Investigating the Preventative Potential of Bioactive Folates in Mitigating Spina Bifida with Hydrocephalus Comorbidity

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A thesis submitted to the National University of Sciences and Technology, Islamabad,

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Supervisor: Prof. Dr. Naila Naz

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MS THESIS WORK

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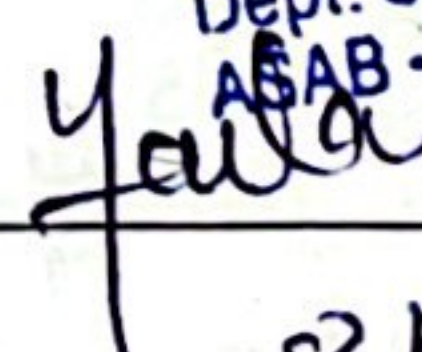
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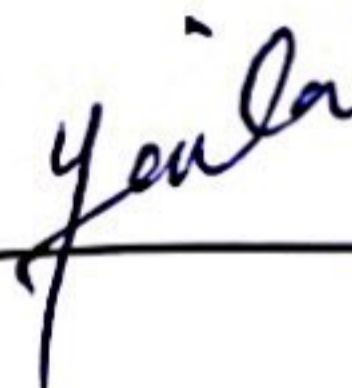**Head of Department**

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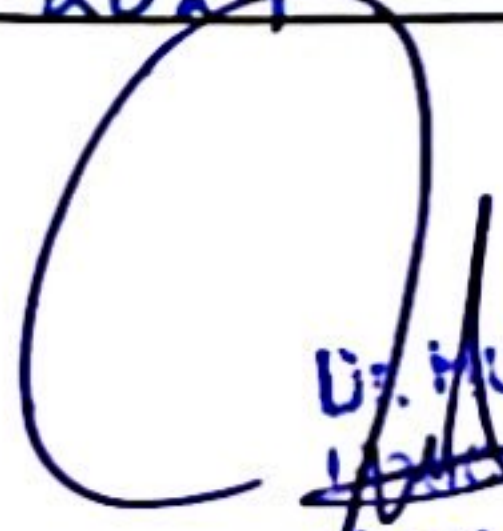Biosciences (ASAB), NUST, Islamabad

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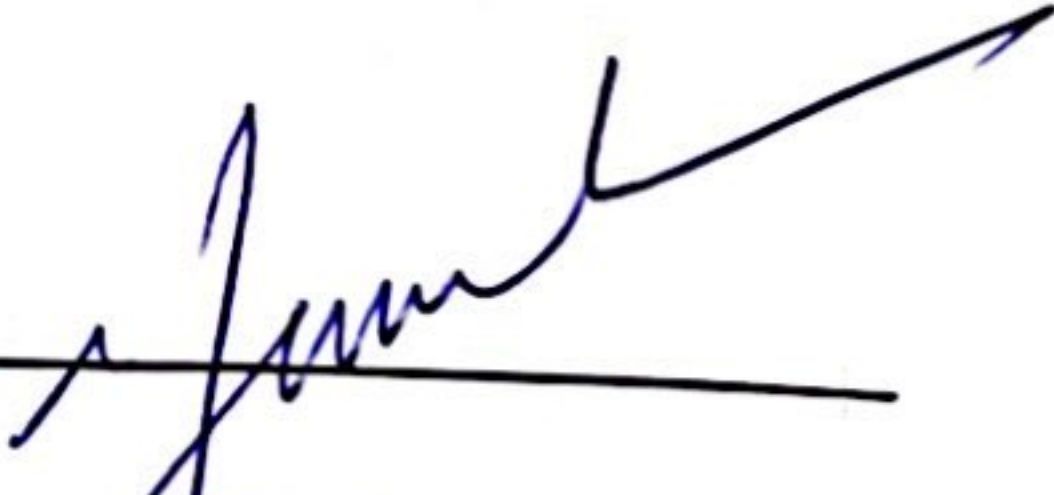
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DEDICATION

This dissertation is dedicated to my beloved late father, to whom I owe all my success

and whose teachings and guidance always enlightened my path.

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Tooba Asif

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5-fTHF	5-formyl tetrahydrofolate
5-mC	5-methylcytosine
5-mTHF	5-methyl tetrahydrofolate
ACM	Arnold-Chiari Malformation
ALDH1L1	Aldehyde dehydrogenase
ANOVA	Analysis Of Variance
BHMT	Betaine-homocysteine methyltransferase
CBS	Cystathionine B-synthase
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
E20	Embryonic day 20
FA	Folic Acid
FDH	10-formyl tetrahydrofolate dehydrogenase
FOLR1	Folate receptor alpha
FR	Folate Receptor
FRα	Folate Receptor A
GABA	Gamma amino butyric acid
H&E	Hematoxylin and Eosin
Hcy	Homocysteine
HC	Hydrocephalus
HDAC	Histone deacetylase

LV	Lateral ventricle
MTHFR	Methylenetetrahydrofolate reductase
MTR	Methionine synthase
NFDM	Non-Fat Dry Milk
NTDs	Neural Tube Defects
PABA	Para-aminobenzoic acid
PCFT	Proton-Coupled Folate Transporter
PFRs	Placental Folate Receptors
PNS	Peripheral Nervous System
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay
RFC	Reduced Folate Carrier
ROS	Reactive Oxidative Species
RT	Room Temperature
SAM	S-Adenosyl Methionine
SB	Spina Bifida
THF	Tetrahydrofolate
UMFA	Unmetabolized Folic Acid
VPA	Valproate or Valproic Acid

ABSTRACT

Folates are crucial in the entire process of pregnancy, and their deficiency can result in congenital diseases such as spina bifida (SB), which are often comorbid with hydrocephalus (HC). Maternal folic acid supplements can precipitate the incidence of hydrocephalus in neonates, and any fault in the folic acid metabolism can prevent the growing embryo from receiving the bioactive folates required for cellular processes. This highlights a need to look for something more promising than folic acid supplementations. We aimed to identify and compare the preventative impact of synthetic and bioactive maternal folate supplements on CNS in an animal model of SB-HC comorbidity generated through valproic acid. Three maternal folate supplements were selected: folic acid (synthetic folate), folinic acid (bioactive folate), and folinic acid+ 5-methyltetrahydrofolate (5-mTHF) (mixture of bioactive folates). The gross appearance and morphological study of the fetuses revealed hydrocephalus precipitation in the folic acid group, which was best prevented by the treatment of folinic acid+5-mTHF. Cerebral folate expression was increased, and methylation levels were decreased in the folic acid group. This indicates that the folates have not been utilized in the DNA and RNA methylation, which is necessary for cerebral development. The activity of cerebral folate receptor alpha was also reduced in the folic acid group. The best preventative effects regarding folate utilization, methylation, and folate receptor activity were observed in the case of folinic acid+5-mTHF. The data indicates that bioactive folates, especially a mixture of folinic acid and 5-mTHF, outperform synthetic folate in the prevention of SB-HC comorbidity.

Keywords: Spina bifida, Hydrocephalus, Folic acid, Bioactive folates, Folinic acid, 5-mTHF.

CHAPTER 1:   INTRODUCTION

1.1   Background

Folate, also known as vitamin B9, is a group of water-soluble B9 vitamins that are a natural part of several foods, such as leafy green vegetables, legumes, and certain fruits (Naderi & House, 2018). Folic acid, a synthetic folate, is primarily utilized in medications and dietary supplements. It is the most oxidized folate form, and to be physiologically active in the body, it must be reduced (Blom et al., 2006). All natural and synthetic folates must be converted to 5-methyl tetrahydrofolate (5-mTHF), which is the circulating folate form, Figure 1.1. 5-mTHF is the most common physiological folate, readily accessible as a dietary component and without metabolization. It is most commonly delivered to peripheral tissues for cellular processes. 98% of all the folates in human plasma are present in the form of 5-mTHF (Page et al., 2017).

The primary biological function of folates is to act as coenzymes in one-carbon metabolism, which is critical for synthesizing nucleotides, methylation reactions, and amino acid metabolism (Ducker & Rabinowitz, 2017). These biological processes are vital for cellular growth and proliferation and the proper functioning of the nervous system. For this reason, folates are particularly essential during embryonic development, especially in the formation of neural tubes (Balashova et al., 2018). Folate deficiency before or during pregnancy is responsible for neural tube defects (NTDs) such as hydrocephalus and spina bifida in infants (Wald, 2022). Spina Bifida develops due to failure in the fusion of neural tubes at the spinal level during the third week of gestation, thus resulting in a portion of the spinal cord and its surrounding tissues being exposed (Botto et al., 1999). Spina bifida

varies widely concerning severity, from mild forms with little to no symptoms to severe forms associated with physical and neurological impairments (Apkon et al., 2014). Hydrocephalus secondary to spina bifida is most likely to occur in developing countries where maternal malnutrition and folate deficiency are common (Warf, 2011). Hydrocephalus is the abnormal accumulation of cerebrospinal fluid (CSF) within the brain ventricles, which increases intracranial pressure and damages brain tissues. The unifying theory on how spina leads to hydrocephalus is that the Chiari II malformation is associated with the obstruction of CSF flow around the lower brain stem that leads to hydrocephalus (McLone & Knepper, 1989).
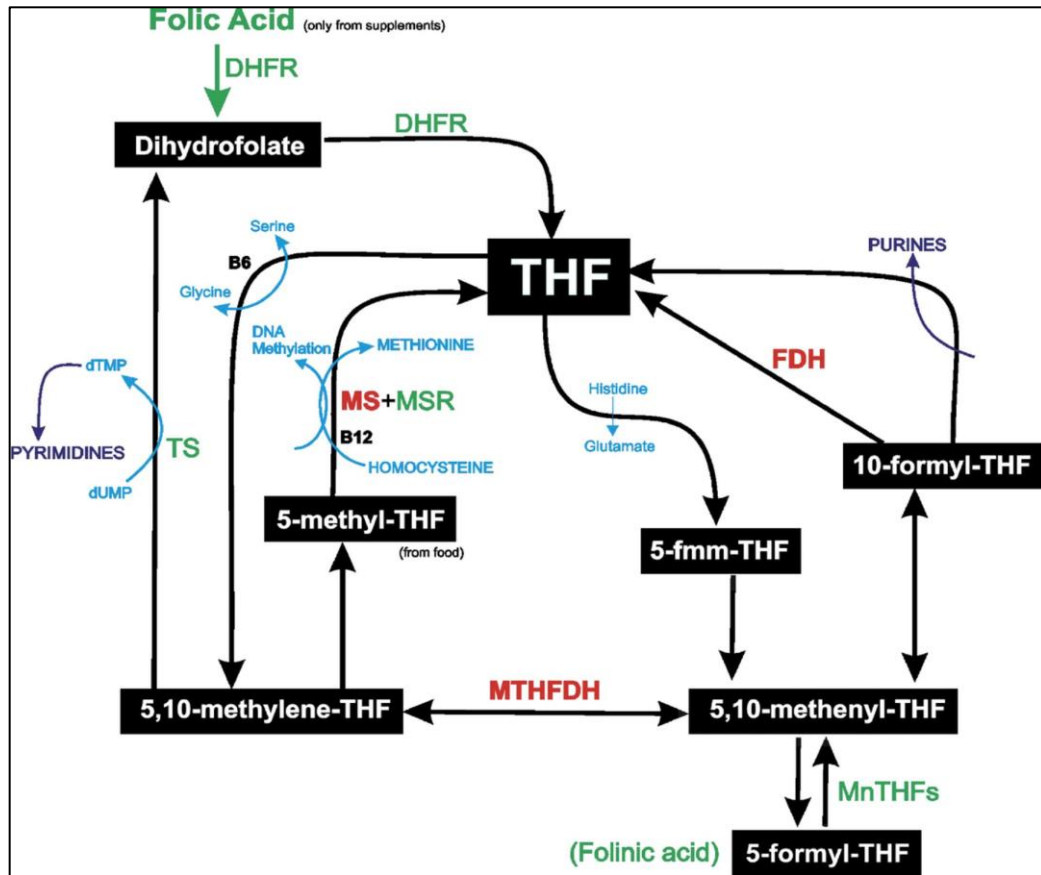
Figure 1.1: Diagrammatic Illustration of Key Events in Folate Metabolism and Relationship among the Metabolites.

DHFR= Dihydrofolate reductase, THF= Tetrahydrofolate, FDH= Folate dehydrogenase, TS= Thymidylate synthase, MTHFDH= Methyltetrahydrofolate dehydrogenase (Cains et al., 2009).

1.2   Prevalence and Prevention

NTDs such as spina bifida are among the most common neurological disorders in neonates (Christianson et al., 2006). The incidence of NTDs worldwide is two in every 1000 live births (Kancherla, 2023). In developing non-income countries, NTDs are responsible for 29% of neonatal deaths (Adeleye et al., 2010; de Paul Djientcheu et al., 2008). In Pakistan, there is no formal data available to indicate the prevalence of NTDs. However, some studies suggest it to be 12 to 14 cases per 1000 live births (Goswami et al., 2015; Saleem et al., 2010). Spina bifida comorbid with hydrocephalus presents a complex clinical challenge, as both conditions are reported to severely affect the individual's quality of life and functional abilities. (Bendt et al., 2020). Current treatment approaches primarily involve the management of symptoms through surgical and rehabilitative interventions (Blount et al., 2020). However, these methods do not tackle the underlying developmental deficiencies that contribute to these conditions.

The most effective preventative measure against NTDs in neonates is maternal folate supplementation before and during the pregnancy. Folate consumed by the mother is preferentially transported to the placenta, where it is supplied and absorbed by the growing embryo via a network of folate transporters and receptors (Yasuda et al., 2008). Folate supplementation rescues these conditions due to its potential to restore cell development in the neuroepithelium of the neural plate and neural fold. Folic acid is given to women of childbearing age as a dietary supplement. Folic acid supplementations have been shown to prevent up to 75% of NTD cases with the daily standard dose of 400mcg throughout the periconceptional period (Berry et al., 1999; Czeizel & Dudas, 1992; De Wals et al., 2007).

1.3   Problem Statement

Maternal folic acid supplementation has been proven to lessen the occurrence of NTDs for many years substantially (Blencowe et al., 2010; de Paul Djientcheu et al., 2008).However, an individual study has revealed that it can precipitate the incidence of hydrocephalus (Cains et al., 2009). Moreover, any disruption in normal folate metabolism or transport can prevent the fetus from receiving the bioactive form of folate even though the FA supplementation was given during the periconceptional period. Bioactive folates such as 5-mTHF deficiency are associated with increased homocysteine (Hcy) levels in the circulation that have been associated with multiple pregnancy-related issues such as recurrent pregnancy loss, NTDs, and premature birth (Serrano et al., 2018; Tinelli et al., 2019).

The rationale of the current research is to confirm the superior efficacy of natural and active folate forms to prevent congenital neural tube defects like spina bifida and associated comorbidity of hydrocephalus. While folic acid has shown effectiveness in averting these disorders, any disruption in folate metabolism that hinders the fetus from accessing the active folate form can lead to severe consequences. Hence, it is crucial to identify a secure alternative to mitigate the risk of these life-threatening disorders.

1.4   Research Objectives

- To generate a non-genetic rat model of spina bifida with hydrocephalus comorbidity using valproic acid.
- To assess changes in fetal cerebral folate metabolism in a rat model of spina bifida with hydrocephalus comorbidity.

- To assess and compare the preventative impact of different maternal folate supplements on spina bifida with hydrocephalus comorbidity.

1.5   Scope and Expected Contributions

The current study will explore the therapeutic potential of bioactive folates, such as 5-methyltetrahydrofolate (5-mTHF) and folinic acid, in an animal model to prevent spina bifida with hydrocephalus comorbidity. Due to the complexity of these conditions, a controlled experimental setting using a rat model will be used to explore the effects of bioactive folates in a systematic and reproducible manner.

Folic acid has been proven effective in preventing neural tube defects such as spina bifida. However, it imposes the risk of precipitating the incidence of hydrocephalus. Moreover, any error in folate metabolism can deprive the fetus of the active form of folate, which will have drastic consequences. Hence, the notion is not the need for folate supplementation but which form of folate is to be given. Addressing this challenge is imperative, prompting the exploration of alternative active forms of folic acid that can efficiently bypass the intricacies of the folic acid cycle as well as have the capacity to reduce the incidence of both disorders at the same time. Hence, this study will serve as the first comparative assessment of bioactive folates in mitigating the incidence of spina bifida with hydrocephalus comorbidity.

CHAPTER 2: LITERATURE REVIEW

2.1 Neural Tube Defects (NTDs)

The most frequent and severe congenital abnormalities are neural tube defects (NTDs), which occur when the neural tube fails to close during embryogenesis. Neural tube development begins at day 16 of gestation when the neural plate is formed and ends with neural tube closure at about day 26-28. The neural tube serves as the predecessor to the central nervous system (CNS) and most of the peripheral nervous system (PNS). The etiology of NTDs is complicated, resulting from interactions between genetic abnormalities and environmental factors (Sever, 1995; Wallingford et al., 2013). The growing neural tube undergoes several processes, such as cell loss, neural crest cell migration, neuroepithelial proliferation, apical cytoskeletal microfilament contraction, and dorsolateral bending. If these developmental pathways do not occur during a critical period of embryogenesis, neural tube closure fails, and the embryo forms an NTD. Different types of NTDs include anencephaly, encephalocele, craniorachischisis, iniencephaly, and spina bifida.

2.1.1 Spina Bifida

Spina bifida (SB), a congenital NTD, develops due to the failure of the neural tube to fuse at the spinal level during the third week of gestation. Spina bifida is further differentiated into four defect-specified subclasses: occulta, closed spinal dysraphism, meningocele, and myelomeningocele. The first kind of spina bifida, occulta, is an asymptomatic, closed NTD caused by a vertebral abnormality that allows for an opening in the spinal column. A layer

of skin often covers the defect and may be accompanied by a hairy patch or dimple. Closed spinal dysraphism, the second kind of spina bifida, is another closed NTD characterized by a lack of at least two vertebral arches as well as fat, bone, or meningeal abnormalities that cover the spinal cord. The third kind of spina bifida is meningocele, which is a closed NTD caused by the extrusion of a meningeal-enclosed pocket of cerebrospinal fluid through the posterior vertebrae or skull. A meningocele lacks all neural components within the extrusion and might be visible or covered with skin. Myelomeningocele, the fourth kind of spina bifida, can be an open or closed NTD caused by the extrusion of the meninges, neural components, and cerebrospinal fluid through the posterior vertebrae, which may or may not be surrounded by the meninges (Copp et al., 2015). Myelomeningocele is the frequent and severe form of spina bifida, as the protruding neural tissues are exposed to amniotic fluid. SB is generally not only limited to the spinal cord but in 85-95% of cases, infants with SB also have a comorbid brain defect (Briner & Lieske, 1995). Spina bifida is frequently associated with conditions such as corpus callosum agenesis, cortical migration problems, hydrocephalus, Chiari malformation type II, vertebral abnormalities, and various genitourinary and gastrointestinal issues (Greene & Copp, 2014).

2.1.2 *Arnold-Chiari Malformation Type II (ACM Type II)*

Arnold-Chiari Malformation Type II (ACM Type II) is a severe congenital brain and spinal anomaly that is closely related to spina bifida, especially to the myelomeningocele (Mahajan, 2021). This condition is characterized by the cerebellar tonsils, vermis, and brainstem being displaced downwards through the foramen magnum into the spinal canal. The malformation usually leads to a range of neurological abnormalities, such as hydrocephalus. The open connection between the spinal cord and the surrounding tissues

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in SB results in a tethering effect, which affects the downward development of brain structures, causing herniation. This displacement can compress the brainstem as well as the upper spinal cord in one way or another, destabilizing the normal CSF dynamics and leading to a series of pathological processes.

2.1.3 Hydrocephalus

Hydrocephalus (HC) refers to the accumulation of CSF in the ventricles and spaces around the brain. HC is associated with an increase in intracranial pressure due to an imbalance in CSF production, absorption, and drainage (Naz et al., 2016). Spina bifida in the presence of ACM is one of the most significant causes of hydrocephalus. The downward displacement of cerebellar tonsils, brainstem, and fourth ventricle through the foramen magnum can obstruct the flow of CSF (Fons & Jnah, 2021). The compression of the fourth ventricle hinders the CSF flow from the ventricular system into the subarachnoid space, which leads to CSF accumulation within the ventricles. This obstruction produces a distal flow block as the choroid plexus is still producing CSF. However, it cannot be efficiently drained, which results in a progression of ventricular dilation and intracranial pressure, which are hallmarks of hydrocephalus (Thomale, 2021).

2.1.4 Folates and NTDs

Maternal nutrition plays a significant role in the complicated etiology of NTDs. Using folic acid supplements during preconception reduces the incidence of NTD-affected pregnancies (Blom et al., 2006). The lack of folate and its metabolites stops the proliferation and migration of neural tissue during neurulation, which leads to NTDs (Isaković et al., 2022). Although the majority of NTD cases are caused by a lack of dietary folate in the absence

of proper supplementation, some pregnant women may still give birth to children with NTDs due to genetic changes in folate metabolism, even if they receive adequate folate supplementation before and during pregnancy. Defects in folate metabolism include the MTHFR C677T gene mutation, which reduces the activity of enzymes essential for folate metabolism, hence lowering blood folate levels (Yang et al., 2015). Exogenous folate antagonists, such as phenytoin, carbamazepine, and other antiepileptic medications, restrict folate absorption or promote folate breakdown. Other antagonists, such as methotrexate, sulfasalazine, and trimethoprim, reduce the activity of DHFR, preventing folate from transforming into active metabolites (Hernández-Díaz et al., 2000). This promotes aberrant apoptosis of neural tissue, which contributes to NTD formation.

Maternal immune responses are thought to alter folate transport, which may impact embryonic development. Rothenberg et al. identified auto-antibodies to the folate receptor in 75% of women who had given birth to NTD-affected babies, compared to just 10% of mothers who had normal infants (Rothenberg et al., 2004). The difference in frequency was associated with a more than 25-fold increase in the incidence of NTDs in babies of women who had these antibodies. This suggests that maternal auto-antibodies that bind to the folate receptor and interfere with folate absorption by target epithelial cells may induce NTDs. This can explain the benefit of periconceptional folate supplementation.

2.1.5 *Folates and Hydrocephalus*

Folate is considered to be an essential factor in the development of a normal brain. This developmental process depends on the supply and transport of the correct folate forms. Any impairment in folate metabolism can lead to hydrocephalus in infants.

FDH is a folate-binding protein that is secreted into the CSF in the brain, where it transports the folate toward the developing cerebral cortex through the fluid (Cains et al., 2009). A study by (Naz et al., 2016) discovered that a lack of FDH causes the inaccessibility of available forms of folate in CSF for the cells in the cortex. The study also established that either tetrahydrofolate (THF) or 5-formyl tetrahydrofolate (5fTHF) supplementation or both in synergy can prevent hydrocephalus and improve the development of the brain in a rat model.

5mTHF is needed in the brain at the choroid plexus and neuroepithelia during the process of differentiation. This was confirmed in a study by (Naz et al., 2016), where 5mTHF was found to be localized in vesicles with FDH and FRα in the lateral ventricle (LV). Such vesicles were also described by (Bachy et al., 2008; Harrington et al., 2009), which transport FDH-5mTHF and FRα-5mTHF complexes to various parts of the brain.

Nuclear folate, a critical cofactor, plays an important role in the synthesis, repair, and methylation of DNA (YOON et al., 1976). The first product of DNA methylation is 5mC, which is involved in gene regulation. A study by (Naz et al., 2016) found that the nuclear FDH was linked to low or none of the nuclear folate and an increased expression of 5mC, which is suggestive of folate transport into the nucleus via FDH and its utilization in DNA methylation. The study showed reduced nuclear FDH in the hydrocephalic rats with an increase in nuclear folate. It diminished 5mC which indicated a failure of DNA methylation due to a lack of FDH even though the folates were present. This establishes the critical requirement of FDH for DNA methylation, as its reduction or absence will reduce the DNA methylation even with the presence of folates, which will remain unused in the nucleus of the cells.

2.2　Folates

Folate is a broad term for a group of water-soluble B-complex vitamins, also known as vitamin B9. The term folate originated from the Latin word folium, meaning leaf; hence, folates are abundant in green leafy foods (Ifergan & Assaraf, 2008). Folates are present in different forms within the cell that can be interconverted via multiple complex biochemical reactions catalyzed by numerous enzymes (Wagner, 2001).

2.2.1　Cellular Functions of Folates

Folate is a rate-limiting vitamin involved in many cellular processes, such as DNA biosynthesis and methylation, protein and lipid methylation, the urea cycle, and the biosynthesis of neurotransmitters (Lucock, 2000; McKay et al., 2004)Folate is fundamental for accurate DNA synthesis due to its involvement in the synthesis of purines and pyrimidines. It is also critical in genome regulation and development as it provides one carbon required in the methylation of DNA.

2.2.2　Folate Variants

Folate molecules share a standard central structure that consists of three chemical moieties: a pteridine ring, which is able to oxidize or reduce; a central para-aminobenzoic acid (PABA), which, along with the pteridine ring, forms the attachment sites for 1C units, and a polyglutamate chain which determines the molecule length and its intracellular localization. (Ducker & Rabinowitz, 2017). Tetrahydrofolate consists of a reduced pteridine and thus is the physiologically active form of folate. The reduced folate form, typically 5-mTHF, is naturally present in the diet and the body in humans (Wright et al.,

2007). The bioavailability and metabolism of folate variants depend on their chemical composition. The folate group contains structurally similar substances such as folic acid (FA), 5-mTHF, and natural folates (Carboni, 2022). FA is the oxidized and mono-glutamate folate that was primarily synthesized as pure crystals in the 1940s. It is included in dietary supplements and foods such as cereals, pasta, bread, and fruit juice. Folic acid is an artificial form of folate that cannot perform any biological processes. In order to exert its biological activity in the human body, it is reduced first to dihydrofolate (DHF) and then tetrahydrofolate (THF) so that it can enter the folate cycle. Food contains natural folates, as do various artificial polyglutamate forms. In the gut, food folates are first hydrolyzed to mono glutamate and then, through active transport, are absorbed across the intestinal mucosa. As a result, before it enters the circulation, the dietary folate is reduced to tetrahydrofolate (THF) and then methylated (5-mTHF). The bioactive form 5-mTHF is the most common folate present in blood as well as in the umbilical cord blood. It is readily accessible as a dietary component and does not need further metabolization. As a result, it is the form most commonly delivered to cells for cellular processes. 5-mTHF makes up for around 98% of the folate in human plasma (Page et al., 2017).

Aldehyde dehydrogenase (ALDH1L1) or 10-formyl tetrahydrofolate dehydrogenase (FDH) is the central enzyme of folate metabolism and mainly exerts its expression in the brain and liver (Krupenko & Oleinik, 2002). FDH is critical for the maintenance of folate levels and the balance of folate metabolism, (CHAMPIoN et al., 1994).

2.2.3   One-Carbon Metabolism

One-carbon metabolism refers to a complicated metabolic system based on the biochemical interconversion of folate molecules. Folic acid functions as a one-carbon metabolic hub, activating and transferring methyl groups for the synthesis of purine and thymidine (Locasale, 2013). One-carbon metabolism consists of the folate and homocysteine pathways. Folic acid in plasma reaches tissue cells via folate receptor (FR), and dihydrofolate reductase (DHFR) converts it to tetrahydrofolate Figure 2.1. Next, tetrahydrofolate is converted to 5, 10-methylenetetrahydrofolate. Then, methylenetetrahydrofolate reductase (MTHFR) transforms 5, 10-methylenetetrahydrofolate into 5-mTHF, which provides a methyl group for methionine synthase (MTR) to catalyze the homocysteine conversions into methionine. This step requires a cofactor of vitamin B12. 5-mTHF can enter the cells via a reduced folate carrier (RFC). An end-product of choline oxidative metabolism is betaine, which provides a substitute methyl donor for the betaine-homocysteine methyltransferase (BHMT)-catalyzed methylation of homocysteine reaction. Cysteine is formed by cystathionine β-synthase (CBS) through the action of vitamin B6 as a coenzyme in the transfer-sulfuration pathway (Obeid & Herrmann, 2012). 5-mTHF donates a methyl group in the remethylation pathway, where Hcy is methylated to methionine by methionine synthase, with vitamin B12 acting as a cofactor (Obeid & Herrmann, 2012). Methionine further converts to S-adenosyl methionine (SAM), which is a critical methyl donor and a common cofactor involved in several epigenetic and biosynthetic processes (Su et al., 2016). The cross-linking of folate and the homocysteine cycle causes an inverse relation between the levels of Hcy and folate.
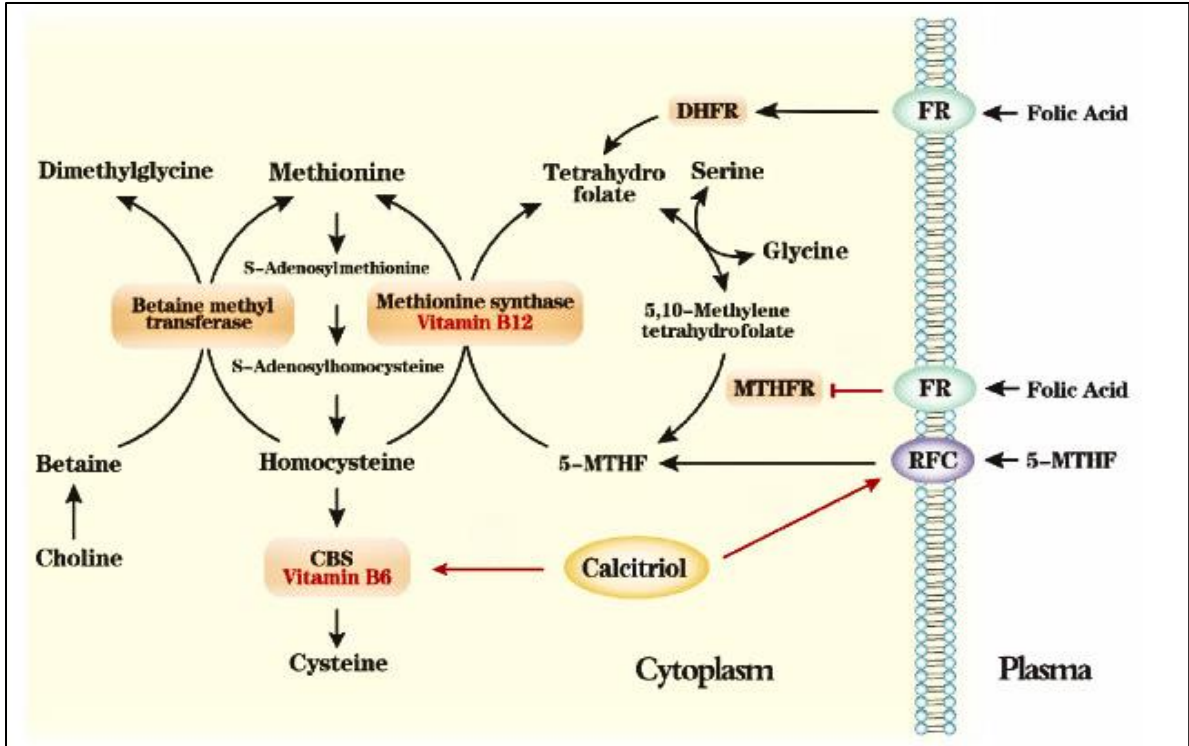
Figure 2.1: Diagrammatic Illustration of One-carbon Metabolism.

FR (folate receptor); RFC (reduced folate carrier); 5-mTHF (5-methyl-tetrahydrofolate); MTHFR (methylenetetrahydrofolate reductase); DHFR (dihydrofolate reductase); BMT (betaine– homocysteine methyltransferase); CBS (cystathionine β-synthase); MTR (methionine synthase) (He & Li, 2023).

2.3   Folate Supplements

Folate supplementation can reverse DNA hypomethylation, indicating folate's effect on DNA methylation (Pufulete et al., 2005). Abnormal DNA methylation causes various diseases, including neurological conditions (Urdinguio et al., 2009). Cerebral folate deficiency is associated with a variety of neurological disorders, such as hydrocephalus (HC) and neural tube defects (NTDs), including spina bifida (SB) and exencephaly.

2.3.1 Folic Acid (FA)

Folic acid is commonly used as a dietary supplement and to fortify food because of its low cost, high thermostability, and significant bioavailability (Molloy, 2002). However, folic acid does not have a coenzyme function, and it must be converted into the metabolically active form, tetrahydrofolate, within cells. The enzyme dihydrofolate reductase (DHFR) first reduces FA to dihydrofolate, which is subsequently converted to tetrahydrofolate. This is a rate-limiting phase, resulting in low DHFR activity in humans, with significant inter-individual variation.

2.3.1.1 Side Effects of Folic Acid (FA)

High FA dosages can cause fast saturation or inhibit the activity of the DHFR enzyme, which leads to the buildup of unmetabolized FA (UMFA) and UMFA syndrome (Scaglione & Panzavolta, 2014). Furthermore, some persons have genetic variants that reduce the activity of DHFR. UMFA competes with natural folate (5-mTHF) for the folate transporter and receptor, reducing the activity of bioactive folates in metabolic cycles. A study found that 86% of UMFA in the hepatic portal vein, while practically all bioactive folate is successfully converted (Patanwala et al., 2014; Pietrzik et al., 2010). UMFA has been found in the umbilical cord and baby blood, raising concerns about potential health risks in neonates (Menezo et al., 2022; Sweeney et al., 2005).

Genetic polymorphisms of methyltetrahydrofolate reductase (MTHFR) can decrease the subsequent metabolization of dietary folates and folic acid into 5-mTHF. MTHFR is eminently polymorphic in the general population, with several MTHFR gene variations found. Today, 35 uncommon but detrimental MTHFR mutations, polymorphisms, and nine

common variations have been identified. The two most prevalent are A1298C and C677T. A polymorphic MTHFR enzyme may work partially at 55% to 70% efficiency in comparison to a normal MTHFR enzyme. Globally, around 40% of individuals have an MTHFR polymorphism. This polymorphism is linked to an increase in thermolability and a decrease in the specific activity of MTHFR in vivo, which results in 65% residual enzyme activity for heterozygous carriers and just 30% for homozygous carriers (van der Put et al., 1998; Verhoef et al., 2017).

2.3.2   *Bioactive Folates*

Bioactive folate supplementation of 5-mTHF avoids the complete folate cycle, which may be affected by the MTHFR polymorphism. In this case, 5-mTHF is directly absorbed and utilized in biological processes. As a result, adopting 5-mTHF as a dietary supplement rather than FA is strongly advised (Scaglione & Panzavolta, 2014). Supplementation with 5-mTHF does not appear to create UMFA since it is immediately utilized by cells and undergoes the one-carbon metabolism necessary for DNA synthesis and red blood cell formation (Servy et al., 2018). Bailey et al. confirmed this discovery, demonstrating that 5-mTHF allows folate utilization more rapidly and evenly than folic acid and without the production of UMFA (Bailey & Ayling, 2018). Some studies have investigated the response of 5-mTHF supplementation on people with MTHFR polymorphisms. Prinz-Langenohl et al. found that the MTHFR gene polymorphism did not affect 5-mTHF supplementation (Prinz-Langenohl et al., 2009). Other studies demonstrate that 5-mTHF supplementation can alleviate folate deficiencies caused by MTHFR deficiency (Litynski et al., 2002; Vidmar Golja et al., 2020).

Disregarding the source of 5-mTHF, methyl folate, along with vitamin B12, enters the one-carbon cycle. One carbon cycle is a network of interconnected biochemical events that occur in all of the body's cells and is necessary for a variety of cellular processes. Low levels of 5-mTHF disrupt one-carbon metabolism, significantly contributing to an increase in the levels of circulating homocysteine (Hcy) and toxic buildup in the circulation (Tinelli et al., 2019). Prolonged exposure to Hcy, also known as hyperhomocysteinemia, is known to damage blood vessels, causing blood clots, leading to the development of cardiovascular illness, as well as neuronal diseases. Healthy blood vessels and levels of folate are required for conception and pregnancy. Excessive Hcy can make it challenging to conceive and sustain a pregnancy (Blom & Smulders, 2011). Many studies have found that hyperhomocysteinemia is linked to a variety of pregnancy issues, including recurrent pregnancy loss, NTDs, preeclampsia, premature birth, placental abruption, fetal growth restriction, and gestational diabetes mellitus (Serrano et al., 2018; Yadav et al., 2021; Zhao et al., 2020). Cawley et al. also discovered that newborn birth weight and mother Hcy levels were adversely associated (Cawley et al., 2020).

2.4   Role of Folates during Pregnancy

Folate plays a vital role in cell development, division, synthesis, and DNA repair. During pregnancy, folate needs to rise not just to promote fetal development and growth of maternal tissue but also to lower the risk of low birth weight, preterm birth, high Hcy levels, and other adverse pregnancy issues. The recommendation is to take folate three months ahead of conceiving and for the first three months of pregnancy to avoid neural tube defects (NTDs) in neonates. NTDs are developed in the first 28 days of pregnancy (van der Put et al., 1998). Pregnant women with MTHFR gene variants are at higher risk to develop a

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variety of birth abnormalities due to an inability to metabolize FA. Furthermore, increased Hcy due to folate insufficiency is another risk factor for a variety of pregnancy-related conditions.

The placenta is responsible for concentrating folates in the fetal circulation (Giugliani et al., 1985; Thorand et al., 1996). Therefore, the level of folates in the umbilical cord is retained even when the levels of maternal folate are low (Wallace et al., 2008). Placental folate receptors (PFRs), namely FR-α, in the syncytiotrophoblast's microvillus membrane facilitate folate transport across the placenta (Bisseling et al., 2004). Maternal folates bind to FR-α with high affinity and are internalized via receptor-mediated endocytosis (Keating et al., 2009). Other transporters, such as the reduced folate carrier (RFC) and the proton-coupled folate transporter (PCFT), play critical roles in folate absorption in the placenta (Prasad et al., 1995). This leads to an increased intervillous blood folate content in the placenta, which may be delivered to the fetus via passive diffusion and the RFC (Henderson et al., 1995). This transport system is developed during the first trimester of pregnancy (Solanky et al., 2010).

2.5 Placental Folate Transport during Pregnancy

The placenta acts as a protective barrier that provides a site for nutrients and waste exchange between the mother and the fetus through transporters (Baumann et al., 2002; Cetin, 2003). Folate receptor α (FRα) is a glycosylphosphatidylinositol-linked glycoprotein that binds folates with high affinity at the surface of the membrane and regulates the unidirectional flux after the internalization of the receptor-folate complex (Antony et al., 1985; Sabharanjak & Mayor, 2004). Reduced folate carrier (RFC/SLC19A1) binds to a

membrane transporter to regulate internalization through membranes, which in turn mediates a bidirectional flux (Ganapathy et al., 2004). These are pH-sensitive carriers and are expressed in the intestine as well as in the placenta (Damaraju et al., 2005; Rajgopal et al., 2001). The placenta develops during a short time, and with the progress of gestation, its function changes. The levels of expression of the transporters that transfer the nutrients and waste between the mother and the fetus vary throughout the pregnancy (Novak et al., 2006; Yamaguchi et al., 1996).

Yasuda et al. (2008) confirmed the involvement of FRα and RFC in the transport of folates from maternal blood to placental villi as the RFCs are localized on the apical side of the villi. FR α and RFC showed involvement in folate transport in the placental brush border membranes; however, not all folate transport was mediated by these two. HCP1 is another proton-coupled, pH-sensitive folate in the placenta that regulates the folate transport mechanism (Qiu et al., 2006; Yasuda et al., 2008).

2.5.1   *Folates in the Development of Neural Tube and Nervous System*

Folate is significantly involved in the development and maturing of the neural tube and nervous system in the embryo. Folate is transported into the neural cells through the folate receptor 1 (FOLR1) or reduced folate carrier (RFC) Figure 2.2. Once inside the cell, folate is utilized in the biosynthesis of DNA and RNA, DNA repair, and one-carbon metabolism. These cellular processes are required for the division of neural cells during embryogenesis, a period of rapid growth and neural tissue repair. Folates interact with their receptors and trigger a specific signaling cascade that is responsible to shape the neural plate during the

formation of the neural tube. It also mediates the synaptic activity, thus facilitating neural function (Balashova et al., 2018).
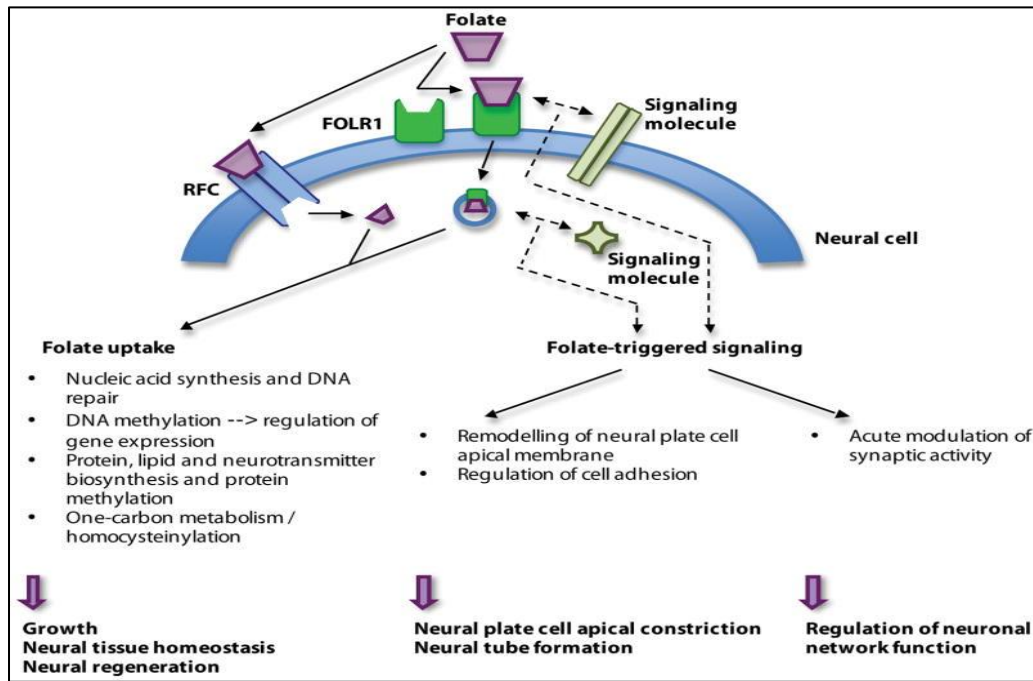
Figure 2.2: Role of Folates in the Development of Neural Tube and the Nervous System.

RFC= Reduced folate carrier (Balashova et al., 2018).

2.6   Valproic Acid (VPA)

Valproate or valproic acid (VPA) is an anti-epileptic drug used to treat all forms of seizures (Romoli et al., 2019). VPA is also used to manage other neurological diseases, such as headaches, migraine, bipolar disorder, psychological disturbances, and neuropathic pain (Tomson et al., 2016)Valproate's anti-convulsant action is due to an increase in the synaptic level of gamma amino butyric acid (GABA), which inhibits the production of action potentials. VPA also blocks the sodium and calcium channels, reducing seizures. The therapeutic formulations of VPA are available as tablets, capsules, oral solutions, and intravenous injections.

Valproate is a short-chain, branched fatty acid derived from natural valeric acid. Consumption of valproic acid to control seizures during pregnancy is associated with teratogenesis in neonates, as VPA can cross the placental barrier and disrupt the normal process of organogenesis (Shakya et al., 2020). VPA can induce a wide range of congenital disorders, such as limb anomalies, exencephaly, lip and palate cleft, craniofacial cleft, fused ribs, fused vertebrae, and spina bifida (Hughes et al., 2018; Tanoshima et al., 2015). Teratogenic effects of valproate are produced due to an increase in apoptosis that leads to an imbalance between cell proliferation and cell death (Manthou et al., 2020). Oxidative stress due to the overproduction of reactive oxidative species (ROS) and mitochondrial and lysosomal dysfunction is associated with a decrease in cell viability (Salimi et al., 2022). VPA can interfere with the polycomb group proteins that modulate the chromatin structure during normal fetal development, which can lead to teratogenicity (Okada, Aoki, et al., 2004). VPA also causes restricted intrauterine growth and a low number of live births due to post-implantation losses (Lin et al., 2019).

2.6.1   Role of Folic Acid

Folic acid is critical for normal metabolic and biochemical processes in the human body, such as cellular growth, gene regulation, production of red and white blood cells, renewal of epithelium, and synthesis of chemicals that regulate brain function (Gazzali et al., 2016)Folic acid is especially needed during embryogenesis for the normal development of tissues associated with rapid cell division.

A study by Rahman and Madkour (2023) co-administered FA with VPA in mice during the gestation period, which resulted in a significant improvement against all the adverse

effects of VPA (Rahman & Madkour, 2023). FA can cross the placental barrier to promote the normal development of the fetus during organogenesis (Ebara, 2017). FA negates the teratogenic effect of VPA by alleviating the oxidative stress caused by VPA (Hsieh et al., 2012). FA also mitigates the inhibition of histone deacetylase (HDAC), an enzyme required for normal DNA replication, caused by VPA (Dawson et al., 2006). The protective effect of FA was attributed to its ability to improve the closure of lumbosacral neural folds by promoting normal neurogenesis and stem cell proliferation. FA also suppresses VPA-induced apoptosis, preserves neuroplasticity and neuronal function in the area of the growing spinal cord, and supports appropriate histogenesis of the developing vertebral arches of the vertebral column (Ichi et al., 2010). Folic acid supplementation during early pregnancy enhances the growth of the fetus and placenta by increasing cell proliferation, DNA and RNA production, and amino acid metabolism (Papadopoulou et al., 2013).

2.6.2 *Animal Model of Spina Bifida Using VPA*

Several animal models of SB have been developed using various teratogens, such as vitamin A, trypan blue, retinoid, and folic acid antagonists. The first successful and reliable animal model of SB was developed in mice through the administration of valproic acid during the period of neural tube development (Ehlers et al., 1992). (Briner & Lieske, 1995) replicated this model in rats by treating the animals with 1200 mg/kg of VPA at day 10 of gestation. The VPA treatment caused the vertebral arches to widen. The embryotoxicity of VPA was also observed as the litter size got smaller with the high dose of VPA. Rat fetuses treated with VPA also produced Arnold-Chiari malformation (ACM). The animals, however, did not exhibit the hydrocephalus that is associated with ACM as in humans, which might be due to the inability of their hindbrains to be rotated ventrally as in humans.

This could result in the drainage of CSF even if the contents of the posterior fossa were crowded in the rats.

VPA administration during the early gestation period is attributed to faulty osteogenesis and chondrogenesis of skull bones and cartilage. Intrauterine exposure to VPA can also cause spina bifida as it interferes with the spinal neural groove closure and prevents the fusion of vertebral arches (Ceylan et al., 2001). VPA can induce skeletal malformations as it alters gene expression, leading to abnormal development of intra-embryonic mesoderm (Okada, Kurihara, et al., 2004). Alteration of gene expression of placental transporters due to VPA can lead to defective transport of nutrients and gases across the placenta to the fetus, which causes growth restrictions and fetal death (Jinno et al., 2020).

CHAPTER 3: MATERIALS AND METHODS

3.1 Animals

Female and male rats of the Sprague-Dawley strain were used to generate the animal model. The rats, female and male, were obtained from the animal house (National Institute of Health, Islamabad) with an average weight of 200g and about 10-12 weeks of age.

3.2 Animal husbandry and breeding procedure:

The ethical approval for performing experiments and animal handling was approved by the Internal Review Board of Atta Ur Rahman School of Applied Biosciences, National University of Science and Technology, Islamabad. All the experiments were conducted according to the ethical guidelines provided by the Laboratory Animal House (LAH), Atta ur Rahman Scholl of Applied Biosciences, National University of Science and Technology, Islamabad. They were kept in the animal house for a week to acclimate before mating. The female rats were divided randomly into five groups: Group I, "Control"; Group II, "Diseased"; Group III, "Treatment 1 (Folic Acid)"; Group IV, "Treatment 2 (Folinic Acid)"; and Group V, "Treatment 3 (Folinic Acid+ 5 methyltetrahydrofolate)". The rats were provided with the standard feed and tap water. The animals were housed in a suitable room maintained at 25± 1 °C with 12 hours of darkness and 12 hours of artificial light. Female rats from each group were mated with males of the same age and strain for 36 hours. The animals were examined for the presence of a copulatory plug, and the first 24 hours after conception was allotted embryonic day 0, with the following calendar day as embryonic day 1. After mating, the females were removed and housed separately.

3.3 Drugs and Drug Administration

3.3.1 Group I (Control)

The female rats received a standard diet and tap water and were administered sterile saline at embryonic day 10 (E10).

3.3.2 Group II (Diseased)

The female rats received 600 mg/kg sodium valproate via subcutaneous injection once in the morning and once in the evening, with an interval of 7 hours, for a total dose of 1200 mg/kg (Briner & Lieske, 1995).

3.3.3 Group III (Treatment 1: Folic Acid)

Along with sodium valproate, the female rats were also administered a subcutaneous dose of 2.25mg/kg folic acid in an aqueous solution one week before mating and throughout pregnancy (Cains et al., 2009).

3.3.4 Group IV (Treatment 2: Folinic Acid)

Along with sodium valproate, the female rats were also administered a subcutaneous dose of 2.25mg/kg folinic acid in an aqueous solution one week before mating and throughout pregnancy (Cains et al., 2009).

3.3.5 Group V (Treatment 3: Folinic Acid+5 methyltetrahydrofolate)

Along with sodium valproate, the female rats were also administered a subcutaneous dose of 2.25mg/kg, an equal mixture of folinic acid and 5 methyltetrahydrofolate in an aqueous solution one week before mating and throughout pregnancy (Cains et al., 2009).

3.4   Specimen and Tissue Collection

On embryonic day 20 (E20), dams were sacrificed, and their abdomens were opened to collect fetuses. The litter size for each group was observed. The fetuses were examined for changes in their gross appearance and external malformations. In terms of specimen and tissue collection, the total litter was divided into two sections. The fetuses in the first section were preserved in a solution of 10% formalin in 0.1M phosphate buffer until being examined for internal malformations. The fetuses in the second section were first subjected to CSF collection via lumbar puncture using a glass capillary and then dissected to harvest the brains. The brains were treated with liquid nitrogen and later preserved at -80ºC for protein analysis.

3.5   Histochemistry

Histochemistry was performed using hematoxylin and eosin (H&E) stain on 15-20 µm sagittal section of fetuses to demonstrate the changes in brain and spine morphology as well as in the ventricular size and cortical thickness. The changes in brain and spine morphology were observed under 40X magnification, and images were captured. The ventricular size and cortical thickness were examined under 100X magnification, and

images were captured. The ventricular size and thickness of cortical layers, marginal zone, cortical plate, sub-plate, and intermediate zone were measured using ImageJ software.

3.6   Total Protein Extraction

Total protein was extracted from the brain tissues of fetuses using RIPA buffer (50 Tris HCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1% Tween 20, 150mM NaCl, and 1mM EDTA) with 1mM phenylmethylsulfonylfluoride (PMSF) (bioworld) protease inhibitor. Brain tissues were homogenized using a Hielscher homogenizer and centrifuged at 16000 rpm for 20 min at 4ºC. The supernatant was collected and stored at -20ºC.

3.7   Protein Determination and Western Blots

Protein concentration was determined using a Nanodrop instrument (Titertek-Berthold). A concentration of 50 µg of protein was loaded into each well of 12% (w/v) polyacrylamide 10 well Mini-PROTEAN Tetra cell (BIO-RAD) for SDS PAGE electrophoresis. Protein wet transfer was carried out using the Mini Trans-Blot cell (BIO-RAD) for 90 min. The PVDF membrane was stained with Ponceau solution for 15-20 seconds to verify the success of the transference. The membrane was washed 3×15 min with 1x TBS-Tween 20. For the western blot, the membrane was blocked with 5% non-fat dry milk (NFDM) in 1x TBST for one hour at room temperature (RT). The primary antibodies (Table 1) were diluted in 5% non-fat dry milk (NFDM) in 1x TBS-Tween 20. Primary antibody incubation was performed overnight at 4ºC on a rotating shaker. The membrane was washed with 1x TBS-Tween 20 for 4×15 min with shaking at room temperature. Secondary antibodies were diluted with 5% non-fat dry milk (NFDM) in 1x TBS-Tween 20. Secondary antibody

incubation was carried out for one hour at room temperature with shaking. The membrane was washed with 1x TBS-T for 4×15 min with shaking at room temperature.

Table 3.1: Antibodies used in Western Blot and Dot Blot.

Antibody	Company	Catalogue No.	Dilution
	Western Blot		
Anti-rabbit FR alpha	proteintech®	29472-1-AP	1: 5000
Anti-mouse METTL3	proteintech®	67733-1-IG	1: 5000
	Dot Blot		
Anti-mouse 5mC	proteintech®	68301-1-IG	1: 5000
Anti-rabbit Folic Acid	Cloud-Clone Corp.	PAA610Ge01	1: 5000

3.8   Dot Blots

Dot blot was performed for folic acid on the brain protein sample and 5-methylcytosine (5mC) on the brain protein sample. A PVDF membrane was first activated in methanol for 5 minutes at 4ºC and then air dried at room temperature. Folic acid and 5-methylcytosine (5mC) were analyzed by dot blot for the qualitative analysis of the differential concentration of these compounds across the five experimental groups. 2µl of each sample containing 50µg protein concentration was pipetted onto pre-determined locations on the

PVDF membrane and air dried. Membranes were blocked in 5% non-fat dry milk (NFDM) in 1x TBS-Tween 20 for one hour. Membranes were washed for 3×15 min with 1x TBS-T. The membranes were incubated with primary antibodies (Table 1) at 4ºC overnight. Membranes were washed for 3×15 min with 1x TBS-T and then incubated with secondary antibody for one hour. Both primary and secondary antibodies were diluted in the 5% non-fat dry milk (NFDM) in 1x TBS-Tween 20. All the steps were performed with continuous shaking on a rocking table.

An ECL kit (SuperSignal ™ West Pico PLUS Chemiluminescent Substrate) by thermoscientific was used to expose the membranes for both western and dot blot. Results were obtained using the chemidoc instrument (company) and were recorded using ImageLab 5.2.

3.9   Statistical Analysis

Western blot and dot blot quantification were performed using the Image J software. Multiple comparisons for mean comparisons between the five groups were made using one-way analysis of variance (ANOVA) using GraphPad Prism 8.

CHAPTER 4: RESULTS

4.1　Changes in Litter Size

The number of pregnant rats in each group and their average litter size are described in Table 4.1. Figure 4.1 illustrates the difference in the litter size across the five groups. The litter size in the diseased group was significantly smaller as compared to control ($p<0.05$). This validates the teratogenic effect of valproic acid and the potential presence of NTD, which is responsible for the termination of pregnancy. Treatment 3 (Folinic acid+ 5mTHF) best prevents this teratogenic effect and potential presence of NTD in comparison with the diseased group ($p<0.05$).

Table 4.1: Number of Pregnant Rats and average Litter Size

Group	No. of Pregnant Rats	Average Litter Size
Control	04	11
Diseased	06	07
Folic Acid	06	08
Folinic Acid	06	09
Folinic Acid+5mTHF	06	11
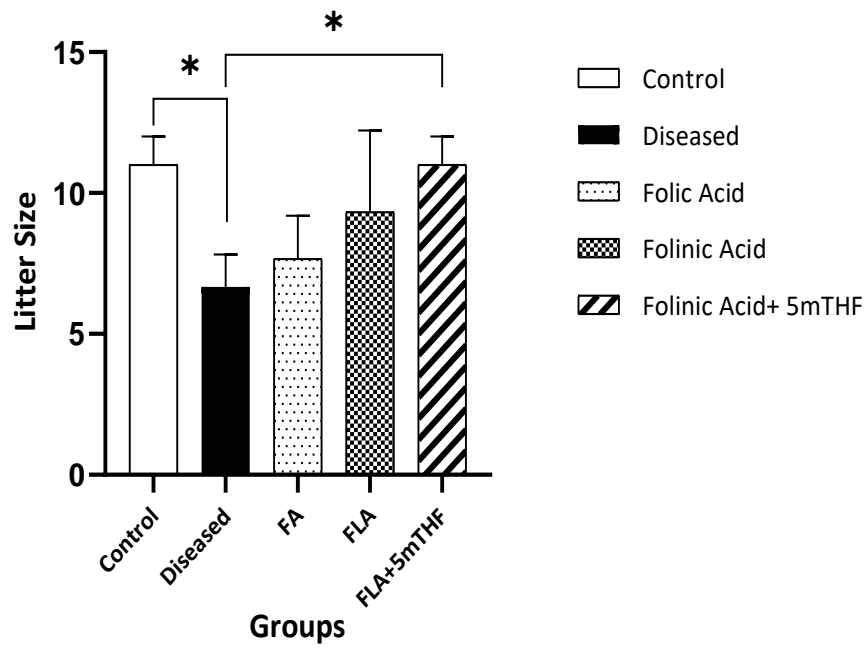
Figure 4.1: Average litter size across the five experimental groups.

Each value depicts the mean ± SEM. * illustrates $p < 0.05$.

4.2   Changes in Gross Appearance

The gross appearance of fetuses of each group was examined to check the presence of spina bifida and hydrocephalus comorbidity Figure 4.2. As compared to the control, diseased fetuses show a hump in the spine region, indicating the presence of spina bifida, along with a dome-shaped head, indicating the presence of hydrocephalus comorbidity Figure 4.2 (b). Treatment 1 (Folic acid) improved the incidence of spina bifida indicated by the lack of hump in the spine region; however, a dome-shaped head indicated the precipitation of hydrocephalus Figure 4.2 (c). Bioactive folates in both Treatment 2 (Folinic acid) and Treatment 3 (Folinic acid+ 5mTHF) proved effective in preventing this comorbidity Figure 4.2 (d, e).
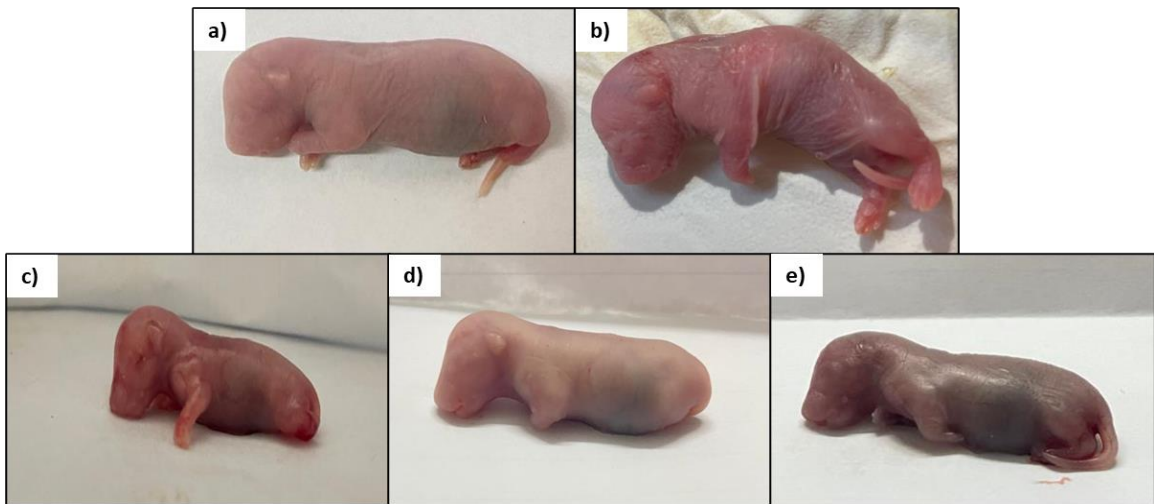
Figure 4.2: Gross appearance of the fetuses at E20.

(a) Control: shows a normal head and spine appearance; **(b)** Diseased: shows an enlarged and dome-shaped head and a hump in the spine region; **(c)** Treatment 1 (Folic acid): shows a dome-shaped head with a normal spine; **(d)** Treatment 2 (Folinic acid): shows a normal head and spine; **(e)** Treatment 3 (Folinic acid+5mTHF): shows a normal head and spine appearance.

4.3   Changes in Spine and Brain Morphology

Hematoxylin and eosin staining was performed on the sagittal sections of the five groups' fetuses to examine the differences in spine and brain morphology (Figures 4.3 and 4.4). The spine morphology revealed distorted cells and sac in the diseased fetuses, thus validating the presence of spina bifida Figure 4.3 (b). Treatment 1 (Folic acid) and Treatment 2 (Folinic acid) rescued the normal morphology to some extent Figure 4.3 (c, d). Treatment 3 (Folinic acid+ 5mTHF) best prevented the incidence of spina bifida Figure 4.3 (e).
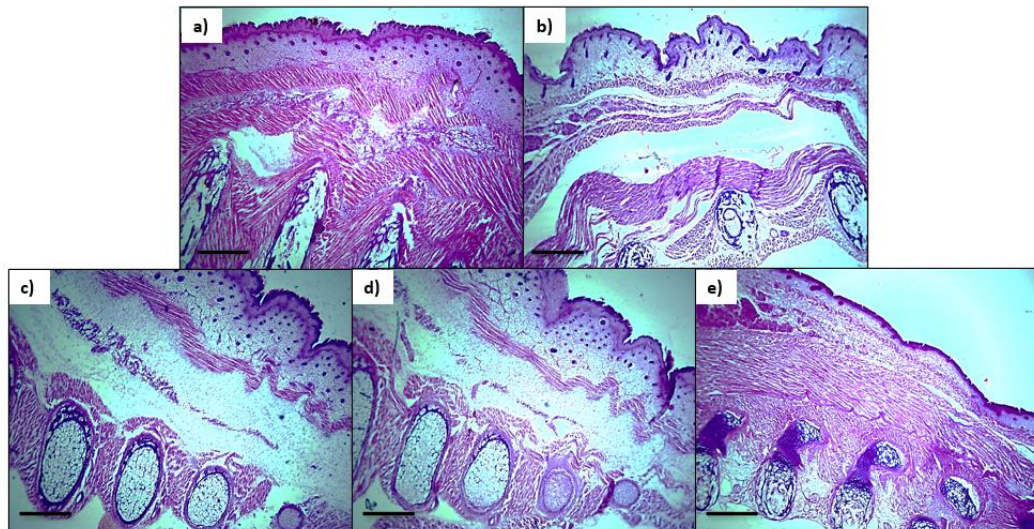
Figure 4.3: Representative images of the fetal spine at E20.

Hematoxylin and eosin-stained sagittal sections; Magnification= 40X, Scale bar=200μm. **(a)** Control: shows normal spine morphology; **(b)** Diseased: altered cellular density and presence of a sac; **(c)** Treatment 1 (Folic acid): altered cellular density; **(d)** Treatment 2 (Folinic acid): altered cellular density; **(e)** Treatment 3 (Folinic acid+5mTHF): normal spine morphology.

H & E staining of sagittal sections of the whole brain revealed distorted tissues and ventricles in the diseased fetuses, thus validating the comorbidity of hydrocephalus Figure 4.4 (b). Treatment 1 (Folic acid) also shows altered brain morphology with increased ventricle size and increased subarachnoid space, indicating the precipitation of hydrocephalus Figure 4.4 (**c**). However, the best preventive results were observed in the case of Treatment 2 (Folinic acid) and Treatment 3 (Folinic acid+ 5mTHF) Figure 4.4 (d, e).
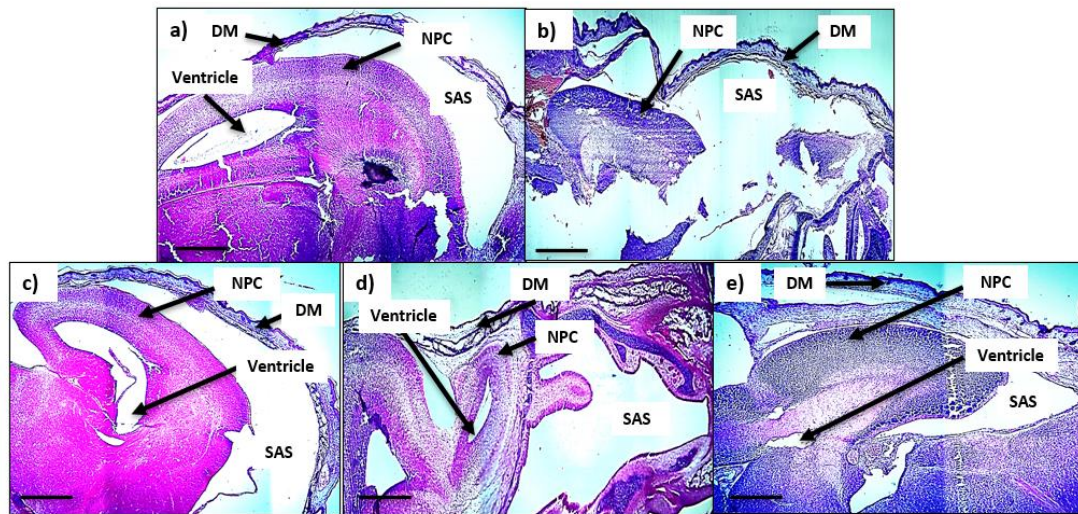
Figure 4.4: Representative images of the fetal brain at E20.

Hematoxylin and eosin-stained sagittal sections; Magnification= 40X, Scale bar=200μm. **(a)** Control: normal brain morphology; **(b)** Diseased: distorted brain morphology and ventricles; **(c)** Treatment 1 (Folic acid): increased ventricle size and increased SAS; **(d)** Treatment 2 (Folinic acid): normal brain morphology; **(e)** Treatment 3 (Folinic acid+5mTHF): normal brain morphology. DM= Dura mater; NPC= Neopallial cortex; SAS= Sub arachnid space.

4.4   Changes in Cortical Cellular Density

Figure 4.5 illustrates the differences in the cortex's cellular density in the five groups. The diseased fetuses displayed a reduced cellular density in the cortical region, which indicates low cell proliferation, maturation, and migration Figure 4.5 (b). The cellular density was improved to some extent in Treatment 1 (Folic acid group) and Treatment 2 (Folinic acid). However, Treatment 3 (Folinic acid+ 5mTHF) best improved the cellular density to a comparable level to that of the control group Figure 4.5 (c, d, e).
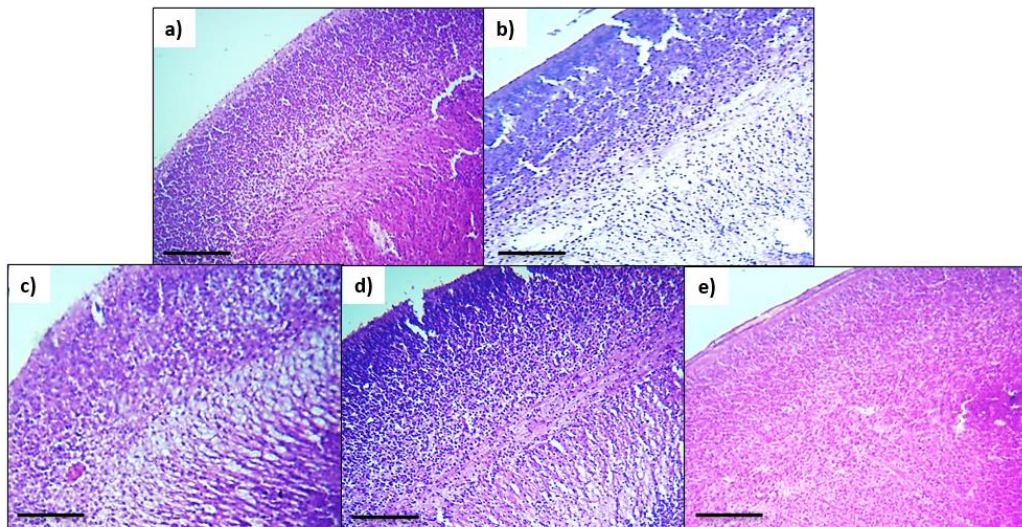
Figure 4.5: Representative images of fetal cortical cellular density at E20.

Hematoxylin and eosin-stained sagittal sections; Magnification= 100X, Scale bar=100µm. **(a)** Control: normal cellular density; **(b)** Diseased: altered cellular density; **(c)** Treatment 1 (Folic acid): altered cellular density; **(d)** Treatment 2 (Folinic acid): improved cellular density; **(e)** Treatment 3 (Folinic acid+5mTHF): normal cellular density.

4.5   Changes in Thickness of Cortical Layers

Hematoxylin and eosin staining was performed for the comparison of the thickness of cortical layers in the brain, such as the marginal zone, cortical plate, subplate, and intermediate zone, across the five experimental groups at E20. Cortical layers were measured using the ImageJ software to examine the differences in their thickness Figure 4.6, and the graphs for each layer were plotted to compare the differences among the five groups Figure 4.7.

4.5.1   Marginal Zone

Control pups demonstrate the thickest marginal zone. In the diseased group, the marginal zone was significantly reduced ($p < 0.001$). All three folate treatments were equally effective in preventing the reduction of marginal zone Figure 4.7 (a).

4.5.2   Cortical Plate

The cortical plate, like the marginal zone, was significantly decreased in the diseased group ($p < 0.001$) Figure 4.7 (b). Treatment 1 (Folic acid) and Treatment 3 (Folinic acid+ 5mTHF) best prevented the decrease in the cortical plate.

4.5.3   Sub Plate

There was not much difference between the sub-plate thickness in the control and diseased groups Figure 4.7 (c). An increase in the thickness of the sub-plate was observed in Treatment 1 (Folic acid) and Treatment 2 (Folinic acid). In Treatment 3 (Folinic acid+ 5mTHF), the sub-plate was comparable to the control group.

4.5.4 Intermediate Zone

An increase in the intermediate zone was observed in the diseased group as compared to the control group. However, this increase was not indicative of a healthy brain, as the cortical morphology revealed a significant reduction in cellular density and a decrease in layered cell organization. The intermediate zone was decreased in Treatment 1 (Folic acid), which may indicate the precipitation of hydrocephalus. A normal intermediate zone was observed in Treatment 2 (Folinic acid) and Treatment 3 (Folinic acid+ 5mTHF) Figure 4.7 (d).
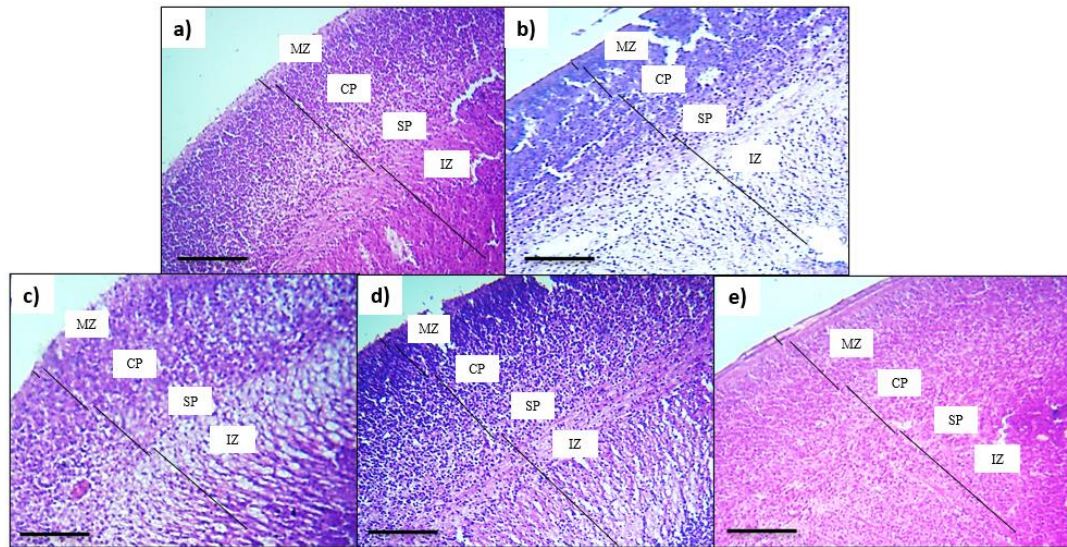
Figure 4.6: Representative images of the thickness of the fetal cortical layers at E20.

Hematoxylin and eosin-stained sagittal sections; Magnification= 100X, Scale bar=100μm. **(a)** Control: normal cortical layer thickness; **(b)** Diseased: reduced marginal zone and cortical plate, increased intermediate zone; **(c)** Treatment 1 (Folic acid): increased cortical and subplate; **(d)** Treatment 2 (Folinic acid): normal cortical layer thickness; **(e)** Treatment 3 (Folinic acid+5mTHF): normal cortical layer thickness. MZ= marginal zone, CP= cortical plate, SP= subplate, IZ= intermediate zone.
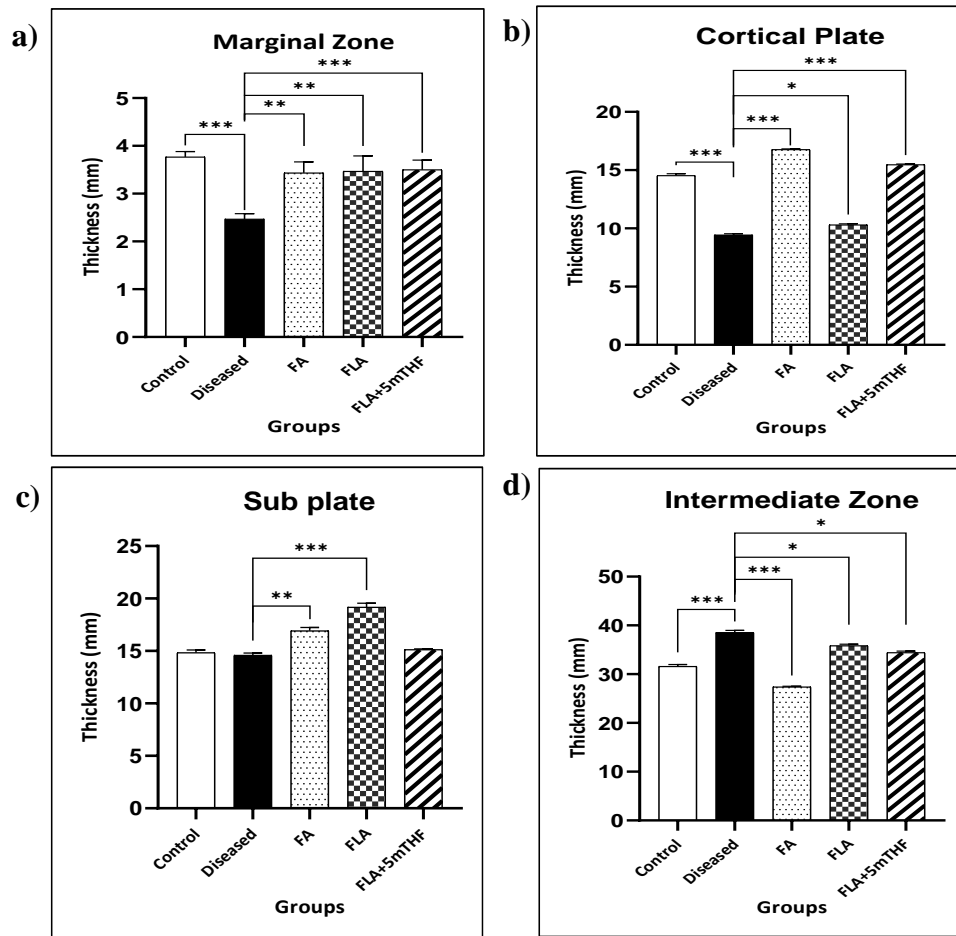
Figure 4.7: Differences in the thickness of the cortical layers in rat fetuses at E20.

(**a**) Marginal Zone; (**b**) Cortical Plate; (**c**) Sub Plate; (**d**) Intermediate Zone. Each value depicts the mean ± SEM. * illustrates $p < 0.05$, ** illustrates $p < 0.01$, *** illustrates $p < 0.001$.

4.6   Folate Expression in CSF and Brain

Dot blot analysis was performed on the proteins extracted from the brain and CSF to determine changes in folate expression. Densitometry analysis revealed an increase in CSF folate expression and a significant increase (p<0.001) in cerebral folate expression in the diseased group. This indicates that the folates are available but not being utilized. CSF folate expression was significantly reduced in all three folate treatments (p<0.001). In the brain, however, Treatment 3 (Folinic acid + 5mTHF) showed the slightest folate expression, revealing the quick utilization of these bioactive folates (p<0.0001).
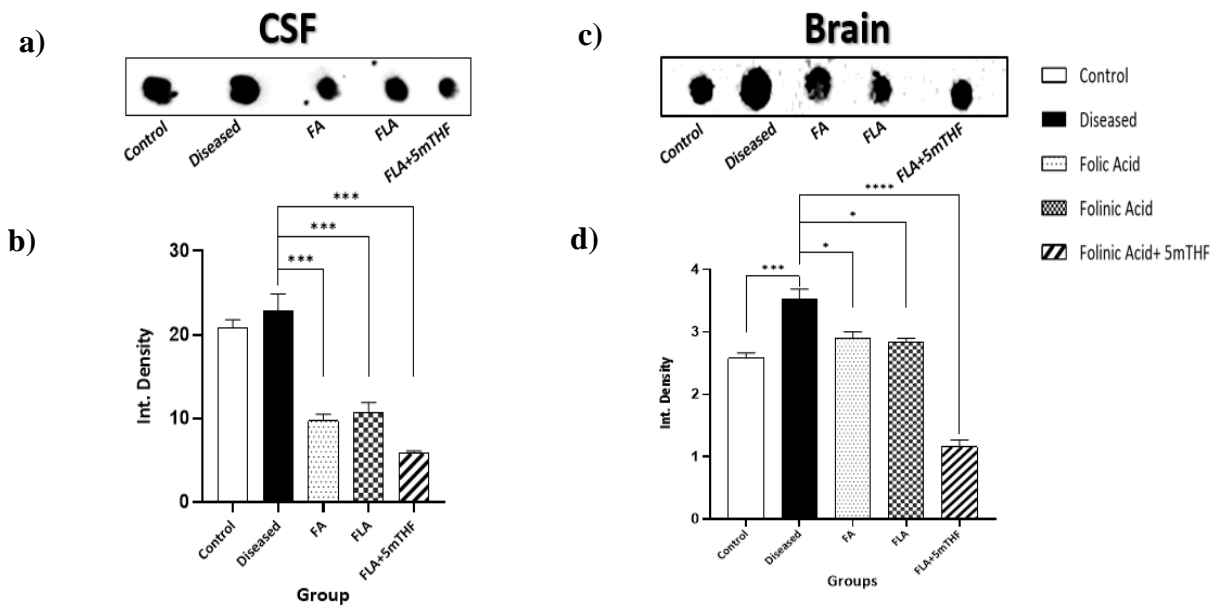
Figure 4.8: Dot blot analysis of fetal CSF and brain at E20.

(a) Dot blot of CSF folates; **(b)** Graphical representation of folate expression in CSF; **(c)** Dot blot of cerebral folates; **(d)** Graphical representation of folate expression in brain. Each value depicts the mean ± SEM. * illustrates p < 0.05, *** illustrates p < 0.001, **** illustrates p < 0.0001.

4.7   Folate Receptor Expression in CSF and Brain

Western blot analysis of fetal CSF proteins indicated a significant decrease in FOLR1 (Folate receptor α) expression in the diseased group. Bioactive folate treatment indicates a significant up-regulation of FOLR1, indicating an increase in folate transport.
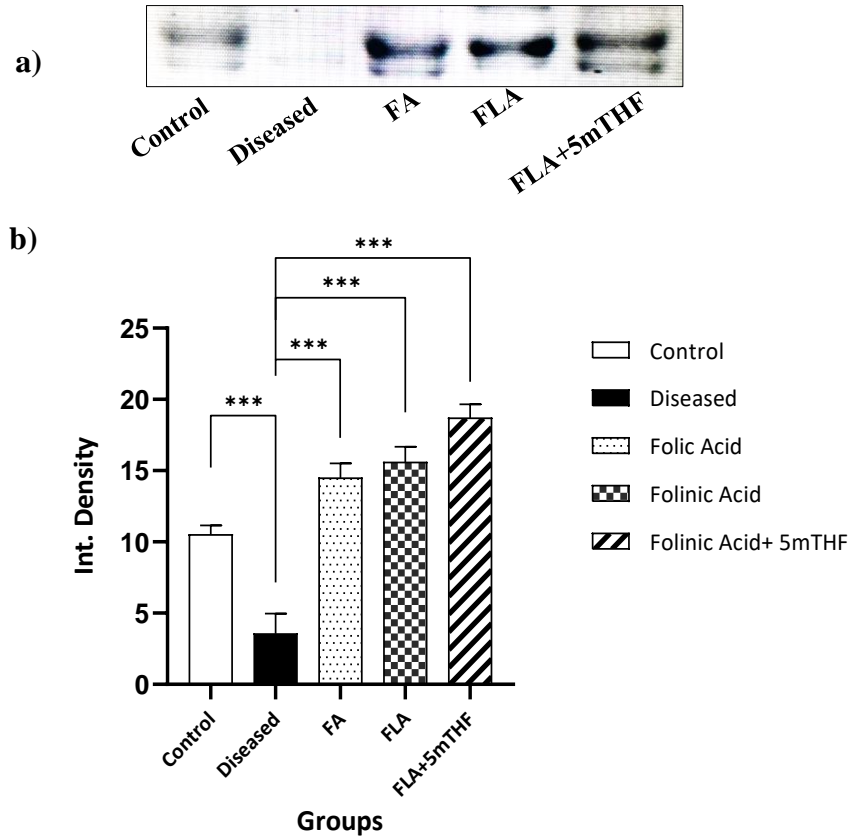
Figure 4.9: Western Blot of FOLR1 expression in fetal CSF at E20.

(**a**) Western blot of CSF FOLR1; (**b**) Graphical representation of FOLR1 expression in CSF. Each value depicts the mean ± SEM. *** illustrates $p < 0.001$.

4.8   Methylation Status in the Brain

Dot blot analysis of cerebral proteins for 5mC revealed a significant decrease in its expression (p< 0.0001) in the diseased group, indicating a decreased DNA methylation. Methylation was slightly increased in the case of Treatment 1 (Folic acid). However, both Treatment 2 (Folinic acid) and Treatment 3 (Folinic acid + 5-mTHF) showed an increased expression of 5mC.
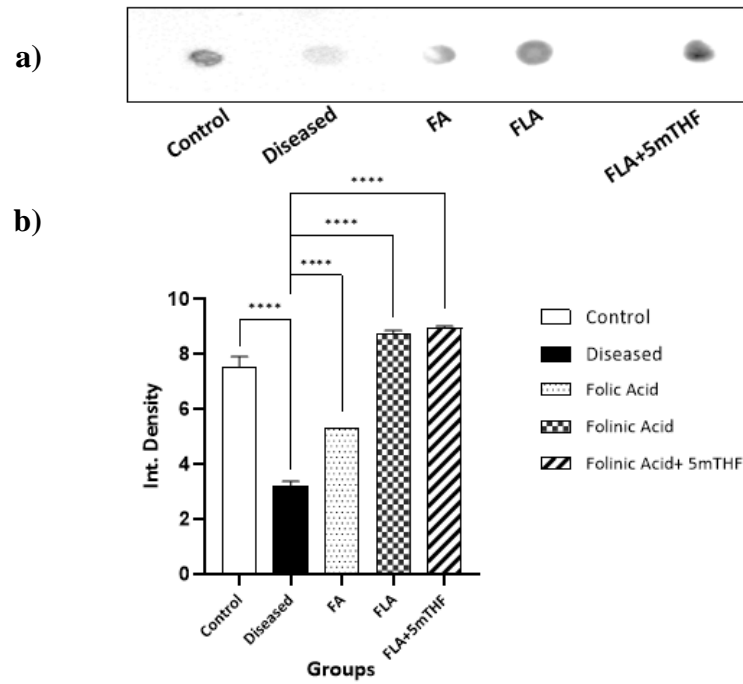
Figure 4.10: Dot blot analysis of cerebral 5mC at E20.

(a) Dot blot of fetal cerebral 5mC; **(b)** Graphical representation of cerebral 5mC expression. Each value depicts the mean ± SEM. **** illustrates $p < 0.0001$.

Western blot analysis of cerebral proteins for METTL3 revealed a significant decrease in the expression in the diseased group, indicating a reduction in RNA methylation. Treatment 1 (Folic acid) increased the METTL3 expression to some extent. However, treatment with bioactive folates, both in the case of Treatment 2 (Folinic acid) and Treatment 3 (Folinic acid+ 5mTHF) showed a significant increase in METTL3 expression.
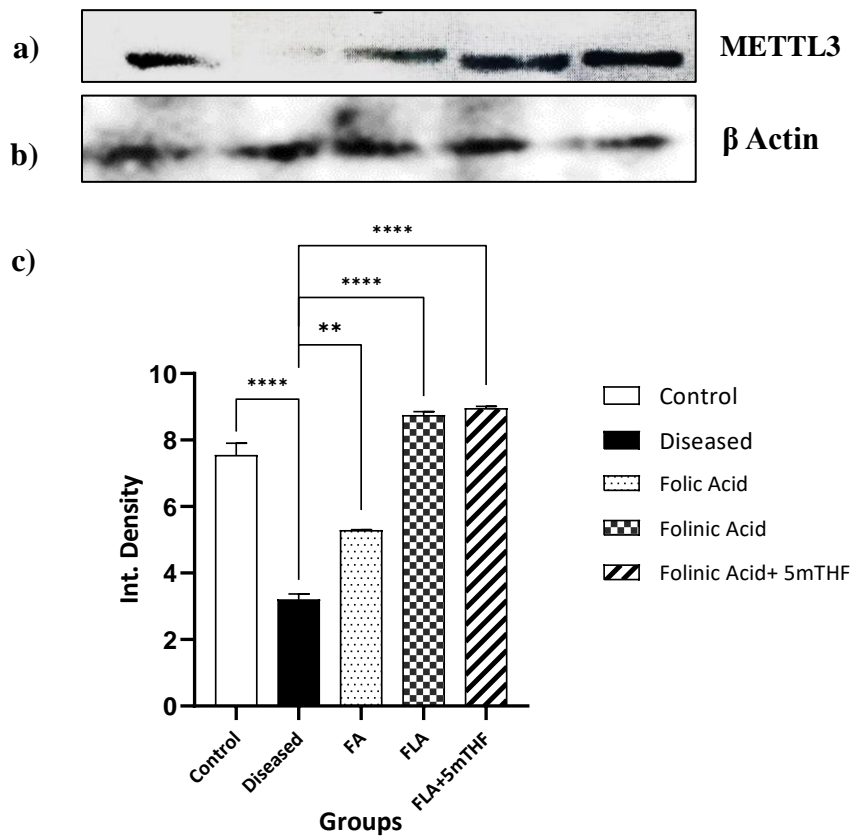
Figure 4.11: METTL3 expression in fetal brain at E20.

(a) Western blot of METTL3 of the fetal brain; MW= 60-70kDa (b) Western blot of β actin of the fetal brain; MW= 30-40kDa (c) Graphical representation of cerebral METTL3 expression. Each value depicts the mean ± SEM. ** illustrates $p < 0.01$, **** illustrates $p < 0.001$.

CHAPTER 5: DISCUSSION

Spina bifida is an NTD that is often comorbid with other neurological conditions such as hydrocephalus. There is no cure for these congenital conditions, and the treatment options available to manage these conditions are limited (Blount et al., 2020). The best course of action, in this case, is to prevent these congenital abnormalities in neonates. Maternal folate deficiency during pregnancy is the primary cause of spina bifida with hydrocephalus comorbidity in neonates (Urdinguio et al., 2009). The routine clinical practice is to recommend folic acid supplements during the periconceptional period to prevent the occurrence of neural tube defects in neonates. However, folic acid is an oxidized folate, and to exert cellular activity, it must be converted into reduced bioactive forms via the folic acid cycle. Any interruption in the folic acid cycle causes a maternal folate deficiency, which prevents the fetus from receiving the bioactive folates from the process of embryogenesis, leading to NTDs. The purpose of our study was to avoid this comorbidity caused by the reduced folate bioavailability by replacing the synthetic folate supplement with bioactive folate supplements.

Valproic acid (VPA) is an anticonvulsant drug used to treat a variety of neurological conditions (Romoli et al., 2019). However, if used during pregnancy, VPA acts as a mutagen and teratogen for the growing embryo (Hughes et al., 2018). Our study exploits this teratogenic activity of VPA to induce spina bifida with hydrocephalus comorbidity in a rodent model and to check the efficacy of synthetic and bioactive maternal folate supplements on the incidence of this comorbidity. Although FA has been proven to be potent in preventing spina bifida for many years, a study by Cains et al., 2013, indicated

that it can precipitate the incidence of hydrocephalus. So, the notion is to use the correct folate supplement to avoid the incidence of not just spina bifida but hydrocephalus as well.

The gross appearance of fetuses (Figure 4.2) and histochemistry analysis (Figures 4.3 and 4.4) confirmed the success of our animal model of spina bifida with hydrocephalus comorbidity. The significant decrease in litter size in the diseased group was consistent with the teratogenic effect of valproic acid, which is responsible for the termination of pregnancy in humans. Moreover, fetuses with neural tube defects are known to terminate before they are even born, which was also observed in our study. The hump in the spine region observed in the diseased group of fetuses was covered with skin which indicated the presence of closed spina bifida or spina bifida occulta. The dome-shaped head confirmed the presence of hydrocephalus comorbidity. The comorbidity usually occurs due to the formation of Arnold Chiari II malformation. Hematoxylin and eosin staining of the sagittal section of the fetuses confirmed the presence of a sac and altered cellular density in the diseased group. Similarly, the altered brain morphology in the diseased group of fetuses revealed a distorted cortex and ventricle. The imbalance in CSF production and drainage in hydrocephalus causes the ventricles to enlarge, and it pushes the cortex outwards towards the skull. This pressure most likely was the reason for the presence of distorted brain tissues. The brain morphology of Treatment 1 (Folic acid) was altered as well, and along with the gross appearance of a dome-shaped head, it confirmed the precipitation of hydrocephalus, although the spina bifida was absent. Maternal bioactive folate supplements, both in Treatment 2 (Folinic acid) and Treatment 3 (Folinic acid+5mTHF), prevented this comorbidity. However, the best prevention was observed in the case of a mixture of bioactive folates, i.e., Treatment 3. This indicates that the bioactive folates were

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readily available for cellular processes such as normal cell proliferation and cell division compared to synthetic folate treatment, as folic acid first must undergo the folate cycle.

Cortical thickness is another factor that was considered when observing the changes in cerebral health due to maternal folate supplements. In hydrocephalus, the development of the cerebral cortex occurs abnormally, and the cortical thickness is reduced (Cains et al., 2009). In our study, we observed the thickness of individual cortical layers. MZ is the outermost cortical layer and consists of Cajal-Retzius cells, which are the earliest neurons to appear during cortical development. This zone plays a critical role in neuronal migration and layering. MZ is also a site for synaptic connections and information processing in the mature brain (Meyer, 2007). The MZ was significantly reduced in the diseased group, confirming the presence of hydrocephalus. However, this reduction was prevented with equal efficacy in all the folate treatments. The cortical plate consists of densely packed neurons that migrate from the ventricular and subventricular zones (Meyer, 2007). This layer was also considerably decreased in the case of diseased fetuses. The subplate consists of migrating neurons and interneurons and is essential for proper cortical wiring, guiding axons to their correct targets, and influencing the maturation of cortical circuits (Meyer, 2007). No difference was observed in the thickness of this layer. However, examination of H&E staining revealed a low cellular density in this area in the diseased group as well as in Treatment 1 (Folic acid). The intermediate zone (IZ) consists of migrating neurons and intermediate progenitor cells and is a site for neuronal migration and axon growth. This zone transforms into the white matter of the mature brain. It was significantly increased in the diseased control group. However, this increase was not subjective to a healthy brain. Cortical morphology revealed a reduced cellular density, cell proliferation, and cell

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migration. All the folate treatments were able to prevent the alteration in the cortical layers. However, it was best prevented in the case of Treatment 3 (Folinic acid, 5mTHF).

We also observed the effect of different maternal folate supplements on cerebral health. If used during pregnancy, VPA can disrupt the maternal folate cycle. Moreover, it can cross the placental barrier and disrupt the folate metabolism in the fetus (Shakya et al., 2020). In this case, folic acid, even if present, cannot be metabolized and converted into its bioactive forms, which are responsible for carrying out critical cellular processes. Folic acid expression would increase, and the same thing was observed in our study. The accumulation of folates in the brain indicates that the folates were not being utilized in the critical cellular processes, thus leading to the precipitation of hydrocephalus. This observation further confirmed the presence of neural tube defect comorbid with hydrocephalus.

Folates are responsible for critical cellular processes such as DNA and RNA methylation (Nazki et al., 2014). During the folate cycle, when 5mTHF is converted into THF, it releases a methyl donor that undergoes a methionine cycle and is eventually utilized for methylation. This establishes a reverse relation between the folate's expression and methylation status. Folate deficiency is associated with a lack of DNA methylation that inhibits the genes required in embryogenesis, thus disrupting gene regulation and cortical development in hydrocephalus. 5mC is the first product of DNA methylation (Crider et al., 2012), and its cerebral expression was significantly reduced in the diseased group. As the folates were pooled in the brain and not utilized, this decreased DNA methylation as well. This decrease may be attributed to cell cycle arrest and low cell proliferation.

The same pattern was observed in the case of METTL3, which indicates the RNA methylation status. Depletion of METTL3 decreases cell proliferation and increases cell apoptosis (Kim & Jang, 2021). METTL3 is also involved in cerebral development, and its reduction affects mRNA stability and cortical neurogenesis. In our study, treatment with bioactive folates decreased the folate levels in both the CSF and the brain, indicating the utilization of folates. Similarly, increased cerebral expression of 5mC and METTL3 indicated the normal methylation of DNA and RNA that eventually prevented the precipitation of hydrocephalus.

FRα acts as a folate transporter that binds and delivers 5mTHF to various regions of the brain, and its activity is altered in a hydrocephalic brain (Naz et al., 2016). Any alterations in folate receptor genes, which in our case were due to VPA, can result in cerebral folate transport deficiency. So, even though the folates are present, their transport inside the cell will be limited. In the current study, we observed a decrease in the folate receptor activity in the CSF of the diseased group, which validates the presence of hydrocephalus. In Treatment 1 (Folic acid), the receptor expression was only partially increased, precipitating hydrocephalus. The maximum expression of FRα was observed in Treatment 3 (Folinic acid+5-mTHF), which proves the superior efficacy of bioactive folates.

Bioactive folates are essential regulators of neural development and function, and these molecules may have the ability to modulate repair processes and positively prevent the incidence of spina bifida and hydrocephalus. The best-known and the most relevant derivative of folate is 5-methyltetrahydrofolate (5-mTHF) – the form that does not require further metabolic transformations in the organism. Although folic acid has proven to be effective in the prevention of spina bifida, the same response has not been observed in the

incidence of hydrocephalus. Studies suggest that bioactive folates such as 5-formyl THF (folinic acid) and 5-mTHF can reduce the incidence of hydrocephalus to a better extent as compared to folic acid (Cains et al., 2009; Naz et al., 2016). Folinic acid is a derivative of tetrahydrofolate, which is an active form of folate and is a precursor for 5-mTHF. Hence, the notion is not the need for folate supplementation but which form of folate is to be given. Addressing this challenge is imperative, prompting the exploration of alternative active forms of folic acid that can efficiently bypass the intricacies of the folic acid cycle as well as have the capacity to reduce the incidence of both disorders at the same time.

Our study analyzed the effect of different maternal folate supplements on spina bifida with hydrocephalus comorbidity on morphological as well as molecular levels. To the best of our knowledge, it is the first study that compared the efficacy of synthetic and bioactive maternal folate supplements on a model of spina bifida with hydrocephalus comorbidity. According to our research, a mixture of bioactive folates consisting of folinic acid and 5-mTHF outperforms the synthetic folate, i.e., folic acid, in preventing this comorbidity. Also, these bioactive folates proved to be best in rescuing the VPA-induced faults in cerebral DNA and RNA methylation. Further studies on gene profiling of fetuses and placenta in order to identify VPA-induced gene modification and how bioactive folates rescued the expression of those genes will further validate our study. Considering the global incidence of NTDs and associated neurological conditions, it is imperative to establish novel and more efficient preventative measures, and the use of bioactive folates might prove to be the first step toward mitigating these conditions.

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ABSTRACT

Folates are crucial in the entire process of pregnancy, and their deficiency can result in congenital diseases such as spina bifida (SB), which are often comorbid with hydrocephalus (HC). Maternal folic acid supplements can precipitate the incidence of hydrocephalus in neonates, and any fault in the folic acid metabolism can prevent the growing embryo from receiving the bioactive folates required for cellular processes. This highlights a need to look for something more promising than folic acid supplementations. We aimed to identify and compare the preventative impact of synthetic and bioactive maternal folate supplements on CNS in an animal model of SB-HC comorbidity generated through valproic acid. Three maternal folate supplements were selected: folic acid (synthetic folate), folinic acid (bioactive folate), and folinic acid+ 5-methyltetrahydrofolate (5-mTHF) (mixture of bioactive folates). The gross appearance and morphological study of the fetuses revealed hydrocephalus precipitation in the folic acid group, which was best prevented by the treatment of folinic acid+5-mTHF. Cerebral folate expression was increased, and methylation levels were decreased in the folic acid group. This indicates that the folates have not been utilized in the DNA and RNA methylation, which is necessary for cerebral development. The activity of cerebral folate receptor alpha was also reduced in the folic acid group. The best preventative effects regarding folate utilization, methylation, and folate receptor activity were observed in the case of folinic acid+5-mTHF. The data indicates that bioactive folates, especially a mixture of folinic acid and 5-mTHF, outperform synthetic folate in the prevention of SB-HC comorbidity.

Keywords: Spina bifida, Hydrocephalus, Folic acid, Bioactive folates, Folinic acid, 5-mTHF.

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