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RESEARCH

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Retinoic acid inhibition of cell proliferation via activation of *CDKN1B* signaling in the forebrain and spinal cord during mouse embryonic development

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Abstract

Background The active metabolite of vitamin A (retinol) is retinoic acid (RA). RA is essential for developing several organs as a signaling molecule that is tightly regulated during embryogenesis. We explored the teratogenic effects of RA on forebrain and spinal cord development modified by cyclin-dependent kinase inhibitor 1B (*CDKN1B*), as the mechanism underlying RA's teratogenic impacts requires further investigation. The study involved four groups of pregnant mice: the negative control group, the positive control group treated with dimethyl sulfoxide (DMSO) diluted in sunflower oil, the RA-treated group receiving a low dosage (5 mg/kg), and the RA-treated group receiving a high dosage (10 mg/kg). The treatment groups received daily intraperitoneal RA dissolved in DMSO and diluted with sunflower oil on gestational days 10.5, 11.5, and 12.5. On day 13.5 of pregnancy, the pregnant mice were euthanized by cervical dislocation, and immunohistochemical analyses of brain and spinal cord tissues were performed.

Results Morphologically, we observed a decrease in the number of implantation sites and the presence of hematomas in several uterus areas in the high-dose RA (10 mg/kg) group. Additionally, RA was shown to cause adverse changes in uterine weight and length. RA treatment indicated elevated levels of *CDKN1B* expression in spinal cord development, the diencephalon, and the telencephalon.

Conclusion Our findings demonstrated that by activating *CDKN1B* as an RA target gene for cell cycle arrest, an excess of RA during brain development in mouse embryos can induce cell undifferentiation during development.

Keywords Diencephalon, Telencephalon, Spinal cord, *CDKN1B*, Retinoic acid

1 Background

"Neurulation" refers to the coordinated morphogenetic stages of chordate embryogenesis that form the neural tube, which subsequently develops and differentiates into the brain and spinal cord. Impulses from the primitive node induce the surrounding embryonic ectoderm to grow and form the neural plate around embryonic day (E) 7.5 [1]. By E8.5, the fusion of the cephalic neural tube initiates at two additional locations: closure 2, which occurs at the forebrain-midbrain boundary, moving bidirectionally, and closure 3, which occurs at the caudally

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progressing rostral extremity of the forebrain. As the cortical neuroectoderm advances through the early phases of differentiation, the cerebral cortex wall stratification becomes evident around E13.5 – E14.0. Cortical layering arises through the migration of neuroblasts from the ependymal layer, known as the ventricular zone (VZ), and the subventricular zone (SVZ) radially into the adjacent marginal zone. These superficial nucleated cell layers are known as the cortical plate and subplate. The intermediate zone, or fiber layer, separates these layers from the underlying VZ and SVZ. This intermediate zone is generally anuclear and comprises cellular processes and transient migratory cells [2–4]. Massarwa and Niswander developed a live imaging system to track the development of various embryonic tissues in the mid-gestation mouse embryo. This system has shown independent cranial neural tissue movements and the dynamics of neural tube closure during mouse embryogenesis [5]. Figure 1 highlights the major structures involved in neurogenesis [2].

The active metabolite of vitamin A (retinol) is retinoic acid (RA). Retinal aldehyde dehydrogenases are essential for converting retinol to RA within target cells (aldehyde dehydrogenases 1A [ALDH1A] isotypes). Nuclear retinoic acid receptors (RARA, RARB, and RARG isotypes) are ligand-dependent transcriptional regulators that mediate RA activity. These receptors typically function as heterodimers with retinoid receptors to regulate the expression of target genes located on specific DNA sites known as retinoic acid response elements (RARE). Like steroid nuclear receptors, RARs can trigger nongenomic activation processes at the cell membrane [6]. Retinol, or vitamin A, is a crucial substance with various functions. It regulates multiple developmental and metabolic processes in vivo and is primarily found in the retina of animal livers, blood, and eyes [7]. RA is tightly regulated during embryogenesis as a signaling molecule essential for forming organs such as the spinal cord, body axis, eyes, limbs, heart, and kidneys [8, 9].

The cyclin-dependent kinase inhibitor 1B (*CDKN1B*) gene encodes the cyclin-dependent kinase inhibitor (CDKi) p27, which is part of the kinase inhibitory protein (Kip) family. CDKis impede the cell cycle. P27 is widely expressed and regulates the normal cell cycle by integrating mitogenic and growth-inhibitory signals [10]. Phosphorylation levels in normal cells are tightly controlled during the cell cycle. A 2- to threefold increase in p27 can completely suppress G1–S-phase cyclin-CDKs [11]. Furthermore, p27 is expressed and critical in embryonic development [12]. Since p27Kip1 is haploinsufficient, deleting a single p27Kip1 allele can increase tumor risk [13]. Reduced p27Kip1 protein levels, particularly

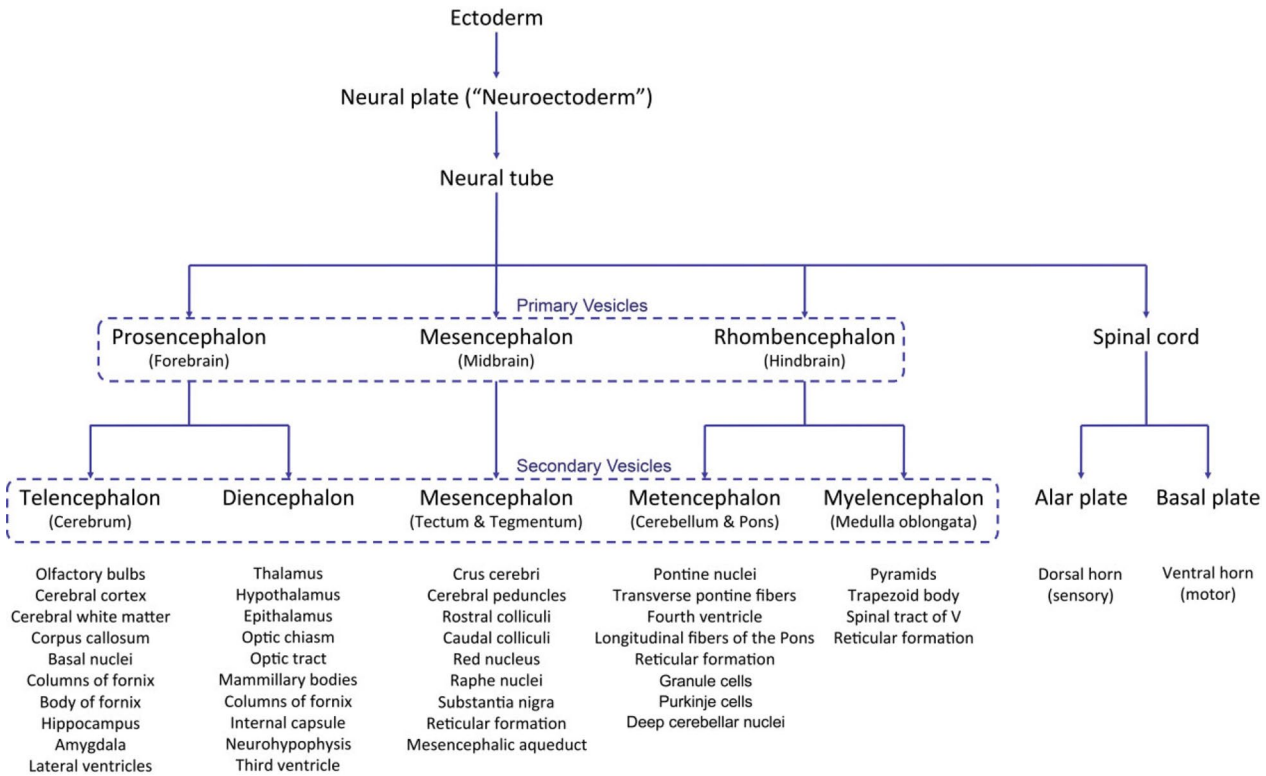

Fig. 1 A flow diagram depicting the stages at which certain brain structures are derived in the developing central nervous system [2]

nuclear-expressed p27Kip1, have been associated with the prognosis and progression of lung, head and neck, colon, breast, ovarian, and prostate cancers [14]. Previous findings have shown that RA inhibits proliferation and induces cell cycle arrest in embryonic palatal mesenchyme (EPM) cells, resulting in palatal dysplasia [15, 16].

Because the mechanism of RA's teratogenic effects requires further study, the present research investigated RA 's teratogenic effects on the cell cycle during cell proliferation and migration in the embryonic brain and spinal cord by manipulating *CDKN1B* as a cell cycle arrest gene.

2 Methodology

2.1 Animals and experimental design

Adult male ($n=10$) and female ($n=20$) albino mice of the Swiss strain were obtained from Cairo University's National Cancer Institute Experimental Animal Unit. The animals were housed under rigorous conditions, including 12-h light/dark cycles, low luminosity, a constant temperature of 25 °C, and an automated exhaust system providing multiple air changes daily. When the male mice reached 6–8 months, adult virgin females (2–3 months old, weighing 25–28 g) were mated with viable males. Mating was conducted at a 1:2 male-to-female ratio in each cage during the night, and vaginal plugs were checked the next morning to confirm pregnancy. Day 0.5 of pregnancy was identified as the morning a vaginal plug was observed, after which the pregnant females were housed individually.

A stock solution of RA (in powder form from SIGMA, St. Louis, MO, USA) was prepared by dissolving it in dimethyl sulfoxide (DMSO) (1:20, v/v) at doses of 5 mg/kg for low and 10 mg/kg for high dosage, then diluted with sunflower oil for in vivo experiments. In a dark bottle, the RA stock solution was stored in aliquots at 8 °C. Twenty pregnant mice were divided into four groups of five animals each: (1) the control group; (2) the positive control group treated with DMSO diluted with sunflower oil; (3) the RA treatment group receiving a low dose of 5 mg/kg; and (4) the RA treatment group receiving a high dose of 10 mg/kg. The sample size calculation followed ARRIVE guidelines. On gestational days (GDs) 10.5, 11.5, and 12.5, the treatment groups received daily RA intraperitoneal (IP) injections. On gestational day 13.5, the animals were euthanized by cervical dislocation. The uterine horns were excised and photographed, and the weights and lengths of viable fetuses and the total number of implants were recorded.

2.2 Immunohistochemical analysis

Tissues were fixed in Carnoy's solution prior to immunostaining. They were dehydrated and washed with 70%, 90%, and 100% ethanol, followed by clearing in xylene and embedding in paraffin. Embryonic tissues were sectioned to a thickness of 5 µm, mounted on glass slides, and deparaffinized in xylene. After being hydrated through a series of ethanol solutions (100%, 90%, 80%, 70%, and 50%) for five minutes each, the slides were immersed in water for another five minutes. The enzyme was quenched by treating the sections with 3% H_2O_2 (v/v) for five minutes. The preparations were then rinsed twice in 0.1 M phosphate-buffered saline (PBS) for five minutes each.

The sections were immersed in a protein-blocking solution (EconoTek Superblock) for 10 min at room temperature to prevent nonspecific staining. Subsequently, the preparations were incubated at room temperature for two hours with the primary antibody (1:200 dilution in phosphate buffer). Following this, the preparations were rinsed twice for five minutes in 0.1 M PBS before being treated for thirty minutes at room temperature with biotinylated secondary antibodies (EconoTek Biotinylated Anti-Polyvalent). After another thirty-minute incubation with HRP conjugate, the preparations were washed twice for five minutes in 0.1 M PBS.

The formulations were subjected to 3,3'-diaminobenzidine hydrochloride (DAB) for five to fifteen minutes to visualize the response. The slides were then counterstained with hematoxylin. Xylene was used to clean and dehydrate the sections, and the slides were mounted with DPX [17]. A Toupcam camera was utilized to inspect and photograph the individual sections.

2.3 Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 25. The Student's F-test was utilized to assess the data. P-values of less than 0.05 or 0.01 were considered statistically significant.

3 Results

3.1 Effect of RA on pregnancy outcomes

Because the results from both control groups (non-treated and dimethyl sulfoxide [DMSO]-treated) were nearly identical, we combined the control pregnant mice from both groups. Our study investigated the effects of retinoic acid (RA) on fetal development at gestational day (GD) 13.5. Morphological and immunohistochemical approaches were employed to describe the effects of two distinct RA doses. The uteri of control pregnant mice exhibited a typical size of implanted embryos between the two horns (Fig. 2, panel a). In the RA low-dose and high-dose treatment groups, the uterine horns displayed evident hematomas in various locations, as well as abnormally sized implanted embryos.

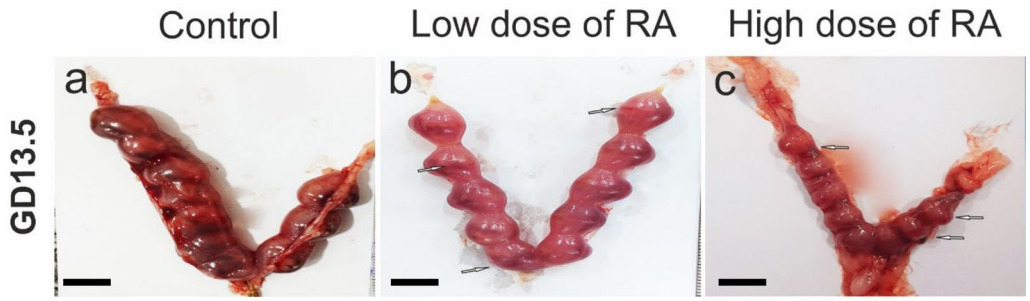

Fig. 2 Photographs of mouse uteri on gestational day (GD) 13.5 showing the lengths of the uteri and implanted embryos in the control group (**a**), low-dose retinoic acid (RA) treated group (**b**), and high-dose RA treated group (**c**). Note that the photographs of the low and high-dose RA groups display some hematomas and smaller sizes of implanted embryos (white arrows). Scale bar: 10 mm

Furthermore, no significant reduction was observed in the number of embryos in both treatment groups (Fig. 3, panel A) compared to the control group (Fig. 2, panels b and c). In contrast, the uteri of pregnant mice treated with a low dose of RA showed no significant loss in weight or length compared to the control group. However, we detected a substantial reduction in the weight and length of the uterus in the RA high-dose treatment group ($P < 0.01$) (Fig. 3, panels B and C).

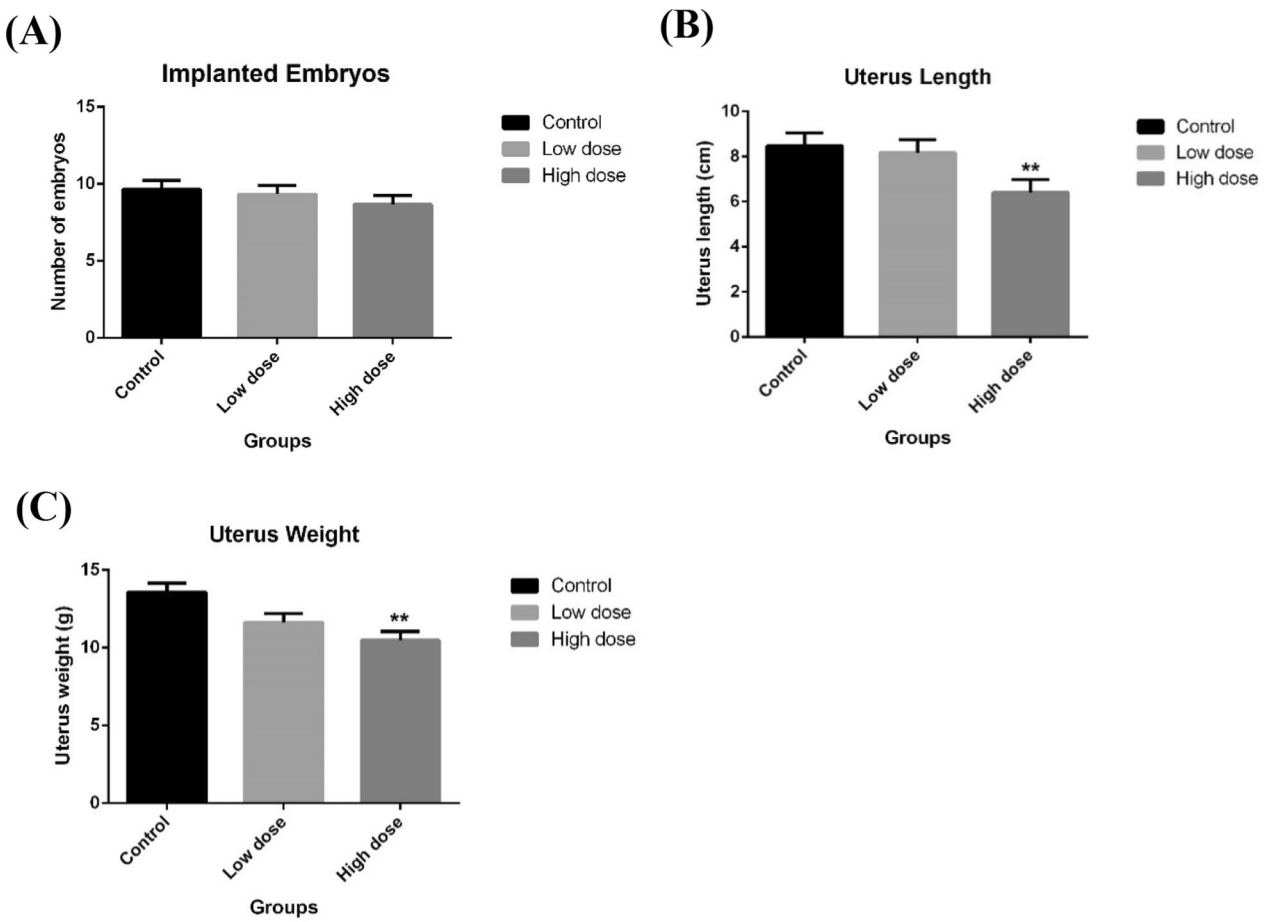

Fig. 3 Histograms representing **A** the mean number of implanted embryos, **B** the mean length of the uterus, and **C** the mean weight of the uterus on gestational day (GD) 13.5. $^{**}P < 0.01$ compared with the control group

3.2 Impacts of RA on brain development modulated by *CDKN1B* immunolocalization

The effect of RA on neurogenesis was examined in the forebrain region (telencephalon and diencephalon). An anti-*CDKN1B* antibody was utilized to assess cell proliferation. Our findings indicated that the transverse section in the control group revealed that one of the brain regions is the forebrain (telencephalon and diencephalon). The telencephalon, located at the most rostral end of the neural tube, is divided into two parts: dorsal and ventral. Additionally, the VZ is situated on the perimeter of the lateral ventricle, while the SVZ, specific to the telencephalon, extends from the basal region of the VZ. Three notable intraventricular bulges are present: the septum, medial ganglionic eminence (MGE), and lateral ganglionic eminence (LGE). At this stage, the choroid plexus in the lateral ventricles of the telencephalon extends from the falx cerebri (FC) (Fig. 4, panels A and D).

Furthermore, in the diencephalon, we observed that the thalamus, hypothalamus, and epithalamus continue to grow and differentiate into distinct nuclei. The two parts of the thalamus nearly overlap at the midline, reducing the volume of the third ventricle. Additionally, the spinal cord's VZ decreases in size and is gradually replaced by the mantle and marginal zones. The size of the central canal diminishes. However, treatment with both doses of RA resulted in disorganized cell migration in the dorsal and ventral telencephalon and degeneration of cells in

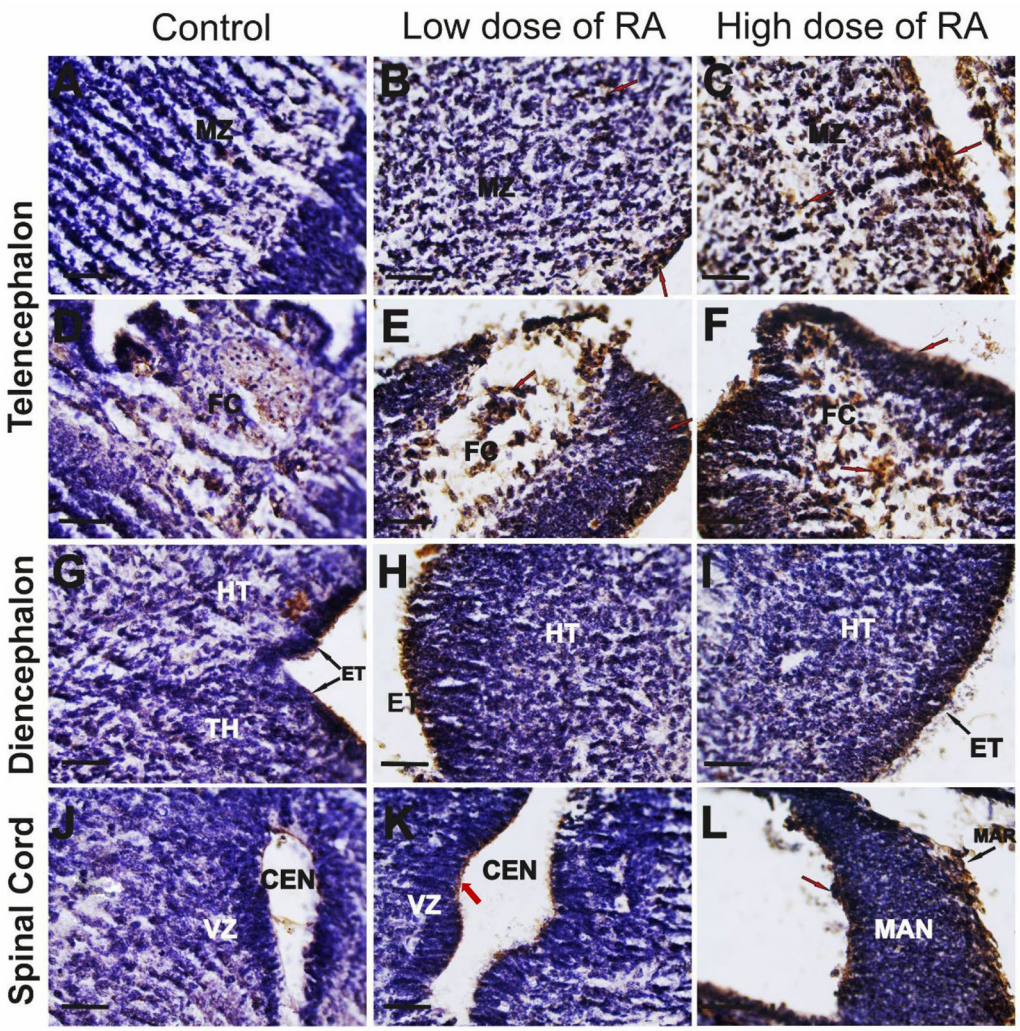

Fig. 4 Photomicrographs illustrating the expression levels of cyclin-dependent kinase inhibitor 1B (CDKN1B), counterstained with hematoxylin, in the mouse brain and spinal cord on gestational day (GD) 13.5 from the control (**A**, **D**, **G**, and **J**), low-dose retinoic acid (RA) treated (**B**, **E**, **H**, and **K**), and high-dose RA treated (**C**, **F**, **I**, and **L**) groups, showing varying expression levels of CDKN1B (red arrows). Labels include epithalamus (ET), central canal (CEN), falx cerebri (FC), hypothalamus (HT), mantle layer (MAN), marginal layer (MAR), mantle zone (MZ), thalamus (TH), and ventricular zone (VZ). Scale bar = 10 µm

the MGE and LGE regions. There was a notable growth retardation of the choroid plexus, which failed to extend from the falx cerebri.

Furthermore, we found that the thalamus and hypothalamus were not enlarged in the diencephalon compared to the control group (Fig. 4, panels H and I). Interestingly, the central canal of the spinal cord did not decrease in size in the low-dose RA group. In contrast, the neural tube remained open in the high-dose RA group, indicating that the VZ was not forming.

On the other hand, immunolocalization of *CDKN1B* in the control group revealed negative expression in the marginal zone, with a faint signal observed in the falx cerebri of the telencephalon (Fig. 4, panels A and D and Table 1). Interestingly, upon magnification, our results demonstrated that RA treatment gradually increased *CDKN1B* activation across most regions of the telencephalon. Specifically, moderate expression was observed in the low-dose RA group, while strong expression was evident in the high-dose RA group. Additionally, the falx cerebri exhibited strong signals in both the low- and high-dose RA groups compared to the control (Fig. 4, panels B, C, E, and F, and Table 1).

Furthermore, a faint signal of *CDKN1B* was detected in the proliferative cells of the hypothalamus within the diencephalon in both the low- and high-dose RA groups, in contrast to the control group, which showed negative signals for *CDKN1B* (Fig. 4, panels H and I, and Table 1). Upon examining the spinal cord, we found that the control group displayed negative expression of *CDKN1B* in the ventricular zone. However, expression gradually increased slightly in this region in both the low- and high-dose RA groups compared to the control group (Fig. 4, panels K and L, and Table 1).

Interestingly, upon further magnification of the images (1000x), we identified several small foci of necrosis and cell undifferentiation in the falx cerebri region of the telencephalon, where *CDKN1B* expression was strong in both the low- and high-dose RA groups compared to the control (Fig. 5, panels A–C, and Table 1). Although a faint signal of *CDKN1B* was detected in the hypothalamus of the diencephalon in both the low- and high-dose RA groups, we observed a strong signal of *CDKN1B* in the epithalamus (ET) (Fig. 5, panels D–F, and Table 1). Moreover, robust expression of *CDKN1B* was detected in the mantle layer of the spinal cord in the high-dose RA group; however, no expression of *CDKN1B* was observed in the VZ of both the control and low-dose RA groups (Fig. 5, panels G–I, and Table 1).

4 Discussion

It is essential to examine the effects of nutrients such as vitamin A on embryonic brain development to understand how maternal nutrition affects fetal growth and organogenesis [18]. This study investigates the effects of RA during a critical period in brain development, from GD 10.5 to GD 12.5, with dissection and analysis conducted at GD 13.5. We evaluated the effects of varying RA dosages by administering low-dose (5 mg/kg) and high-dose (10 mg/kg) treatments. The impact of RA at midgestation on GD 13.5 pregnancy outcomes was assessed, revealing distinct hematomas in the uterine horns at multiple locations, as well as smaller implantation sites, particularly in the high-dose RA group compared to the low- and control-dose groups. Additionally, a single intraperitoneal injection of RA (15 mg/kg/day) on GD 18.5 resulted in reabsorption, reducing the number of implanted fetuses, as RA induced fetal miscarriage during the final stages of pregnancy [9].

Furthermore, we examined how RA affected cell migration and differentiation in the telencephalon, diencephalon, and spinal cord. Our findings indicated that RA treatment at both doses impaired cell migration in the telencephalon's falx cerebri, LGE, and MGE regions. Furthermore, unlike the control group, no enlargement of the thalamus or hypothalamus was observed in the diencephalon region. Previous studies have shown that RA plays a crucial role in forebrain development (telencephalon and diencephalon regions), which aligns with our findings. Altering the expression patterns of the three retinaldehyde dehydrogenases (RALDHs), the primary local sources of RA in the developing embryo plays a critical role in telencephalon development [19].

Additionally, RA responsiveness in the brain is widespread, with at least one RA receptor, often several, expressed at each site. However, RALDH expression is

Table 1 Reciprocal intensity of cyclin-dependent kinase inhibitor 1B (*CDKN1B*) immunostaining in fore-brain and spinal cord

Groups	Telencephalon		Diencephalon		Spinal cord	
	Marginal zones	Falx cerebri	Hypothalamus	Epithalamus	Ventricular zone	Mantle layer
Control	–	+	–	+ +	–	–
Low-dose of RA	+ +	+ + +	+	+ + +	+	+
High-dose of RA	+ + +	+ + +	+	+ + +	Not formed	+ + +

+ + +, Strong expression; + +, moderate expression; +, faint expression; –, no expression; RA, retinoic acid

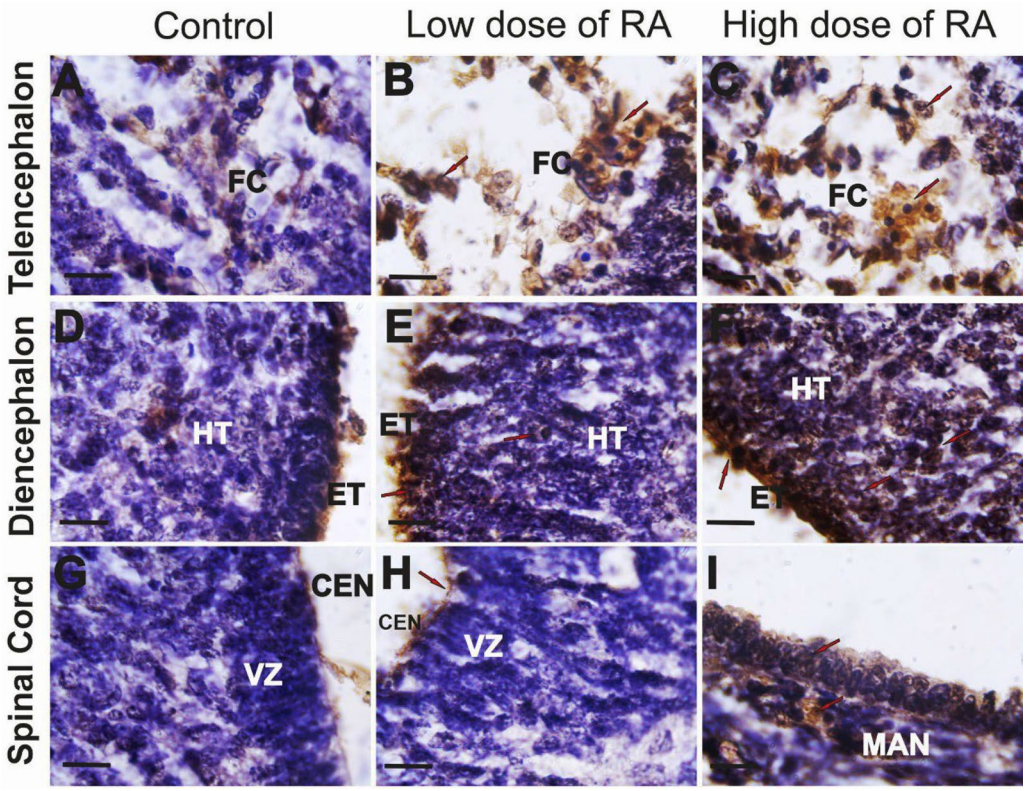

Fig. 5 Photomicrographs illustrating the expression levels of cyclin-dependent kinase inhibitor 1B (CDKN1B), counterstained with hematoxylin, in the mouse brain and spinal cord on gestational day (GD) 13.5 from the control (**A**, **D**, and **G**), low-dose retinoic acid (RA) treated (**B**, **E**, and **H**), and high-dose RA treated (**C**, **F**, and **I**) groups, showing the differentiation of neurocytes and varying expression levels of CDKN1B (red arrows). Labels include epithalamus (ET), central canal (CEN), falx cerebri (FC), hypothalamus (HT), mantle layer (MAN), and ventricular zone (VZ). Scale bar = 2 µm

surprisingly limited, and RA production is absent from most telencephalic areas [19]. The cellular structure and organization of the telencephalon are shaped by optimal RA concentration, which stimulates neuronal development and the radial migration of neurons into the cerebral cortex [19].

However, many studies have indicated that vitamin A concentrations exceeding those necessary for normal cell function can have detrimental effects, such as disrupting the redox environment, causing mitochondrial malfunction, and inducing cell death [20]. Vitamin A and its derivatives are essential for developing and maintaining central nervous system activity. According to our research, RA administration at either a low or high dose may interfere with these brain regions' normal growth and organization, as evidenced by the disarray of cell migration in the telencephalon and degeneration of cells in the MGE and LGE regions. Previous studies have shown that effective cell migration is critical for forming functional neuronal circuits and establishing connections between various brain regions [21]. Disruption of cell migration can lead to neuronal misplacement and functional impairments [22]. Our findings suggest that RA treatment disrupts this essential process, potentially affecting the normal development and function of the telencephalon.

Additionally, the presence of necrotic foci in the falx cerebri region of the telencephalon supports the hypothesis that RA treatment can harm developing brain cells. Necrosis, a type of cell death, is characterized by the rapid rupture of the plasma membrane and leakage of cellular contents, triggering an inflammatory response [23]. Previous research has shown that early RA exposure in cortex neurogenesis reduces telencephalic size. The presence of necrosis in this region suggests that RA treatment may adversely affect the survival and integrity of cells in these brain regions. Conversely, RA exposure at the end of this period has no discernible impact on the brain's overall size. Mice exposed to RA and grown to adulthood achieved a normal body size, but their skulls were visibly smaller and malformed [19]. As RA binds to nuclear RA receptors (RARs) and regulates gene expression, our findings align with previous studies demonstrating RA's role in early embryonic development and

emphasizing the importance of RA signaling in forebrain development [20].

To investigate the histological abnormalities in brain development caused by RA administration, we performed immunohistochemistry analysis to assess *CDKN1B* expression in both the low-dose and high-dose RA groups compared to the control group. The cell cycle inhibitor *CDKN1B* regulates cell division and proliferation [24]. Interestingly, our research demonstrated that RA treatment activates *CDKN1B* expression. In both RA-treated groups, *CDKN1B* expression was elevated in most telencephalon regions, particularly in the marginal zone and falx cerebri. Elevated *CDKN1B* expression in proliferative cells led to ventricular zone deformation in the diencephalon's epithalamus and the spinal cord's marginal zone. The increased expression of *CDKN1B* in response to RA treatment suggests a potential role for *CDKN1B* in mediating RA's effects on brain development. RA-induced *CDKN1B* activation may influence the regulation of cell proliferation and differentiation in the telencephalon, potentially explaining the observed disruptions in cell migration and cellular changes.

Further research is needed to clarify the precise mechanisms underlying this phenomenon. Our findings align with earlier studies, which showed that vitamin A and its derivatives regulate cell division, proliferation, survival, and death by inducing apoptosis, a process critical to shaping the embryo's morphology, organogenesis, and tissue physiology [20].

Moreover, previous studies have reported that RA increases *CDKN1B* levels, which are crucial for cell cycle arrest by inhibiting the association of CD-CDK4 and CE-CDK2, thereby coordinating actions to halt the cell cycle [25]. Consequently, the upregulation of *CDKN1B* in response to RA treatment may contribute to cell cycle disruption during mouse embryo organogenesis, impeding fundamental processes such as cell proliferation, differentiation, fate determination, and division. Abnormalities in organ development, including brain development, can result when the cell cycle is interrupted during embryogenesis. In the brain cortex, the overexpression of cell cycle inhibitors (CDKIs) due to RA treatment affects cortical development, as overexpression of p57 and p27 promotes cortical progenitors to exit the cell cycle [26].

5 Conclusion

In conclusion, our study provides evidence that excess RA during brain development in mouse embryos can induce various abnormalities, particularly in the forebrain and spinal cord, on GD 13.5 due to its teratogenic effects, which disrupt cell migration and induce cell degeneration. This is accompanied by the upregulation of *CDKN1B*, which halts the cell cycle necessary for differentiation, emphasizing the potential adverse impact of RA on brain development. Further research is required to clarify RA exposure's underlying mechanisms and long-term consequences during critical developmental periods. Given these findings, avoiding excessive vitamin A supplementation in maternal nutrition is advisable, as it could unintentionally lead to developmental disturbances in the embryo.

Abbreviations

ALDH1A	Aldehyde dehydrogenases 1A
CDKN1B (CDKip27)	Cyclin-dependent kinase inhibitor 1B
DMSO	Dimethyl sulfoxide
E	Embryonic day
ET	Epithalamus
FC	Falx cerebri
EPM	Embryonic palatal mesenchyme
GD	Gestational day
IP	Intraperitoneal
LGE	Lateral ganglionic eminences
MGE	Medial ganglionic eminences
RA	Retinoic acid
RALDHs	Retinaldehyde dehydrogenases
RAR	Retinoic acid receptor
RARE	Retinoic acid response elements
SVZ	Subventricular zone
VZ	Ventricular zone

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Author contributions

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by [Ahmed Said], [Amira S. AbdElkhalek], and [Karima M. Nasraldin]. The practical work was performed by [Ahmed Said], [Karima M. Nasraldin], [Mariam Sherief], [Lydia Amir], [Maysem Samy], and [Mariam S. Nabil]. The first draft of the manuscript was written by [Ahmed Said], and [Karima M. Nasraldin]. The final draft was reviewed by [Gehan Safwat], and [Ayman A. Diab] and all authors commented on previous versions. All authors read and approved the final manuscript.

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Availability of data and materials

All the data are already presented in the manuscript.

Declarations

Ethics approval and consent to participate

The study received approval from the Al-Azhar University Faculty of Science's Ethics Committee for Animal Experimentation (Permit Number: AZHAR9/2023).

Consent for publication

Written informed consent for publication was obtained from the participant.

Competing interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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