

## The effect of exclusive breastfeeding on synaptogenesis and cell survival in mouse offspring

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**ABSTRACT:** The first thousand days of life are critical for determining a child's cognitive development in humans. Breastfeeding may provide the nutrients needed for brain development. Cognitive function has been widely associated with neuronal turnover, driven by synaptogenesis and apoptosis. To explore this hypothesis, this study aimed to examine the effect of exclusive breastfeeding (EBF) on cognitive function and neuronal turnover in the dentate gyrus of the mice hippocampus. We conducted cognitive function assessments using the Morris Water Maze (MWM) test. We also examined neuronal apoptosis with TUNEL assay and synaptogenesis using PSD-95 antibodies. The newborn mice (mixed gender) aged 0 days were randomly divided into two groups. The first group received exclusive breastfeeding (EBF, n = 14), while the second group (MF, n = 14) received mixed breast milk and formula milk. At postnatal day 21 (PND21), the MWM test was performed, followed by an assessment of neuronal apoptosis and synaptogenesis. In the MWM test, the escape latency of the EBF group was shorter than the MF group. There was a significant increase in PSD-95 expression and a decrease in TUNEL expression in the EBF group ( $p < 0.001$ ) than in the MF group. In conclusion, exclusive breastfeeding is associated with higher cognitive function. Exclusive breastfeeding affects neuronal turnover by increasing synaptogenesis and decreasing apoptosis in the dentate gyrus of the hippocampus. This has both scientific and clinical implications, pointing to nutritional practices early in life that could optimise the attainment of cognitive potential.

**Keywords:** Breastfeeding; Neurogenesis; Cognitive development.

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## 1.0 INTRODUCTION

The ability of a child to grow and develop optimally is determined by the nutrition in the first thousand days of life in humans. The World Health Organization (WHO) and United Nations Children's Fund (UNICEF) recommend that babies should be breastfed exclusively during the first six months of life ([Salim & Stones, 2020](#)). The American Academy of Pediatrics also suggests breastfeeding for at least 12 months ([Meek et al., 2022](#)). Exclusive breastfeeding (EBF) refers to giving infants only breast milk to acquire adequate nutrition ([Sánchez-infantes et al., 2018](#)). EBF can increase the life expectancy of children ([Sankar et al., 2015](#)), protect against various allergic diseases ([Nuzzi et al., 2021](#)), and have been connected to gut health ([van den Elsen et al., 2019](#)). Moreover, several studies have found that breastfed participants were linked to higher scores on neurodevelopmental and cognition tests ([Plunkett et al., 2021](#)). Thus, EBF may provide the nutrients needed for brain development.

The decision to breastfeed is often influenced by several factors, resulting in many infants being unable to obtain exclusive breast milk ([Balogun et al., 2015](#)). Globally, only about 44% of babies aged 0–6 months receive EBF ([Gizaw et al., 2017](#)). Mothers commonly choose formula milk as a nutrition source, with cow milk as the base ingredient ([Griebler et al., 2016](#)). Formula milk is enriched with arachidonic acid (AA), docosahexaenoic acid (DHA), iron, and fat mixtures to mimic the composition of breast milk ([Lemaire et al., 2018](#); [Lorenzo et al., 2019](#)). Various formula milk is comparable to breast milk in many components; however, there is no ideal equivalent for breast milk. For example, heat processing used in the production of formula milk can affect the structure of proteins ([Krishna et al., 2021](#)), while whey and casein may form thermal-induced aggregates affecting the solubility ([O'Kennedy & Mounsey, 2006](#)). Although formula milk may also contain substances that help protect infants from illness, it cannot fully replicate the nutrition of breast milk, which contains thousands of beneficial components, including antibodies, hormones, white blood cells, prebiotics, and enzymes that support immune and digestive systems ([Lorenzo et al., 2019](#)). Furthermore, formula milk has higher quantities of vitamins and minerals, rendering it more difficult to digest ([Yang et al., 2018](#)). Thus, formula-fed babies often have digestive and immune problems, as well as problems with brain development ([Jewell et al., 2004](#)).

In recent years, the benefits of breast milk have attracted various neuroscience studies. Breast milk has

been suggested to contain one or more constituents to facilitate cognitive development ([Sánchez-infantes et al., 2018](#)). Previous studies have shown differences in cognitive development between breastfed and formula-fed babies ([Jewell et al., 2004](#)). Mental development and IQ scores of breastfed children were better than others ([Plunkett et al., 2021](#)). Studies in humans showed cognitive development affected by the development of white matter, as well as the maturation of basal ganglia and thalamus ([Isaacs et al., 2011](#)). Because cognitive scores are related to neurodevelopment, we hypothesised that breast milk mediates cognitive effects by affecting neuronal turnover.

To explore this hypothesis, this study aimed to examine the effect of EBF on cognitive function and neuronal turnover in the dentate gyrus of mice hippocampus. Among brain areas showing neurogenesis throughout life, we focused on the dentate gyrus of the hippocampus, which plays a vital role in controlling cognitive functions ([Irfannuddin et al., 2021](#)). We carried out cognitive function assessments using the MWM test. In the present study, we also examined neuronal turnover by assessing neuronal apoptosis and synaptogenesis in the dentate gyrus of the mice hippocampus. Various methods can measure neuronal apoptosis. Among such methods, the TUNEL assay can identify fragmented DNA in apoptotic cells ([Moore et al., 2021](#)). On the other hand, PSD-95 is an excellent marker for determining synaptic size and strength ([McEachern et al., 2020](#)). Thus, we studied the effects of EBF on the neuronal turnover in the dentate gyrus of mice hippocampus with TUNEL assay and immunohistochemistry (IHC) staining using PSD-95 antibodies.

## 2.0 MATERIALS AND METHODS

### 2.1 Animals

The subjects were mice offsprings from the mother strain Balb/C obtained from the animal house of the Faculty of Medicine, Universitas Sriwijaya. The mice were kept in a cage measuring 500 cm<sup>2</sup>, provided with *ad libitum* access to food and water, and then maintained in a room with a temperature of 20–24°C and a dark-light cycle for 12 hours.

The offspring mice (mixed gender) aged 0 days were randomly divided into two groups. The first group received exclusive breastfeeding (EBF, n = 14), while the second group received mixed breast milk and formula milk (mixed feeding/MF, n = 14). The formula milk was Nutri Growth KMR<sup>®</sup>, thawed through sonde for up to 0.5 cc/day. On PND21, mice were subjected to the MWM

test. Then, euthanasia was performed by cervical decapitation. Brain tissue was collected immediately and placed in a container containing 10% formalin solution. Histopathological examination was carried out through TUNEL assay and IHC staining.

Animal experiments were conducted in accordance with protocols approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Sriwijaya (protocol number: 200-2021). All procedures adhered to established criteria for the design and statistical analysis of research involving laboratory animals.

## 2.2 MWM Test Analysis

At PND21, the MWM test was conducted to assess spatial memory. The test used a circular open swimming arena with a diameter of 100 cm and was performed daily between 9:00 am and 12:00 pm. Mice underwent a three-day habituation period to the MWM apparatus ([Khan et al., 2019](#)). The test pool contained a cylindrical platform (10 cm diameter, 40 cm height) placed in the 3rd quadrant. The water level was maintained at 45 cm, with the platform submerged 0.5 cm below the surface. Both room and water temperatures were kept at  $22 \pm 2^\circ\text{C}$ . The MWM test consisted of place navigation trials (days 1-5) and a spatial probe trial (day 6). During the first five days, mice completed four trials per day. In each 90-second trial, mice swam freely to locate the hidden platform. The time taken to find and stand on the platform with all limbs was recorded as the escape latency. If a mouse failed to find the platform, it was guided to it, and the escape latency was recorded as 90 seconds. On day 6, the probe test was conducted. The platform was removed, and animals were placed in the pool at a designated location to swim freely for 90 seconds. The proportion of time spent in each quadrant was recorded.

## 2.3 TUNEL Assay Analysis

The brain was collected, fixed in 10% formalin, and embedded in paraffin. The paraffin block was sectioned with a microtome (3–4  $\mu\text{m}$  thick), mounted on glass slides, deparaffinised, and used for TUNEL Assay and IHC analysis ([Preissmann et al., 2012](#)). Apoptotic cells in brain tissue sections were identified using a cell *in situ* death detection kit (Roche, Switzerland) as described previously ([Chen et al., 2013](#)). In brief, after deparaffinization, the slides were incubated with proteinase K in 10 mM Tris (pH 8) for 20 minutes at room temperature. The slides were rinsed with 1X PBS, and then reacted with 3%  $\text{H}_2\text{O}_2$ , followed by incubation with 1X Reaction Buffer. End Labeling Reaction & Detection

were carried out with Complete Labeling Reaction Solution for 1 to 1.5 hours at  $37^\circ\text{C}$ . Subsequently, the slides were incubated with blocking buffer and antibody solution followed by DAB (3,3-diaminobenzidine tetrahydrochloride) chromogen (Solarbio, China). The reaction was terminated by rinsing with  $\text{H}_2\text{O}$ .

## 2.4 Immunohistochemistry (IHC)

Immunohistochemical analysis was conducted on hippocampal tissue using a multi-step process. First, the tissue was dehydrated by soaking twice in xylol and then in graded alcohol (96%, 90%, 80%, 70%). The sections were then washed in PBS (pH 7.4) three times for 5 minutes each, followed by immersion in 3%  $\text{H}_2\text{O}_2$  (in distilled water) for 20 minutes. After another PBS wash cycle, the tissues were soaked in 1% BSA for 10-30 minutes at room temperature and washed again in PBS. The primary antibody (ScyTek, USA) was applied to the sections for 1 hour at room temperature, followed by overnight incubation. After washing, the sections were incubated with a secondary antibody labeled with Strep avidin horseradish peroxidase (SA-HRP) for 1 hour at room temperature. Following another wash cycle, chromogen DAB was added dropwise and incubated for 5-10 minutes. The sections were then washed in distilled water, counterstained with hematoxylin for 5 minutes, and washed again. Finally, the slides were mounted with Entellan, and immunoreactivity was observed using an Optilab microscope.

## 2.5 Quantitative Analysis

Quantitative analysis of IHC preparations focused on five fields of view in the dentate gyrus, observed at 400x magnification using Olympus BX51 and CX22 light microscopes equipped with an Indomicro HDMI camera, and the Future Win Joe application. Digital image processing was employed to analyze synaptic plasticity by calculating the percentage of PSD-95-positive tissues, while the TUNEL assay was used to assess cell death. Using ImageJ software (version 1.50i), the percentage of positive cells was calculated in 5 Regions of Interest (ROI). Observations concentrated on cell nuclei, with average values computed across the five fields of view. The results for each group were then prepared for statistical analysis.

## 2.6 Statistical Analysis

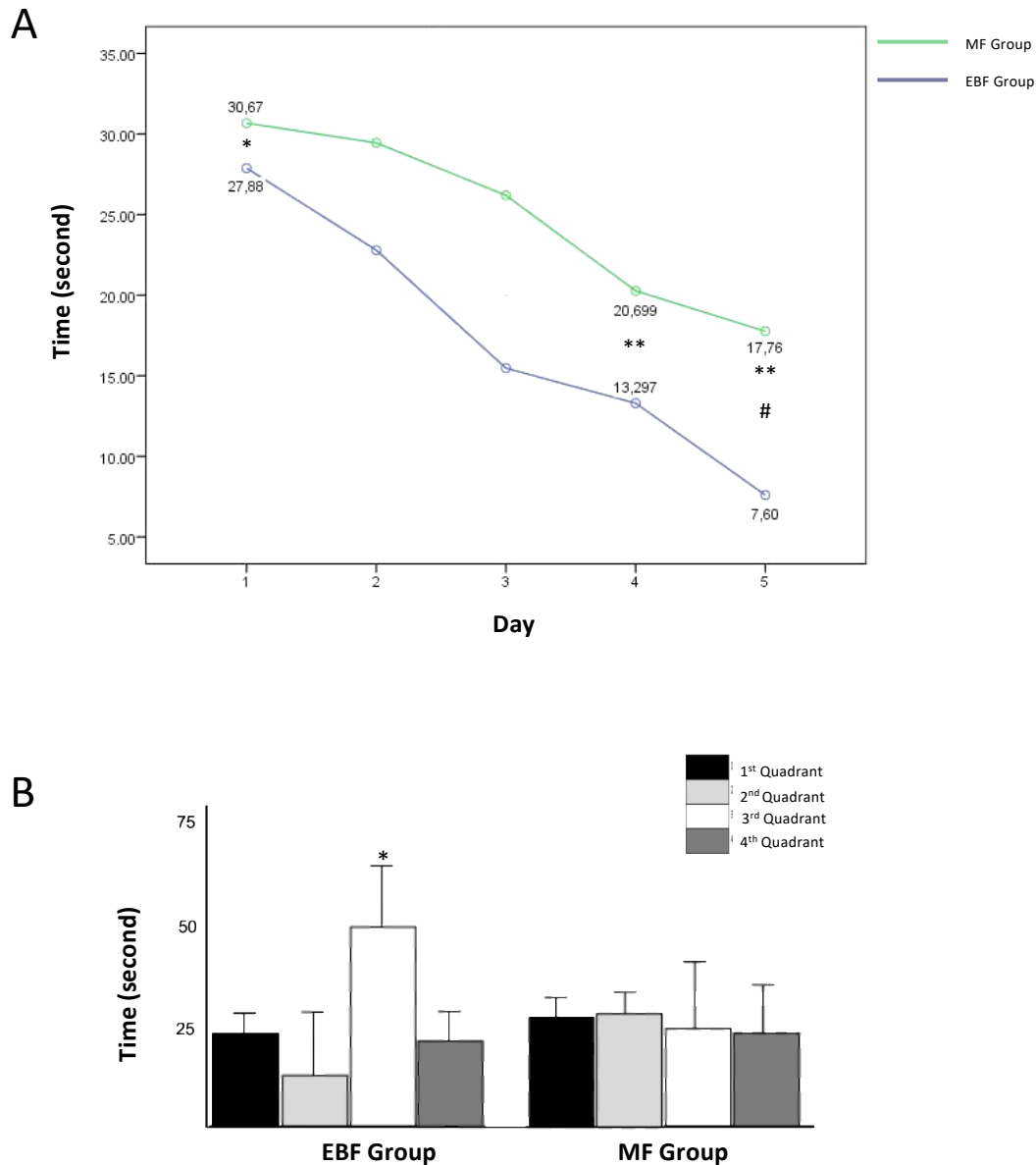
To examine the treatment effect of EBF, a Repeated Measure, Multiple Comparison with GLM-Repeated Measure test was employed. Additionally, individual differences in the percentage of TUNEL assay and PSD-95 positive areas were compared using independent T-test analysis.

### 3.0 RESULTS

#### 3.1 Spatial memory

Analysis of spatial memory acquisition and retention in the MWM test revealed significant differences between the EBF and MF groups (**Figure 1A**). GLM-Repeated Measure analysis showed significant differences in mean escape latency between day 1 vs day 4 and

between day 4 vs day 5. On the spatial probe trial (day 6), EBF mice demonstrated a preference for the target quadrant (3rd quadrant) where the platform was originally located [ $F(5,25)=3.62$ ,  $p=0.012$ ]. In contrast, MF mice failed to show such a preference [ $F(5,25)=0.87$ ,  $p=0.213$ ] (**Figure 1B**).



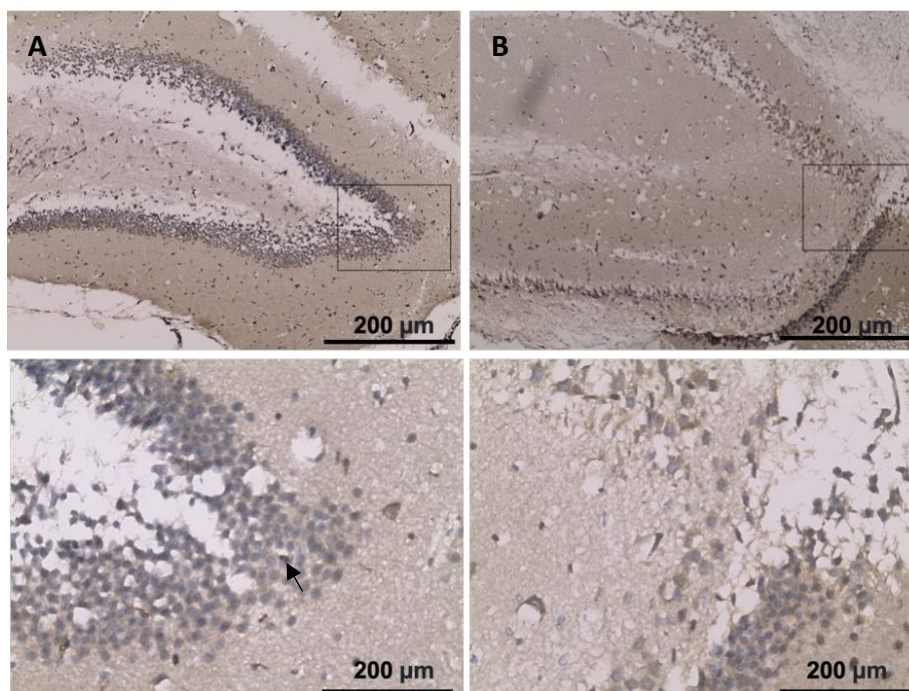
**Figure 1:** Performance of mice in the MWM test. **(A)** Escape latency test (mean  $\pm$  SD in second), a Repeated Measure Multiple Comparison with GLM-RM test showed a significant difference between EBF and MF groups. Multiple comparisons of the EBF and MF groups showed a significant difference in mean escape latency between day 1 vs day 4 ( $*p = 0.044$ ) and between day 4 vs day 5 ( $**p = 0.049$ ). **(B)** The proportion of total time spent in each quadrant. Mice in the EBF group showed a preference for the 3rd quadrant [ $F(5,25)=3.62$ ,  $p=0.012$ ].  $*p<0.05$  compared to day 5;  $\#p<0.05$  compared to MF group.

### 3.2 PSD-95 immunoreactivity

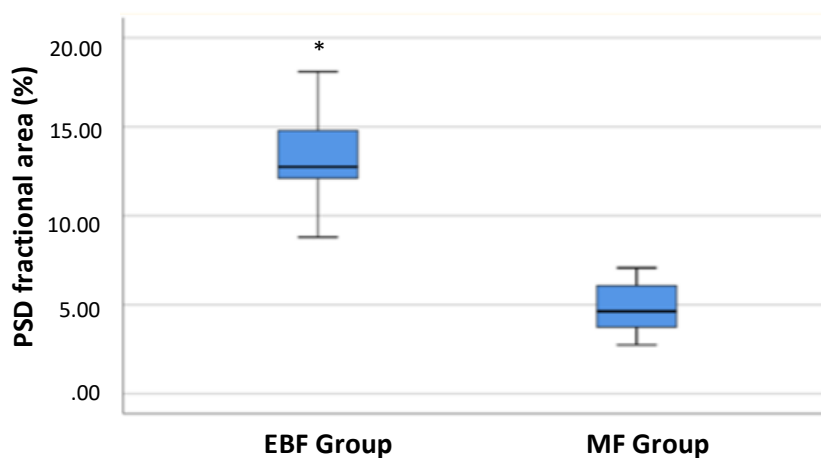
PSD-95 immunoreactivity in the dentate gyrus was assessed to evaluate neuroplasticity. A representative photomicrograph is shown in **Figure 2**, while **Figure 3** depicts the percentage area of PSD-95 immunoreactivity. A significant difference was observed between the EBF and MF groups ( $P < 0.05$ ), suggesting that exclusive breastfeeding enhances neuroplasticity in newborn mice.

### 3.3 Cell survival of the dentate gyrus

Apoptosis was evaluated using TUNEL staining. **Figure 4** presents a representative photomicrograph, and **Figure 5** shows the average percentage of TUNEL-positive areas. The EBF group exhibited significantly fewer TUNEL-positive cells compared to the MF group ( $P < 0.05$ ), indicating that exclusive breastfeeding may inhibit apoptosis in the dentate gyrus.

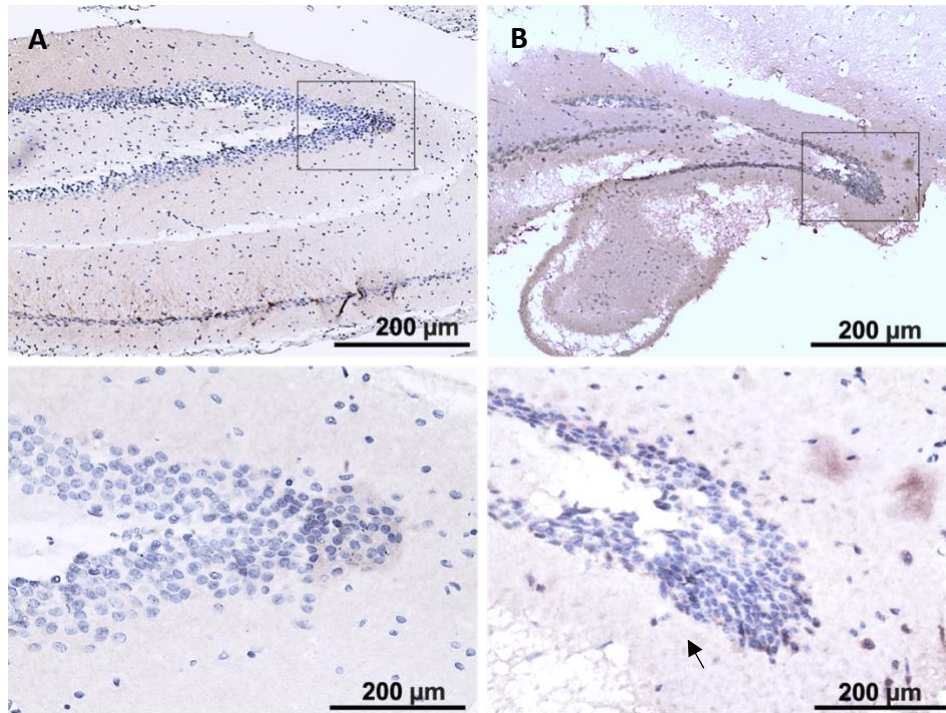


**Figure 2:** Immunohistochemistry of PSD-95 in the dentate gyrus. The black arrow pointed to positive cells. The PSD-95-positive tissues were seen more in the (A) EBF group than in the (B) MF group.

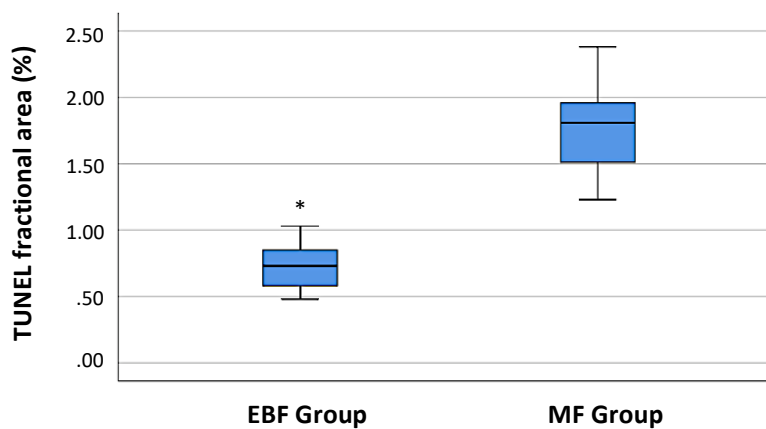


**Figure 3:** Percentage of PSD-95-positive tissues in the dentate gyrus of the hippocampus. The PSD-95-positive tissues were higher in the EBF group. The independent t-test showed a significant difference,  $t(26) = 10.42$ ,  $*p < 0.000$ . The data is expressed as average  $\pm$  SEM.  $n = 14$ .





**Figure 4:** TUNEL's expression in the dentate gyrus. The black arrow pointed to positive cells. The TUNEL-positive tissues were less visible in the (A) EBF group than in the (B) MF group.



**Figure 5:** Percentage of TUNEL-positive tissues in the dentate gyrus of the hippocampus. The TUNEL-positive tissues were lower in the EBF group. The independent t-test showed significant differences between the EBF group and the MF group,  $t(26) = 10.13$ ,  $p < 0.0001$ . The data is expressed as average  $\pm$  SEM.  $n = 14$ .

#### 4.0 DISCUSSION

This study compared the effects of EBF and MF on cognitive function and neuronal turnover in the dentate gyrus of the hippocampus in newborn mice. Our results demonstrate that EBF mice exhibited shorter escape latencies in the MWM test and showed a preference for the target quadrant during the spatial probe trial. These findings suggest that exclusive breastfeeding before weaning age (i.e., in the first six months of life) may enhance cognitive function. We observed that apoptosis

in the dentate gyrus, measured by the TUNEL assay, decreased in the EBF group compared to the MF group, indicating that apoptosis was inhibited by EBF but not by MF. Additionally, the number of PSD-95-positive cells increased significantly in the EBF group compared to the MF group, suggesting that EBF enhanced synaptogenesis. Collectively, these results indicate that EBF alters both synaptogenesis and apoptosis in the dentate gyrus of the hippocampus.

The hippocampus is critical for learning and memory and has been the brain structure most frequently studied as a substrate for these functions ([Irfannuddin et al., 2021](#)). The MWM is a reliable test to evaluate cognitive functions in rodents ([Khan et al., 2019](#)). Our study showed that the escape latency of the EBF group was shorter than that of the MF group. Furthermore, on the spatial probe trial on day 6, mice in the EBF group showed a preference for the target quadrant where the platform had been originally located. These results indicate that the EBF animals used a more focused search strategy in the probe trial, potentially indicating a more precise memory trace for the platform location. These findings align with a previous study on Sprague Dawley rats given breast milk from PND3 until weaning, which showed improved performance on cognitive behavioral tests ([Docq et al., 2020](#)). Together, these results suggest that EBF has the potential to improve learning and memory.

Cognitive function has been widely associated with neuronal turnover, which is driven by neurogenesis, synaptogenesis, and neuronal loss or apoptosis ([Gorecki, 2022](#)). Our study showed that EBF mice had decreased apoptosis as assessed by TUNEL assay and increased synaptogenesis as assessed by PSD-95 expression compared to the MF group. Many previous studies have shown decreased hippocampal neurons in cognitively impaired animals ([Irfannuddin et al., 2021](#); [Talpos et al., 2008](#)). The TUNEL assay detects fragmented DNA in apoptotic cells ([Moore et al., 2021](#)), while PSD-95 is an excellent marker for determining the size and strength of synapses ([McEachern et al., 2020](#)).

Decreased neurogenesis in cognitive decline has been reported in the granule cell layer and hilus of the dentate gyrus between 2 and 18 months in C57BL/6 mice. Computational studies using various neural characteristics have consistently found that synaptic weight change and neurogenesis algorithms improve the ability of information-carrying networks to learn new information ([Chambers et al., 2007](#)). Taken together, our results suggest that EBF mediates cognitive effects presumably by affecting neuronal turnover.

In this study, cognitive function and neuronal turnover assessment were carried out at PND21, which corresponds to the golden period age in humans ([Semple et al., 2013](#)). This timing makes our model particularly useful for examining the effect of early nutrition on various organ functions, including the brain.

Previous research has shown that exclusive breastfeeding increases brain size and white matter ([Isaacs et al., 2011](#)). Breast milk contains bioactive molecules essential for brain and cognitive development ([Dror & Allen, 2018](#)). A major advantage of breast milk is that its composition changes with the growth needs of infants. In the first five days of life, breast milk called colostrum contains more protein and less lactose ([Dror & Allen, 2018](#)), suggesting a protective role at the beginning of life. Colostrum production decreases at 7–14 days after calving as the composition changes to mature milk fluid ([Lubetzky et al., 2015](#)). Mature milk contains more lipids and higher concentrations of casein. In contrast, formula contains more casein than breast milk ([Thakkar et al., 2016](#)), but this casein can coagulate in the stomach, prolonging the digestion process. This problem rarely arises with breast milk because the casein is easily digested and efficiently used. Casein is a protein that plays a significant role in brain activity and autonomic nerve activity ([Nakamura et al., 2010](#)). Given the composition of breast milk, breastfeeding for the first six months of life has the potential to improve cognitive function.

## 5.0 CONCLUSION

The present evidence supports the hypothesis that the effects of exclusive breastfeeding are associated with a higher cognitive level. Our study helps provide a framework for a probable mechanism of the impact of exclusive breastfeeding on neuronal turnover. Exclusive breastfeeding affects neuronal turnover by increasing synaptogenesis and decreasing apoptosis in the dentate gyrus of the hippocampus, which is known to be beneficial to brain function. This has both scientific and clinical implications, pointing to nutritional practices early in life that could optimize the attainment of cognitive potential.

**Author contributions:** II was responsible for proposing the grant. II and SSFP were responsible for the concept, design of experiments, and interpretation of data. II, SSFP, KM, YK, HD, and NK were responsible for experimentation procedures, data collection, and data analysis. All authors edited, revised, read, and approved the final manuscript.

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**Conflicts of interest:** The authors declare no conflict of interest.

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