The impact of *in ovo* administration of folic acid on gga-mir-let-7k-5p, Cyclin D1 (CCND1) and nerve growth factor (NGF) expression in the cerebral cortex of chicken embryos

T. Khoshkenar†, F. Mashayekhi, M. Mottaghi† and Z. Salehi*

Abstract

Folate is essential for the proper development of the brain. The gene CCND1 is responsible for the synthesis of the cyclin D1 protein, which facilitates the differentiation and proliferation of neurons. Nerve growth factor (NGF) is critical for the survival and maturation of neurons. MicroRNAs are involved in regulating various phases of neurogenesis, including the proliferation of neural stem cells and differentiation of neurons. Given the important role of folic acid (FA) in the regulation of gene expression, the aim of this study was to evaluate the effects of in ovo injection of FA on expression levels of gga-mir-let-7k-5p, CCND1, and NGF in the cerebral cortex of chicken embryos. This study involved a total of 120 hatching eggs. Of these, 40 eggs were administered FA injections into the amniotic fluid at a dosage of 150 µg per egg, another 40 eggs received Phosphate buffered saline (PBS) injections (Sham) on embryonic day 11, while the remaining 40 eggs served as controls without any injections. Cortical tissues were subsequently collected on embryonic day 19, and Real-time PCR was employed to analyse the expression of mir-let-7k-5p, CCND1, and NGF. The results revealed that the expression levels of gga-mir-let-7k-5p in the FA-treated, Sham, and control groups were 0.46±0.09, 0.73±0.18, and 0.76±0.17-fold change, respectively. Additionally, the expression levels of CCND1 were recorded at 1.77±0.38, 1.18±0.25, and 1.11±0.28-fold change, respectively, while NGF levels exhibited similar patterns at 1.92±0.50, 1.12±0.25, and 1.04±0.21-fold change, respectively ($P<0.05$). Statistical analysis indicated a significant increase in the expression of CCND1 and NGF, alongside a reduction in gga-mir-let-7k-5p expression in

Tara KHOSHKENAR, MSc, Farhad MASHAYEKHI*, (Corresponding author, e-mail: mashayekhi@guilan.ac.ir), PhD, Professor, Mahshad MOTTAGHI, Zivar SALEHI, MD, PhD, Professor, Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran

†The first and third authors contributed equally to this study

cortices treated with FA compared to the control. This suggests that the in ovo administration of FA enhances the expression of CCND1 and NGF, while decreasing gga-mir-let-7k-5p expression in the developing cerebral cortex of chicks. Enhancing our understanding of how folate impacts the activation of genes and microRNAs related to the development of the cerebral cortex, and its potential connection to neural tube defects, will improve our knowledge of brain development, and could result in new treatment approaches to further reduce the occurrence of these abnormalities..

Key words: *folate; miRNA; gene expression; brain; chick embryo*

Introduction

Folate, also known as vitamin B_9, plays a crucial role in the closure of the neural tube. The mechanism by which a deficiency in folic acid leads to neural tube disorders is currently an active subject of research. Folic acid (FA), the synthetic form of folate, is a vital nutrient involved in regulating DNA synthesis during brain cell division (Barbour et al., 2012), and is also essential for regulating DNA methylation. Additional evidence supporting the importance of epigenetic mechanisms in proper neural tube development comes from findings demonstrating that manipulating histone-modifying enzymes (acetyltransferases, deacetylases, demethylases) leads to neural tube defects (Murko et al., 2013). The occurrence of 25–30% of human neural tube birth defects could be potentially reduced by increase the folate ingested by pregnant women. Therefore, the U.S. Public Health Service advises women of childbearing age to consume 0.4 milligrams of folic acid daily (Czeizel and Dudás, 1992). Inside the cell, both folate and FA go through a series of conversion processes to produce 5-methyltetrahydrofolate, the biologically active form utilised in cellular processes (Greenberg et al., 2011). Folate requirements in the body increase during pregnancy to support the rapid growth of maternal and foetal tissue. The available research, both in clinical and preclinical settings, indicates that a lack of maternal folate can lead to complications in offspring, such as changes in brain development and structure, including reduced brain size and decreased thickness in certain brain regions (Zou et al., 2021). Studies in clinical settings have also demonstrated that the folate status of the mother can influence the neurodevelopment of her offspring and contribute to neuropsychological disorders. Providing sufficient folic acid supplementation during pregnancy and throughout gestation is linked to positive effects on child neurodevelopment (Caffrey et al., 2019). Folate plays a crucial role in cell metabolism, acting as a coenzyme in the synthesis of purines and pyrimidines, DNA repair, and methylation reactions that impact gene expression. Folate deficiency in pregnant women has been recognised as a cause of neural issues during pregnancy and after birth, though the specific mechanism is still unknown (Ducker and Rabinowitz, 2017). Folate intake during pregnancy can also influence the activity of genes and proteins related to brain function (Naninck et al., 2019).

Genes such as nerve growth factor (NGF) play a significant role in the development of the brain. In vertebrates, NGF is crucial for the growth and survival of sympathetic neurons and sensory neurons found in the dorsal root ganglion. It promotes the outgrowth of neurites, increases the size of neuron cells, and is essential for brain development. The en-

largement of chick embryo sensory and sympathetic ganglia as a result of NGF treatment is attributed to the improved survival of neurons that would otherwise deteriorate. There might also be an increase in glial cell mitosis. Throughout embryonic development, the survival of most neurons relies on NGF and its receptor NTRK1 (also called TrkA) (Akassoglou, 2005). The gene expression of neurotrophins, such as NGF, and their signalling molecules can be affected by FA and vitamin B12 (Sable et al., 2014).

Normal cell proliferation is essential for proper brain development, with factors like cyclins playing a crucial part in this process. Positive regulators of the mid-G1-transition are cyclin D1 (Sherr and Roberts, 2004). During mouse brain development, it is anticipated that cyclin D1 expression will be high and have a significant impact. In the embryonic brain, a high level of cyclin D1 expression has been observed (Chen et al., 2005).

Many developing cells possess specific regulatory RNAs known as microRNAs (miRNAs) that play a role in controlling protein synthesis and influencing networks of cellular events. Additionally, certain miRNAs are linked to specific developmental processes. Research has indicated that a small subset of miRNAs in neural stem cells may be influenced by ethanol, with some of these miRNAs being crucial for neuron formation during development (Shaikh and Doshi, 2024). Brain miRNAs are critical in processes such as neurogenesis, synapse formation, neural stem cell proliferation, and differentiation. Furthermore, miRNAs can be impacted by epigenetic mechanisms, such as DNA methylation, which can also regulate the expression of other miRNAs. The miRNA levels and their genetic targets can be regulated by the interplay of these two mechanisms. This means that environmental factors like folate might impact miRNA profiles by changing the DNA methylation of genes encoding miRNA, or by adjusting the expression of genes situated before the miRNA signalling pathways (Beckett et al., 2017).

According to the Mirdb database, two target genes for the selected miRNA (gga-mir-let-7k-5p) are Cyclin D1 (CCND1) and nerve growth factor (NGF). Considering the significance of folate, NGF, CCND1, and miRNAs in brain development, the aim of this study was to investigate the impact of *in ovo* feeding of FA on gga-mir-let-7k-5p, CCND1, and NGF expression in the cerebral cortex of chick embryos.

Materials and Methods

Fertilised eggs

In the experiment, 120 hatching eggs of *Gallus gallus domesticus* from the ROSS 308 line of 42-week-old parental broiler stock were utilised. These eggs were of normal shape and weight, with a mean weight of 65 ± 4.50 g. They were subjected to an incubation temperature of 37.5°C and a relative humidity of 70%.

Folic acid treatment

In total, 120 fertilised eggs were chosen and divided into three groups. Egg surfaces were disinfected with 70% ethanol before a small hole (2 mm diameter) was created using an 18G needle at the wider end of the egg. In the first group (n=40), FA (Sigma-Aldrich, Darmstadt, Germany) was injected into the amniotic fluid at a dose of 150 μg per egg. FA was dissolved in 0.2 mL Phosphate buffered saline (PBS) and then injected into the amniotic sac using a method previously described (Foye et al., 2006). The Sham group (n=40) received an injection of the same volume (0.2 mL PBS), while the

control group eggs (n=40) were not injected. The holes were sealed with hot paraffin after manipulation, and the eggs were placed back into incubation. On embryonic day 19 (E19), the cortices were collected and stored at -70ºC for the analysis of gga-mir-let-7k-5p, CCND1, and NGF expression. All procedures involving animals were conducted in compliance with the Animals (Scientific Procedures) Act (1986). The animal protocols used in this study were approved by the University of Guilan's institutional animal experimentation committee and Review Board (Approval code: IR.GUILAN.REC.1401.005).

Analysing mir-let-7k-5p, CCND1, and NGF expression using Real-time PCR

RNA was extracted from cortices using TRIzol reagent (Invitrogen, USA) as per the manufacturer's guidelines. The Nanodrop UV-Vis spectrophotometer was used to measure the purity and concentration of total RNA at 260 and 280 nm. The integrity of the RNA samples was assessed with 2.0% agarose gel electrophoresis. Following the manufacturer's protocol, cDNA was synthesised using the PrimeScript 1st strand cDNA Synthesis Kit (Takahara, Japan). The resulting cDNA was then stored at -20ºC until future analysis. The Roche lightcycler® 96 Instrument (Roche Molecular Systems) was used for the qPCR with Green Hot Master Mix (BioRon, Germany). Table 1 contains the gene primer sequences. The forward and reverse primers were developed based on DNA sequences sourced from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank) using Oligo-primer analysis software (Version 7.54, Molecular Biology Insights, USA) and were manufactured by Bioneer Company (Daejeon, South Korea). The real-time PCR procedure followed the method described previously (Zhao et al., 2021).

Statistical analysis

The gene expression quantity was assessed following the method outlined by Livak and Schmittgen (2001). Statistical analysis was carried out using GraphPad Prism version 8.0 software, and the results were expressed as mean ± standard deviation. The values were subjected

Table 1. Gene symbol and primer sequence of selected candidate references genes

Gene symbol	Primer sequence (5´→3´)	Product size bp
gga-CCND1	Forward: 5´-CCCGACGAGTTACTGCAAATGG-3´ Reverse: 5´-GATGATCTGTTTGGTGTCCTCTGC-3´	135
gga-NGF	Forward: 5´-GGTTTCTCAGCAGTGCAGTCTC-3´ Reverse: 5´-CCTCTTTGCCTTTGATGTCGGTG-3´	153
gga-GAPDH	Forward: 5´-AGTCGGAGTCAACGGATTTGG-3´ Reverse: 5´-CGCTCCTGGAAGATAGTGATGG-3´	225
gga-mir-let-7k-5p	Forward: 5´-CCGTCGTGAGGTAGTATTGA-3´ Reverse: 5´-AGGGTCCGAGGTATTCGC-3´	83
gga-U6	Forward: 5´-CACATATACTAAAATTGGAACGATACAGAG-3´ Reverse: 5´-GCGTCGACTAGTACAACTCAAG-3´	149

to one-way ANOVA for comparison, and the results were presented as Mean ± SD. A significance level of $P \leq 0.05$ was considered statistically significant.

Results

mir-let-7k-5p relative expression

In this study, a total of 120 hatching eggs were used, with 40 eggs in each of three groups (FA treated, Sham, and control). On embryonic day 11 (E11) FA was injected to amniotic fluid (FA group) while the sham group was received only PBS on E11, and the control group were not received any injection. On E19, cerebral cortices of all groups were collected, total RNA extracted for the study of CCND, NGF, and gga-mir-let-7k-5p expression using Real-time PCR (Figures 1, 2). The results indicated a decrease in the expression level of mir-let-7k-5p in the cortices treated with FA (0.46 ± 0.09-fold) compared to the Sham and control groups, which had a 0.73 ± 0.18, and 0.76 ± 0.17-fold change, respectively (Figure 3). Statistical analysis revealed a significant decrease in mir-let-7k-5p expression in the cortices of FA treated groups compared to either the Sham or control group (P=0.001 and 0.0004, respectively). However, there was no significant difference in mir-let-7k-5p expression in the cerebral cortex between the Sham and control groups (P=0.72).

CCND1 and NGF relative expression

The Real-time PCR analysis also included the study of CCND1 and NGF, the target genes of mir-let-7k-5p. Upon analysis, it was discovered that the expression levels of both CCND1 and NGF

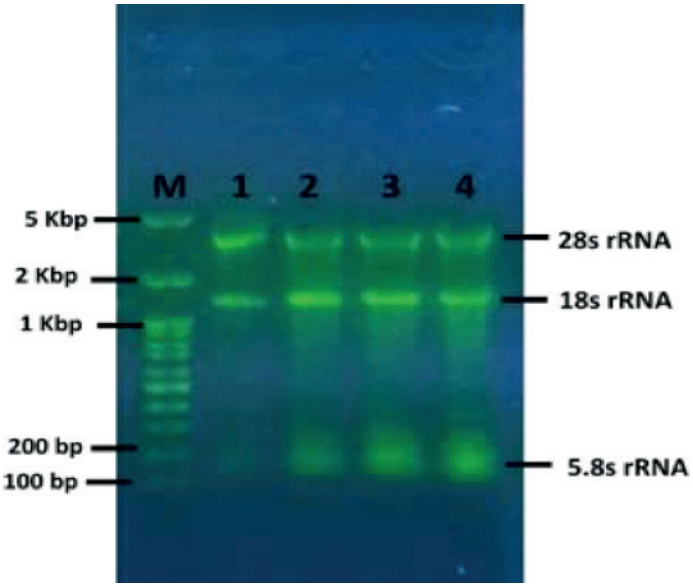

Figure 1. Total RNA purified with Trizol was subjected to agarose gel electrophoresis, along with mRNA extracted from cerebral cortex using Trizol-purified total RNA. Abbreviations: bp= base pair; kbp = kilobases pair; M = molecular weight marker; 28S, 18S and 5S = 28S, 18S and 5S ribosomal RNA.

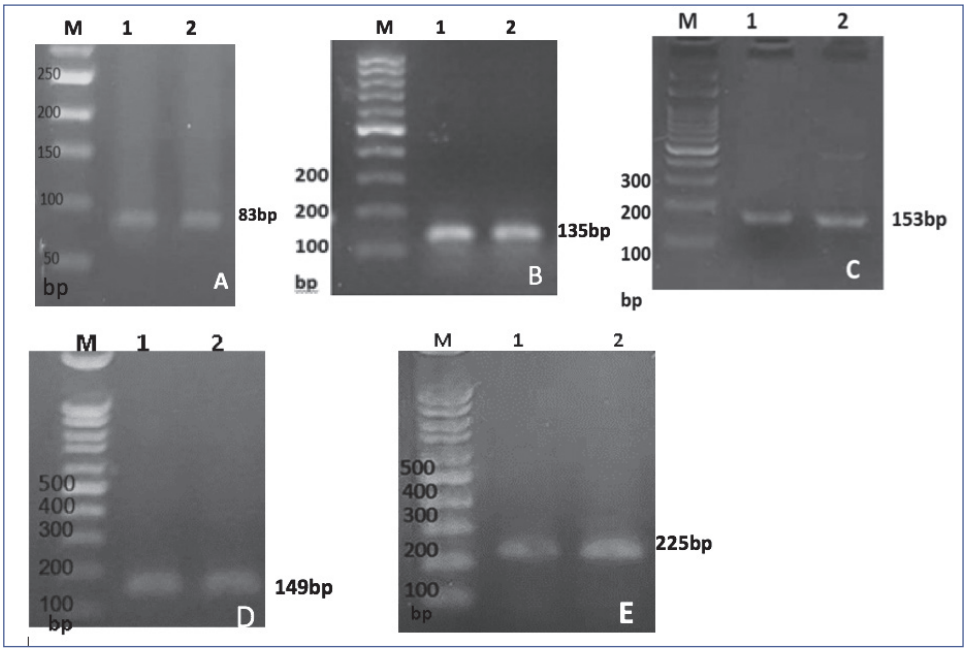

Figure 2. Agarose gel electrophoresis of PCR products of cerebral cortical samples (A-D). (A): gga-let-7k-5p, 83 bp, 1-4=samples; (B): CCND1, 135bp, 1-4= Samples. (C): NGF, 153bp, 1-2= Samples. (D): U6, 149bp, 1-4=Samples. (E): GAPDH, 225bp, 1-4=cerebral cortical Samples.

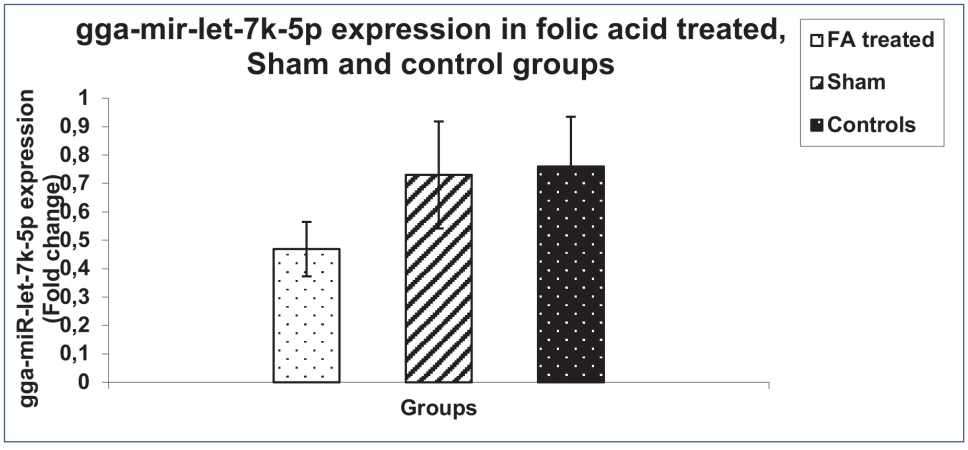

Figure 3. The expression of gga-miR-let-7k-5p in the cerebral cortices showed values of 0.46 ± 0.09, 0.73 ± 0.18, and 0.76 ± 0.17-fold change for the folic acid treated (FA) (on E11), Sham, and control groups, respectively. The folic acid treated cortices exhibited a decreased expression of gga-miR-let-7k-5p compared to both the Sham and control groups (*P*=0.001 and 0.0004, respectively), while no significant change was observed between the Sham and control groups (*P*=0.72).

were higher in the cortices treated with FA compared to both the Sham and control groups. Specifically, the expression levels of CCND1 in the FA treated, Sham, and control groups were measured at 1.77 ± 0.38, 1.18 ± 0.25, and 1.11 ± 0.28-fold change, respectively (Figure 4). Similarly, the expression levels for NGF in the FA treated, Sham, and control groups were 1.92 ± 0.50, 1.12 ± 0.25, and 1.04 ± 0.21-fold change, respectively (Figure 5). Statistical analysis demonstrated a significant increase in the expression of CCND1 and NGF in the cerebral cortices of the FA treated group compared to both the Sham and control groups ($P<0.001$). However, there was no significant difference in the CCND1 and NGF expression in the cerebral cortices between the Sham and control groups ($P=0.60$ and $P=0.50$, respectively).

Discussion

Embryonic development requires the essential nutrient folate. A deficiency in folate can lead to embryonic lethality, neural tube defects, and orofacial anomalies. Folate receptor 1 (Folr1) is a protein that binds to folate and helps in the absorption of dietary folate at the cellular level. Research indicates that almost half of orofacial clefts could be prevented by supplementing with folic acid before and during early pregnancy, highlighting the crucial role of folate in normal orofacial development. These findings collectively suggest that any changes in optimal folate levels or folate utilisation during early pregnancy could negatively affect normal orofacial development (Seelan et al., 2021). Researchers are interested in the potential contribution of folate in preventing disorders related to CNS development, dementia, including Alzheimer's disease, and aging. The precise process by which folate functions in the development of the neural tube and brain is still not fully comprehended.

The family of growth factors, including NGF, plays a vital role in brain de-

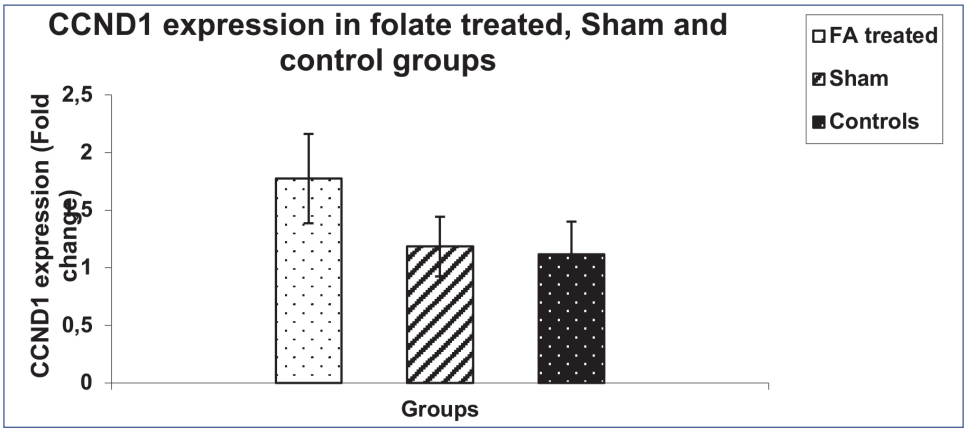

Figure 4. In the cerebral cortices of folic acid treated (FA) embryos on E11, the expression of CCND1 was 1.77 ± 0.38-fold change, while in the Sham and control groups, it was 1.18 ± 0.25 and 1.11 ± 0.28-fold change, respectively. Comparatively, the CCND1 expression in the folic acid treated cortices showed a significant increase compared to both the Sham and control groups ($P=0.001$ and 0.0008, respectively). However, there was no significant difference in CCND1 expression between the Sham and control groups ($P=0.6$).

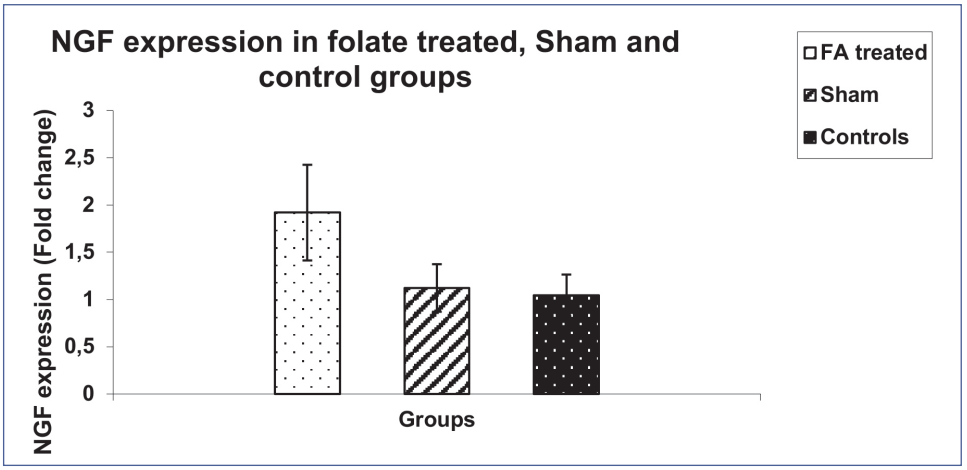

Figure 5. NGF expression in the cerebral cortices of folic acid treated (on E11), Sham, and control groups was 1.92 ± 0.50, 1.12 ± 0.25, and 1.04 ± 0.21-fold change, respectively. The folic acid treated cortices showed increased NGF expression compared to the Sham and control groups (P=0.0006 and 0.0002, respectively); however, there were no significant differences between the Sham and control groups (P=0.5).

velopment by supporting neural cell proliferation, maintaining specific neuronal populations, and promoting axonal growth through the activation of the tyrosine kinase TrkA and p75 receptors (Aloe et al., 2016). NGF supports the survival, growth, and regulation of the neuron life cycle, as well as the differentiation of neuronal cells in both the central nervous system and the peripheral nervous system during development and adulthood. There is a suggestion that FA might enhance the repair of peripheral nerve injuries by stimulating the proliferation and migration of Schwann cells and promoting the secretion of NGF (Amadoro et al., 2021).

This study investigated the effects of administering FA *in ovo* on the expression of mir-let-7k-5p, CCND1, and NGF in the developing chicken cerebral cortex. Previous findings demonstrated that supplementing folate during the early stages of life impacted gene expression in weaning mice and resulted in long-term behavioural deficiencies in adult mice in a dose-dependent manner (Chu et al., 2019). Studies have suggested that folate induces alterations in promoter methylation within the CpG island of the TSSK3 gene. It has been noted that providing FA supplementation during the early stages of life could prevent changes in promoter methylation status and gene expressions in the livers of animal models with intrauterine growth restriction (IUGR) (Jing-Bo et al., 2013). The expression of genes involved in regulating food intake may be influenced by maternal FA supplementation, according to Huot et al. (2016). Research revealed that injecting FA on the first day of incubation can enhance gene expression and performance in broilers (Gamboa Gonzales et al., 2023). Additionally, a study found that moderate FA supplementation throughout pregnancy and lactation results in sex-specific changes in gene expression in the brains of mouse embryos at day 17.5 and in 30-day-old mouse pups, affecting genes related to

angiogenesis, neurotransmission, and neuronal growth and development. It has also been shown to cause changes in gene expression and behaviours in mouse female offspring (Yang et al., 2021). Furthermore, research has found that vitamin B12-FA supplementation can regulate the expression of neuronal immediate early genes (nIEGs) and enhance the dendritic arborisation of hippocampal neurons and memory in elderly male mice (Barman et al., 2021). Lastly, supplementation with FA during late gestation or throughout pregnancy has been linked to a significant decrease in Er-α gene expression in the liver. Moreover, maternal FA supplementation impacts tissue folate concentrations, DNA methylation, and gene expression in the offspring in a manner that depends on the gestation period and the specific organ (Ly et al., 2016). Supplementing maternal FA could significantly improve the expression of DNMT-1 and Bcl-2 genes while reducing the expression of p53, Bax, Mpg, and Apex-1 in the jejunum section of IUGR piglets. IUGR hinders intestinal development and the expression of apoptosis-related genes in piglets, but maternal FA supplementation can enhance the expression of apoptosis-related genes in the jejunum section of the intestine (Liu et al., 2011). FA has the ability to modify the gene expression of neurotrophins, including BDNF (Razeghi et al., 2023).

Additionally, multiple research studies have indicated that FA can induce changes in miRNA expression. Researchers have demonstrated that the administration of FA led to alterations in the expression of gga-miR-182-5p and gga-miR-190a-3p in the cerebral cortex of chicken embryos (Razeghi et al., 2023; Heydari et al., 2024). It has been proposed that FA may influence the epigenetic regulation of hepatic microRNAs (miR-21, -34a, and -122) and the expression of their target genes (HBP1, SIRT1, and SREBP-1c) in rats (Salman et al., 2022). A study by Li et al. (2018) suggested that maternal FA deficiency triggers neuronal apoptosis through microRNA-34a associated with Bcl-2 signalling in rat offspring. Moreover, it was found that FA protected neuronal cells from aluminium-maltolate-induced apoptosis by averting the decrease of miR-19 and adjusting the miR-19 related downstream PTEN/AKT/p53 pathway (Zhu et al., 2016). In the brains of mice, FA has the ability to decrease the expression of miR-106a-5p, miR-200b-3p, and miR-339-5p (Liu et al., 2015). There is a suggestion that miRNA mis-regulation and the down-regulation of mir-10a might be associated with FA deficiency and NTDs (Shookhoff and Gallicano, 2010). It has been proposed that the folate receptor alpha (FRα) operates by directly stimulating the production of miRNAs that target these genes or their effector molecules (Mohanty et al., 2016). Research has shown that paternal folate could influence lipid and glucose metabolism in the offspring of broiler chickens over multiple generations, and the transmission of epigenetic changes might involve modifications in sperm miRNAs and lncRNAs (Wu et al., 2019).

One of the primary events in brain development is cell proliferation. Cyclin-D1 (CCND1) is widely recognised for its role in promoting the G1-S transition of actively dividing cells in partnership with CDK proteins. It has been documented that CCND1 enhances the proliferation of cerebellar granule cell progenitors (GCPs) (Pogoriler et al., 2006). Previous studies have demonstrated that adding FA to the diets of cows led to an increase milk production and levels of insulin-like growth factor-1 and oestradiol in the serum. These findings suggested that FA

stimulates the proliferation of bovine mammary gland epithelial cells (BMECs) by amplifying the expression of proliferating cell nuclear antigen (PCNA), cyclin A2 and cyclin D1 (CCND1), and cyclin A1 (Zhang et al., 2023). The absence of FA has been observed to lead to a reduction in cell proliferation, disruption of the cell cycle, and the onset of cellular senescence in myoblasts, suggesting that FA deficiency affects the development of skeletal muscle (Hwang et al., 2018). Experiments conducted *in vitro* have demonstrated that culturing cells in folate-depleted media leads to decreased cell proliferation, differentiation, and survival in the rat H19-7 hippocampal cell line (Akchiche et al., 2012). Furthermore, the addition of FA has been shown to enhance the proliferation of embryonic neural stem cells (NSCs). Embryonic NSCs respond to FA by increasing Notch signalling and cell proliferation. This mechanism could potentially explain the impact of FA supplementation on neurogenesis in the embryonic nervous system (Liu et al., 2010).

Proper brain growth requires normal cell proliferation, in which factors such as cyclins play an important role. Cyclin D1 (CCND1) expression is highly elevated in the developing brain, as reported by Chen et al. (2005). Folate, a member of the vitamin B family, plays a role in methylation reactions necessary for nucleotide synthesis, supporting rapid growth by enabling DNA synthesis in rapidly dividing cells. Therefore, this study aimed to assess the effect of *in ovo* administration of folate on mir-let-7k-5p, CCND1, and NGF expression in the cerebral cortex of chicken embryos.

The investigation into how FA deficiency leads to NTDs is currently a significant area of study. FA is a crucial nutrient involved in regulating DNA synthesis during brain cell proliferation and plays a key role in regulating DNA methylation. Epigenetic mechanisms are essential for normal development of the neural tube and brain. NTDs may be caused by impaired DNA methylation (Barbour et al., 2012). Regardless of the mechanism, it has been suggested that FA supplementation in pregnant women could prevent 20-30% of NTDs in humans.

In summary, the study's findings indicated that administering FA *in ovo* led to a decrease in gga-mir-let-7k-5p expression and an increase in the expression of its two target genes, CCND1 and NGF, in the developing cerebral cortex of chickens. Gaining a better understanding of how FA might impact the expression of genes and miRNAs related to cerebral cortex development, and whether this could contribute to NTDs, will enhance our understanding of brain development and potentially pave the way for new treatments to further reduce their occurrence.

Acknowledgements

The authors appreciate the Vice President for Research of the University of Guilan for providing research facilities.

References

1. AKASSOGLOU, K. (2005): Nerve growth factor-independent neuronal survival: a role for NO donors. Mol. Pharmacol. 68, 952-965. 10.1124/mol.105.017277.
2. AKCHICHE, N., C. BOSSENMEYER-POURIÉ, R. KEREK et al. (2012): Homocysteinylation of neuronal proteins contributes to folate deficiency-associated alterations of differentiation, vesicular transport, and plasticity in hippocampal neuronal cells." FASEB. J. 26, 3980-3992. 10.1096/fj.12-205757.
3. ALOE, L., M. L. ROCCO, B.O. BALZAMINO et al. (2016): Nerve growth factor: role in growth, differentiation and controlling cancer cell development. J. Exp. Clin. Cancer. Res. 35, 116. 10.1186/s13046-016-0395-y.
4. AMADORO, G., V. LATINA B. O. BALZAMINO et al. (2021): Nerve Growth Factor-Based Therapy in Alzheimer's Disease and Age-Related Macular

Degeneration. Front. Neurosci. 15, 735928. 10.3389/fnins.2021.735928. eCollection 2021.
5. BARBOUR, R. S., M. MACLEOD, G. MIRES et al. (2012): Uptake of folic acid supplements before and during pregnancy: focus group analysis of women's views and experiences. J. Hum. Nutr. Diet. 25, 140-147. 10.1111/j.1365-277X.2011.01216.x.
6. BARMAN, B., A. KUSHWAHA, M. K. THAKUR (2021): Vitamin B12-folic acid supplementation regulates neuronal immediate early gene expression and improves hippocampal dendritic arborization and memory in old male mice. Neurochem. Int. 150, 105181. 10.1016/j.neuint.2021.105181.
7. BECKETT, E. L., M. VEYSEY and M. LUCOCK (2017): Folate and microRNA: Bidirectional interactions. Clin. Chim. Acta. 474, 60-66. 10.1016/j.cca.2017.09.001.
8. CAFFREY, A., H. MCNULTY, R. E. IRWIN et al. (2019): Maternal folate nutrition and offspring health: evidence and current controversies. Proc. Nutr. Soc. 78, 208-220. 10.1017/S0029665118002689.
9. CHEN, Z., R. S. DUAN, Y. ZHU et al. (2005) Increased cyclin E expression may obviate the role of cyclin D1 during brain development in cyclin D1 knockout mice. J. Neurochem. 92, 1281-1284. 10.1111/j.1471-4159.2004.02934.x.
10. CHU, D., L. LI L, Y. JIANG et al. (2019): Excess Folic Acid Supplementation Before and During Pregnancy and Lactation Activates Fos Gene Expression and Alters Behaviors in Male Mouse Offspring. Front. Neurosci. 13, 313. 10.3389/fnins.2019.00313.
11. CZEIZEL, A. E. and I. DUDÁS (1992): Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N. Engl. J. Med. 327, 1832-1835. 10.1056/NEJM199212243272602.
12. DUCKER, G. S. and J. D. RABINOWITZ (2017): One-Carbon Metabolism in Health and Disease. Cell. Metab. 25, 27-42. 10.1016/j.cmet.2016.08.009.
13. FOYE, O. T., Z. UNI Z, P. R. FERKET (2006): Effect of in ovo feeding egg white protein, beta-hydroxy-beta-methylbutyrate, and carbohydrates on glycogen status and neonatal growth of turkeys. Poult. Sci. 85, 1185-1192. 10.1093/ps/85.7.1185.
14. GAMBOA GONZALES, N. F., A. P. ALVES LEÃO, R. RIBEIRO ALVARENGA et al (2023): The effects of in ovo injection with sulfur amino acids and folic acid on the gene expression, relative organ weights, hematologic parameters, performance, and carcass characteristics of broiler chickens. Anim. Biotechnol. 34, 2207-2218. 10.1080/10495398.2022.2081578.
15. GREENBERG, J. A., S. J. BELL, Y. GUAN et al. (2011): Folic Acid supplementation and pregnancy: more than just neural tube defect prevention. Rev. Obstet. Gynecol. 4, 52-9.
16. HEYDARI, B., F. MASHAYEKHI and M. H. G. KASHANI (2024): Effect of in ovo feeding of folic acid on Disabled-1 and gga-miR-182-5p expression in the cerebral cortex of chick embryo. J. Anim. Physiol. Anim. Nutr. (Berl) 108, 285-290. 10.1111/jpn.13889.
17. HUOT, P. S., A. LY, I. M. SZETO, et al. (2016): Maternal and postweaning folic acid supplementation interact to influence body weight, insulin resistance, and food intake regulatory gene expression in rat offspring in a sex-specific manner. Appl. Physiol. Nutr. Metab. 41, 411-20. 10.1139/apnm-2015-0503.
18. HWANG, S. Y., Y. J. KANG, B. SUNG, et al. (2018): Folic acid is necessary for proliferation and differentiation of C2C12 myoblasts. J. Cell. Physiol. 233, 736-747. 10.1002/jcp.25989.
19. JING-BO, L., Y. YING, Y. BING et al. (2013): Folic acid supplementation prevents the changes in hepatic promoter methylation status and gene expression in intrauterine growth-retarded piglets during early weaning period. J. Anim. Physiol. Anim. Nutr. (Berl) 97, 878-886. 10.1111/j.1439-0396.2012.01333.x.
20. LI, Y., Q. FENG, M. GUO et al. (2018): Genome-wide survey reveals dynamic effects of folate supplement on DNA methylation and gene expression during C2C12 differentiation. Physiol. Genomics. 50, 158-168. 10.1152/physiolgenomics.00094.2017.
21. LIU, H., G. W. HUANG, X. M. ZHANG et al. (2010): Folic Acid supplementation stimulates notch signaling and cell proliferation in embryonic neural stem cells. J. Clin. Biochem. Nutr. 47, 174-180. 10.3164/jcbn.10-47.
22. LIU, H., T. TIAN T, S. QIN, et al. (2015): Folic acid deficiency enhances abeta accumulation in APP/PS1 mice brain and decreases amyloid-associated miRNAs expression. J. Nutr. Biochem. 26, 1502-1508. 10.1016/j.jnutbio.2015.07.020.
23. LIU, J., D. CHEN, X. MAO et al. (2011): Effects of maternal folic acid supplementation on morphology and apoptosis-related gene expression in jejunum of newborn intrauterine growth retarded piglets. Arch. Anim. Nutr. 3, 76-85. 10.1080/1745039x.2011.594352.
24. LIVAK, K. J. and T. D. SCHMITTGEN (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 25, 402-408. 10.1006/meth.2001.1262.
25. LY, A., L. ISHIGURO, D. KIM et al. (2016): Maternal folic acid supplementation modulates DNA methylation and gene expression in the rat offspring in a gestation period-dependent and organ-specific manner. J. Nutr. Biochem. 33, 103-110. 10.1016/j.jnutbio.2016.03.018.
26. MOHANTY, V., A. SHAH, E. ALLENDER et al. (2016): Folate Receptor Alpha Upregulates Oct4, Sox2 and Klf4 and Downregulates miR-138 and miR-let-7 in Cranial Neural Crest Cells. Stem. Cells. 34, 2721-2732. 10.1002/stem.2421.
27. MURKO, C., S. LAGGER, M. STEINER et al. (2013): Histone deacetylase inhibitor Trichostatin A induces neural tube defects and promotes neural crest specification in the chicken neural tube. Differentiation 85, 55-66. 10.1016/j.diff.2012.12.001.
28. NANINCK, E. F. G., P. C. STIJGER and E. M. BROUWER-BROLSMA (2019): The Importance

of Maternal Folate Status for Brain Development and Function of Offspring. Adv. Nutr. 10. 502-519. 10.1093/advances/nmy120.
29. POGORILER, J., K. MILLEN, M. UTSET M, et al. (2006): Loss of cyclin D1 impairs cerebellar development and suppresses medulloblastoma formation. Development 133, 3929-3937. 10.1242/dev.02556.
30. RAZEGHI, E., F. MASHAYEKHI, F. GHASEMIAN, et al. (2023): Effect of in ovo feeding of folic acid on brain derived neurotrophic factor (BDNF) and gga-miR-190a-3p expression in the developing cerebral cortex of chickens. Res. Vet. Sci. 154, 73-77. 10.1016/j.rvsc.2022.11.010.
31. SABLE, P., A. KALE, A. JOSHI et al. (2014): Maternal micronutrient imbalance alters gene expression of BDNF, NGF, TrkB and CREB in the offspring brain at an adult age. Int. J. Dev. Neurosci. 34, 24-32. 10.1016/j.ijdevneu.2014.01.003.
32. SALMAN, M., M. A. KAMEL, S. E. H. EL-NABI, et al. (2022): The regulation of HBP1, SIRT1, and SREBP-1c genes and the related microRNAs in non-alcoholic fatty liver rats: The association with the folic acid anti-steatosis. PLoS. One. 17, e0265455. 10.1371/journal.pone.0265455.
33. SEELAN, R. S., P. MUKHOPADHYAY, J. PHILIPOSE et al. (2021): Gestational folate deficiency alters embryonic gene expression and cell function. Differentiation 117, 1-15. 10.1016/j.diff.2020.11.001.
34. SHAIKH, M., and G. DOSHI (2024): Epigenetic Aging in Major Depressive Disorder: Clocks, Mechanisms and Therapeutic Perspectives. Eur. J. Pharmacol. 17, 176757. 10.1016/j.ejphar.2024.176757.
35. SHERR C. J., and J. M. ROBERTS. (2004): Living with or without cyclins and cyclin-dependent kinases. Genes Dev. 18, 2699-711. 10.1101/gad.1256504.
36. SHOOKHOFF, J. M. and G. I. GALLICANO. (2010): A new perspective on neural tube defects: folic acid and microRNA misexpression. Genesis 48, 282-294. 10.1002/dvg.20623.
37. WU, S, W. GUO, X. LI, Y. LIU et al. (2019): Paternal chronic folate supplementation induced the transgenerational inheritance of acquired developmental and metabolic changes in chickens. Proc. Biol. Sci. 286, 20191653. 10.1098/rspb.2019.1653.
38. YANG, X., W. SUN, Q. WU et al. (2021): Excess Folic Acid Supplementation before and during Pregnancy and Lactation Alters Behaviors and Brain Gene Expression in Female Mouse Offspring. Nutrients. 14, 66. 10.3390/nu14010066.
39. ZHANG, J., Y. P. LIU, L. J. BU, et al., (2023): Effects of dietary folic acid supplementation on lactation performance and mammary epithelial cell development of dairy cows and its regulatory mechanism. Anim. Biotechnol. 34, 3796-3807. 10.1080/10495398.2023.2228842.
40. ZHU, M., B. LI B, X. MA X, et al. (2016): Folic Acid Protected Neural Cells Against Aluminum-Maltolate-Induced Apoptosis by Preventing miR-19 Downregulation. Neurochem. Res. 41, 2110-2118. 10.1007/s11064-016-1926-9.
41. ZOU, R., H. EL MARROUN, C. CECIL, et al. (2021): Maternal folate levels during pregnancy and offspring brain development in late childhood. Clin. Nutr. 40: 3391-3400. 10.1016/j.clnu.2020.11.025.

Učinak *in ovo* primjene folne kiseline na ekspresiju gga-mir-let-7k-5p, ciklina D1 (CCND1) i faktora rasta živaca (NGF) u cerebralnom korteksu embrija pilića

Tara KHOSHKENAR, MSc, Farhad MASHAYEKHI, PhD, Professor, Mahshad MOTTAGHI, MSc, Zivar SALEHI, MD, PhD, Professor, Department of Biology, Faculty of Sciences, University of Guilan, Rasht, Iran

Folat je ključan za prikladan razvoj mozga. CCND1 gen je odgovoran za sintezu ciklin D1 bjelančevine, koja olakšava diferencijaciju i proliferaciju neurona. Faktor rasta živaca (NGF) ključan je za preživljavanje i sazrijevanje neurona. MikroRNK (miRNK) su uključene u reguliranje različitih faza neurogeneze, uključujući proliferaciju neuralnih matičnih stanica i diferencijaciju neurona. S cbzirom na važnu ulogu folne kiseline (FA) u regulaciji ekspresije gena, naš je cilj jest procijeniti učinke *in ovo* injektiranja FA na razine ekspresije mir-let-7k-5p, CCND1 i NGF u cerebralnom korteksu embrija pilića. U ovoj studiji bilo je uključeno ukupno 120 jaja za valjenje. Od toga, u 40 jaja su ubrizgane injekcije FA u amnionsku tekućinu pri dozi od 150 μg po jajetu, 40 jaja je primilo injekcije PBS-a (placebo) na embrionalni dan 11, dok je preostalih 40 jaja služilo kao kontrola skupina bez injekcija. Prikupljena su kortikalna tkiva na embrionalni dan 19, a PCR je korišten u stvarnom vremenu za analizu ekspresije mir-let-7k-5p, CCND1 i NGF. Rezultati su pokazali da su razine ekspresije gga-mir-let-7k-5p u skupini koja je primala FA, onoj koja je primala placebo te u kontrolnoj skupini bile 0,46±0,09,

0,73±0,18, odnosno 0,76±0,17-struka promjena. Uz to, razine ekspresije CCND1 su zabilježene pri 1,77±0,38, 1,18±0,25, odnosno 1,11±0,28-strukoj promjeni, dok su razine NGF pokazale slične obrasce pri 1,92±0,50, 1,12±0,25, odnosno 1,04±0,21-strukoj promjeni (P<0,05). Statistička analiza pokazala je značajno povećanje ekspresije CCND1 i NGF, uz smanjenje ekspresije gga-mir-let-7k-5p u korteksima skupine tretirane s FA u usporedbi s kontrolnom skupinom. To ukazuje da *in ovo* primjena FA povećava ekspresiju CCND1 i NGF, uz istovremeno smanjenje ekspresije gga-mir-let-7k-5p u cerebralnom korteksu pilića u razvoju. Povećavanje našeg znanja kako folat utječe na aktivaciju gena i miRNK povezano s razvojem cerebralnog korteksa i njegova potencijalna veza s oštećenjima neuralne cijevi (NTD), unaprijedit će naše znanje o razvoju mozga, a moglo bi rezultirati i novim pristupima liječenju za dodatno smanjenje pojavnosti ovih abnormalnosti.

Ključne riječi: *folat, miRNK, ekspresija gena, mozak, embrij pilića*