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Original Article

# Effects of maternal vitamin deficiency on the microstructure of the maternal hippocampus and behavior in offspring

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**الملخص**

**أهداف البحث:** تلعب التغذية دورا مهما في عمل الدماغ وتطوره. فيتامين ب 6 في شكل فوسفات بيريدوكسال مطلوب للتخليق الحيوي للعديد من الناقلات العصبية. نظرا لأن فيتامين ب 6 لا يتم تصنيعه داخليا، يصبح توفر المصادر الغذائية أمرا ضروريا. بسبب مساهمته في الوظائف العصبية، يؤدي النقص الحاد في فيتامين ب 6 إلى زيادة خطر الإصابة بالاضطرابات النفسية والخرف واضطرابات النمو العصبي. هدفت الدراسة الحالية إلى إنشاء نموذج يعاني من نقص فيتامين ب 6 في حيوانات التجارب وتقييم آثار النمو العصبي في ذريتهم.

**طريقة البحث:** تم استخدام إناث فئران "س 57 ب ل/6 ج" عمرها شهرين إلى ثلاثة أشهر في الدراسة. تم تقسيمهم بشكل عشوائي إلى المجموعة الضابطة وفيتامين ب 6 الناقص. تم تغذية المجموعة الضابطة بنظام غذائي منتظم يحتوي على 6 ملغ من فيتامين ب 6 / كغ من النظام الغذائي وتم تغذية المجموعة التي تعاني من نقص فيتامين ب 6 بنظام غذائي مخصص يحتوي على صفر ملغ من فيتامين ب 6 / كغ غذاء لمدة خمسة أسابيع (عدد=6). بعد خمسة أسابيع، تم تقدير فوسفات بيريدوكسال في البلازما. تم تربية الحيوانات لتولد ذرية. تم التضحية بالفئران الأمهات أيضا بعد الفطام، وتم تحديد كمية الخلايا العصبية في قرن آمون باستخدام صبغة الكريسيل البنفسجي. تم تخصيص النظام الغذائي للنسل بعد الفطام حتى عمر شهرين. تم تقييم التعلم والذاكرة باستخدام اختبار متاهة موريس المائية.

**النتائج:** أكدت مستويات البلازما فوسفات بيريدوكسال النقص في المجموعة الناقصة بالمقارنة مع المجموعة الضابطة. أظهرت الخلايا العصبية الهرمية القابلة للحياة في منطقة "س أ 3" من قرن آمون فرقا كبيرا بين المجموعة الضابطة والمجموعة الناقصة. أظهر النسل المولود للفئران ذات النقص زيادة كبيرة في زمن الوصول للوصول إلى الربع المستهدف أثناء تجربة المسبار مقارنة بالمجموعة الضابطة.

**الاستنتاجات:** يقلل نقص فيتامين ب 6 من الذاكرة في الفئران وذريتهم، مما يشير إلى أهمية فيتامين ب 6 لكل من وظائف الدماغ وتطوره. يجب القيام بالمزيد من البحث في هذه المعرفة والموقف مع مرور الوقت وتنفيذها الفعلي في الممارسة.

**الكلمات المفتاحية:** فوسفات البيريدوكسال؛ فيتامين ب 6؛ الحصين؛ نقص؛ التعلم والذاكرة

**Abstract**

**Objectives:** Nutrition plays a critical role in the brain's function and development. Vitamin B6 in the form of pyridoxal phosphate (PLP) is required for the biosynthesis of several neurotransmitters. As vitamin B6 is not endogenously synthesized, the availability of dietary sources becomes imperative. Due to its contribution to neurological functions, severe vitamin B6 deficiency leads to an increased risk of psychiatric disorders, dementia, and neurodevelopmental disorders. This study aimed to establish a vitamin B6-deficient model in experimental animals and assess the neurodevelopmental effects in their offspring.

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**Methods:** Two- to three-month-old female C57BL/6J mice were used in the study. They were randomly divided into control and vitamin B6-deficient groups. The control group was fed a regular diet containing 6 mg vitamin B6/kg and the vitamin B6-deficient group was fed a customized diet containing 0 mg vitamin B6/kg, for 5 weeks (n = 6). After 5 weeks, plasma PLP was assessed. The animals were bred to generate offspring. The dams were killed following weaning, and the hippocampal neurons were quantified using cresyl violet staining. The offspring were assigned the respective diet post-weaning up to 2 months of age. Learning and memory were assessed using the Morris water maze test.

**Results:** The plasma PLP levels confirmed the deficiency in the deficient group compared to the control group. The viable pyramidal neurons in the cornu ammonis 3 (CA3) region of the hippocampus showed a significant difference between the control and deficient groups. Offspring born to deficient dams showed a substantial increase in latency to reach the target quadrant during the probe trial compared to the controls.

**Conclusion:** Vitamin B6 deficiency reduces memory in dams and their offspring, suggesting the importance of vitamin B6 for both brain function and development.

**Keywords:** Deficiency; Hippocampus; Learning; Memory; Pyridoxal phosphate; Vitamin B6



## Introduction

Nutrition is critical for the brain's function and development.[1] Vitamin B plays a contributing factor to brain function. Vitamin B6 is available in multiple forms including pyridoxine, pyridoxal, and pyridoxamine. Pyridoxal 5′-phosphate (PLP) is a biologically active coenzyme. More than 140 enzymes, including those involved in the metabolism of amino acids, decarboxylation of neurotransmitters, folate, protein and polyamine synthesis, carbohydrate and lipid metabolism, mitochondrial function, and erythropoiesis, require PLP as a cofactor.[2]

As vitamin B6 is not endogenously synthesized, the availability of dietary sources becomes imperative. The deficiency of dietary vitamin B6 can hamper optimal health by altering the physiological metabolic reactions. Due to its contribution to neurological functions, severe vitamin B6 and other B complex deficiencies increase the risk of psychiatric disorders, dementia, and neurodevelopmental disorders.[3]

Studies have shown the effects of vitamin B6 deficiency on the brain, particularly the hippocampus.[4–7] A recent study revealed that CL57BL/6J mice fed a vitamin B6-deficient diet for 8 weeks showed a decrease in Ki67 and doublecortin-positive cells in the subgranular region of the dentate gyrus, suggesting the reduction of proliferative cells and differentiating neuroblasts cells, respectively.[8] A study also revealed that vitamin B6 deficiency causes hyperhomocysteinemia, leading to microvascular changes in the hippocampus.[9]

It is well established that numerous micronutrient deficiencies exist among women of reproductive age in developing countries. The demand for these micronutrients further increases during pregnancy. A major focus of nutrient supplementation is on iron, folic acid, vitamin B12, and calcium during pregnancy to prevent anemia, neural tube defects, and bone deformation. Although vitamin B6 is known to play a potential role in gamma-aminobutyric acid (GABA) synthesis, vitamin B6 supplementation during pregnancy is barely emphasized.

This pilot study aimed to establish vitamin B6 deficiency in mice and observe its effects on the microstructure of the hippocampus, particularly the cornu ammonis (CA) region, as well as understand the behavioral changes in the offspring.

## Materials and Methods

### *Animals*

Twelve female C57BL/6J mice of about 2 months old were used in the study and housed in a controlled environment, with a 12-h light and dark cycle and food and water provided *ad libitum*. After receiving approval from the Institutional Animal Ethics Committee [IAEC/KMC/24/2019], all experiments were carried out with conventional animal care. Twelve mice were randomly divided into two groups. A regular diet containing 6 mg vitamin B6/kg diet was fed to the control group, which was designated the mother mice control (MMC) group; a customized diet containing 0 mg vitamin B6/kg diet was fed to the deficient group, which was designated the mother mice-deficient (MMD) group, for 5 weeks. After 5 weeks, the female mice were bred and continued with the assigned diet during pregnancy until weaning. The dams were sacrificed post-weaning to study the microarchitecture of the hippocampus. At weaning, six male pups were randomly selected from both groups and continued with the same diet assigned to the dams until 2 months of age for the behavioral analyses. The male pups was selected based on previous studies, which showed that males perform better in behavioral tests involving spatial learning and memory compared with females due to factors such as varying estrous cycles in each mouse and also less effective orientation strategies.[10]

#### *Blood collection and analysis of plasma PLP in the dams*

After 5 weeks of administration of the customized diet, 0.5 mL blood was collected from the female mice in EDTA-coated Eppendorf tubes from periorbital sinuses, by puncturing the medial canthus of the eye with the capillary tube. The blood samples were centrifuged at 3500 rpm for 20 min. The plasma was collected and stored at −80 °C until further use.

*Liquid chromatography–mass spectrometry analysis of PLP*

The instrument utilized was the Dionex Ultimate 3000 liquid chromatography (LC) system coupled to a linear ion trap analyzer by an electron spray ionization (ESI) source (Thermo Fisher Scientific, Waltham, MA, USA). The LTQ Orbitrap XL mass spectrometer and Chromeleon chromatography data system (both from Thermo Fisher Scientific) were used for tandem mass spectrometry (MS/MS) and analysis of the chromatographic results, respectively. XCalibur software was used for batch analysis and LCquan was used for quantification (both from Thermo Fisher Scientific). Separation was accomplished at 25 °C using the Acclaim 18 column (150 × 4.6 mm, 3 μm; Thermo Fisher Scientific). The mobile phase had a flow rate of 0.1 mL/min and included methanol and formic acid at a 70:30 v/v ratio. MS analyses were performed in positive ion mode (ESI+) using single reaction monitoring with isoniazid serving as an internal standard.

*Collection of brain tissues from the dams*

After creating the deficiency model, the female mice were allowed to breed. Following the generation of pups and their weaning, the MMC and MMD groups were sacrificed. The left ventricle of these dams was transcardially perfused with heparinized saline before being fixed in 10% formalin at a rate of 1 mL/min, and the brains were fixed. Then tissues were dehydrated through a series of increasing concentrations of ethanol, embedded in paraffin wax, cut into blocks, and labeled.

*Tissue processing*

In accordance with the procedure outlined in 'Principles and Techniques in Histology, Microscopy, and Photomicrography' by Singh et al., the brain sections from the mice (n = 6 mice/group) were fixed on slides.[11] According to the methodology described by Shetty et al., sections were also stained with cresyl violet. The slides were incubated in 0.1% cresyl violet solution for 20–25 min at 60 °C before being stained with 0.1% cresyl violet stain for 20–25 min at 60 °C.[12]

*Histological assessment of the hippocampus of the dams*

Stained sections were examined for healthy neurons. The number of remaining nerve cells was determined by light microscopy (MLX Plus; Magnus Opto Systems India Pvt. Ltd., Delhi, India). Each brain was divided into six regions for measurement. Stained slices were examined and photographed at 10× and 100× magnifications using the Olympus Research Microscope (Model cx21i; Olympus, Tokyo, Japan). After calibrating the light microscope with an ocular and stage micrometer, quantification was performed (Erma Inc., Tokyo, Japan). An ocular micrometer was used to observe the CA1 and CA3 regions of the hippocampus in at least three random microscopic fields spaced 250 μm apart. In these regions of the hippocampus, the number of viable brain cells with an obvious and distinct nucleus was measured. Quantification was not performed on neurons that had abnormal nuclei, reduced cell bodies, or dark staining.

*Behavioral analysis of the pups: Morris water maze*

The Morris water maze (MWM) was employed to assess memory and spatial learning in the offspring. The MWM for mice consists of a circular pool of water, with a diameter of 1.1 m, height of 57 cm, and water depth of 30 cm, maintained at a temperature of 26 °C (±2 °C). The 10 cm diameter escape platform was lowered 1–1.5 cm below the water's surface during the spatial acquisition trial. Two principal axes divided the water pool into four equal quadrants or zones. The experiment was conducted in two phases: the spatial acquisition trial and the retention trial.

The mice were subjected to the spatial acquisition trial to evaluate their learning ability. All of the selected offspring, i.e., breed mice control (BMC) and breed mice-deficient (BMD), received MWM training sessions for four consecutive days using a four trial/day regime with an intertrial interval of 20 min. In each trial, the mice were placed in the pool at random starting points of each quadrant. The time taken by the mice to locate the escape platform was recorded. If the mouse could not locate the platform within 60 s, it was manually led to the platform and granted 15 s to remain on the stage. A video camera recorded the escape latency, which was analyzed using ANY-maze video tracking software (Stoelting Co., Wood Dale, IL, USA). A retention trial was performed 1 day after the last acquisition day, i.e., on the fifth day, to estimate spatial memory retention. In this phase, mice were administered a probe trial for 30 s and the escape platform was removed from the pool. Latency to reach the desired quadrant where the platform was placed during the acquisition trial was noted. The latency needed to reach the escape platform and the target quadrant in the spatial acquisition and retention trials was provided by specialized maze software.

## Statistical analyses

The GraphPad Prism statistical program was used to analyze the data using the unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant, and the results are presented as the mean ± standard deviation (SD) and are also presented as mean ± SEM, as appropriate.

## Results

*Plasma PLP (in the dams) could not be detected in the deficient group*

Based on estimation of PLP levels in the plasma collected from the mice after 5 weeks of diet, PLP levels in the control group were estimated to be 8.11 ± 0.90 ng/mL, whereas PLP levels in the deficient group were below the detectable limit (Figure 1).

*Quantitative assessment of hippocampal neurons in the CA1 and CA3 regions of the dams*

On examination under a microscope, there were fewer viable neurons in the CA1 subregion of the hippocampus in

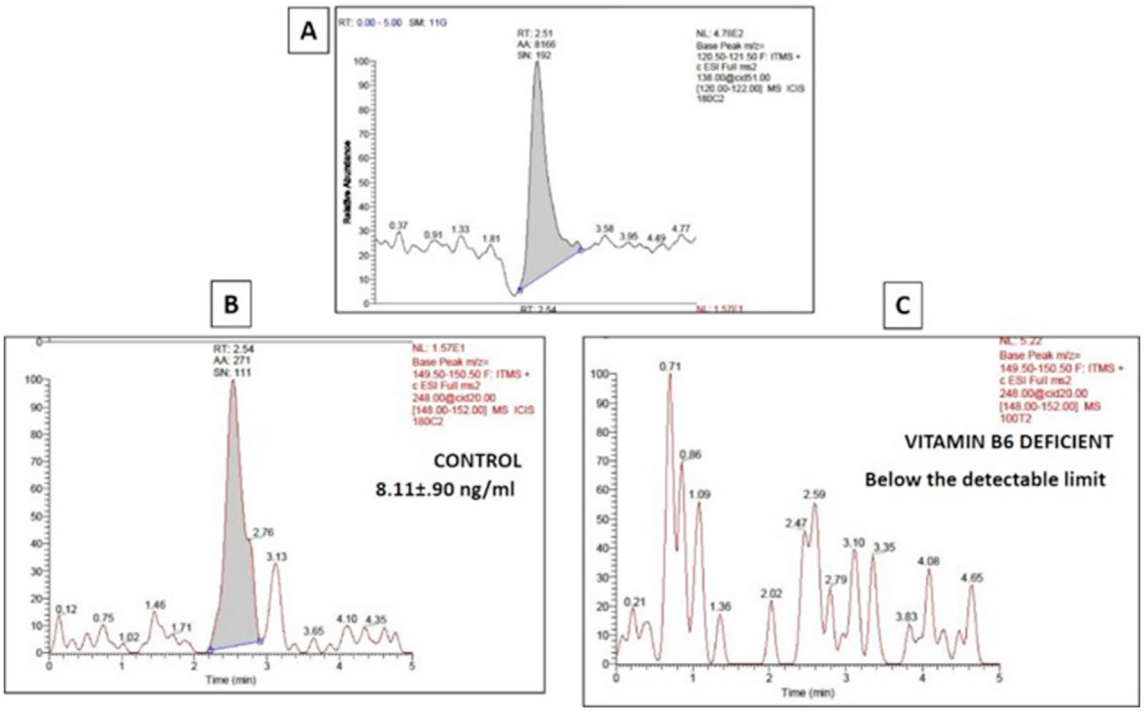


**Figure 1:** Chromatograms of plasma pyridoxal phosphate: (A) shows the isoniazid internal standard, (B) represents plasma PLP in the control mice, (C) represents plasma PLP in the vitamin B6-deficient mice.

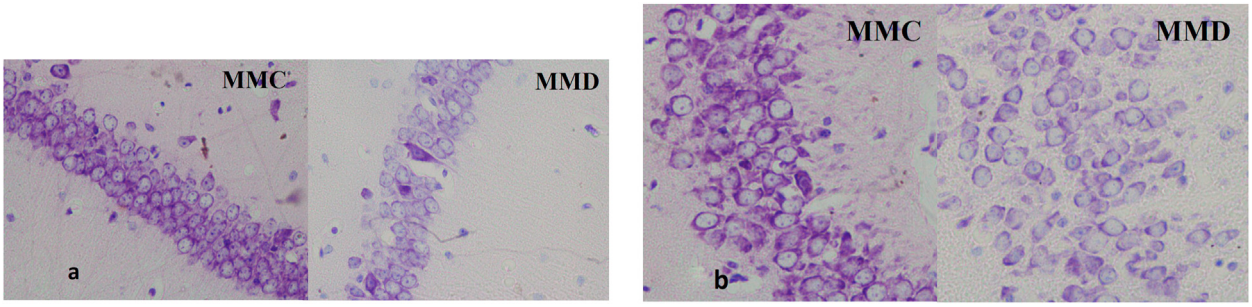


**Figure 2:** Cresyl violet stain of the cornu ammonis (CA) subregion of the hippocampus: (a) represents the CA1 region, (b) illustrates the CA3 region of the hippocampus. MMC, mother mice control; MMD mother mice-deficient.

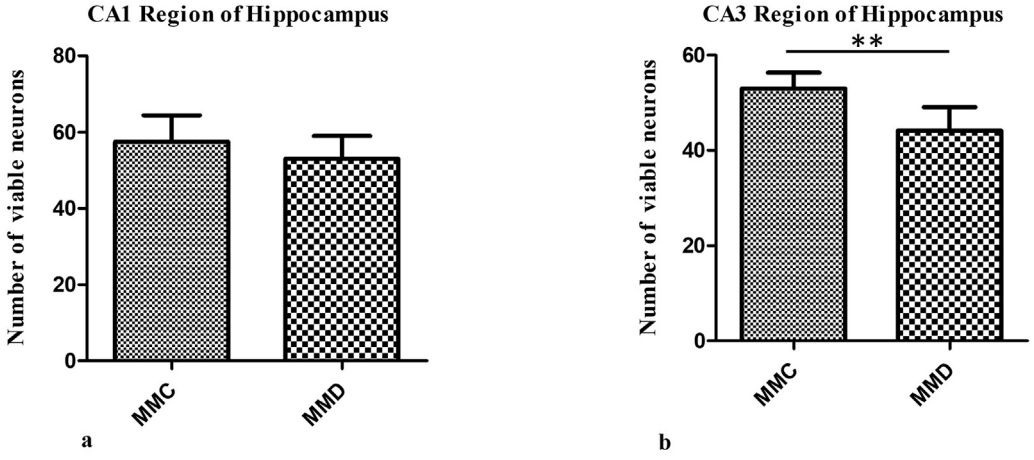


**Figure 3:** Graphical representation of the number of viable neurons in the cornu ammonis (CA) of the hippocampus: (a) represents neurons in the CA1 region, (b) represents neurons in the CA3 region. MMC, mother mice control; MMD, mother mice-deficient.

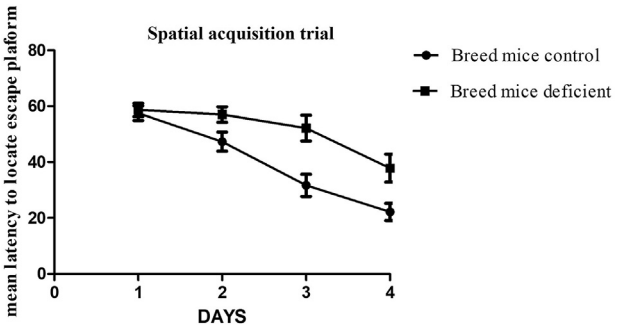


**Figure 4:** Graphical representation of the mean latency of the breed mice to reach the escape platform during spatial acquisition from day 1 to day 4.

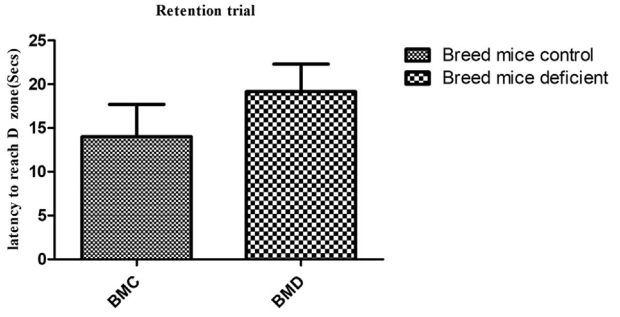


**Figure 5:** Graphical representation of the time taken by the mice to reach the target quadrant during the probe trial.

the MMD group (53.00 ± 2.43; Figure 3a) than in the MMC group (57.50 ± 2.8; Figure 3a), but this difference was not statistically significant ($P > 0.05$; Figure 2a). However, the number of viable neurons in the CA3 area of the MMC group was much higher than that of the MMD group ($P \leq 0.05$; Figures 2b and 3b).

*Spatial memory and learning in the offspring*

During the spatial acquisition trial, the average escape latency decreased from day 1 to day 4 in the BMC (mean ± SEM; 39.67 ± 7.89) and BMD (mean ± SEM; 51.42 ± 4.73) groups. Mice in both groups showed improved performance by taking a shorter duration with an increased number of trials and training sessions (see Figure 4). However, the latency to reach the escape platform was significantly higher in the BMD group than in the BMC group on all 4 days ($P < 0.05$; Figure 4).

During the retention trial on the fifth day, mice in the BMC group took a shorter time to reach the target quadrant (14 ± 3.68) compared with the BMD group ($P < 0.05$), suggesting memory deterioration in the BMD group compared with the BMC group (Figures 5 and 6).

## Discussion

Nutrition is essential for the regular maintenance of life; however, the demand for good nutrition increases during pregnancy and lactation to provide optimum nutrition for growth and well-being.[13] Vitamin B6, one of the micronutrients, acts as a coenzyme in more than 120 enzymatic reactions that involve energy metabolism and neurotransmitter synthesis.[14]

Various studies have shown the effects of vitamin B6 deficiency on the brain, particularly the hippocampus.[4–7] In this study, the number of viable neurons was lower in the CA1 and CA3 hippocampal areas, particularly in the CA3 region of deficient mice compared to the controls, indicating the significance of vitamin B6 in the development and maintenance of hippocampal neurons.

The role of vitamin B6 in neurodevelopment has also been seen in the literature.[4] Vitamin B6 also plays a major role during the development of the fetus, particularly brain development and its functions. Wang et al. studied the effects of vitamin B supplementation, i.e., B6, B12, and folic acid, on a pregnant mouse exposed to particulate matter 2.5, which is known to cause neurodevelopmental impairment in offspring.[15] The authors concluded that vitamin B supplementation alleviated the mitochondrial damage in the hippocampus, prevented neuronal apoptosis, and improved neurogenesis in the hippocampus. In another study, the newborns of Wistar dams fed a vitamin B6-deficient diet showed dysregulation of glutamate metabolism, wherein upregulation of glutamine synthase and

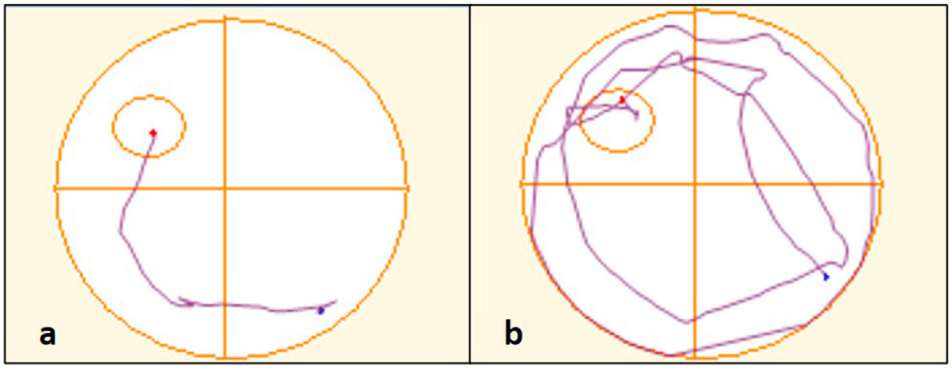


**Figure 6:** Swim tracks of the mice in the Morris water maze during the probe trial wherein (a) illustrates the tracking of the breed mice control to reach the island zone (the zone where the escape platform was placed during the acquisition trial) and (b) illustrates the swim track of breed mice-deficient to reach the island zone.

downregulation of glutaminase caused a disturbance in the concentration of glutamate in the brain.[4] Changes in gene expression associated with neurotransmitters such as glutamine, serotonin, and GABA leading to an imbalance in the concentrations of these neurotransmitters may be associated with autism, schizophrenia, and other neuropsychological and neurodevelopmental disorders in humans. In the present study, on behavioral analysis of mice, employing the MWM test for spatial learning and memory, the offspring of vitamin B6-deficient mice took significantly longer than control mice, suggesting impairment in the ability to learn and recall information during the retention or probe trial. This suggests that maternal vitamin B6 deficiency could hamper potential adult hippocampal neurogenesis in its offspring, resulting in poor spatial navigation.[16]

Although pyridoxine or vitamin B6 deficiency is rare due to its availability in various food sources including animal products, fruits, and vegetables, the demand for vitamins increases during pregnancy and lactation. Women in middle- and low-income countries may be deprived of these sources, and their deficiency could exacerbate potential adverse effects on the developing fetus.[17] The nutritional status of the mother is crucial, as it contributes to the biochemical, structural, and functional parameters of the developing fetal brain.[18] This study also suggests the need for supplementing vitamin B6 in the diets of pregnant and lactating mothers apart from other supplements such as iron and folic acid.

*Limitations of the study*: As this was a preliminary study, the parameters assessed were restricted to the maternal hippocampal microstructure and behavioral assessment, which only included assessment of spatial memory in offspring. Further assessments of behavioral parameters for testing object recognition memory and avoidance memory in offspring along with assessments of hippocampal cytoarchitecture could have strengthened the results of the study.

*Strength of the study*: Our study established a vitamin B6-deficiency model by administering a customized diet that did not contain vitamin B6. Although some studies have used a customized diet to create vitamin B6 deficiency, they were not validated as was done in the current study, wherein we confirmed the deficiency by assessing the levels of plasma PLP using LC–MS. Furthermore, the results obtained in this study add to the existing literature.

*Contribution of these findings to the literature and their possible clinical implications*: This preliminary study forms a basis for future research, wherein various parameters such as levels of GABA and quantification of GABAergic interneurons can be analyzed. Furthermore, the attainment of significant results regarding the impact of maternal vitamin B6 deficiency will provide a benchmark to modify guidelines on the requirements for vitamin B6, a vital micronutrient during pregnancy, apart from existing micronutrients such as iron and folic acid.

## Conclusion

This study successfully established a vitamin B6-deficient model by feeding the mice with a customized diet, which was confirmed by estimating the plasma PLP levels. Although the plasma levels of PLP were minimal in the mice, the method was sensitive, linear, and accurate for detecting the PLP levels. Furthermore, the study confirmed that the vitamin B6 deficiency impacted the hippocampal neuronal cells, indicating the possible damage to healthy neurons, and decreased hippocampal neurogenesis. These results emphaises the need to conduct additional studies on the neurodevelopmental effects of vitamin B6 deficiency as the offspring showed alterations in learning and memory. This study also demonstrates the need for vitamin B6 supplementation during pregnancy and lactation along with other supplements such as iron and folic acid.

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## Conflict of interest

The authors have no conflict of interest to declare.

## Ethical approval

The study was conducted after receiving approval from the institutional animal ethics committee (IAEC/KMC/24/2019).

## Authors' contribution

AS conceived and designed the study and prepared the original draft. AK curated the data. NB interpreted the data. SM and AR analyzed the data. PK collected and analyzed the data. SS reviewed and finalized the final manuscript. 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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