50x10^{-4}$ m/L) show a stage increase compared to the control. The embryos in the EGCG environments were at stage 20 after incubation while the control was at stage 19. The data does not determine that curcumin and EGCG combined has a greater effect than their individual effects. After incubation, the embryo incubated in C- $8.72x10^{-4}$ +E- $3.50x10^{-4}$ m/L combination solution was at the same stage as the control (stage 19). The embryo incubated in C- $8.72x10^{-4}$ alone was at stage 20 and the embryo incubated in E- $3.50x10^{-4}$ m/L alone was also at stage 20. After incubation, the embryo incubated in C- $4.36x10^{-4}$ m/L + E- $1.75x10^{-4}$ m/L had the same stage as the control (stage 19). The embryo incubated in C- $4.36x10^{-4}$ m/L alone was at stage 19 and the embryo incubated in E- $1.75x10^{-4}$ m/L alone was also at stage 20. The embryo incubating in EGCG concentrations alone had a stage increment compared to the control. The embryos in curcumin and EGCG only environments were the same as the control or 1 stage ahead of the control just as the embryos in the combination of treatments. There was a variation among the trails.

From this experiment, the curcumin and EGCG concentrations did not disturb the development of *Xenopus laevis* embryos. The stage number of the embryos after incubation was not less than that of the control. Also, there were no morphological changes found with the collected data.

Among curcumin's solvents we chose to use Dimethyl sulfoxide (DMSO). Many investigators found DMSO to be an effective anti-infammatory agent, an aid to muscle relaxation, a vasodilator, and a nerve blockader. Curcumin properties also include anti-inflammation, anti-oxidative stress, and tumor reduction. DMSO is an organic solvent which

aids other drugs in permeating cell membranes (23). We experimented with both ethanol and DMSO from the choices Sigma Aldrich provided for curcumin's solubility. We found that the curcumin powder did not fully dissolve in ethanol as it did in DMSO.

DMSO is a clear, colorless liquid with a bitter odor and has been widely used in industry as a solvent (24). Curcumin is insoluble in water. However, in 40mL aged tap water curcumin in DMSO measured in micro liter is soluble when mixed and boiled. In a molecular dynamics study, results are compared with many experimental data to conclude that in most cases components in water-DMSO mixture maintain their structure upon dilution. DMSO is a widely used cryoprotectant for biological structures such as membranes and proteins (25). DMSO is also the standard solvent for preparing stock solutions of compounds for drug discovery (26).

It is essential to schedule the ordering *Xenopus* embryos needed for the experiment accordingly. It is essential to have fertilized embryos arrive from the *Xenopus*, the company; working with non fertilized embryos can alter results. All other materials and solutions need to be available when the embryos arrive. When conducting experimental research organization is important in such a way all factors were taken into consideration and every detail has been polished to avoid delays. Also, making sure the equipment such as a microscope or camera is working and available for use is essential for collecting data. Malfunctioning equipment can cause a setback in the experiment. It is also important to keep temperatures that are suitable for the living specimens to avoid fatal organisms. In addition, it is vital to make sure experimenters know the exact concentrations they will be using before they carry out the experiment. This removes delays in performing trials and errors of determining accurate concentration.

Further research in this study should include close analysis of the specific stages in which the *Xenopus* embryos are first placed into their curcumin, EGCG, or combination treatment. The influence of the treatments on the development of *Xenopus* embryos based on their stage upon exposure should be evaluated. Further tests can also include investigating *Xenopus* spinal cord development. Also, we can test how curcumin and EGCG affects amphibian development in later stages with a range of curcumin and EGCG concentrations. Expanding the number of *Xenopus* embryos per trial can provide a stronger analysis of *Xenopus* development based on the treatment in which they were incubated in. Removing the vitelline membrane will allow the solutions to better penetrate the embryos, possibly leading to a greater effect on the rate of neurulation. Incubating embryos at an earlier stage could also be done to test if the rate of neurulation can be greater that embryos incubated in treatment at later stages. Testing development rate using other concentrations of curcumin and EGCG solutions can provide a range of data to analyze in supporting the hypothesis. Photographing and recording the initial and post stages of incubation at various times can also expand analysis.

Literature on the combination of dietary factors and its effects on neurodegenerative disease are scarce compared to dietary factors alone. However, scientific literature supports that Vitamin E, Vitamin C, beta-carotene, omega-3 fatty acids, curcumin, and fish oil alone lower the risk of neurodegenerative disease. Research shows, that Vitamin E and C combined, berry fruit and fatty acids from walnuts and fish oils combined, and curcumin synergized with fish oil lower the risk of neurodegenerative disease. Antioxidant beta carotene is found in kale, carrots, broccoli, and spinach. Flavonoids are in cranberries, green and black tea. Vitamin C can be obtained when consuming citrus fruits, kiwi, sprouts, broccoli, and cabbage. Lastly vitamin E is in grain, nuts, milk, and egg yolk (2). Further research is necessary to support the hypothesis that

a combination of dietary factors has a greater effect in lowering the risk of neurodegenerative diseases.

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