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Original Article

Determining the effect of folate diets during pregnancy and lactation on neurobehavioural changes in the adult life of offspring

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الملخص

أهداف البحث: أظهرت الدراسات الحيوانية والبشرية أن حمض الفوليك ضروري للجهاز العصبي وتطور المخ. في البشر، من المعروف أن انخفاض حمض الفوليك لدى الأمهات يتسبب في عيوب الأنبوب العصبي، وطيف التوحد وغيرها من اضطرابات النمو العصبي لدى الأطفال. تهدف هذه الدراسة إلى تحديد تأثير مكملات حمض الفوليك عند الأمهات على المهارات الحركية النفسية، والتعلم، ووظائف الذاكرة لذريتهن، وخاصة بعد البلوغ.

طرق البحث: تم تقسيم ثمانية عشر أنثى من جرذان ويستار بشكل عشوائي إلى ثلاث مجموعات. تم تغذية الفئران على ثلاثة تراكيز مختلفة من حمض الفوليك من قبل الحمل، خلال الحمل وأثناء الرضاعة. بعد البلوغ، تم تقييم النسل للتغيرات السلوكية العصبية والتأكيد النسيجي عن طريق القياس الكمي للخلايا العصبية للحُصَين.

النتائج: أظهر تقييم السلوكيات العصبية عددا أقل بكثير من البدائل، والمزيد من التحيز بالنسبة المئوية، وعدد أكبر من أخطاء الذاكرة العاملة والمرجعية. وأشار زيادة الوقت الذي تقضيه في مقصورة مظلمة في مجموعة مكملات حمض الفوليك العجز في ذاكرة التعلم. كما أظهر القياس الكمي العصبي للحصين أن عدد الخلايا العصبية القابلة للحياة في منطقة قرن آمون في المجموعة الضابطة كان أعلى (منطقة قرن آمون ١، ٣١.٢ ±٣.٢؛ ومنطقة قرن آمون ٣، ٢٣.٢ ± ٣.٢) مع نواة مميزة في كلا المنطقتين وأقل في مجموعة مكملات حمض الفوليك (منطقة قرن آمون١، ٢٤.٢ ± ٣.١؛ منطقة قرن آمون٣، ١٥.٢ ±٢.٢).

الاستنتاجات: تدعم دراستنا التأثير السلبي المحتمل لارتفاع مكملات حمض الفوليك للأمهات أثناء الحمل والرضاعة. من المحتمل أن تؤدي مثل هذه التغييرات إلى تغييرات في السلوك العصبي في نسلهم في وقت لاحق.

الكلمات المفتاحية: السلوك العصبي؛ حمض الفوليك؛ الحُصَين؛ التعلم والذاكرة

Abstract

Objectives: Animal and human studies have demonstrated that folic acid (FA) is essential for nervous system and brain development. In humans, insufficient maternal FA intake is known to cause neural tube defects, autism spectrum, and other neurodevelopmental disorders in children. The present study aimed to determine the impact of maternal FA supplementation on psychomotor skills and learning and memory functions in their adult offspring.

Methods: Eighteen female Wistar rats were randomly divided into three groups. The animals were fed three different concentrations of FA from preconception to pregnancy and during lactation. The adult offspring were assessed for neurobehavioural changes and histological confirmation by hippocampal neuron quantification.

Results: Neurobehavioural assessment revealed a significantly smaller number of alternations, a higher percentage bias, and a greater number of working and reference memory errors. The increased time spent in the dark compartment in the FA-supplementation group indicated deficit(s) in learning memory. Hippocampal neuron quantification revealed a higher mean number of viable neurons in the *cornu ammonis* (CA) region in the control group (CA1 region, 31.2 ± 3.2; CA3 region,

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23.2 ± 3.2), with a distinct nucleus in both regions, and least in the FA-supplementation group (CA1 region, 24.2 ± 3.1; CA3 region, 15.2 ± 2.2).

Conclusion: Results of this investigation support the possible negative effect of high levels of maternal FA supplementation during pregnancy and lactation. Such alterations potentially lead to neurobehavioural changes in the adult offspring of Wistar rats.

Keywords: Folic acid; Hippocampus; Learning and memory; Neurobehaviour

Introduction

Although neural tube defects are preventable with adequate folic acid (FA) supplementation, the World Health Organization estimates that 300,000 newborn deaths occur worldwide annually, particularly in low- and middle-income countries.[1] Pregnancy demands higher FA requirements due to greater demand from the growth of the foetus and the uteroplacental organs, rapid plasma clearance, increased FA breakdown into functional substrates, and urinary FA excretion.[2] All women of reproductive age should obtain 400 mcg (micrograms) of FA each day, irrespective of the amount of FA from a varied diet.[1] Women who are planning pregnancy or could become pregnant should be advised to obtain 800 mcg per day.[3,4]

FA is a cofactor for enzymes involved in nucleic acid biosynthesis and supplies the methyl group to the methionine cycle. Deficiency in FA compromises cellular ability to methylate proteins, lipids and myelin, resulting in impaired cellular function and neural tube defects.[4–6] However, an excess of FA in pregnancy has been reported to result in the T allele of methylene-tetrahydrofolate in infants. Infants with this allele exhibit a higher tendency to abort spontaneously or to develop neurological disorders in adult life.[7–9]

The available literature suggests that either a deficiency or excess of FA, especially during the critical periods of development, affects embryos, which are unable to successfully repair damage or undergo catch-up growth after a toxic insult.[10] Alterations in maternal dietary FA levels during pregnancy and lactation are associated with an increased risk for cardiovascular, renal, and metabolic diseases later in the life of the infant.[11] Additionally, an association exists between maternal plasma FA levels and the incidence of cardiovascular diseases,[12] structural brain changes and neurodegenerative conditions,[13–15] insulin resistance,[16] and childhood asthma[17] in their offspring in later periods of life. A few clinical studies have implicated the incidence of decreased cognitive behaviour,[18] glomerular sclerosis,

systemic blood pressure,[11] and conotruncal defects[10] in postnatal life.

To date, few, if any, studies have systematically evaluated the effects of maternal FA levels, from preconception to the time of weaning, on their offspring's cognitive performance in childhood or adult life. The purpose of the present study was to evaluate evidence regarding the impact of maternal FA supplementation and restriction diets on psychomotor, learning, and memory functions in their offspring, especially in adult life.

Materials and Methods

Group allocation: The animals were randomly divided into three groups, with six animals per group. The first set (normal control [NC]) was fed a normal diet containing the normal recommended dose of FA (2 mg/kg). The second set was fed a diet containing no FA (F-Ab) (purchased from Vinod Ramkrishna Kulkarni (VRK) nutritional solutions, Miraj, India). Addition of 1% succinyl sulfathiazole to the diet with no FA content helped to reduce/inhibit the relevant gut flora, which prevents a source of FA during pregnancy because rats have a habit of eating their own faeces. Finally, the third set was orally fed a diet containing an excess of FA (F-Inc [40 mg/kg]). This was the highest dose experimented on albino Wister rats after which no change was noticed by the author.[12]

All rats were housed in separate cages in a dedicated room (alternating 12 h light and 12 h of darkness per day, and 25 °C and 35% humidity), with ad libitum access to food and water. All rats were housed and fed in the central animal research facility in accordance with the animal handling protocol of the authors' university. The FA-specific diets (i.e., NC, F-Inc, F-Ab) were fed to the different groups from five weeks before mating and continued until pregnancy and 3 weeks after delivery. The male rats used for mating were fed the same diet as the females in the same cage. Female rats could complete their pregnancy. After delivery, the size and number of live offspring (pups) were recorded. All pups from each experimental group were weaned from their mothers at 21 days of age. After weaning, the pups were separated into male and female cages until adult age.

Neurobehavioural assessment: Behavioural testing commenced at approximately postnatal day 75, as per the standard protocol described in earlier studies.[19–21] Neurobehaviour was assessed using the following three tests:

(a) *T maze test*[19]: This test included both spontaneous and rewarded alternation tests. Spontaneous alternation test results were analysed as alternations were outnumbered and a lower percentage bias were considered to be an index of improvement in learning ability. Rewarded alternation test results were analysed as an increase in correct response percentage, which was considered to be an index for improved learning and memory.

(b) *Passive avoidance test*[20]: This test included three experiments: exploration; aversive stimulation and learning (passive avoidance acquisition); and retention. The results were analysed as the decrease in time spent in the smaller compartment during the retention test,

which was considered to be good memory retention performance.

(c) *Eight-arm radial maze*[21]: This experiment was used to as a test to assess spatial learning and memory.

Hippocampal neuron quantification: One day after the behavioural tests, young-adult offspring rats from each group were euthanised by placing them in a closed jar containing ether for 10 min. Brain tissues were carefully removed and the hippocampus was dissected. Serial frozen coronal sections (60 microns thick) were cut using a cryostat and stored at 4 °C. The sections were processed for cresyl violet and immunohistochemical staining.

Cresyl violet staining: Before staining, slide-mounted hippocampal sections were chosen according to a systematic random sampling method. The section sampling fraction followed in the present study was 1/10 (i.e., one section was chosen and stained for every 10 continous sections). Slides were differentiated in 95% ethyl alcohol for 2 h at room temperature, and later rinsed in 75% ethyl alchohol for 5 min and washed with distilled water. The slides were stained with 0.1% cresyl violet (Sigma Aldrich, St Louis, MO, USA) solution for 5 min and quickly rinsed in distilled water. The sections were decoloured with 75% ethyl alcohol for 5 s, and dehydrated in 95% and absolute ethyl alcohol for 5 min. The stained sections were mounted using permount (Thermo-Fisher Scientific, Waltham, MA, USA). Slides were observed under a fluorescence microscope (Olympus, Tokyo, Japan) equipped with Image Pro-Premier software. The *cornu ammonis* (CA) CA1 and CA3 regions were identified, and viable and non-viable neurons, identified by their cellular architecture, were counted in five different regions of CA1 and CA3 by a senior histopathologist as per a standard protocol.[13]

The average number of neuronal cells from all five sites were calculated. Hippocampal neuron survival in offspring born to mothers fed with F-Ab, F-Inc, and NC diets was quantified by counting them in the pyramidal cell layer, and both CA1 and CA3 regions (from at least five different sites in each of the slides and each region) using a stereological approach. Neurons with intact neurites and a cellbody with a smooth rounded appearance were considered to be viable (i.e., healthy), whereas neurons with irregular and vacuolated cell bodies, along with absent or fragmented nuclei, were considered to be nonviable.

Immunohistochemistry technique

Blocking of non-specific antibodies: A 2% blocking solution was prepared by dissolving 0.1 g of bovine serum albumin (BSA) in 5 ml of 0.1 M PBS-TX. The equilibrated slide was incubated for 4 h in the freshly prepared 2% blocking solution to block non-specific antigens. The slides were washed three times with PBS-TX before further incubation with primary antibody.

Primary antibody incubation: Primary antibody (ab13847 Abcam, USA), which was stored at −20 °C, was transferred to 4 °C. A thin layer of Vaseline Petrolium Jelly (Unilever, HPC-USA) was applied to the border of the slides to contain the antibodies on the sections. The primary antibodies were diluted (1:200 [i.e., 4 μl of antibody was added to 1.6 ml of PBS-TX]) in working buffer and approximately 200 μl of diluted

antibody was added to each slide and incubated at room temperature (23−27 °C) for 4 h, and then at 4 °C for 36 h.

Secondary antibody incubation: After 36 h of incubation with primary antibody, the slides were gently washed three times with working buffer (PBS-TX) and transferred to a dark room for incubation with cyanine-3 fluorescence-tagged secondary antibodies. The secondary antibodies (Sigma Aldrich, St. Louis, MO, USA) were diluted (1:100 [i.e., 8 μl of antibody was added to 1.6 ml of PBS-TX]) in working buffer, and approximately 200 μl of diluted antibody was added to each slide and incubated at room temperature for 2 h and then at 4 °C for 12 h.

Mounting the slides: A 70% glycerol solution was prepared with 0.1 M PBS without TX for mounting. The diluted secondary antibody solution was drained, and the slides were washed three times with working buffer. Glycerol (70%) was added to all slides, which were covered with coverslips without introducing air bubbles. Nail paint was used to seal the edges of the coverslip to prevent the evaporation of glycerol. The slides were then carefully preserved and photomicrographic images were captured using a confocal microscope. The slides were first scanned using a fluorescence microscope and the area of interest was located. Because the secondary antibody was tagged with fluorochrome cyanin-3, the images were visualized using a confocal microscope (Leica SP8 Spectral Confocal Microscopy model DMi8 Heidelberg, Germany), which was first used to scan with 10 × /0.30 HC PL FLUO-TAR objectives, then the confirmed images were captured in Argon/2 (488 nm) laser using a 100 × /1.40 HCX PL APO OIL CS objective, later in *Software:* LAS X for image acquisition, processing, and quantification. The values obtained for each parameter were adjusted to achieve the same signal intensity between the control and experimental groups.

Results

a. Gross findings in pregnant mothers fed a specific FA diet and their pups
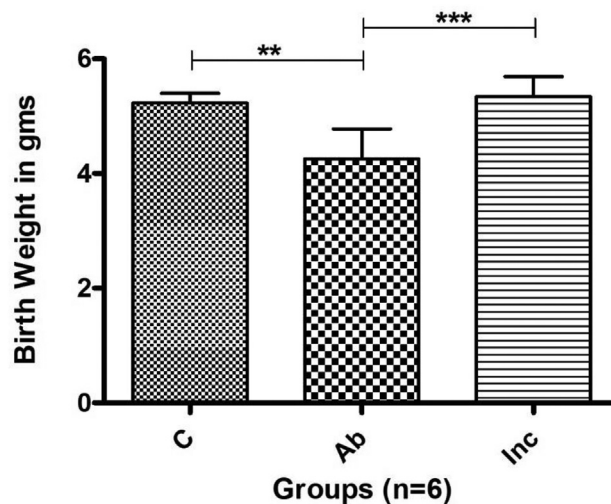
Figure 1: Birthweight of pups born to pregnant dams fed specific diets. Data expressed as mean ± standard deviation. (Tukey's HSD test, multiple comparison of variables). **p < 0.05; ***p < 0.001. C=Control; Ab = Folic acid absent; Inc = Folic acid increased.

According to Kruskal–Wallis test results, there was no statistical difference in the number of pups born to mothers fed the specific diets (i.e., normal FA, FA-supplemented, and FA-absent). However, the mean birth weight of pups born to mothers fed the FA-supplemented diet was higher (mean weight, 5.49 g) and the lowest in pups born to mothers in the FA-absent diet group (mean, 4.28 g). Two-way ANOVA testing to compare the body weight of pups in the three groups revealed statistically significant differences, but were not significant when compared within groups. Pairwise comparison (Tukey HSD test) of the birth weight of pups born (Figure 1) to pregnant rats fed with specific diet demonstrated significant values in the FA-supplemented and FA-absent groups (p˂0.002).

No gross malformations of any type, either external or within the body of the pups, were noted in any of the three groups.

b. Neurobehavioural analysis

T-maze test analysis: In the spontaneous alternation test, the F-Inc group exhibited a significantly fewer number of alternations (Figure 2a) and more percentage bias (Figure 2b) compared with the NC group, whereas in the reward alternation test, rats in the FA-Inc group demonstrated a higher percentage of correct responses compared with that of the NC group.

Eight-arm radial maze test: Analysis of these test results revealed a higher number of working memory errors (Figure 3a) in the FA-Inc and F-Ab groups compared with the NC group. However, the reference memory errors (Figure 3b) were significantly higher in the FA-Inc group.

Passive avoidance test: Twenty-four hours after administration of foot shock in the dark compartment, the total time
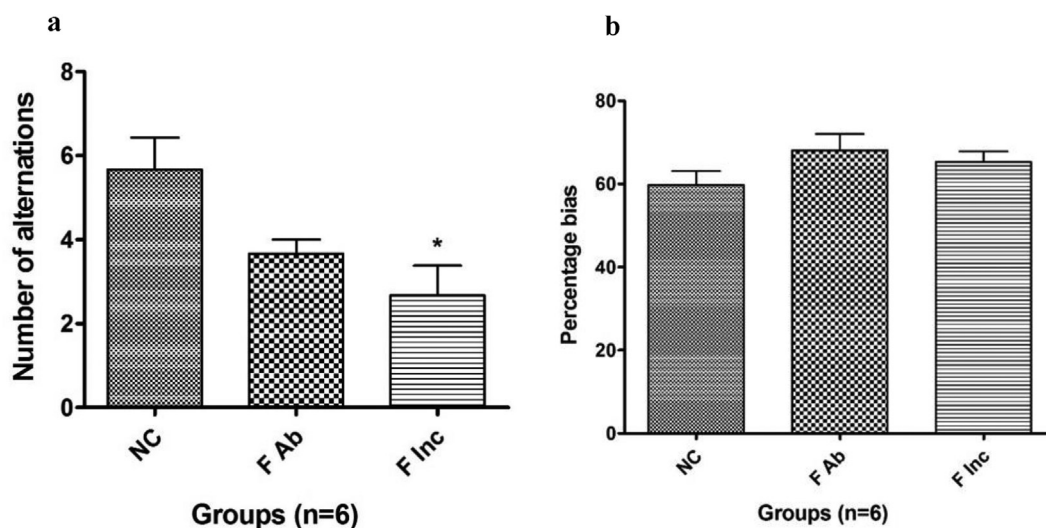
Figure 2: T-maze analysis. 2a. Spontaneous alternation test. 2b. Percentage bias. Number of alternations in spontaneous alternation (2a) and percentage bias in spontaneous alternation (2b) task performance. Data expressed as mean ± standard deviation. (One-way ANOVA, Bonferroni's test). C versus F Inc, *p < 0.05. C=Control; F Ab = Folic acid absent; F Inc = Folic acid increased.
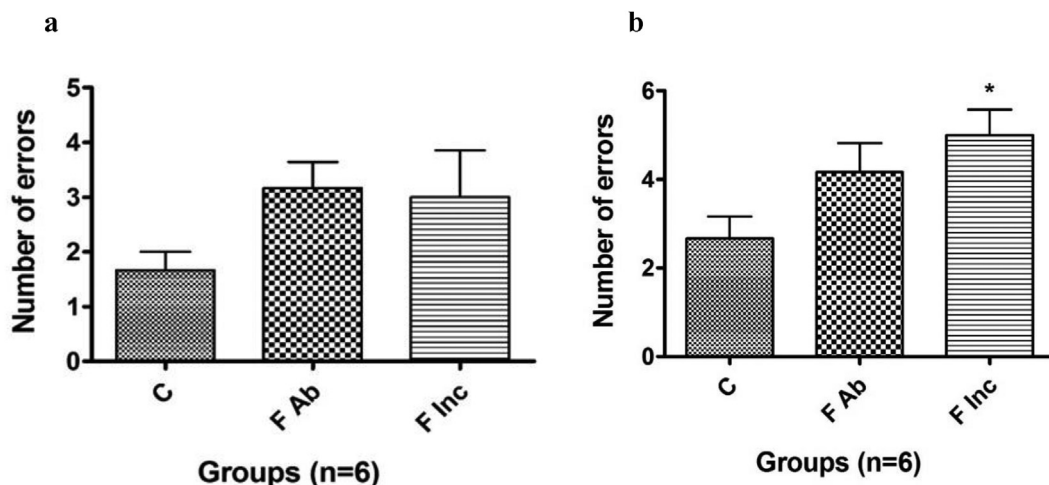

Figure 3: Eight-arm radial maze. 3a. Working memory errors. 3b. Reference memory errors. Mean number of working memory errors (3a) and reference memory errors (3b) during radial arm maze retention trails. Data expressed as mean ± standard deviation. (One-way analysis of variance, Bonferroni's test, C vs. F Inc, *p < 0.05). C=Control; F Ab = Folic acid absent; F Inc = Folic acid increased.

spent (Figure 4a) was found to be higher in the FA-Inc group, which demonstrated a lower latency time (Figure 4b) to enter the dark compartment compared with other groups.

c. Microscopic details of the hippocampus

The number of viable neurons were counted according to normal morphology in both CA1 and CA3 regions of the hippocampus (Figure 5). The NC group exhibited a greater number of viable neurons (CA1 region, 31.2 ± 3.2; CA3 region, 23.2 ± 3.2) with distinct nucleus in both regions, followed by the F-Ab (CA1 region, 27.2 ± 3.7; CA3 region, 20.0 ± 1.2), and the least in the FA-Inc group (CA1 region, 24.2 ± 3.1; CA3 region, 15.2 ± 2.2). Statistical analysis revealed significant values in the number of neurons in the CA1 region compared to the NC and F-Inc groups. The number of neurons in the CA3 region was highly significant when compared with the NC group versus the FA-Inc group, similarly with F-Ab group versus the F-Inc group.
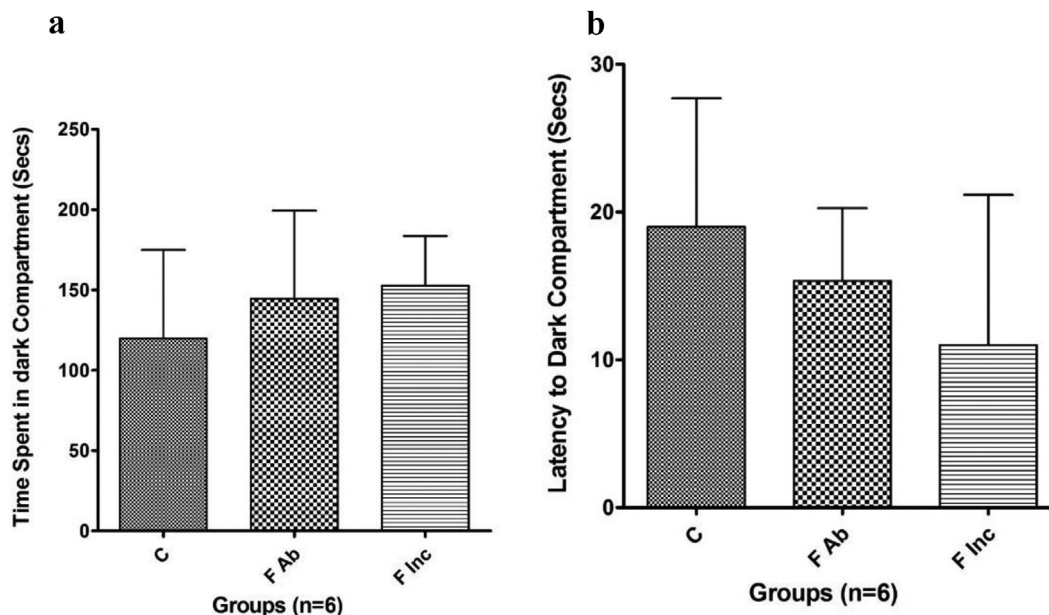
Figure 4: Passive avoidance test. 4a. Time spent in dark compartment. 4b. Latency to dark compartment. Time spent in the dark compartment (4a) and during passive avoidance retention (4b) trails. Data expressed in mean ± SD. (One-way analysis of variance, Bonferroni's test). C=Control; F Ab = Folic acid absent; F Inc = Folic acid increased.
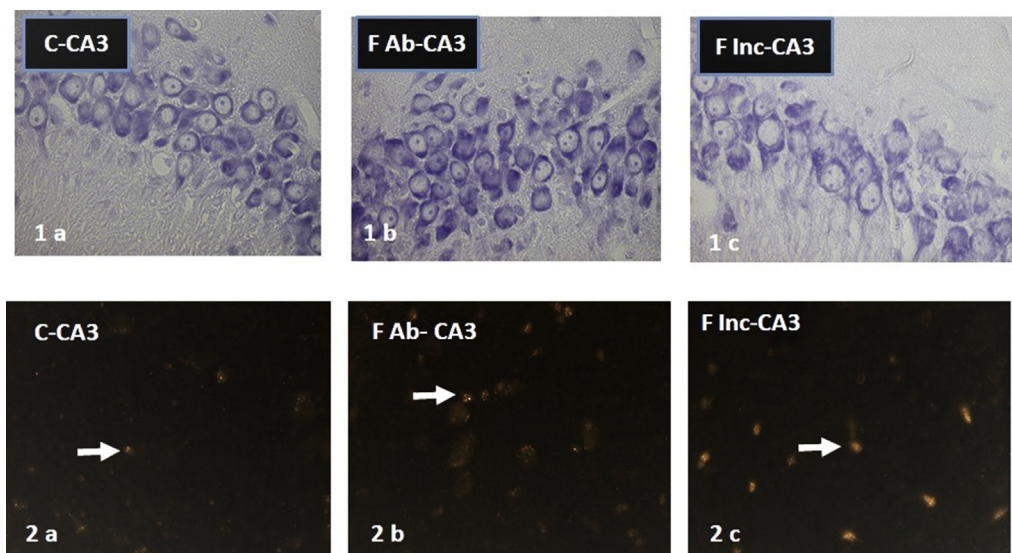
Figure 5: Histological (Figure 1a, 1b and 1c) and immunohistochemistry (Figure 2a, 2b, and 2c) photomicrographs of the *cornu ammonis* (CA)3 neurons showing signals of caspase-3 expression (arrow) in adult rats born to mothers fed folic acid-specific diets (N, normal amount of folic acid in the diet, F-Ab, Folic acid absent diet, and F-Inc, folic acid increased diet).

d. Immunohistochemical analysis

Immunohistochemical analysis revealed that the CA3 sub region of the hippocampus of adults born to mothers fed the control FA diet exhibited very few and random signals of caspase-3 expression. Whereas, the caspase-3 expression in CA3 neural cells of adult (72-day-old) rats born to mothers fed the F-Ab diet exhibited a higher number compared with the same in the NC group. The signals of caspase-3 expressions in CA3 neural cells of the hippocampal section was higher in the hippocampus of adults (72-day-old) born to mothers fed the FA-Inc diet.

Discussion

Animal and human studies have shown that FA is essential for nervous system and brain development. In humans, decreased maternal FA status during pregnancy is known to cause neural tube defects, autism spectrum and other neurodevelopmental disorders in children.[2] Due to the lack of epidemiological findings, the reference value for serum FA or daily recommended intake for women of reproductive age remains unclear.[22] Values for daily recommended FA intake varies widely, especially in pregnancy (250−520 mcg) across the world. The Centers for Disease Control and Prevention (Georgia, USA) recommends 400 mcg of FA daily for all women of reproductive age, apart from FA in the diet. The CDC recommends higher doses of FA (4000 mcg) only in high-risk cases.[23]

FA plays a dual role: its deficiency has been associated with abnormalities in both mother (anaemia in pregnancy and peripheral neuropathy) and foetuses (neural tube, cardiovascular and orofacial defects); however, excess levels in blood have been associated with vitamin B_{12} deficiency, cancer, depression, and cognitive impairment.[2] Although FA supplementation to higher levels than the recommended dose has demonstrated many benefits to pregnant women and foetuses, concerns have been raised about the harmful effects of unmetabolized FA in both mothers and their offspring.[24]

Foetal birth weight is the best predictor of pregnancy outcome and, hence, growth restriction has been associated with high morbidity and mortality.[2] Results of the present study revealed that the birth weight of offspring born to mothers fed a FA-supplemented diet was found to be significantly increased compared to those fed with a normal amount of FA in the diet, and least was noticed in offspring born to mothers fed with a diet devoid of FA. Few studies have addressed the importance of the correlation of foetal head size and body size (weight) with maternal FA status.[18] However, they found larger prenatal head size with pre-conceptional maternal FA supplementation. The relationship between birth weight and indicators of brain development were investigated by Walhovd et al.,[25] who found that birth weight exerted more positive effects on regional cortical surface area in multiple regions, total brain, and caudate volumes, as cranial volumes correlated well with body size.[25]

Brain development begins with the formation of neuronal cells and ends with the network of synapses to enable cells to communicate with one another.[26] Maternal FA deficiencies restrict myelination, dendritic arborisation, tissue levels of neurotransmitters, and synaptic connectivity prenatally and continues through school age.[26] Myelination, once retarded in infancy, leads to delayed acquisition of cognitive skills, and brain atrophy leads to regression of these skills.[26] Thus, reduction in the number of neurons can have an exponential impact on the number of neuronal connections within the brain and predisposes to cognitive and behavioural impairment in postnatal life.[18] Results of the present study revealed a reduced number of viable neurons in the CA1 and CA3 regions in rats born to mothers fed the F-Inc diet. Low maternal FA concentrations are directly related and limit the availability of FA to foetal cells, which results in impairment of cell division and, in turn, impaired growth.[27]

Documented evidence has demonstrated poor memory and maze learning ability in adult offspring born to mothers fed a diet with reduced FA and high homocysteine concentrations in pregnancy.[28] Neurobehavioural assessment revealed a significantly fewer number of alternations, more percentage bias, and a greater number of working and reference memory errors, evidenced by increased time spent in the dark compartment in the FA-Inc group, indicating learning memory deficit(s). This could be due to supplementation of FA during pregnancy, which alters the morphology of the hippocampus (in terms of area and number of viable neurons) leading to deficit(s) in short-term learning and memory.[29]

Lucock and Yates reported that excess FA supplementation in pregnancy increases the incidence of children born with the T allele of methylene-tetrahydrofolate. Infants with this allele are at greater risk for developing neurological disorders such as depression,[8,14] schizophrenia,[15] bipolar disorders,[24] neural tube defects[16] and, rarely, Down syndrome[20,30,31] in their adult life. The Valencia cohort study found that excess maternal FA was associated with reduced foetal growth, which in turn reduces infant psychomotor development.[32] Results of the present study found increased body weight at birth as well as growth, but revealed neurobehavioural findings in the offspring.

Neurons in the brain are critical for learning and memory. Neuronal death can caused by the production and accumulation of amyloid β-peptide (Aβ), which induces oxidative stress and disrupts cellular homeostasis. A maternal FA-deficient or -absent diet in pregnancy increases the incidence of homocysteine levels and sensitizes the neurons of their offspring's hippocampus, resulting in Aβ-induced neuronal cell death.[33] Immunohistochemistry was performed to estimate neuronal loss as a result of FA supplementation using the apoptosis marker caspase-3, which is based on the principle of antigen−antibody interaction. In this technique, the hippocampal sub regions were targeted with rabbit anti-caspase-3 primary antibodies, which bind to expressed caspase-3 enzymes. After appropriate processing, the secondary sheep antibody was tagged with fluorochrome cyanin-3, and the fluorescence images were captured using a confocal microscope.[34]

Veena et al., tested the association between concentrations of maternal plasma levels of FA and cognitive ability in their offspring, maternal socioeconomic status and child's head size. The authors found no association between maternal vitamin B_{12} and homocysteine concentrations and childhood cognitive functions. Additionally, they noted

better cognitive performance in children born to mothers fed an FA-supplemented diet in their pregnancy.[35]

Study limitations: First, comparative assessment of maternal serum FA status at preconception, during pregnancy, and at weaning for each FA-specific diet was not performed. Second, dose-dependent effects of maternal FA during each phase of pregnancy on the development of vital organs was not assessed morphologically or histologically. Finally, the small sample size used in this study, due to institutional ethical concerns, may have affected the results.

Conclusion

The present study demonstrated that excess FA supplementation in pregnancy contributed to neuronal loss in offspring, especially in the CA1 and CA3 regions of the hippocampus, and resulted in deficient short-term learning and memory. Results of our study correlate positively with declining cognitive performance in children born to mothers with excessive FA supplementation during pregnancy. Further research is essential to evaluate the FA-sensitive period of foetal brain development with its effect on specific brain regions and dose-dependent effects of FA during different phases of gestation on foetal brain development and its association with the development of neurological diseases later in life.

Source of funding

Conflict of interest

The authors have no conflict of interest to declare.

Ethical approval

Eighteen female Albino Wister rats (strain 94-PO-ReBi-S-99-CPC SEA), weighing approximately 120 g, were issued on approval from the authors' animal research facility and the Institutional Animal Ethics Committee. This experimental study was conducted from June 2017 to February 2018, after approval letter was obtained by our Institutional Animal Ethical Committee (Ref no: IAEC/KMC/06/2017), Kasturba Medical College, Manipal Academy of Higher Education, Manipal (India).

Authors contributions

LCP conceived and designed the study, AK conducted research, provided research materials, and collected the data, NV organized data, analysed and interpreted data. LCP & LSQ wrote the initial and final drafts of article, and provided logistical support. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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