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Resveratrol mitigates diazinon-induced neurotoxicity during fetal brain development

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ABSTRACT

Objective(s): Disruption of transcriptional programs governing fetal neurogenesis represents a critical but underexplored mechanism of diazinon (DZN)-induced developmental neurotoxicity. This research examines whether resveratrol (RV) influences the expression of genes associated with neurogenesis and survival after prenatal exposure to DZN.

Materials and Methods: Twenty-four pregnant Wistar rats were divided into DZN (40 mg/kg) group, RV (10 mg/kg), DZN+RV (40 mg/kg + 10 mg/kg), and Control. On day 21 of pregnancy, rats were cesarean sectioned, and the neonatal brains were examined using HPLC, GC-MS, real-time PCR, and histology techniques to assess RV's neuroprotective effects against DZN-induced toxicity. Data were statistically analyzed using SPSS and GraphPad Prism.

Results: DZN significantly reduced neuronal survival and altered gene expression in fetal brains, with lower Ptf1α, SOX2, BDNF, and BCL2 levels and higher BAX levels compared to controls. The simultaneous administration of RV partially restored these molecular changes. Histological findings indicated that RV mitigated neuronal damage associated with DZN, resulting in decreased dark neuron formation and preserved myelin integrity in the hippocampus and thalamus.

Conclusion: Collectively, these findings suggest that DZN exposure correlates with changes in the molecular and structural composition of the fetal brain, and that RV may help regulate these effects.

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Introduction

Diazinon (DZN) (O, O-diethyl-O-[2-isopropyl-6-methyl-pyrimidin-4-yl] phosphorothioate) is a highly toxic organophosphate (OP) insecticide widely used as an agricultural pesticide (1).

Excessive use of OPs can cause significant environmental pollution and serious health risks. Exposure to OPs is linked to both acute and chronic adverse effects, including cancer, physiological disorders, neurobehavioral and cognitive abnormalities, endocrine disruption, immunotoxicity, teratogenicity, respiratory paralysis, cardiac arrest, respiratory failure, allergies, nausea, headaches, and damage to the central and peripheral nervous systems (2). The central nervous system (CNS) is particularly vulnerable to the toxic effects of OP compounds (3). Previous studies suggest multiple mechanisms underlying the developmental neurotoxicity of organophosphates, including direct targeting of key processes in the developing brain (4).

Long-term exposure to DZN can also lead to gene mutations, alter the expression of genes involved in neural cell development, induce chromosome damage and apoptosis, negatively affect cell differentiation, trigger neuronal death, arrest mitosis in embryonic cells, and reduce DNA synthesis (5, 6).

Exposure to DZN has been shown to decrease the expression of neurotrophic factors in the newborn rat brain (4). This includes alterations in the expression of neural growth factor genes, such as brain-derived neurotrophic factor (BDNF), a member of the neurotrophic superfamily that promotes neuronal development, growth, and survival

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(7). Additionally, exposure affects the expression of Sex-determining region Y box 2 (SOX2), a transcription factor essential for maintaining the pluripotency of early embryonic cells and critical for the development of various cell types and tissues in the embryo (8, 9). Ptf1α, a helix–loop–helix (bHLH) transcription factor essential for pancreatic development and also expressed in the brain, spinal cord, and enteric nervous system, is sufficient to reprogram human fibroblasts into tripotent neural stem cells (10). Neurotrophic factors play a crucial role in brain development during critical periods of development by promoting cell proliferation, migration, differentiation, survival, synaptogenesis, and myelination (11). These processes can be disrupted by the damaging effects of DZN exposure during the fetal period (5).

DZN can induce neuronal death via apoptosis by affecting the Bax/Bcl-2 protein balance (12, 13) as well as caspase-1 (casp1) and caspase-4 (casp4) (12). An increased Bax/Bcl-2 ratio leads to mitochondrial membrane permeabilization and the release of cytochrome c into the cytoplasm, triggering a caspase cascade that results in the apoptotic phenotype (14).

RV (trans-3,5,4′-trihydroxystilbene) is a phenolic phytoalexin found in grapes, peanuts, pistachios, mulberries, and raspberries. Previous studies have suggested that RV possesses anticancer, anti-inflammatory (15, 16), antioxidant, and anti-apoptotic properties (17).

According to past studies, evidence exists for the neuroprotective properties of resveratrol (RV) (18, 19), and, interestingly, it can cross the blood-brain barrier and the placenta (20). This compound has been shown to exert neuroprotection through various mechanisms. The neuroprotective effects of RV are linked to improved neuron cell survival through the induction of neurotrophic factor activity, including BDNF, and RV also reduces neuroinflammation and apoptosis (21).

Given the fact that 1) RV can cross the placental barrier and 2) the neuroprotective properties of RV, in this paper, we aimed to evaluate the neuroprotective role of RV injected into the DZN-exposed mothers during pregnancy on the fetal brain. The originality of this study lies not in assessing individual neurotrophic markers but in examining whether prenatal DZN exposure disrupts coordinated transcriptional responses that govern neurogenesis, cell fate maintenance, and survival, and whether RV intervention can restore this regulatory balance during a critical developmental window. Despite extensive evidence linking organophosphate exposure to developmental neurotoxicity, it remains unclear whether disruption of neurogenesis-associated transcription factors represents a primary mechanistic driver of fetal brain vulnerability and whether such transcriptional injury is therapeutically reversible during gestation.

Materials and Methods

Animals

In this study, 24 two-month-old female Wistar rats weighing 200-250 Grams were used. After mating and observation of the vaginal plug, the pregnant rats were divided into four groups of 6, as shown in Table 1. The animals were kept under standard conditions for temperature, humidity, water, and food. All procedures were performed in accordance with the ethical code (IR.NUMS.REC.1402.005) at Neyshabur University of Medical Sciences. Drugs, including DZN (40 mg/kg) (22) and RV (10 mg/kg) (23), were administered by gavage to the rats. On pregnancy day 21, six pregnant rats from each experimental group were anesthetized, and cesarean sections were performed to obtain the fetuses. Thereafter, anesthesia was also administered to 18 male pups per cohort. The fetuses used for molecular and histological investigations were sourced from all six dams in each cohort, ensuring that samples were allocated across various mothers to mitigate maternal bias. Brains were extracted from these specimens for a range of analytical methodologies: six pups' brains were designated for histological assessment, another six for real-time PCR analysis, and the remaining six for HPLC and GC-MS evaluations.

Table 1. Experimental design and group assignment for prenatal exposure to diazinon (DZN) and resveratrol (RV) in rats

Groups	Treatment	Route of exposure	Duration of exposure
1	Control (no intervention)	-	1-20 pregnancy (daily)
2	DZN (40 mg/kg)	gavage	1-20 pregnancy (daily)
3	RV (10 mg/kg)	gavage	1-20 pregnancy (daily)
4	DZN+RV	gavage	1-20 pregnancy (daily)

DZN: Diazinon, RV: resveratrol

Sample preparation

HPLC

Quantification of RV in brain tissue followed the methodology described by Emília Juan (24). In this procedure, a 1 g portion of fetal brain tissue was minced and transferred to a homogenizer. The tissue was then treated with 80% (v/v) methanol containing 2.5% (v/v) acetic acid as an acidifying agent. The brain specimens were immersed in 3 ml of acidified methanol and homogenized with a manual glass homogenizer for 30 complete strokes. To ensure complete transfer, an additional 1 ml of the solution was used to rinse any residual material from the glass container, and the rinse was combined with the initial 3 ml. The homogenizer was then rinsed twice with 1 ml of acidified methanol, and the rinses were added to the previous collection, bringing the total volume to 6 ml. The homogenates were transferred into 10 ml conical glass tubes and centrifuged at 3000 rpm for 30 min at 4 °C. After centrifugation, the supernatant was carefully collected and transferred to a clean tube. The remaining pellet underwent two additional extraction cycles, each involving mixing with 4 ml of acidified methanol for 5 min, followed by repeated centrifugation. The collected supernatants were ultimately transferred into specialized vials for HPLC analysis.

GC-MS

The method used to prepare the samples was based on the AOAC (Association of Official Analytical Chemists) digestion or extraction method (25). According to this method, 1 g of dried and powdered fetal brain tissue was transferred into a 250 ml Erlenmeyer flask. To determine the amount of pesticide, 10 ml of N-hexane (an organic solvent) was added to the Erlenmeyer flask containing the sample. The contents of the flask (sample and solvent) were mixed for 20 min at a medium speed using a stirrer. Then, the Erlenmeyer flask containing the sample was kept at ambient temperature for 24 to 48 hr. After this period, the sample was filtered through Whatman No. 42 filter paper, and the filtrate was transferred to the round-bottom flask of a rotary evaporator. After the solvent had completely evaporated, the remaining contents in the flask were

Table 2. Primer sequences used for quantitative real-time PCR (qRT-PCR) analysis of target genes in rat fetal brain tissue

Gene	Accession no.	Primer sequence (5'->3')	Amplicon (bp)	Melting temp (^{0}C)	Annealing temp (^{0}C)
Ptf$_{1\alpha}$	NC_000068.8	F-AAGCCGACTTGCCACTGC	132	58.9	58.9
		R- GCGAACGGGTGCCTCGAT		60.9	
BDNF	NC_000068.8	F-GTTCCACCAGGTGAGAAGAG	104	57.54	58.9
		R- CGTGGACGTTTGCTTCTTTCAT		60.03	
SOX$_2$	NC_000069.7	F- TGGTCTTGTTTAGGGCAAACG	92	59.05	58.9
		R- ACGATATCAACCTGCATGGAC		58.16	
BAX	NC_000073.7	F- TTTGCTACAGGGTTTCATCCAG	107	60.3	58.9
		R- CGCTCAGCTTCTTGGTGGAT		60.5	
BCL$_2$	NC_000067.7	F- AGTTCGGTGGGGTCATGTG	135	59.6	58.9
		R- CATCCCAGCCTCCGTTATC		57.39	
β actin	NC_000071.7	F-CGCGAGTACAACCTTCTTGC	199	59.56	58.9
		R-ATCCCACCATCACACCCTG		59.00	

BDNF: Brain derived neurotrophic factor; Ptf1α: Pancreas associated transcription factor 1α; SOX2: Sex-determining region Y box 2; BAX: Pro-apoptotic; BCL2: B- cell lymphoma 2

brought to a final volume of 5 cc with methanol and poured into special Eppendorf vials, which were then tightly closed. The vials containing the prepared samples were ready for injection into the gas chromatograph, and the target OP was measured using the relevant standard.

Preparation of standard solutions

HPLC

The RV solution was prepared by transferring the RV reference material into methanol. Standard solutions of RV with concentrations of 7.0-500 µg/ml were used, and 20 µl were injected.

Instrumental parameters

HPLC Configuration

Analytical measurements were performed using a DanChrom HPLC system (Tehran, Iran). The system consisted of a DanChrom P-400 pump with low-pressure gradient capability, an integrated vacuum degasser, a DU-800 UV-Vis detector, and a manual injector with a 20 µl sample loop. Methanol was used as the mobile phase at a flow rate of 1.0 ml/min. The analyte was detected via UV absorption at 306 nm.

GC-MS Specifications

Analyses were carried out using a Shimadzu-QP2010SE gas chromatograph coupled to a mass spectrometer (GC-MS). The system was equipped with an Rtx-5MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The column temperature program was as follows: 45 °C (held for 1 min), ramped at 10 °C/min to 110 °C (held for 5 min), then ramped at 10 °C/min to 170 °C (held for 5 min), and finally ramped at 10 °C/min to 250 °C (held for 5 min). Helium was used as the carrier gas at a constant flow rate of 0.9 ml/min. The mass spectrometer was operated with an ionization energy of 70 eV.

Brain histology

Fixation and embedding of the brains were performed in 10% formalin and paraffin, respectively. A microtome was then used to section tissues at 5 micrometers. Tissues were stained using Luxol fast blue (LFB) protocols. In the tissue sections, the hippocampus and thalamus were selected for their known sensitivity to neurotoxic agents and their essential roles in neurodevelopment. Histological sections were examined under light microscopy, and images were captured at 40x magnification from the hippocampus and thalamus. Analyses were performed by two blinded observers across four fields of view per sample for DNs in the hippocampus and thalamus. Myelin assessment in the thalamus was conducted using ImageJ software under standardized imaging conditions, with pixel-based measurements. DNs are degenerative cells characterized by hyperbasophilia and hyperelectron density on histological examination, often seen in specific neural injuries.

Quantitative real-time PCR analysis

Total RNA was extracted from brain tissue using an RNA extraction kit (ROJE Technologies, Tehran, Iran). The quality and quantity of the extracted RNA (260/280 and 260/230 ratios) were assessed using a Nanodrop (PCRmax Lambda, England). Next, using a cDNA synthesis kit (Parstous, Mashhad, Iran), the extracted RNA was reverse transcribed to cDNA according to the manufacturer's protocol. Quantitative real-time PCR was performed on a LightCyclerVR 96 Real-Time System (Rotorgen 6000, QIAGEN, Germany).

As shown in Table 2, primer sequences were designed for the following genes: *Ptf1α*, *BDNF*, *SOX2*, *BAX*, *BCL2*, and *β-actin*. To evaluate the relative mRNA expression, the fold change ($2^{-\Delta\Delta Ct}$) method was employed. The *β-actin* gene was utilized as the reference gene to normalize the sample data (27).

Statistical methods

Data analysis was conducted using SPSS and GraphPad Prism. For data evaluation, we employed one-way ANOVA followed by Tukey's *post hoc* test. Results are presented as mean values with standard error of mean (SEM). Statistical significance was established at $P<0.05$.

Results

Amount of DZN and RV in the fetuses' brains

According to Figure 1 and Table 3, the amount of DZN

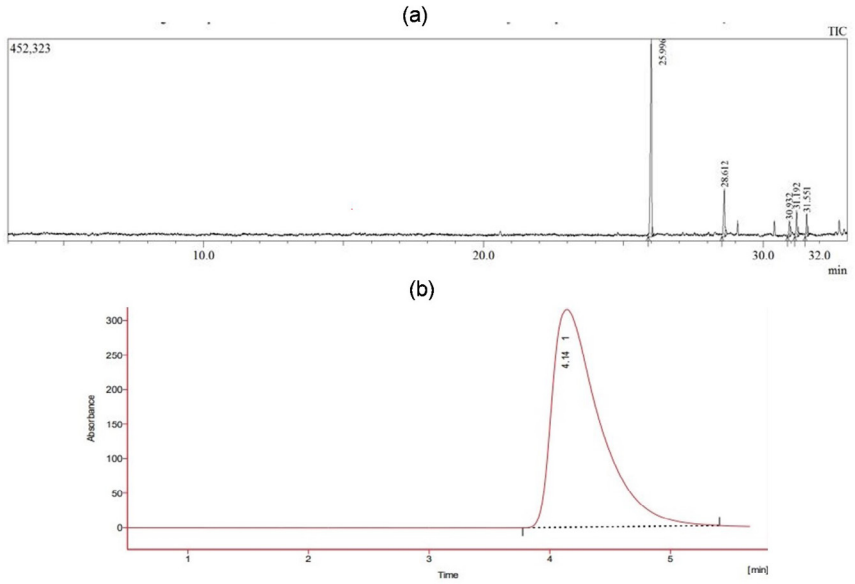

Figure 1. Representative chromatograms of diazinon (DZN) and resveratrol (RV) in rat fetal brain tissue
(a) Gas chromatography–mass spectrometry (GC–MS) spectrum of DZN (retention time: 25.996 min); (b) high-performance liquid chromatography (HPLC) chromatogram of RV (retention time: 3.642 min).

Table 3. Diazinon (DZN) and resveratrol (RV) concentration in rat fetal brain tissue

Tissue	Diazinon (GC/MS)	Resveratrol (HPLC)
Brain	115 µg/ml	89.83 µg/ml

HPLC: High performance liquid chromatography; GC/MS: Gas chromatography-mass spectrometry

and RV in the brains of the fetuses was 115 µg/ml and 89.83 µg/ml, respectively.

Effect of DZN and RV on the gene expression of Ptf$_{1\alpha}$, SOX$_2$, BDNF, BAX, and Bcl$_2$

To determine whether prenatal DZN exposure disrupts transcriptional programs essential for fetal neurogenesis and survival, we quantified the expression of key regulatory genes implicated in neural progenitor maintenance, differentiation, and apoptosis (Figure 2). Cell proliferation in the brain was also assessed by measuring Ptf1α gene expression. Data showed up-regulation in the RV group (6.163±0.593) and down-regulation in the DZN group (2.122±0.552). Significant differences were detected between DZN vs. control (P=0.0001), DZN vs. RV (P=0.0001), and DZN vs. RV+DZN (P=0.0001).

Meanwhile, SOX2 expression is shown in Figure 2 across 4 groups, with the highest and lowest expression observed in the RV (3.507±0.459) and DZN (0.901±0.429) groups, respectively. Also, there were statistically significant differences between the DZN and control groups (P=0.0043) and between the DZN and RV groups (P=0.0001). However,

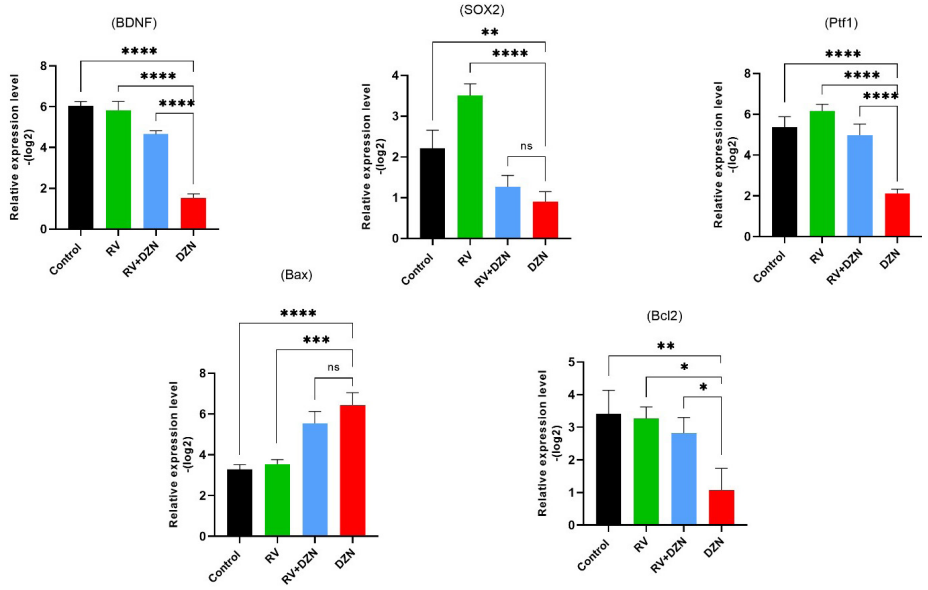

Figure 2. Effect of DZN and RV on gene expression in rat fetal brain tissue
Relative expression levels of BAX, BCL2, SOX2, BDNF, and Ptf1α are shown in the studied groups (Control, RV (resveratrol), RV+DZN (resveratrol + diazinon), and DZN (diazinon)). Data are expressed as mean±SEM (n=6). *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001; ns: not significant.
DZN: Diazinon; RV: Resveratrol; BCL2: B- cell lymphoma 2; BAX: Pro-apoptotic; SOX2: Sex-determining region Y box 2; BDNF: Brain derived neurotrophic factor; Ptf1α: Pancreas associated transcription factor 1α

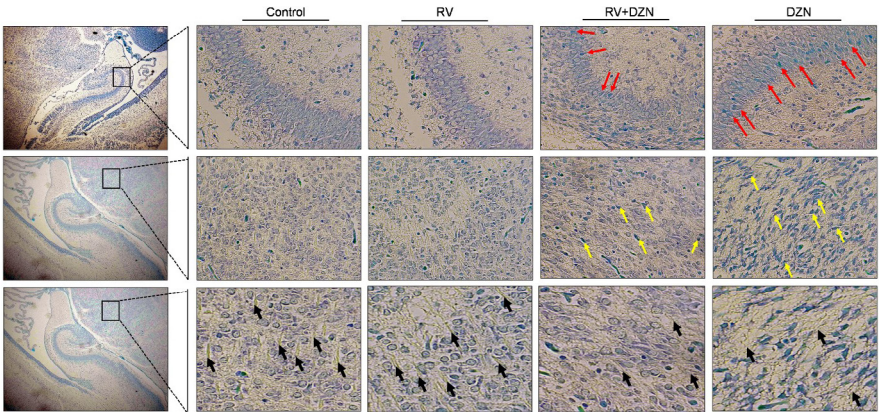

Figure 3. Representative transverse sections of rat fetal brain stained with Cresyl violet–Luxol fast blue in different experimental groups
Red arrows indicate dark neurons (DNs) in the hippocampus (HPC), yellow arrows indicate DNs in the thalamus, and black arrows indicate myelin sheaths in the thalamus. Groups include Control, RV (resveratrol), RV+DZN (resveratrol + diazinon), and DZN (diazinon). Magnifications: 4× and 40×.

the difference between the groups of DZN vs. RV+DZN (P=0.3940) was not significant.

Additionally, BDNF analysis showed down-regulation in the DZN group (1.536±0.387). Statistically significant differences were observed between the DZN group and the control group (P=0.0001), the RV group (P=0.0001), and the RV+DZN group (P=0.0001).

The quantitative RT-PCR data revealed that treatment with DZN and RV increased BAX expression in the DZN group (6.430±0.648) and decreased it in the RV group (3.552±0.672). The significant differences were found between the groups of DZN vs. control (P=0.0001) and DZN vs. RV (P=0.0003), while there was no significant difference between the groups of DZN vs. RV+DZN (p=0.1825).

Regarding Bcl2 gene expression, the highest expression was observed in the control group (3.276±0.747), and the lowest in the DZN group (0.958±0.814). Tukey's *post hoc* test showed statistically significant differences between the DZN and control groups (P=0.0072), between DZN and RV (P=0.0108), and between DZN and RV+DZN (P=0.0390).

Morphologic changes following treatment of DZN and RV

To examine histological changes in brain tissue, ImageJ was used. The results showed that the mean highest number of neurons was observed in the RV group in the HPC (152±9) and thalamus (163.33±9.01), whereas the lowest number of neurons was observed in the DZN group in the HPC (62.33±6.65) and thalamus (48±8.54). A significant difference was found between groups in the HPC for control vs. RV+DZN (P=0.004), control vs. DZN (P=0.0001), and RV+DZN vs. DZN (P=0.004) (Figures 3 and 4a). A significant difference was also observed in the thalamus between groups for control vs. DZN (P=0.0001), RV vs. RV+DZN (P=0.027), and RV+DZN vs. DZN (P=0.0001) (Figures 3 and 4d).

Regarding the mean of dark neurons, the highest number was observed in the HPC (92.66±4.93) and the thalamus (99.66±5.03) of the DZN group. Also, the lowest values were observed in the HPC (3.33±3.51) and the thalamus (8.66±2.51) in the RV group. The results reported a significant difference between groups of control vs. RV+DZN (P=0.001), control vs. DZN (P=0.0001), and RV+DZN vs. DZN (P=0.0001) in the HPC (Figure 3, Figure 4b), also between groups control vs. RV+DZN (P=0.016), control vs. DZN (P=0.0001), and RV+DZN vs. DZN (P=0.0001) in the thalamus (Figures 3 and 4e). No obvious

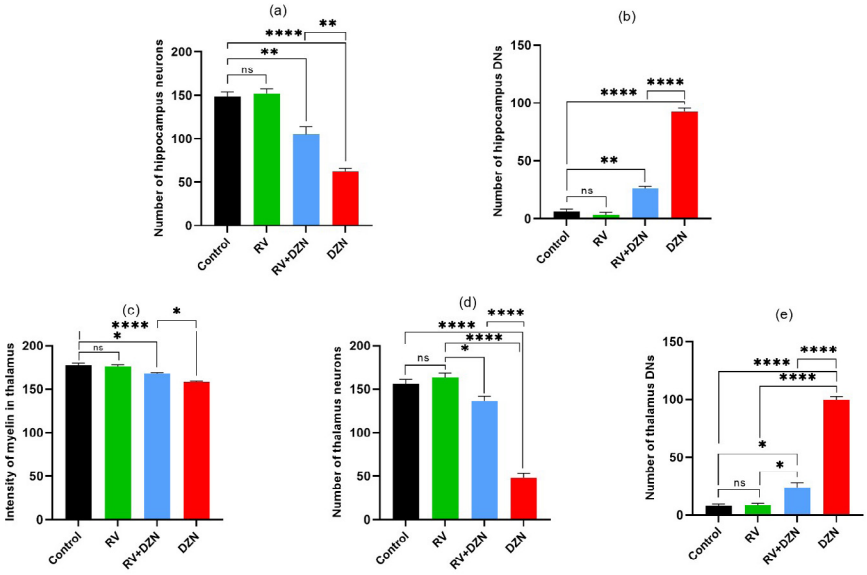

Figure 4. Quantitative analysis of morphological changes in rat fetal brain tissue across experimental groups
The number of neurons (a) and dark neurons (DNs) (b) in the hippocampus (HPC), as well as neurons (d) and DNs (e) in the thalamus, and the amount of myelin in the thalamus (c) are shown. Groups include Control, RV (resveratrol), RV+DZN (resveratrol + diazinon), and DZN (diazinon). Data are expressed as mean±SEM (n=6). *P≤0.05, **P≤0.01, ****P≤ .0001; ns: not significant.

morphological abnormalities were identified in the RV-treated group relative to controls.

After examining myelin levels in the thalamus of the studied groups using ImageJ, the data indicated the highest intensity in the control group (177.88±4.27) and the lowest in the DZN group (158.67±1.49). Comparison of myelin intensity between groups showed statistically significant differences between the control and RV+DZN groups (P=0.017), the control and DZN groups (P=0.0001), and the RV+DZN and DZN groups (P=0.022) (Figures 3 and 4c).

Discussion

The dosages in this research were chosen to use established experimental frameworks. In developmental neurotoxicity research, a dosage of 40 mg/kg of DZN has commonly been employed. This concentration effectively induces observable changes at both the molecular and histological levels, while minimizing significant harm to mothers and fetuses. RV doses between 5 and 20 mg/kg have also been observed in *in vivo* studies, with 10 mg/kg proving effective for antioxidant and neuroprotective benefits.

This study aimed to examine the impact of these compounds at a specific exposure level, rather than determine dose–response relationships. Therefore, without a multi-dose approach, we cannot determine threshold effects or optimal dosages.

Furthermore, while a group receiving only RV was included to examine its independent effects, a comprehensive assessment of developmental toxicity was not within the purview of this investigation. Consequently, although the results point to a regulatory function for RV, additional research is necessary to thoroughly understand its safety during pregnancy.

Quantification of DZN and RV in fetal brain

Gas chromatography–mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) analyses detected fetal brain concentrations of 115 µg/ml DZN and 83.9 µg/ml RV, indicating passage across the placental and blood–brain barriers. These findings collectively indicate that a neurotoxic organophosphate pesticide and a compound that may protect the nervous system coexist in the fetal brain, underscoring the need to understand their synergistic effects on neurodevelopment. DZN's lipophilicity (log P = 4.3) and relatively low molecular weight (304 g/mol) suggest that it accumulates in fetal brain tissue via passive diffusion or transporter-mediated uptake. The detected concentration of DZN is noteworthy given its established neurotoxic effects, including oxidative stress, impaired neuronal development, and disrupted neurotransmission (24). The reported concentrations should be interpreted as semi-quantitative estimates rather than fully validated quantitative measurements. Furthermore, multiple studies have demonstrated that organophosphate pesticides, such as DZN, cross the placental barrier and accumulate in fetal tissues, as evidenced by residues detected in human and rodent meconium and fetal brain. By contrast, RV enters the fetal brain and exerts neuroprotective and antioxidant activities that may counteract DZN's toxic effects (23). HPLC is preferable for RV analysis due to its sensitivity in detecting thermolabile polar molecules without causing degradation (28, 29).

Effect of DZN and RV on Ptf1α transcription factor expression

Our results demonstrate that DZN suppresses Ptf1α expression, whereas RV enhances it. Based on previous studies, DZN exposure may influence the expression of neurodevelopmental genes by interacting with cyclic AMP signaling pathways, including changes to adenylyl cyclase and protein kinase A, as observed in PC12 cell models. However, the present study did not directly examine these mechanisms. Thus, while the observed reduction in Ptf1α expression may correlate with the disruption of intracellular signaling pathways, the underlying molecular processes require further elucidation (30).

Ptf1α is essential for nervous system development, including specification of neuronal inhibitory identity, neuronal subtype differentiation and migration, target gene activation, and integration into upstream regulatory networks. Consequently, Ptf1α deficiency causes profound developmental disruption. Ptf1α regulates expression of the immunoglobulin superfamily proteins Nephrin and Neph3 in the developing CNS (31). In the present study, RV co-administration was associated with increased Ptf1α expression, suggesting a potential modulatory effect on neurodevelopment-related pathways. Research has previously indicated that RV affects several intracellular signaling pathways, including SIRT1, Nrf2, and NF-κB, in the context of neural and oxidative stress. Although these mechanisms weren't directly assessed in this study, a specific link to Ptf1α regulation remains unproven (32). Therefore, while the observed up-regulation of Ptf1α may reflect a broader protective response, the precise molecular mechanisms underlying this effect remain unclear and require further investigation.

Effect of DZN and RV on SOX2 transcription factor expression

Our findings show that RV up-regulates SOX2 expression, whereas DZN down-regulates it. Recent research indicates that organophosphate exposure may cause nerve damage through oxidative stress and changes in gene expression affecting genes crucial for brain development. Although these mechanisms might play a role in the observed SOX2 down-regulation, they weren't directly investigated in this study, making them purely theoretical (8, 33, 34).

Given SOX2's vital role in sustaining neural progenitor cell proliferation and their capacity to generate neurons, these alterations might indicate a shift in the balance between stem cell maintenance and differentiation during fetal brain development (35, 36).

RV possesses neuroprotective qualities and influences various signaling pathways. RV's influence on SOX2 expression could be mediated by SIRT1 activation (37). As a NAD+-dependent deacetylase, SIRT1 regulates SOX2 acetylation, which reduces SOX2 degradation and helps maintain stem cells. It has been proposed that this system improves how SOX2 controls neural progenitor cell functions (38, 39). This study did not directly investigate these pathways. Consequently, the observed rise in SOX2 expression should be viewed as a correlation rather than proof of a specific SIRT1-driven process.

Effect of DZN and RV on BDNF neurotrophic expression

Our research suggests a link between DZN exposure and lower BDNF expression, though RV given alongside it helped to bring those levels back up. Previous studies have

reported inconsistent effects of DZN on BDNF expression, with some showing no significant changes, possibly due to differences in dose, timing, and developmental stage. These differences imply that BDNF regulation when exposed to organophosphates could vary depending on the situation. Post-transcriptional processes are thought to regulate BDNF protein levels without affecting mRNA expression, potentially contributing to discrepancies observed across studies (4, 40).

Our findings, which suggest that DZN exposure correlates with lower BDNF expression, are supported by several studies, although some minor discrepancies remain. For instance, Aliomrani *et al.* found that hippocampal BDNF levels decreased after DZN exposure (7), and Rodríguez-Carrillo *et al.* showed that DZN reduces neurotrophic factor production in specific regions of newborn rat brains, with hints that TLR4 signaling may play a role in this modulation (41). These findings indicate that DZN-induced alterations in BDNF may depend on both developmental stage and molecular context.

RV, on the other hand, is extensively documented to increase BDNF expression through several pathways. These mechanisms involve the activation of ERK1/2–CREB signaling (42, 43), regulation of AMPK/SIRT1/PGC-1α pathways, and their anti-inflammatory actions (44).

While not directly investigated here, these pathways might play a role in re-establishing neurotrophic support during stressful periods.

Given the central role of BDNF in neuronal maturation and synaptic plasticity, these shifts could have consequences for brain development; however, the functional consequences of the observed transcriptional changes remain to be determined (45–47).

Therefore, altered BDNF expression likely influences lifelong behavior and contributes to the pathophysiology of neuropsychiatric disorders such as depression, schizophrenia, and addiction (48, 49).

Effect of DZN and RV on BCL2/BAX apoptosis regulators

The data show a correlation between DZN exposure and decreased BCL2 and increased BAX expression, with RV co-treatment partially attenuating these changes. Although several studies have examined DZN's effects on apoptotic pathways across tissues, few have investigated BCL2 and BAX expression in developing brain tissue. In one study, the authors suggested that DZN-exposed neonates exhibit selective regulation of proapoptotic gene expression through non-cholinesterase-inhibition mechanisms. The absence of BCL2 alteration may indicate a minimal role for this gene in DZN-induced developmental apoptosis or suggest that its regulation occurs post-transcriptionally (50). Studies indicate that organophosphates can affect apoptosis-related pathways and cell-cycle regulation in diverse experimental models (51).

Studies indicate that exposure to DZN might alter the expression of genes involved in apoptosis during nervous system development, potentially affecting the ratio of pro-apoptotic (BAX) to anti-apoptotic (BCL2) signals. In support of a pro-apoptotic shift, a rat study found that DZN-induced apoptosis in cardiac tissue increased the BAX/BCL2 ratio and activated caspase-3, suggesting a potential increase in pro-apoptotic signaling. However, definitive proof of apoptosis requires further functional and protein-level investigations (52). Conversely, Rashedinia *et al.* reported no significant changes in BAX or BCL2 levels in the adult rat brain (13). This discrepancy may reflect differences in dose, exposure duration, or animal age, suggesting that DZN's neurotoxicity in adult rats involves non-BCL2/BAX apoptotic pathways—such as acetylcholinesterase inhibition—or requires higher doses or prolonged exposure.

In our study, RV counteracted DZN's effects by up-regulating BCL2 and down-regulating BAX expression. Prior research has indicated that RV might influence the BCL2/BAX ratio by modulating signaling cascades, such as the PI3K/AKT/mTOR pathway (53), and by regulating p53. These processes have been linked to higher BCL2 expression and lower BAX levels, thereby affecting caspase activation and PARP cleavage. RV reduces BAX via SIRT1 activation—which deacetylates p53—and by inhibiting p53 under stress conditions. RV also suppresses cleavage of caspase-9 and caspase-3, thereby preventing PARP degradation (54). Together, these studies demonstrate that RV reinforces the BCL2/BAX balance within neural environments, thereby modulating the molecular thresholds of apoptotic signaling. RV provides robust neuroprotective support that helps preserve cellular homeostasis against DZN-induced stress. These routes were not specifically investigated in this research, so their relevance here is uncertain. Using only one reference gene (β-actin) for normalization could be a limitation, as its expression may change under stressful conditions. Consequently, future research should include validation using several housekeeping genes.

Histological examination of brain tissue and effects of DZN and RV

Histological analysis using ImageJ quantified neuronal density, identified dark neurons as morphological indicators of neuronal damage, and measured the extent of myelination in brain tissue. The findings suggested that DZN exposure correlated with microscopic signs of neuronal injury. In contrast, when RV was administered concurrently, it appeared to partially mitigate these changes. Consistent with our findings, Hashem *et al.* reported that offspring of DZN-treated rats exhibited cerebellar neurodegeneration, including thickening of the external granular layer and a significant reduction in Purkinje cell numbers (55). Similarly, Saraei *et al.* found that high-dose DZN exposure reduced cortical thickness in fetuses at gestational day 18 (5). Ultrastructural analysis revealed that DZN induces mitochondrial disruption, cristae loss, and axonal and dendritic deformations. These detailed microscopic observations suggest that sublethal levels of DZN could interfere with cellular energy production, potentially leading to impaired nerve function (5, 56). In summary, these results indicate that exposure to DZN during pregnancy could disrupt cortical development.

Even in small amounts, DZN has been linked to damage to nerve cells and changes in tissue structure. These changes include an increased number of dark neurons, which may be due to oxidative stress, mitochondrial dysfunction, and inflammation (57). DZN and its metabolites severely impair astrocyte-driven neurite outgrowth in hippocampal neurons, disrupt astrocytic function, and thereby negatively affect neuronal growth and survival (58). Supplementing mothers with RV has been shown to improve antioxidant and anti-inflammatory reactions, and modulate apoptosis signaling, potentially offering neuroprotection (59). In the DZN-exposed group, we observed reduced myelination.

Although few studies exist, one reported that DZN exposure altered the expression of genes associated with glial function and myelination. Given the white matter's critical role in cognitive and behavioral function, these alterations may have implications for cognitive and behavioral development (50). Maternal RV supplementation also normalized dysregulated oligodendrocyte-related gene expression in the progeny. Specifically, RV was reported to down-regulate oligodendrocyte transcription factor 1 (Olig1) and synapsin-1 (Syn1) mRNA in the hippocampus, suggesting enhanced oligodendrogenesis and myelination in offspring (60). Additional studies indicated that perinatal RV treatment prevented cognitive decline in offspring. Collectively, these data support that RV enhances neuronal survival through its antioxidant and anti-inflammatory effects (61, 62). These findings, viewed collectively, hint at a connection among DZN buildup, molecular shifts, and histological alterations. However, this link hasn't been definitively proven and requires additional research. The lack of functional and protein-level evaluations also restricts our ability to draw firm conclusions about neurodevelopmental results from these histological observations.

Conclusion

The study examined how prenatal exposure to DZN and RV influenced fetal brain development. The results suggest a link between DZN exposure and changes in the expression of key genes involved in brain development and cell death, as well as visible structural changes in brain tissue.

Partial normalization of these alterations was associated with RV co-administration, suggesting a possible modulatory role. However, these conclusions stem mainly from transcriptional and morphological data, not direct functional or mechanistic results. The absence of protein-level validation, dose–response analysis, and long-term functional assessments limits the interpretation of these results. As a result, further investigation is required to elucidate the underlying mechanisms, assess the functional effects, and establish the safety and efficacy of RV during fetal development.

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Authors' Contributions

A D, N A, J B, R B, A S, E F, Y B, and F RA contributed to investigation, methodology, and data curation. A D, R B, AS, and F RA handled Formal analysis, drafted the original manuscript, and participated in review and editing. F RA was responsible for funding acquisition. All authors reviewed the results and approved the final version of the article.

Conflicts of Interest

The authors confirm that there are no financial interests or personal connections that might have influenced the findings presented in this manuscript.

Declaration

We confirm that ChatGPT was used for language correction purposes.

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