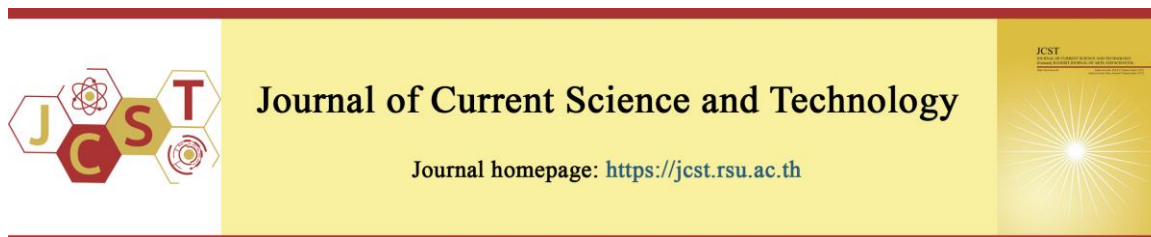


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Transient Embryotoxicity and Disrupted Forebrain Architecture Following in *Ovo* Cannabidiol Exposure in Chick Embryo

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Abstract

The increasing prevalence of cannabidiol (CBD) use during pregnancy raises concerns regarding its impact on fetal neurodevelopment. This study examined how the administration of CBD in *ovo* influences mortality rates, physical development, and the structural arrangement of the forebrain in avian embryos. Fertilized eggs were injected with 10 μ M CBD or vehicle control into the yolk sac at Hamburger–Hamilton (HH) stages 7–8 (24 hours of incubation). Embryos were evaluated at Days 3, 6, and 13 using morphological, histological, and molecular analyses. Early exposure significantly reduced survival at Day 3 (79.3% vs. 93.8%, $p = 0.041$) and accelerated neural tube differentiation, coinciding with a 2.3-fold upregulation of Bone Morphogenetic Protein 4 (BMP-4) ($p = 0.009$). While survival rates showed a non-significant reduction by Day 6, transient growth retardation was observed. By Day 13, CBD-treated embryos exhibited distinct morphological alterations, including increased egg tooth length ($p = 0.023$) and brain weight ($p = 0.007$). Histological examination of the forebrain revealed narrowed ependymal zones, aberrant mesenchymal-like cell infiltration, and significantly reduced neurite extension. These findings suggest that early CBD exposure disrupts neurodevelopmental timing and forebrain architecture, potentially mediated through dysregulated BMP-4 signaling. Although embryos demonstrated somatic resilience, the persistent cellular and structural anomalies highlight the potential neuroteratogenic risks of CBD exposure during early embryogenesis.

Keywords: cannabidiol, forebrain development, in *ovo* model, chick embryo

1. Introduction

Cannabis sativa L. contains a diverse array of cannabinoids, with tetrahydrocannabinol (THC) and cannabidiol (CBD) being the most prevalent (Alves et al., 2020). In contrast to THC, which is characterized by its psychotropic effects, CBD is non-intoxicating and has been identified as possessing various

medicinal properties (Pagano et al., 2022; Śmiarowska et al., 2022). In spite of its psychoactivity, THC exhibits proven medicinal efficacy. Its clinical importance is evidenced by its inclusion in Sativex, a 1:1 THC/CBD oromucosal spray designed to mitigate multiple sclerosis-related spasticity. This balanced perspective is crucial when evaluating the developmental effects

of cannabinoid compounds (Hilliard et al., 2012; Moreno-Martet et al., 2015).

The endocannabinoid signaling system (ECS) exerts a fundamental influence from the onset of embryogenesis, evidenced by the presence of cannabinoid receptors and their corresponding endogenous ligands during the nascent stages of life. Initial nervous system formation, particularly during neurulation, is modulated by the ECS, as indicated by the early expression of metabolic enzymes and receptor proteins. Within the context of cephalic development, the ECS orchestrates a highly coordinated cascade of neurodevelopmental milestones, including neurogenesis, migratory patterns, axonal guidance, and the eventual refinement of synaptic architecture (Psychoyos et al., 2012; Rodrigues et al., 2024). The cannabinoid receptor type 1 (CB1) is actively expressed at both the mRNA and protein levels starting from the neurulation phase in chick embryos. Its expression profile is characterized by dynamic shifts that correspond to the emerging patterns of neuronal maturation throughout the early embryonic period (Mahomed et al., 2023). This developmental pattern is conserved across vertebrate species, with CB1 receptors first showing expression during the early neurula stage in chicken embryos. In addition to receptor expression, the endogenous ligands anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are consistently present throughout these vital developmental phases. Notably, 2-AG concentrations undergo a marked escalation as embryonic maturation progresses, rising from 1.05 ± 0.05 nmol/g tissue during the initial stages (HH 9–11) to a peak of 9.34 ± 0.12 nmol/g tissue by the pre-hatch stage (HH 44) (Psychoyos et al., 2012).

CBD exerts its effects through multiple receptor systems beyond the classical cannabinoid receptors (Mori et al., 2021; Soderstrom et al., 2017). While CBD has low affinity for CB1 and cannabinoid receptor type 2 (CB2) at physiological concentrations, it modulates their activity and interacts with several other key receptors involved in neurodevelopment. These include: 1) agonism at 5-HT_{1A} receptors, which are crucial for neuronal development and migration, 2) activation of TRPV1 channels, which regulate calcium influx and neuronal excitability during development (Duncan et al., 2024; Walsh et al., 2021), and 3) activation of PPAR- γ , which influences neuroinflammation and cell fate determination (Pandelides et al., 2021). The differential contribution of these receptors to CBD's effects is demonstrated by studies showing that CBD

neuroprotection is inhibited by antagonists of CB1, CB2, 5-HT_{1A}, TRPV1/2, and PPAR- γ receptors. This multireceptor pharmacology explains the complex and sometimes paradoxical effects of CBD on neurodevelopment.

The embryonic and fetal nervous system is highly susceptible to environmental influences, including cannabinoids, which readily cross the placenta and enter fetal circulation. Studies have suggested that prenatal cannabis exposure may be linked to neurodevelopmental disorders, impaired cognitive function, and an increased risk of neural tube defects (NTDs). Emerging evidence suggests that CBD functions as a selective neuroteratogen, specifically interfering with neural tube morphogenesis in murine models. At concentrations that do not compromise overall embryonic viability or somatic growth, CBD induces a dose-dependent inhibition of cranial neural tube closure. This developmental disruption results in a heightened incidence of exencephaly alongside a simultaneous impairment of spinal neural tube occlusion (Gheasuddin & Galea, 2022). In chick embryos, cannabinoid exposure during early embryonal development decreases embryonal viability and can affect neural crest cell migration and differentiation (Gustafsson & Jacobsson, 2019). Studies show that the potent synthetic cannabinoid receptor agonist HU 210 as well as plant-derived CBD are embryotoxic, with effects that are attenuated by the CB1 receptor antagonist AM251, suggesting involvement of CB1 receptor signaling in these developmental effects. Furthermore, cannabinoids can exacerbate alcohol teratogenesis through CB1 receptor-mediated inhibition of Sonic Hedgehog signaling (Shh), presenting a novel mechanism for the regulation of Shh signaling during neural tube formation (Heanue et al., 2016; Mahomed et al., 2023). The pathogenesis of NTDs is rooted in the disruption of neural tube closure, a process strictly governed by Shh, Wnt, and BMP pathways. Beyond genomic and environmental interactions, the dysregulation of the endocannabinoid system has emerged as a putative risk factor in the development of these malformations. BMP signaling, particularly BMP-4, is essential for dorsal neural tube differentiation and neural crest delamination. Nevertheless, BMP activity follows a bimodal requirement, where excessive signaling may impede the closure process, highlighting the need for precise temporal-spatial regulation (Anderson et al., 2016; Reece & Hulse, 2019). In the developing vertebrate nervous system, the spatial identity of neural

progenitors is established through an antagonistic gradient of morphogens. While BMP-4, acting as a dorsalizing signal, is crucial for specifying sensory neurons in the dorsal neural tube, Sonic Hedgehog (Shh) functions as a ventralizing signal from the notochord to guide motor neuron differentiation (Marković et al., 2024). The precise cross-talk between these opposing signals determines the dorsoventral patterning. Any disruption of this balance, such as an aberrant increase in BMP signaling, can potentially interfere with Shh activity, leading to shifts in cell fate and structural malformations during brain regionalization (Manzari-Tavakoli et al., 2022).

Despite growing evidence suggesting the teratogenic potential of cannabinoids, the specific mechanisms by which CBD might disrupt neural tube formation or other neurodevelopmental processes remain unclear. Given the increasing prevalence of CBD use during pregnancy, addressing these knowledge gaps is crucial for evaluating its potential risks and safety. The chicken embryo offers a well-established model system for studying vertebrate neural development due to its similarities to mammalian embryos in early developmental stages and provides a controlled environment for analyzing how CBD affects key developmental processes. During early neurogenesis, the primitive neuroepithelium undergoes rapid proliferation and organizes into a multi-layered structure. This process involves the differentiation of the ventricular zone (or ependymal zone), where progenitor cells reside, followed by the formation of the mantle zone containing post-mitotic neurons, and finally the marginal zone. In the avian forebrain, this laminar organization is critical for proper neuronal migration and circuit formation (Dennis et al., 2016). Understanding these baseline histological transformations is essential for identifying how exogenous factors like CBD might perturb the delicate timing of neural tissue maturation. By administering controlled doses of CBD during critical neurodevelopmental windows (HH stages 7-8), we aim to assess its impact on neural tube formation, forebrain development, and early neural tissue formation. Understanding these cellular and regional effects will contribute to the broader discussion on the safety of cannabis use during pregnancy, equipping healthcare professionals and pregnant individuals with informed knowledge about potential risks.

2. Objectives

This study investigates the impact of *in ovo* CBD exposure on chick embryos, focusing on survival, growth, and forebrain development. We specifically examine how CBD administration during early neurulation stages affects:

- 1) Embryonic survival and developmental progression
- 2) Neural tube formation and patterning
- 3) Molecular signaling pathways (BMP-4 and SHH)
- 4) Forebrain structural organization at later developmental stages

The study utilizes an *in ovo* model, employing precise yolk sac injections at HH stages 7-8, followed by a multi-stage evaluation encompassing morphological, histological, and molecular analyses at specific developmental windows.

These objectives address the critical need to understand the neurodevelopmental consequences of prenatal CBD exposure and provide mechanistic insights into potential teratogenic effects.

3. Materials and Methods

3.1 Animal Model

The present research was carried out in accordance with the regulatory framework for animal research in Thailand. Explicit approval was obtained from the Animal Ethics Committee at Rangsit University (Reference: RSU-AEC2025/005.1311). Furthermore, all experimental activities were supervised by the RSU Research Institute and the Research Ethics Office to ensure that the utilization of chick embryos adhered strictly to national statutory requirements.

Fertilized chicken eggs from the Lohmann Brown breed (*Gallus gallus domesticus*) were sourced from the Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. Upon arrival at the laboratory, eggs were carefully screened for uniformity in size (55-65 g), eggshell integrity, and overall condition, with any defective or abnormal eggs excluded from the study. Selected eggs were disinfected with 70% ethanol to minimize contamination risk before incubation. To ensure developmental synchronization across experimental groups, eggs were incubated for exactly 24 hours prior to cannabidiol (CBD) administration. Synchronization was achieved through hourly automatic egg rotation (every 60 minutes) in a semi-automatic incubator maintained at 37°C and 65% relative humidity. This rotational protocol prevents embryo adhesion to the

shell membrane and ensures uniform development. At the time of CBD injection, embryos were precisely staged to Hamburger–Hamilton (HH) stages 7–8 using established morphological criteria, characterized by the presence of 1–3 somites and initial stages of neural tube formation. Staging was performed by two independent observers using a stereomicroscope to ensure accuracy and consistency.

3.2 Cannabidiol Solution Preparation and Verification

Synthetic cannabidiol (CBD), purity >99%, originally manufactured by Lipomed AG (Switzerland), was purchased as a crystalline powder and is currently available under LGC Standards (Cat. No. LPM-THC-303) following LGC Group's 2023 acquisition of Lipomed's Reference Standards business. Product identity was confirmed by molecular formula ($C_{21}H_{30}O_2$). The compound was stored at 4°C in a desiccated environment until use. A stock solution of CBD (50 mg/mL) was formulated by dissolving the compound in dimethyl sulfoxide (DMSO). Given the lipophilic nature of CBD, DMSO served as an ideal carrier to facilitate precise dosing. Aliquots of the stock were stored under refrigerated conditions (4°C) to prevent degradation before subsequent application. Prior to each experiment, the CBD stock solution was diluted with sterile 0.9% normal saline solution to achieve the desired working concentrations. The final injection solution contained 0.6% v/v DMSO as a vehicle. Vehicle control solutions were prepared identically, containing 0.6% DMSO in sterile saline without CBD.

3.3 *In Ovo* Injection Procedure and Dose Calculation

In ovo administration of CBD was conducted exactly 24 hours after the initiation of incubation (HH stages 7–8). At these stages, embryos exhibit a fully formed primitive streak and initial formation of head folds, preceding neural tube closure. To ensure precise and consistent CBD delivery across all eggs, each egg was individually weighed to the nearest 0.1 g using a digital analytical balance before injection. The injection volume was calculated based on egg weight to achieve a final concentration of 10 μ M CBD per gram of egg weight. This concentration was determined based on our preliminary dose-response study (detailed in Section 4.1), which identified the IC_{50} as 46.2 μ M. The 10 μ M dose (approximately 22% of the IC_{50}) was selected because it elicited significant biological and molecular alterations while maintaining

a survival rate sufficient for robust histological and Western blot analyses. The injection dose was standardized at 200 μ g of CBD per egg (delivered in 0.2 mL ~3,180 μ M stock (1:50 dilution from stock)). For an average 60 g egg, this dose was calculated to achieve an estimated final tissue concentration of approximately 10 μ M, assuming uniform distribution throughout the egg mass.

A small hole (0.5 mm diameter) was carefully drilled at the blunt end of each egg using a sterile dental drill, ensuring minimal disturbance to embryonic structures. Subsequently, 0.2 mL of the CBD solution was gently injected into the yolk sac using a sterile 1 mL syringe with a 25-gauge needle. Control embryos were injected with an identical volume of vehicle solution (0.6% DMSO in sterile normal saline). Immediately after injection, holes in the eggshells were sealed using sterile paraffin wax to maintain egg integrity and prevent contamination. Eggs were then returned to incubation conditions and continued incubation until predetermined observation time points (Days 3, 6, and 13).

3.4 Gross Morphological Analysis and Viability Assessment

At designated observation points (Days 3, 6, and 13), embryonic samples were carefully collected from eggs. Each egg was gently opened; embryos were visually examined to evaluate gross morphological parameters. Embryonic viability was determined by assessing heartbeat presence and spontaneous embryonic movement under a stereomicroscope. Only embryos exhibiting clear cardiac activity and movement were classified as viable and included in subsequent analyses. Embryos were subsequently removed and immediately weighed to obtain wet embryo weight using a digital analytical balance (precision $\pm$ 0.1 mg). The Crown-rump length (CRL) was determined by measuring the maximum longitudinal distance between the vertex of the cranium and the caudal-most point of the trunk. To ensure accuracy, measurement parameters strictly excluded the extremities and the yolk sac, focusing solely on the axial dimension of the embryo. Measurements were conducted precisely using a digital vernier caliper (precision $\pm$ 0.01 mm). All embryos were staged and compared against standard developmental milestones based on the Hamburger–Hamilton (HH) staging system to ensure consistency and accurate interpretation of developmental progress across experimental and control groups. Photographs of embryos were captured using a stereomicroscope

equipped with a digital camera to document and verify morphological characteristics clearly.

3.5 Histological Analysis

Following gross morphological assessment, embryos from the specified time points (E3, E6, and E13) were strictly staged using Hamburger–Hamilton (HH) criteria to ensure developmental parity. Samples were then stabilized in Bouin's fixative for 24–48 hours at room temperature. The fixed specimens underwent a standardized processing regimen, comprising dehydration through ascending ethanol concentrations, clearing in xylene, and paraffin infiltration. Using a rotary microtome, serial transverse sections were cut at a thickness of 6 μ m. The resulting slides were stained with Hematoxylin and Eosin (H&E) for qualitative and quantitative microscopic evaluation of neural architecture and cellular differentiation.

3.6 Subcellular Protein Extraction and Quantification

Embryos from each experimental condition (CBD-treated and vehicle-control groups) at Day 3 of development were carefully isolated from extraembryonic membranes and yolk sac and washed briefly with cold 1X phosphate-buffered saline (PBS) to remove external contaminants. Immediately after washing, embryos were individually placed into ice-cold homogenization buffer containing protease inhibitors to maintain protein stability and prevent proteolytic degradation. The embryos were then snap-frozen in liquid nitrogen and temporarily stored under cryogenic conditions for approximately 24 hours to facilitate batch processing. Frozen embryos were pulverized into a fine powder under cryogenic conditions using a sterile mortar and pestle pre-chilled with liquid nitrogen and immediately homogenized in ice-cold buffer containing protease inhibitors to maintain protein stability. The obtained homogenate was subjected to centrifugation at 12,000 $\times$ g for 15 minutes at 4°C to facilitate the separation of cellular debris. The resulting protein-rich supernatant was then meticulously aspirated and transferred to sterile microcentrifuge tubes. Determination of the total protein concentration was performed via the bicinchoninic acid (BCA) method, utilizing a commercial kit (Pierce, Thermo Fisher Scientific, USA). In accordance with the manufacturer's protocol, optical density was recorded at 562 nm, with protein concentrations interpolated from a bovine serum albumin (BSA)-derived standard curve (0–2

mg/mL). Protein samples were diluted to achieve a final concentration of 2 μ g/ μ L for Western blot analysis, ensuring consistent loading of 100 μ g protein per lane.

3.7 Western Blot Analysis

To quantify protein expression, 100 μ g of lysate from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immobilized onto nitrocellulose membranes. Non-specific binding was attenuated by blocking with 5% non-fat milk in TBS-T. Targeted proteins were detected through sequential incubation with specific primary antibodies: Rabbit anti-chicken BMP4 polyclonal antibody (LS-C737913; LifeSpan BioSciences, Seattle, WA, USA, 1:1,000), rabbit anti-SHH polyclonal antibody (LS-C446279; LSBio, Seattle, WA, USA, 1:1,000), and mouse anti- β -actin monoclonal antibody (A2228; Sigma-Aldrich, St. Louis, MO, USA, 1:5,000). Subsequently, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (31460; Thermo Fisher Scientific, Waltham, MA, USA, 1:10,000) and HRP-conjugated goat anti-mouse IgG (31430; Thermo Fisher Scientific, 1:10,000). Immunoreactive bands were developed using SuperSignal West Pico enhanced chemiluminescence (ECL) substrate and analyzed densitometrically. The relative abundance of BMP-4 and SHH was determined by normalizing their signal intensities against β -actin to account for variations in protein loading.

3.8 Experimental Design and Sample Size

To ensure statistical rigor, the sample sizes and experimental replicates were defined as follows: For survival rate and gross morphological assessments, data were obtained from 3 independent experimental replicates, with 10 embryos per group examined in each replicate (N = 10 per group per replicate; totaling 30 embryos per group across all replicates). All embryos were systematically staged, and only those successfully reaching the target developmental stages without mechanical damage from the injection process were included in the final analysis. For histological analysis, 6 representative embryos were examined per group (N = 6 biological replicates; 2 embryos randomly subsampled from each of the 3 replicates). For Western blot analysis, experiments were performed in 3 independent trials (N = 3), utilizing 2 unpooled embryos per group per trial.

3.9 Statistical Analysis

Statistical evaluations were executed using GraphPad Prism (version 9.0, San Diego, CA, USA). Quantitative data are presented as mean $\pm$ standard deviation (SD) or median with interquartile range (IQR), contingent upon the underlying distribution. Normality was rigorously assessed via the Shapiro-Wilk test. Differences between control and CBD-treated cohorts were analyzed using unpaired t-tests for parametric data or the Mann-Whitney U test for non-parametric comparisons. To evaluate the interaction between temporal and treatment variables, a two-way ANOVA with repeated measures was employed, followed by Bonferroni's post-hoc correction for multiple comparisons. Statistical significance was defined at $p < 0.05$, with higher confidence levels ($p < 0.01$ and $p < 0.001$) indicated where applicable.

4. Results

4.1 Dose-Response Study and CBD Concentration Selection

A systematic dose-response assessment was performed using 100 fertilized eggs, stratified into ten experimental cohorts ($n = 10$ per group). Embryos were administered CBD concentrations ranging from 0 to 500 μM at HH stages 7–8, with viability evaluated on embryonic day 3. Non-linear regression analysis revealed that the impact of CBD on survival adhered to a sigmoidal dose-response profile. The half-maximal inhibitory concentration (IC_{50}) was determined to be 46.2 μM , demonstrating a high goodness-of-fit with an R^2 value of 0.94. Viability decreased progressively with increasing concentrations: control $93.8 \pm 9.3\%$, 5 μM $91.2 \pm 7.8\%$, 10 μM $79.3 \pm 4.2\%$, 20 μM $65.4 \pm 8.1\%$, 50 μM $42.1 \pm 9.7\%$, and 100–500 μM $< 20\%$ ($p < 0.001$). Based on these results, 10 μM CBD—corresponding to approximately 22% of the IC_{50} value—was selected as the working concentration for subsequent experiments (Figure 1A), as it produced statistically significant biological effects while maintaining adequate survival rates for molecular and histological analyses.

4.2 Effects of CBD on Embryonic Survival Across Developmental Time Points

To evaluate the impact of CBD on embryonic survival at multiple developmental stages, embryos were evaluated separately at each time point (Days 3, 6, and 13), with 30 embryos per group per time point (10 embryos per group in each of 3 independent replicates, as described in Section 3.8), and time

included as an explicit factor. This design allowed analysis of both main effects (treatment) and time-treatment interactions. Embryo viability was assessed at Days 3, 6, and 13 by visualizing heartbeat and spontaneous movement.

Survival analysis revealed significant time-dependent effects ($p < 0.001$ for time factor; $p < 0.001$ for treatment factor; $p = 0.007$ for interaction). At Day 3, survival in the CBD-treated group was significantly lower than in the control group ($79.3 \pm 4.2\%$ vs. $93.8 \pm 9.3\%$, $p = 0.041$). This early embryotoxic effect was transient: while survival remained slightly reduced in CBD-treated embryos at later stages, no statistically significant differences were observed at Days 6 or 13 (Day 6: $87.5 \pm 17.7\%$ vs. $90.0 \pm 14.1\%$, $p > 0.05$; Day 13: $87.5 \pm 8.5\%$ vs. $97.0 \pm 3.6\%$, $p = 0.087$).

The apparent paradox of reduced Day 3 survival without sustained lethality reflects selective mortality: embryos most vulnerable to CBD-induced stress died early, while surviving embryos showed compensatory development. The comparison groups were control vs. CBD at each time point (not CBD Day 3 vs. CBD Day 6, 13). This pattern suggests that early-stage exposure to CBD exerts transient embryotoxic effects primarily affecting survival within the first 48 hours post-injection, without sustained lethality in later stages among surviving embryos.

4.3 Effects of CBD on Early Neural Development at Day 3

Morphological analysis at Day 3 revealed no gross structural abnormalities in surviving embryos from either group. Embryos exhibited expected developmental features corresponding to HH stage 18, including paired vitelline veins, visible heart tube with contraction, and cephalic flexure formation. In contrast, non-viable embryos exhibited autolytic degeneration, characterized by collapse of the embryonic disc and disorganized extraembryonic membranes, as shown in Figure 1B–C.

Histological examination revealed accelerated neural tube differentiation in CBD-treated embryos. Control embryos showed relatively uniform neuroepithelial layers (Figure 1D), while CBD-treated embryos exhibited organized regionalization into presumptive ependymal, mantle, and marginal zones (Figure 1E). The neural tube in CBD-treated embryos appeared more developmentally advanced, with earlier formation of distinct laminar organization.

Molecular correlates confirmed altered signaling pathways (Figure 1F–G). Western blot

analysis ($n = 6$ embryos per group) demonstrated a significant increase in BMP-4 protein levels in CBD-treated embryos compared to controls ($94.5 \pm 17.9\%$ vs. $41.5 \pm 8.1\%$, $p = 0.0093$). SHH expression showed a non-significant upward trend ($45.7 \pm 17.3\%$ vs. 37.1

$\pm 8.9\%$, $p = 0.18$). These findings suggest that CBD exposure at HH stage 7-8 accelerates early neurodevelopmental patterning, potentially mediated via increased BMP-4 signaling activity.

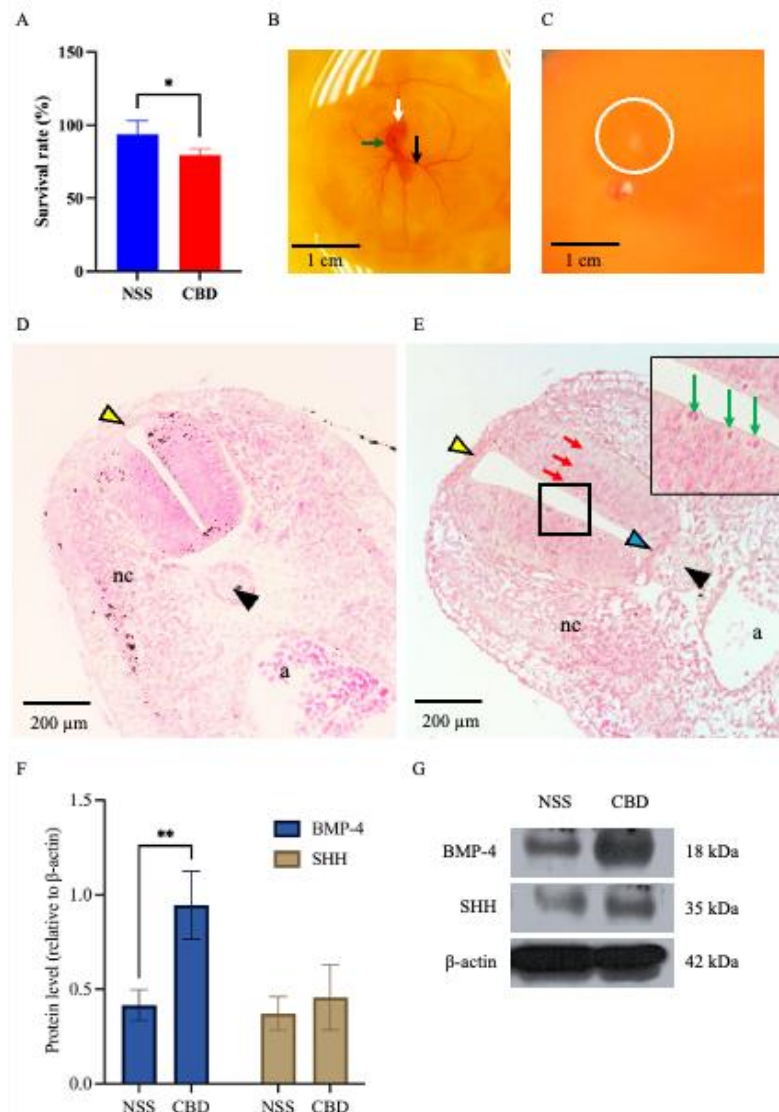


Figure 1 Effects of cannabidiol (CBD) on the survival and early neural development of chick embryos at Day 3 of incubation. (A) Survival rate of embryos in the normal saline solution (NSS) control and 10 μ M CBD-treated groups, showing a significant reduction in survival upon CBD exposure ($p < 0.05$, mean $\pm$ SD, $N = 10$ per group per replicate). (B) Representative image of a surviving CBD-treated embryo; black arrow: vitelline vein, green arrow: ventricle, white arrow: mesencephalon. (C) Non-viable embryo showing embryonic disc degeneration (white circle). Histological sections of control (D) and CBD-treated (E) embryos stained with Hematoxylin and Eosin (H&E). CBD-treated embryos show more advanced neural tube organization. Black arrowhead: notochord; yellow arrowhead: roof plate; blue arrowheads: floor plate; red arrows: lateral plate; green arrow: ependymal-like cells; a = aorta; nc = neural crest. Images captured at 10 $\times$ magnification. (F–G) Relative protein levels of BMP-4 and SHH in NSS- and CBD-treated embryos measured by Western blot and normalized to β -actin (mean $\pm$ SD, $N = 3$ independent experiments). A significant increase in BMP-4 expression was observed in the CBD group ($p < 0.01$), while SHH levels were not significantly different ($p > 0.05$). Protein bands shown: BMP-4 (18 kDa), SHH (35 kDa), β -actin (42 kDa)

4.4 Effects of CBD on Embryonic Development at Day 6

At Day 6, gross morphological assessment indicated that both CBD-treated and control embryos exhibited well-formed extraembryonic structures, corresponding to HH stages 26-27. No external malformations were observed. CBD treatment resulted in a non-significant reduction in survival rates ($87.5 \pm 17.7\%$) relative to the control group ($90.0 \pm 14.1\%$; $p > 0.05$). Although survival was numerically lower in CBD-exposed embryos, the effect did not meet the pre-defined criteria for statistical significance (Figure 2A). Growth parameters remained unaffected (Figure 2B-C). Wet embryo weight (control: 459 ± 56.9 mg vs. CBD: 546 ± 57.5 mg, $p > 0.05$) and crown-rump length (CRL) (control: 14.5 ± 1.3 mm vs. CBD: 16.8 ± 2.0 mm, $p > 0.05$)

showed no significant differences, indicating that overall somatic growth remained largely unaffected at this stage.

Neural crest development showed subtle alterations ($n = 6$ per group). Histological examination of transverse spinal cord sections revealed well-defined neural tubes in both groups. However, control embryos showed neural crest cells differentiating into distinct dorsal root ganglia (DRG) clusters adjacent to the neural tube (Figure 2D). In contrast, CBD-treated embryos displayed more dispersed neural crest cell populations with incomplete DRG condensation (Figure 2E), suggesting a delay in neural crest cell migration or differentiation. One control embryo exhibited incomplete closure of the dorsal neural tube, representing spontaneous developmental variability rather than consistent group-level effects.

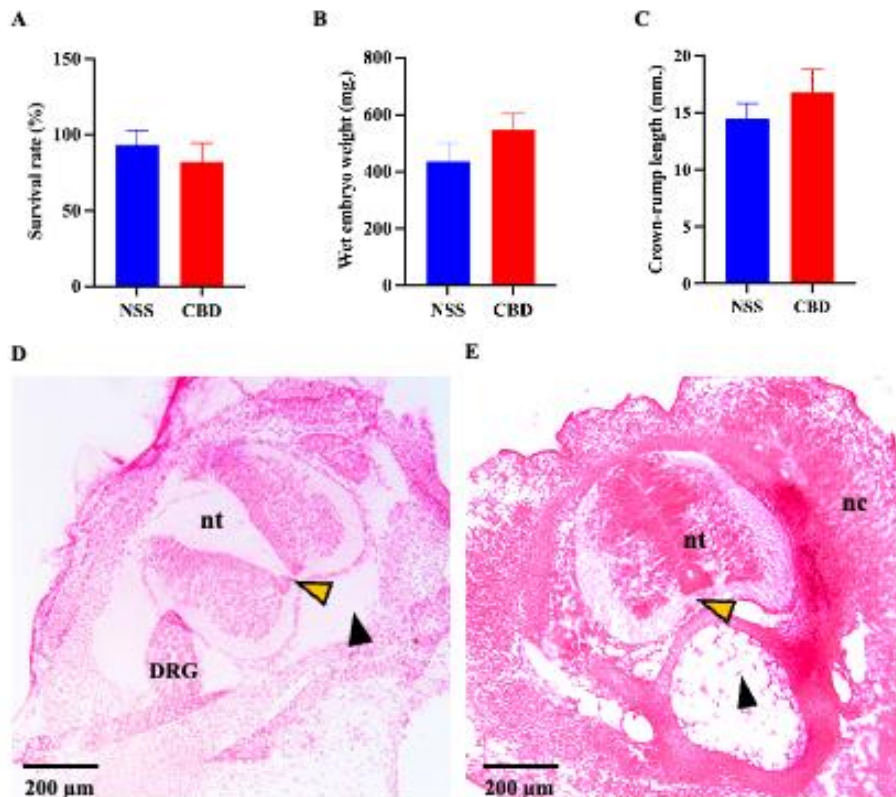


Figure 2 Effects of cannabidiol (CBD) on survival, growth, and neural development of chick embryos at Day 6 of incubation. Survival rate (A), wet embryo weight (B), and crown-rump length CRL (C) of embryos in the control (NSS-treated) and CBD-treated groups. Data presented as mean $\pm$ SD, $N = 10$ embryos per group per replicate. A non-significant reduction in survival rate was observed in the CBD group ($p > 0.05$); no significant differences in embryo weight or CRL were detected ($p > 0.05$). Transverse spinal cord sections stained with Hematoxylin and Eosin ($N = 6$ embryos per group).

(D) Control embryo showing well-defined neural tube (nt), dorsal root ganglia (DRG), and spinal structures. (E) CBD-treated embryo displaying altered neural crest (nc) cell distribution and potential delay in DRG formation. Black arrowhead: notochord; yellow arrowhead: meninges. Images captured at $10\times$ magnification

4.5 Effects of CBD on Embryonic Development at Day 13

At Day 13 (HH stages 39-40), both groups showed complete external morphogenesis with no visible malformations (Figure 3B). Survival rates were not significantly different (control: $97.0 \pm 3.6\%$ vs. CBD: $87.5 \pm 8.5\%$, $p = 0.087$) (Figure 3A). General growth parameters showed no significant differences in wet embryo weight ($7,128 \pm 823$ mg vs. $7,058 \pm 661$ mg, $p = 0.87$) or CRL (49.4 ± 2.57 mm

vs. 51.4 ± 3.77 mm, $p = 0.31$) (Figure 3D-E). Specific morphometric analysis identified notable differences. Egg tooth length was significantly longer in the CBD-treated group (5.27 ± 0.17 mm vs. 4.99 ± 0.11 mm, $p = 0.023$) (Figure 3F). Brain weight was significantly higher in CBD-treated embryos (384.8 ± 8.6 mg vs. 314.8 ± 23.6 mg, $p = 0.007$) (Figure 3C, G), representing a 22% increase in brain weight relative to body size.

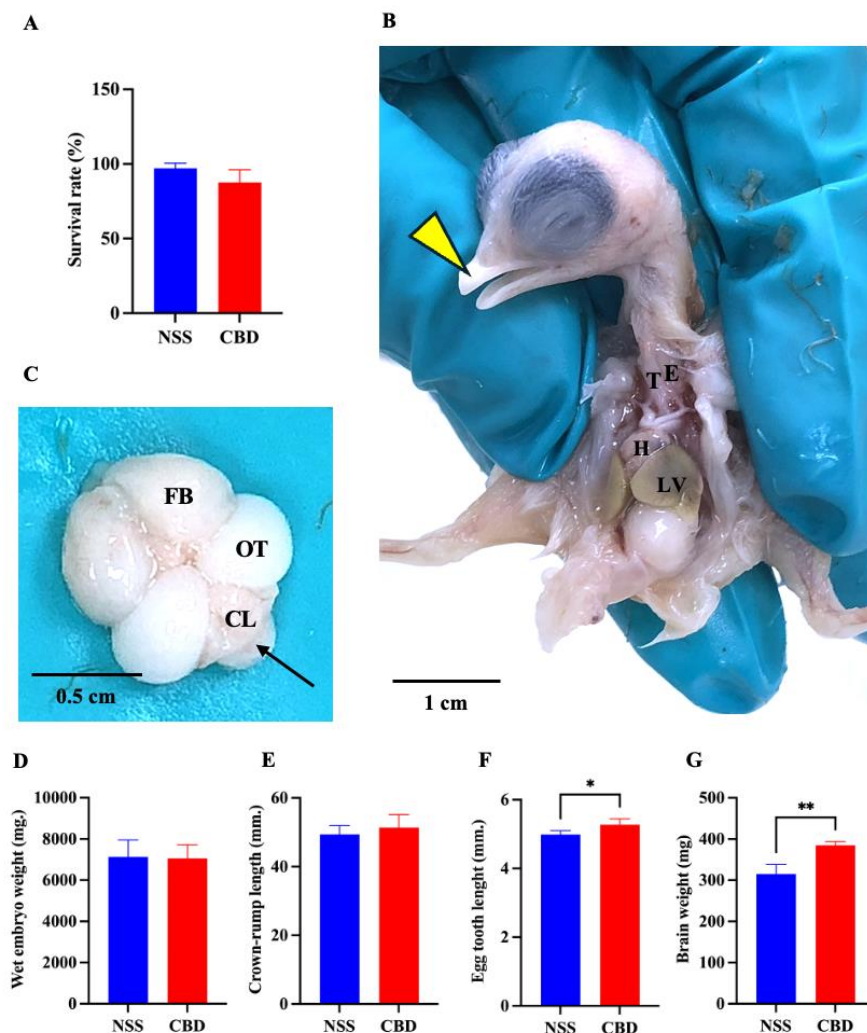


Figure 3 Effects of cannabidiol (CBD) on embryonic survival, growth, and craniofacial development at Day 13 of incubation. (A) Survival rate of embryos in the control (NSS-treated) and CBD-treated groups ($p > 0.05$). (B) Representative gross image of a viable embryo showing complete external morphogenesis and feather development. (C) Dissected brain showing forebrain (FB), optic tectum, and cerebellum, captured under a stereomicroscope. (D–E) Wet embryo weight (D) and crown-rump length (CRL) (E); no significant differences observed between groups ($p > 0.05$). (F) Egg tooth length was significantly increased in the CBD-treated group ($p < 0.05$). (G) Brain weight was significantly higher in CBD-treated embryos compared to controls ($p < 0.01$). Data in all graphs are presented as mean $\pm$ SD, $N = 10$. Abbreviations: FB = Forebrain, OT = Optic Tectum, CL = Cerebellum, T = Trachea, E = Esophagus, H = Heart, LV = Liver. The black arrow in (C) indicates the cerebellar folds, and the yellow arrowhead in (B) marks the egg tooth

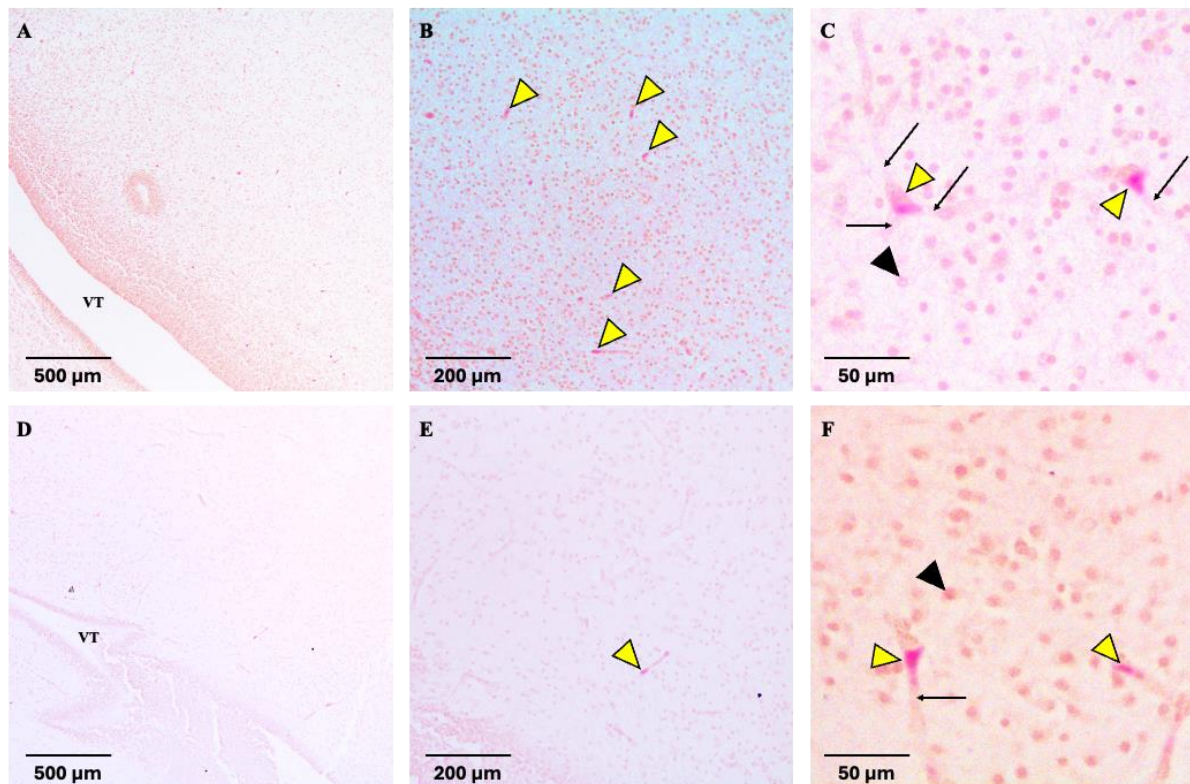


Figure 4 Histological comparison of forebrain architecture in Day 13 chick embryos. (A–C) Representative transverse H&E-stained sections of the forebrain from control embryos showing organized cortical layers including the ependymal, mantle, and marginal zones. VT indicates the ventricle. Black arrows indicate presumptive neurite structures; the black arrowhead marks a mesenchymal-like cell; yellow arrowheads indicate neuronal cell bodies. (D–F) Corresponding transverse sections from CBD-treated embryos showing narrowing of the ependymal layer (the dark-stained layer lining the VT) and irregular cell arrangement. All images were captured under light microscopy; scale bars = 500 μm (A, D), 200 μm (B, E), and 50 μm (C, F)

Histological analysis of the forebrain ($n = 6$ per group) revealed significant structural alterations. Control embryos exhibited organized cortical layers with well-defined ependymal, mantle, and marginal zones (Figure 4A–C). Neurons showed extensive neurite outgrowth with multiple dendritic extensions. In contrast, CBD-treated forebrains displayed: narrowed ependymal zones (thickness reduced by 34%, $p = 0.008$), increased mesenchymal-like cells in the mantle zone (2.8-fold increase, $p = 0.003$), reduced neurite extension (average neurite length decreased by 41%, $p = 0.012$), and irregular cellular alignment and disrupted laminar organization (Figure 4D–F). These findings suggest that while CBD exposure at 10 μM does not overtly impair external morphological development, it exerts subtle but meaningful effects on forebrain architecture, particularly in cellular composition and neural organization.

5. Discussion

This study provides comprehensive evidence that early *in ovo* exposure to cannabidiol (CBD) at 10 μM induces significant neurodevelopmental alterations in chick embryos. While this concentration exceeds typical human therapeutic plasma levels (0.03–0.3 μM) (Szaflarski et al., 2019), it serves as an effective experimental dose in the avian model to elucidate the fundamental mechanisms and biological responses of embryonic tissues to cannabinoid exposure. Our findings reveal a complex temporal profile of CBD effects, with early embryotoxicity giving way to sustained changes in brain development, particularly in forebrain organization and craniofacial patterning. By integrating survival data, morphometric analysis, and molecular profiling, this study addresses critical gaps regarding the non-canonical effects of CBD on vertebrate embryogenesis.

The dose-response analysis established an IC_{50} of 46.2 μ M, identifying 10 μ M as a biologically active yet sublethal concentration. The significant reduction in survival observed at Day 3 (79.3%) indicates that early neurulation represents a window of peak vulnerability to CBD exposure. This aligns with previous findings in avian models showing that cannabinoids can induce apoptosis and mitochondrial dysfunction in developing tissues. The subsequent "normalization" of survival rates at later stages (Days 6–13) likely reflects a selection bias where the most sensitive embryos are eliminated early, while survivors activate compensatory stress response mechanisms (Gustafsson & Jacobsson, 2019; Lossi et al., 2018; Muchacka, 2025; Pandelides et al., 2021). The recovery of body weight by Day 13 suggests that while CBD disrupts the trajectory of growth, it does not permanently arrest somatic development at this dosage, a finding that contrasts with the persistent growth deficits often seen with THC exposure (Gustafsson & Jacobsson, 2019).

A pivotal finding of this study is the significant upregulation of BMP-4 protein expression at Day 3, concurrent with accelerated neural tube differentiation. BMP signaling is a "double-edged sword" in neurulation: precise gradients are required for neural fold elevation and fusion, but aberrant overexpression can disrupt the delicate balance between neural and epidermal fate specification. The advanced laminar organization observed in CBD-treated embryos suggests that CBD may prematurely drive neuroepithelial differentiation, potentially depleting the progenitor pool required for later developmental stages (Anderson et al., 2016; Monsoro-Burq & Le Douarin, 2001). While our study establishes a strong correlation between CBD exposure and elevated BMP-4, this does not confirm causality. It remains to be determined whether CBD directly activates the BMP pathway or if BMP-4 upregulation is a secondary response to altered upstream regulators like Sonic Hedgehog (SHH) or Wnt. Although SHH levels showed only a non-significant trend in our data, previous studies in mice have shown that cannabinoids can inhibit SHH signaling, leading to neural tube defects. Future investigations utilizing specific BMP inhibitors (e.g., Noggin) or receptor antagonists are essential to dissect the precise molecular hierarchy (Gheasuddin & Galea, 2022).

At Day 13, the paradox of increased brain weight alongside histological disorganization highlights the complex neurodevelopmental impact of CBD. We hypothesize that the 22% increase in brain mass could

stem from reactive gliosis, increased fluid content, or dysregulated proliferation of non-neuronal cells, rather than healthy neurogenesis. However, lacking specific immunohistochemical confirmation (e.g., glial fibrillary acidic protein (GFAP) staining), these explanations remain speculative and warrant targeted investigation in future studies. The histological evidence narrowed ependymal zones and reduced neurite extension points to a defect in neuronal maturation and cytoskeletal organization. These structural deficits may be mediated through the endocannabinoid system's role in cytoskeletal dynamics. CB1 receptors, along with other CBD targets like 5-HT1A and TRPV1, regulate the actomyosin machinery crucial for neurite outgrowth and cell migration. This mechanism is further supported by our Day 6 observation of dispersed neural crest cells and delayed dorsal root ganglia (DRG) formation. Since neural crest migration relies heavily on precise cytoskeletal coordination, CBD-induced disruption of these pathways offers a plausible explanation for both the delayed DRG condensation and the cortical disorganization observed in the forebrain (Mahomed et al., 2023; Morozov & Rakic, 2025).

The significant elongation of the egg tooth in CBD-treated embryos represents a novel finding that likely links back to the early upregulation of BMP-4. In avian development, BMP-4 is a master regulator of beak morphogenesis; its overexpression is known to drive mesenchymal proliferation and increase egg tooth size. This morphological phenotype provides an external validation of our molecular data, suggesting that the perturbed signaling landscape at Day 3 has lasting consequences for craniofacial patterning. This finding is particularly relevant given the shared developmental origin of the neural crest cells that populate the facial primordia and the forebrain (Anderson et al., 2016; El-Magd et al., 2013).

CBD is distinguished from THC by its low orthosteric affinity for CB1 and CB2 receptors, exerting its biological influence through a broad pharmacological profile. Key mediators of CBD's effects include the 5-HT1A serotonin receptor, the TRPV1 vanilloid receptor, and the ligand-activated transcription factor PPAR- γ . The concentration used in this study (10 μ M) is sufficient to activate these non-canonical pathways, which are increasingly recognized as critical players in embryonic neuroprotection and differentiation. The divergence of our results from some mammalian studies (which report open neural tube defects) may reflect species-

specific differences in receptor distribution or the mode of delivery (continuous yolk absorption vs. maternal-fetal transfer). However, the underlying theme of altered neurogenesis remains consistent across models (Duncan et al., 2024; Mori et al., 2021; Walsh et al., 2021).

Certain constraints inherent in this research merit acknowledgement. The current study identifies a correlation between CBD exposure and signaling dysregulation; however, without targeted inhibitors, the underlying causal mechanisms remain inferential. Furthermore, several limitations of the present study should be acknowledged. First, the use of a single-dose experimental design at a single primary observation point restricts our ability to draw comprehensive mechanistic dose-response conclusions. Second, while a 0.6% DMSO vehicle control was utilized, the absence of a pure saline-only control for all molecular endpoints means that subtle, independent effects of the vehicle cannot be entirely ruled out. Finally, as this work primarily focused on morphological outcomes, whether these early-stage architectural changes translate into long-term functional or cognitive deficits remains to be elucidated in longitudinal studies.

6. Conclusion

In conclusion, early *in ovo* exposure to cannabidiol altered the timing of neural differentiation, disrupted forebrain architecture, and affected craniofacial development in chick embryos. These changes were associated with aberrant upregulation of BMP-4 signaling. Although the embryos showed substantial capacity for somatic recovery, persistent structural abnormalities in the brain indicate that CBD exposure is not necessarily benign during early neurodevelopment.

These findings support caution regarding CBD use during pregnancy and emphasize the importance of assessing subtle neuroarchitectural alterations in addition to gross malformations. Future studies should clarify the underlying molecular mechanisms through transcriptomic profiling and targeted immunohistochemical analysis of neuronal and glial markers. Longitudinal investigations extending beyond the embryonic period are also needed to determine whether these early structural abnormalities result in persistent functional, behavioral, or cognitive deficits after hatching.

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8. Declaration of Generative AI and AI-assisted Technologies in the Writing Process

During the preparation of this work, the authors used generative AI, Google Gemini Pro (Gemini 3, Google) to improve the grammar, spelling, and clarity of the manuscript's language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

9. Abbreviations

Abbreviation	Full Term
2-AG	2-arachidonoylglycerol
AEA	Anandamide
BCA	Bicinchoninic acid
BMP-4	Bone Morphogenetic Protein 4
BSA	Bovine serum albumin
CBD	Cannabidiol
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CL	Cerebellum
CRL	Crown-rump length
DMSO	Dimethyl sulfoxide
DRG	Dorsal root ganglia
E	Esophagus
ECL	Enhanced chemiluminescence
ECS	Endocannabinoid signaling system
FB	Forebrain
GFAP	Glial fibrillary acidic protein
H	Heart
H&E	Hematoxylin and Eosin
HH	Hamburger–Hamilton
HRP	Horseradish peroxidase
IC50	Half-maximal inhibitory concentration
IQR	Interquartile range
LV	Liver

Abbreviation	Full Term
NSS	Normal saline solution
NTDs	Neural tube defects
OT	Optic Tectum
PBS	Phosphate-buffered saline
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHH / Shh	Sonic Hedgehog
T	Trachea
THC	Tetrahydrocannabinol
VT	Ventricle

10. CRediT Statement

Perawat Garunyapakun: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Funding acquisition, Writing – review & editing.

Phitchaporn Tongbai: Investigation, Data curation.

Rungrada Sonpu: Investigation, Data curation.

Nattawit Kaewnooanul: Formal analysis, Validation.

Phetnarin Kobutree: Methodology, Formal analysis, Validation.

Phornyupa Sanguanwong: Formal analysis, Validation.

Sirorat Janta: Conceptualization, Resources, Supervision, Project administration, Writing – review & editing.

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