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Influence of Folic Acid on Chick Neural Connectivity

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Abstract

Recent studies suggest persons with mild autism show distinctive brain "underconnectivity"[9, 10]. Underconnectivity is described as a lack of communication and coordination via fiber tracts between imperative points of the brain. Folic acid (FA) has been recommended since the 1980s, without limitations, as a perinatal supplement to ensure proper brain development, in particular to prevent neural tube defects. We decided to measure neural outgrowth and synapse formation (connectivity) under FA supplementation, which may contribute to the state of underconnectivity. Dorsal root ganglia (DRG) were dissected from the lumbosacral region of eight day chick embryos and cultured in medium with or without FA or methotrexate, a substance which blocks synthesis of FA to obtain a depleted FA condition. A monoclonal antibody which recognizes synaptic vesicles was used to immunostain developing synapses. The FA cultures showed significantly shorter and fewer neurites compared to control and methotrexate cultures. They also showed a reduction in the number of synapse forming areas. These results suggest developing neurons exposed to excess FA exhibit a reduction in two vital parameters of establishing neural connectivity.

Keywords: Autism, Underconnectivity, Folic Acid

1. Introduction

1.1 Folic Acid

Folic Acid (FA) is a synthetic version of Vitamin B_9, a water-soluble substance needed for synthesis, repair and methylation of DNA and for the synthesis of the vital amino acid methionine. Folate is naturally found in foods like spinach, but because FA is not biologically derived, it depends upon dihydrofolate reductase for conversion into tertrahydrofolate. Folates are imperative in the methylation process for regulation of genes and protection of DNA from harmful cleavage. FA has been recommended as a perinatal supplement (since the 1980s) to prevent neural tube defects, and has been found to decrease neural tube defects[21]. These birth defects occur within the first month of pregnancy, yet women are advised to supplement throughout pregnancy. Very high consumption of FA has been on the rise since 1998 when the FDA required fortification of grain-based foods with FA[20]. Women and teen girls should consume 400 µg FA/d, pregnant teens and women should consume 600 µg FA/d, and breastfeeding teens and women should consume 500 µg FA/d[1]. The safe upper limit of FA consumption in all adults is 1,000 µg FA/d[1]. Between fortification of grains and oral supplementation, overconsumption of FA is a serious issue. It is conceivable that the prophylactic overconsumption of FA for neural tube defects may be related to epigenetic effects, for example DNA or histone modification of the fetal chromatin[7].

While there are known benefits of FA in regards to spinal cord development[21], studies have also suggested that there may be a detrimental upper limit of FA[18]. The Institute of Medicine recommends 1-15 mg/d of FA for adults[20]. Officials recommend a FA supplement when pregnant, but there is no cap on the amount of folic acid recommended.

However, several studies have provided evidence that excessive FA intake may be detrimental to development and that not all neural tube defects may be prevented through FA supplementation[5, 7, 13, 18]. It is at least possible there is some neurotoxic effect of excess FA[6]. One study found healthy volunteers might experience adverse neurological effects. In this never replicated study, participants were administered pharmacological doses of FA (15 mg/d) until the study had to be abandoned due to mental changes, sleep disruption and GI symptoms[7]. DeSoto and Hitlan[4], using a large Centers for Disease Control dataset, found FA supplementation increased the odds of having a child diagnosed with autism by more than 2.5 times compared to mothers who did not supplement. Although folate is imperative for proper neural development, an abundance of FA may be a detrimental factor, and could lead to changes in gene expression and neural formation. Potentially, it could relate to autism risk.

1.2 Methotrexate

The body produces various neurotransmitters and other molecules from amino acids. In doing so, methylation must occur. A neurotransmitter may be a modified amino acid, synthesized by enzymatically attaching one or more methyl groups to the amino acid (a methyl group is one carbon atom plus three hydrogen atoms). For example, this occurs in a cycle to generate the vital amino acid methionine. Within the methylation cycle, methyl groups may also be removed creating homocysteine. This cycle requires folate to provide methyl groups. A shortage or excess amount of folate may have profound effects on the methylation cycle. Methylation is important for proper development[14].

Methotrexate (MTX) is a medically important inhibitor that prevents the conversion of FA into tetrahydrofolate in the folic acid cycle. Wright et al.[23] first used methotrexate for breast cancer remission. There are many clinical applications for MTX, such as slowing the growth of cancer cells and skin cells in psoriasis, controlling the progression of rheumatoid arthritis and other inflammatory diseases, ending an ectopic pregnancy and preventing fetal cell growth[23]. Antibiotics and antimycotics are known to reduce the effectiveness of MTX and increase its toxicity[17]. MTX was used in this study to create a reduced folate control condition.

1.3 Dorsal Root Ganglia, Chick

The neuronal development of dorsal root ganglia (DRG) of the chick embryo in vitro is a faithful model for neurogenesis. Day eight of chick incubation is when DRG neurons begin to innervate the spinal cord and also connect to peripheral receptors. Neurogenesis processes are simultaneously intense in both DRG and spinal cord, as well as in the brain at this time. Ganglion neurons advance neurites outward across the plastic surface from the cultured tissue and these interact to establish synaptic communication and networks. A neurite is an extension from the cell body of an immature neuron; this extension may become either a dendrite or an axon.

1.4 Theory Of Underconnectivity

Prevalence of autism in the United States is estimated at 1 in 88 live births as of 2008, and more recently 1 in 50[19]. The fundamental basis and neurological implications of autism have been pondered for decades and have been studied in humans. A nascent theory of underconnectivity emphasizes a possible underlying neural mechanism of autism. Underconnectivity is described as a lack of communication and coordination via fiber tracts between imperative points of the brain. Just and colleagues studied brain activation in persons with high-functioning autism by use of fMRI[9, 10, 11]. High-functioning autism is the designation applied to people with autism who have an IQ greater than 70 and are deemed "higher functioning" compared to other people with classic autism[3, 16]. High-functioning autism is not a recognized diagnosis in the Diagnostic and Statistical Manual of Mental Disorders, edition IV-TR. Just and colleagues found a pattern of distinct cortical underconnectivity between the frontal and parietal areas, and also found smaller corpus callosum size in high functioning autistic persons. Koshino and colleagues[12] who studied memory of facial stimuli among persons with high functioning autism by use of fMRI conducted a similar study. The autistic group showed distinctly lower and different brain activation, and showed lower connectivity in the frontal lobes than the control group, further supporting the theory of underconnectivity. It has been recently established that white matter is decreased in the fiber tracts in persons with autism[22]. Specifically, these researchers[22] reported that development of fiber tracts in autistic persons at six months was altered, compared to the control group.

Given the potentially deleterious effects of excess FA and the provocative correlation of post-first-trimester FA dietary supplementation with higher risk for autism among offspring, along with the existing evidence of white matter underdevelopment, it seems important to investigate the direct effects of FA on neurogenesis. Beard and colleagues[2] noted that the increase in autism prevalence was observed at the same time that increase in folic acid intake occurred. The study of cultured DRG described here provides some evidence for a significant inhibitory effect of FA on neurite outgrowth and synaptogenesis.

2. Methods and Materials

2.1 Experimental Design

Chicken eggs (obtained from Sunray Chicks of Hazelton, Iowa) were incubated at 38 °C with approximately 80% humidity until day eight of development. Dorsal root ganglia (DRG) were excised from the lumbosacral region using microdissection tools (Minitool, Inc.) into Falcon Primaria culture dishes. DRGs were positioned into Earle's Balanced Salt Solution (EBSS, Sigma-Aldrich) mediating culture of three conditions: control, FA treated and MTX treated. Excess EBSS was drawn off and 2 mL of Medium 199 (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (Sigma-Aldrich) and 1% antibiotic/antimycotic (Sigma-Aldrich) were added to the FA treated and control cultures. Medium 199 with 10% Fetal Bovine Serum but without antibiotic/antimycotic was added to the Methotrexate treated cultures. We supplemented the medium at 5 µM FA (using a 5 mM stock solution). The recognized average serum concentration of FA is 11.3 nM (5-16 ng/mL). Thus fetal bovine serum FA concentration was augmented by 5 µM. Adults normally circulate 3-15 ng/mL FA, children around 5-21 ng/mL. The level of FA used in this experiment was 442 times higher than the recommended amounts. We used 20 µM MTX by diluting a stock solution (0.9 mg/mL MTX). A total of 3 µL of FA and MTX were added to the treated dishes and they were then incubated at 38 °C with 5% CO_2 for 36 hours.

Thus, there were three conditions: FA treated, control and MTX treated cultures. This study was replicated, with all three conditions, six times. Attachment was observed at 24 hours for all three conditions; those explants not attached were excluded from the experiment. Attachment marks a key event in neural development in vitro. Observing attachment is imperative to control for differences in growth stages.

Immunostaining to reveal localization of synaptic vesicles was started after fixation at 36-hours of explant incubation. The explants were fixed in ice-cold 20% DMSO in methanol for 60 minutes on ice. The explants were then washed in phosphate buffered saline (PBS) three times for two minutes each. Following the washes, explants were treated for ten minutes with peroxidase quenching solution (1mL 30% hydrogen peroxide per 9mL methanol). Then a permeabilizing solution (0.1% Triton X-100) was added to the cultures for 30 minutes. Blocking solution (0.5% Tween-20, 2.5% bovine serum albumen, 0.5% nonfat dry milk) was next added for one hour with gentle agitation. Primary antibody SV2 culture supernatant (Developmental Studies of Hybridoma Bank, University of Iowa Department of Biological Sciences) diluted 1:50 by volume with blocking solution was added and the dishes were incubated overnight at 4 °C. The cultures were then warmed to ambient temperature with gentle agitation and then washed again three times in PBS. Secondary biotinylated antibody from a Histostain SP kit (Invitrogen) was added to dishes for 20 minutes followed by streptavidin-peroxidase conjugate solution, also from the kit, for 15 minutes with gentle agitation. Then a chromagen mixture from the kit was added and color was developed in darkness for ten minutes and immediately rinsed with dH_2O to stop the reaction. The stained cultures were finally post-fixed with 3.7% paraformadahyde solution at 4 °C for eight minutes to stabilize color and were rinsed and stored in dH_2O.

2.2 Microscopy And Analysis

After staining and fixation, images were captured using an Olympus CK2 inverted phase contrast microscope connected to an Optronics CCD digital camera and Macintosh G5 computer using Magnafire© software (Karl Storz Imaging, Inc., Goleta, CA). Twelve radial lines, at 30^o deriving from the center of the explant, were drawn over each image to allow for random cell selection using Open*lab* software (Improvision, Inc., Lexington, MA). Each phase-refractile cell (neurosphere) and each neurite touching one of these twelve lines was enumerated and measured, and this number was used in the pooled analysis (Figure 1). Developing neurons appear bright when viewed in phase contrast optics because the cell body has established a three-dimensional cytoskeleton that refracts light. All neurites

crossing the radial red lines (random sample) outside of the explant were measured. Statistical analysis was conducted using Statistical Package for the Social Sciences (IBM SPSS Statistics). Significance was tested through one-way analysis of variance (ANOVA). The Bonferroni correction was used as a Post Hoc test.

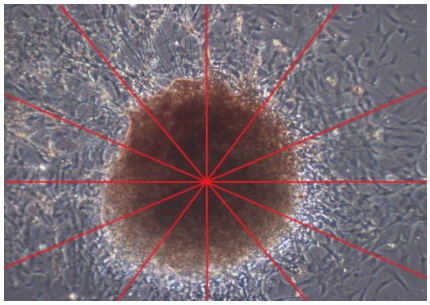

Figure 1. twelve radial line analysis. Random cell selection was achieved by counting or measuring cells or neurites touching the computer-drawn red lines spaced at 30^{o} deriving from the center of the explant (Open*lab* software).

3. Results

The FA treatment significantly reduced synapse formation and the implied neural connectivity in the explanted and cultured DRGs of eight-day chick embryos. FA significantly reduced the length of neurites (and perhaps their numbers) and also reduced the number of synaptic vesicle containing regions of neural nexus, thus providing biological evidence to support the hypothesis of underconnectivity. Post Hoc testing suggested that the MTX treated cultures were not significantly different in these parameters of neurogenesis from controls, indicating a reduced folate condition. Though small amounts of FA are present in fetal bovine serum, MTX presumably blocked the synthesis of new FA in culture. Interestingly, FA, but not MTX, increased the number of emigrated phase-refractile cells, presumably neurons. Many of these overall results are apparent in representative images of the cultured DRGs seen here (Figure 2).

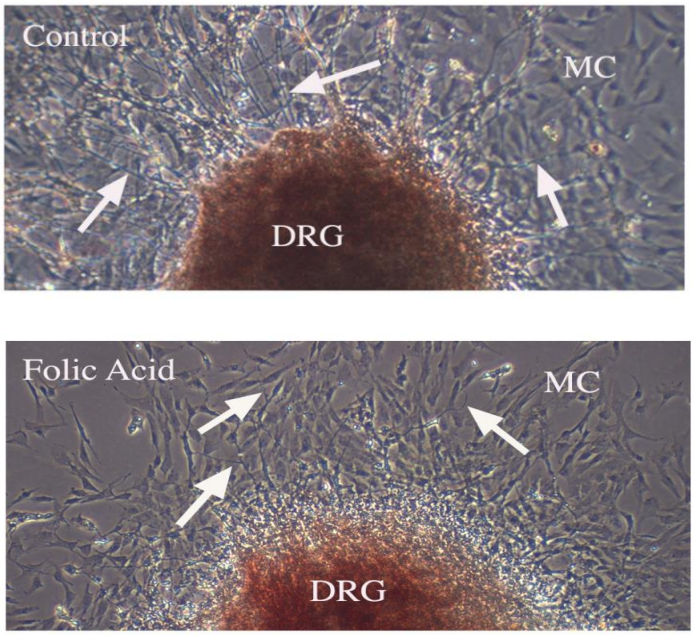

Figure 2. representative chick embryo dorsal root ganglia in culture. Longer, thicker and more numerous neurites (arrows), and abundant red immunostaining were found in the control cultures (top) compared to the FA cultures (bottom). DRG, dorsal root ganglion; MC, migratory cells. Magnification = 80X.

3.1 Effect Of FA On Neurite Length

Images of control and FA treated explants with extending neurites are shown in Figure 2. The length of all neurites in the sample (touching red radial lines) was drawn with computer mouse and measured. The mean neurite length in FA cultures was statistically shorter than the control and MTX cultures [$F(2,479)=10.46$, $p<0.001$]. The Post Hoc test showed that the difference between FA and control conditions was significant ($p< 0.001$). It also showed a non-significant difference trend between FA and MTX ($p=0.22$) treated neurites, and a marginal difference trend between MTX treated and control ($p=0.09$).

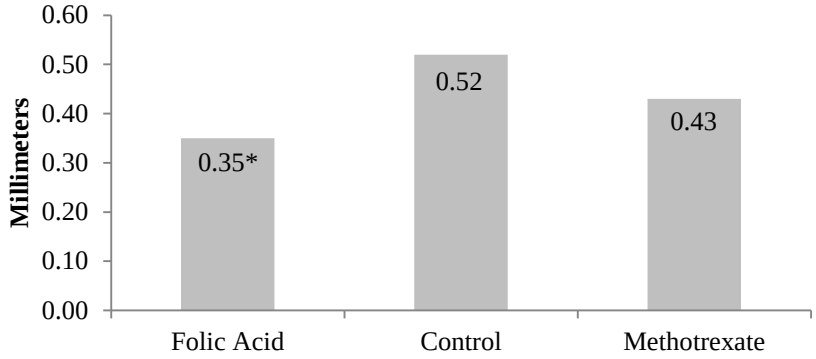

Figure 3. mean length of neurites in DRG explants. FA treatment caused extension of shorter neurites.

3.2 Effect Of FA On Number Of Neurites

The images of control and FA treated explants with extending neurites shown in Figure 2 also reveal a clear difference in the number of neurites sent out from cultured neurons. It is apparent from these representative cultures that FA had a negative impact on neurogenesis. The mean number of neurites observed crossing any red line in all of the FA DRG cultures was less than the control and MTX conditions. A statistically non-significant difference was observed between the numbers of neurites extending from DRGs in culture [$F(2,20)=3.42$, $p=0.05$]. The Post Hoc test showed that a marginal difference trend did exist between FA and control conditions ($p=0.07$). But it showed a non-significant difference trend between FA and MTX ($p=0.38$), and a non-significant difference trend between MTX and control. These results are represented graphically in Figure 4.

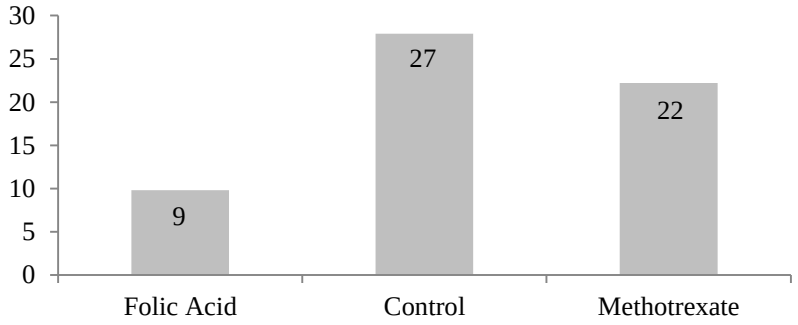

Figure 4. mean number of neurites formed per DRG explant. FA treatment caused a marginal trend toward reducing the number of neurites.

3.3 Effect Of FA On Number Of Phase-Refractile Cells

Many bright, phase-refractile cells were visible in each of the cultures in all three conditions. These cells contrasted vividly with the abundant gray and flat fibroblastic cells (Figure 2). The mean number of phase refractile cells (neurospheres around the the explant tissue) in the control cultures was lowest as compared to the FA and MTX treated cultures (Table 1). FA showed a trend towards significance for a greater number of phase refractile cells in culture [$F(2,27)=3.06$, $p=0.06$]. The Post Hoc test showed that a marginal trend existed between FA treated and control conditions ($p=0.07$). It also showed a non-significant trend between FA and MTX ($p=1.00$), and between MTX and control ($p=0.30$), for mean number of phase refractiles.

Table 1. mean number of neuronal and synaptic vesicle exhibiting cells

	Phase refractile (neurosphere)	**Red (synaptic vesicle positive)**
Folic Acid	16	4
Control	4	12
Methotrexate	14	10

3.4 Effect Of FA On Number Of Synaptogenic Areas

Following immunostaining, areas in the culture dishes where differentiation of neurons had resulted in the production and assembly of synaptic vesicles were apparent as antibody-bound, peroxidase-generated red coloration. These were visible as red patches within the DRG explants, but also as regions of red-tinged nexuses peripheral to the DRGs where cell out-migration and neurite extension had occurred. These were easily visible using phase contrast optics as seen in Figure 2, but could be even more easily discerned using brightfield optics because the red was more obvious against the plain background. The mean number of these synaptogenic areas in the control cultures was 12, but was only 4 for the FA treated DRG cultures, and for the MTX treated DRG cultures it was 10 (Table 1). FA significantly reduced the number of synaptogenic areas [$F(2,27)=15$, $p<0.001$]. Post Hoc testing using the Bonferroni correction showed that a significant difference existed between FA and control conditions ($p<0.001$). A significant difference also existed between FA treated and MTX treated ($p=0.001$), but the difference between MTX and control was not significant ($p=0.52$).

4. Discussion

The results described here add a possible biological rationale for the theory of underconnectivity. Although FA is imperative to ensure proper neural tube development from conception through the first trimester of human pregnancy, this study suggests limitations may need to be placed on FA consumption. With increased FA supplementation in grain-derived foods and the oral FA supplementation push, many women are consuming high amounts of FA. Adults normally circulate 3-15 ng/mL FA, children around 5-21 ng/mL. The level of FA used in this experiment was 442 times higher than the recommended amounts. However, the overconsumption of foods and supplements with FA should be given consideration. Multivitamins taken daily are supplemented with 400 µg FA, and FA only oral supplements contain 400 µg or 80 µg. Common food servings such as one-half cup of spaghetti contain 83 µg FA and a one-cup cereal serving contains 100 µg FA[1].

Fetal Bovine Serum, used in culture medium at 10% by volume, contains small amounts of FA. In these experiments methotrexate was used to block synthesis of FA in order to obtain a depleted FA condition. Because the effects of MTX are inhibited in the presence of antibiotics and or antimicotics, these cultures were carried out without those supplements, extra care being taken to avoid contamination. Although the FA concentration was not measured, some depletion of it compared to the controls was likely, and the results indicated that the MTX cultures were similar in neurogenesis to controls, except for their apparent ability, along with FA, to increase the number of phase refractile cells. Therefore it seems that the FA concentrations considerably higher than those normally present in serum were responsible for the inhibition of neurogenesis. Day eight of chick embryo incubation is the time when DRG neurons begin to innervate the spinal cord and connect to peripheral receptors. Neurogenesis processes are

simultaneously intense in both DRG and spinal cord, as well as in the brain at this time. The neural tube closes at both ends (though later at the caudal neuropore) within the first trimester in humans. It is during this time that folate is imperative for proper neural tube development.

Neurospheres are cell bodies that have not completed differentiation into neurons and have not sent out neurite processes[11]. We observed that neurospheres were more abundant in the FA and MTX treated cultures than in control cultures. This is an interesting finding and may suggest that FA is having an effect on differentiation, interfering with the potential of the neurons. But because the neurosphere numbers were also higher than control numbers in MTX treated cultures, it may be that the failure to send out numerous robust neurites caused the early differentiating neurons to maintain a more three-dimensional shape and thus appear more phase-refractile. Further research should be conducted on the neurospheres and their potential.

Implied in the overall hypothesis of underconnectivity as a contributing cause of autism is the reasonable assumption that underconnectivity would not be necessarily uniform or even widespread because of variation in regional neural developmental sensitivities. Researchers have found, for example, that there is extensive thalamocortical connectivity in persons with autism[15]. Perhaps underconnectivity is more prevalent in the frontal lobes and corpus callosum, while there is extensive connectivity in various portions of the brain. One cannot deny the importance of the presence of FA during conception through the first trimester, but one cannot ignore the possible adverse implications of continuing increased amounts of FA.

Our findings suggest that further research is necessary to establish the FA concentrations at which the neurogenetic inhibition is first evident and whether it is more potent at even higher concentrations of FA. The neurosphere data suggests FA may be having an effect on differentiation, altering the potential of cells to differentiate into neurons. Further investigation is necessary.

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