



Prenatal Progesterin Exposure Induces Neurodevelopmental Alterations in Offspring Rats

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ABSTRACT

Background: Progesterin, a synthetic progesterone analog, is widely used during pregnancy to prevent complications. Considering the cerebellum's critical role in coordinating motor and higher cognitive functions implicated in autism spectrum disorder (ASD), this study examined the consequences of prenatal progesterin exposure on cerebellar development and autism-related behaviors in offspring.

Methods: Twenty-four pregnant rats were randomly assigned to treatment (progesterin 10 mg/kg BW) or control groups (n=12 each), with injections administered on gestational days 1, 7 and 14. Male offspring (n=10/group, randomly selected from different litters) were evaluated at 10 weeks using validated behavioral assays, including marble burying and nestlet shredding, to assess repetitive traits. Cerebellar tissue was processed for histological examination, oxidative stress profiling and immunohistochemical detection of estrogen receptor β (ER β).

Result: Progesterin-exposed offspring exhibited significantly heightened repetitive behaviors. Histological analysis showed marked cerebellar alterations, particularly Purkinje cell degeneration and loss of Nissl bodies. Oxidative stress markers revealed disrupted redox balance, with elevated NADPH oxidase activity and decreased antioxidant enzyme levels. ER β expression was significantly reduced across the cerebellar cortex, especially in Purkinje cells.

Key words: Autism spectrum disorder, Cerebellum, Estrogen receptor β , Neurodevelopment, Oxidative stress, Progesterin.

INTRODUCTION

Autism spectrum disorder (ASD) represents a significant global health concern, with epidemiological studies revealing a growing prevalence worldwide. Recent data indicates a ratio of 1:68 (Sauer *et al.*, 2021). ASD is a complex neurodevelopmental disorder characterized by persistent deficits in social interaction and communication, accompanied by restricted, repetitive patterns of behavior and interests. The disorder has emerged as a leading cause of childhood disability (Sauer *et al.*, 2021).

The etiology of ASD involves multiple contributing factors, including genetic, epigenetic and environmental influences (Yin *et al.*, 2019; Al-Brakati *et al.*, 2021; Mishra *et al.*, 2021; Noura *et al.*, 2022; Journal *et al.*, 2024). Epidemiological research has identified maternal hormonal interventions as a potential risk factor in ASD development (Shukla and Rajput, 2021; Aagaard *et al.*, 2023). Notably, ASD patients demonstrate elevated steroidogenic activity, with the disorder's development linked to abnormalities in vitamin D metabolism, cholesterol metabolism and other steroid-related irregularities (Whitaker-Azmitia *et al.*, 2014).

The cerebellum, located in the posterior cranial fossa beneath the occipital lobes, has emerged as a critical brain region in ASD pathophysiology. Neuroimaging studies consistently reveal cerebellar abnormalities in individuals with ASD, including alterations in volume, connectivity and cellular architecture (van der Heijden and Sillitoe, 2020). A significant investigation by Li *et al.* (2018) identified three critical factors associated with ASD prevalence: progesterin use for threatened abortion prevention, consumption of progesterin-based contraceptives during conception and ingestion of progesterin-contaminated seafood.

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Progesterone serves as a therapeutic agent for various reproductive conditions, including infertility, threatened abortion, recurrent miscarriage, luteal phase maintenance in assisted reproduction and threatened preterm labor prevention (González *et al.*, 2024). Elevated progesterone levels during corpus luteum formation may influence embryonic development, with higher circulating levels potentially accelerating developmental processes (Bullelli *et al.*, 2022). Critically, progesterone crosses the placenta via passive diffusion, allowing maternal hormone levels to directly influence fetal neurodevelopment.

Progestins, synthetic analogs of progesterone, bind to intracellular progesterone receptors and modulate gene transcription in target tissues including the uterus, placenta and fetal brain (Wagner and Quadros-Mennella, 2016). Their use during early pregnancy, particularly during critical windows of brain formation, has raised concerns regarding potential neurodevelopmental impacts. Steroid hormones,

including progesterone, cross the blood-brain barrier and influence neural development through multiple mechanisms: Regulation of gene expression, neuronal migration and synaptic plasticity (Alliende *et al.*, 2018). The presence of progesterone receptors in the developing cerebellum suggests a direct pathway through which progestin exposure might influence cerebellar development and contribute to ASD-related behaviors (Li *et al.*, 2018).

Despite mechanistic insights from animal models, significant knowledge gaps persist regarding progesterone exposure effects on human fetal development. The impact of progesterone dosage on fetal development and the consequences of excessive exposure remain poorly understood. Based on the hypothesis that hormonal alterations during pregnancy influence ASD development, this study investigates progestin supplementation effects on cerebellar development using therapeutic doses, aiming to establish a research trajectory for evaluating the safe use of progesterone during pregnancy with particular attention to neurodevelopmental outcomes.

MATERIALS AND METHODS

Animals and experimental design

Twenty-four female albino rats (12 weeks old, 175-225g) were obtained from the animal facility at Taif University. Animals were maintained under controlled conditions (temperature: 28±7°C; 12:12 hour light/dark cycle) with ad libitum access to standard chow and water. Nulliparous females were paired with mature males for breeding. Pregnancy was confirmed by detecting vaginal copulation plugs or sperm in vaginal flush, with the day of confirmation designated as embryonic day one.

Pregnant females were randomly allocated into two groups (n=12 each):

- **Control group**

Received equivalent volumes of physiological saline following the same schedule.

- **Treatment group**

Received subcutaneous injections of proluton depot (hydroxyprogesterone hexanoate; Schering AG, Germany) at 10 mg/kg body weight on gestational days 1, 7 and 14 (Ahmed *et al.*, 2016).

Maternal body weight was monitored daily to adjust dosages. The selected dose corresponds to therapeutic ranges used in human pregnancy (Dodd *et al.*, 2019). Post-parturition, male offspring were selected and maintained with mothers until weaning (28 days), then housed in groups of 2-3 until behavioral testing.

Behavioral assessment

At 10 weeks of age, male offspring (n=10 per group, randomly selected from different litters) underwent behavioral testing using two validated protocols.

Marble burying test (MBT)

Animals were individually placed in standardized cages (35 × 23 × 19 cm²) containing 5 cm deep wood chip

bedding. Twenty glass marbles (1 cm diameter) were arranged in a 5 × 4 grid pattern on the bedding surface. After 30 minutes, the number of marbles buried (>50% covered) was recorded by a blind observer. This test evaluates repetitive/perseverative behavior characteristic of autism-like phenotypes (Bahi, 2016).

Nestlet shredding test (NST)

Pre-weighed cotton nestlets (5 × 5 cm, ~2.5 g) were placed in test cages with individual animals. After 30 minutes, remaining unshredded material was weighed. The percentage of shredded material was calculated as:

$$\frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100$$

Increased shredding indicates elevated repetitive behavior (Angoa-Pérez *et al.*, 2013). Tests were conducted on consecutive days.

Tissue collection and processing

Following behavioral testing, animals were anesthetized with intraperitoneal ketamine (90 mg/kg) and xylazine (10 mg/kg) (Giroux *et al.*, 2015). After confirming deep anesthesia, animals were transcardially perfused with ice-cold PBS. Cerebellar tissue was rapidly dissected and divided for different analyses.

Analysis of oxidative stress markers

Cerebellar tissue was homogenized in ice-cold PBS (pH 7.4) to prepare 10% (w/v) homogenate, centrifuged at 10,000 × g for 15 minutes at 4°C. Total protein was determined using Bradford method (Kruger, 1994).

NADPH oxidase activity was assessed using lucigenin-enhanced chemiluminescence (Ebrahimian *et al.*, 2006). Reaction mixture contained 50 mM phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose, 5 µM lucigenin and 100 µM NADPH. Photon emission was measured using a luminometer (Biotek, USA). Activity was expressed as nmol superoxide/mg protein/min.

Glutathione peroxidase (GPx) activity was determined colorimetrically by monitoring NADPH decrease at 340 nm, as described by the company method. The reaction mixture contained 50 mM potassium phosphate buffer, 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH and 1 mM glutathione. Activity was calculated using the NADPH extinction coefficient (6.22 mM⁻¹ cm⁻¹) and expressed as units/mg protein.

Superoxide dismutase (SOD) activity was measured using commercial kits (Biodiagnostic, Egypt) following the company's described method. The assay evaluates SOD capacity to inhibit nitro blue tetrazolium reduction and activity was expressed as units/mg protein.

Histological analysis

Cerebellar tissues were fixed in 10% neutral buffered formalin for 24 hours, dehydrated through graded alcohols, cleared in xylene and embedded in paraffin. Sections (5 µm) were cut using a rotary microtome and stained with Cresyl

violet for Nissl substance visualization. This technique reveals detailed cytoarchitecture, with healthy neurons displaying distinct blue-purple coloration of nucleus, nucleolus and Nissl substance (Vollmer, 1977).

Immunohistochemical analysis

Paraffin sections were deparaffinized, rehydrated and subjected to antigen retrieval in citrate buffer (pH 6.0) at 95°C for 20 minutes. After blocking endogenous peroxidase with 3% H₂O₂, sections were incubated overnight at 4°C with anti-ER β antibody (mouse monoclonal, 1:200, Santa Cruz, sc-390243). Detection was performed using avidin-biotin complex method (NovaCastra, UK) with diaminobenzidine as chromogen. Sections were counterstained with Mayer's hematoxylin. Negative controls omitted primary antibody (Pandurangan *et al.*, 2024).

Transmission electron microscopy

Small cerebellar fragments (1 mm³) were fixed in 2.5% glutaraldehyde in phosphate buffer for 2 hours at 4°C, post-fixed in 1% osmium tetroxide for 1 hour, dehydrated through graded ethanols (50%, 70%, 90%, 100%) and embedded in epoxy resin. Ultrathin sections (60-90 nm) were cut using an ultramicrotome, collected on copper grids and double-stained with uranyl acetate and lead citrate. Sections were examined using JEOL JEM-1400 transmission electron microscope at 80 kV, focusing on Purkinje cell ultrastructure (Phillipp, 2012).

Statistical analysis

Data were expressed as mean $\pm$ standard deviation. Statistical comparisons between groups were performed using one-way ANOVA followed by Tukey's honestly significant difference post-hoc test. P-values <0.05 were considered statistically significant. Sample size (n=12/group) was determined based on power analysis from similar published studies involving behavioral and histological evaluations in rats.

RESULTS AND DISCUSSION

Prenatal progesterin exposure induces repetitive-like behaviors in offspring

We used marble burying and nestlet shredding tests to measure repetitive behavior. Treated rats buried more

marbles and tore more nestlets than controls, showing more compulsive behavior.

Increased compulsive and repetitive behaviors following progesterin treatment

The treated group demonstrated significant increases in repetitive and stereotypical behaviors compared to the control group. In the Marble Burying Test (MBT), animals from the treated group buried significantly more marbles (13.3 $\pm$ 2.36) compared to the control group (2.5 $\pm$ 1.35; p <0.00001) (Fig 1), indicating enhanced repetitive behavior. Similarly, the Nestlet Shredding Test (NST) revealed significantly higher shredding activity in the treated group (40.5 $\pm$ 3.6%) compared to controls (16.26 $\pm$ 3.4%; p <0.05) (Fig 1), further supporting the presence of compulsive-like behaviors characteristic of autism-like phenotypes.

Oxidative stress markers are altered in the cerebellum of treated offspring

We measured oxidative stress markers to check for possible damage. The treated group had higher NADPH oxidase activity and lower levels of GPX and SOD, which suggests increased oxidative stress in the brain tissue.

Oxidative stress markers

Since repetitive behavior was observed in the treated group, we next examined oxidative stress levels in cerebellar tissue to investigate possible underlying mechanisms. Biochemical analysis revealed significant alterations in oxidative stress parameters in the treated group. Specifically: NADPH Oxidase Activity: A significant increase was observed in the treated group (10.11 $\pm$ 0.235 nmol/mg protein/min) compared to the control group (4.96 $\pm$ 0.295 nmol/mg protein/min; p <0.05) (Fig 2). Glutathione Peroxidase (GPX): The treated group showed significantly reduced GPX activity (0.204 $\pm$ 0.101 U/mg protein) compared to controls (0.507 $\pm$ 0.17 U/mg protein; p <0.05) (Fig 2).

Superoxide Dismutase (SOD): A marked decrease in SOD activity was observed in the treated group (1.009 $\pm$ 0.56 U/mg protein) compared to the control group (2.95 $\pm$ 0.39 U/mg protein; p <0.05) (Fig 2). These changes in oxidative stress markers indicate significant oxidative damage and compromised antioxidant defense mechanisms in the cerebellar tissue of the treated group.

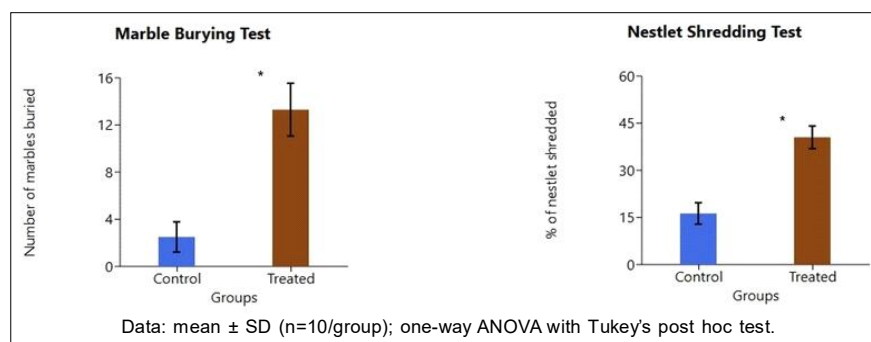


Fig 1: Behavioral assessment showing (A) marble burying test and (B) nestlet shredding test results in control versus progesterin-treated groups.

Histological changes in cerebellar cortex indicate neuronal damage

To explore whether structural damage in the cerebellum contributed to behavioral changes, we performed cresyl violet staining to assess Purkinje cell morphology. We examined cerebellar tissue with cresyl violet stain. Treated rats showed damage in Purkinje cells, including pale cytoplasm and pyknotic nuclei, unlike the healthy structure seen in controls. Light microscopic examination of the cerebellar sections revealed distinct differences between control and treated groups. In the control group, cerebellar cortex comprises three distinct layers: the outer molecular layer (Mo), the middle Purkinje cell layer (Pc) and the inner granular layer (Gr) (Fig 3). The molecular layer showed proper cellular distribution, while the Purkinje cell layer displayed characteristic arrangement of large pyriform cells in a single row. These Purkinje cells exhibited rounded, basophilic vesicular nuclei and possessed a dark basophilic thin rim of cytoplasm concentrated near the plasmalemma, indicating normal distribution of Nissl granules. The granular layer maintained appropriate cellular density with darkly stained neurons. In the treated group, significant alterations in cerebellar histology were

observed. Most notably, there was an apparent reduction in Purkinje cell numbers, with the remaining cells showing distinctive pathological changes (Fig 3). These cells displayed pale, diffuse basophilic cytoplasm, indicating dispersion of Nissl granules within the cytoplasm - a condition known as chromatolysis. Furthermore, many Purkinje cells exhibited darkly stained pyknotic nuclei, suggesting cellular stress and potential degenerative changes.

ER β expression is significantly reduced after progesterin exposure

Since ER β plays a role in neural development and antioxidant defense, we examined its expression in cerebellar tissue using immunohistochemistry. ER β expression in cerebellar sections were tested. The control group showed strong ER β staining, especially in Purkinje cells, while the treated group showed much weaker expression. Immunohistochemical examination for ER β expression demonstrated marked differences between the experimental groups. The control group showed strong positive immunoreactivity for ER β throughout the cerebellar cortex, with notable expression in the molecular and granular layers, as well as in the nuclei of Purkinje cells (Fig 4). The staining pattern was consistent and well-distributed

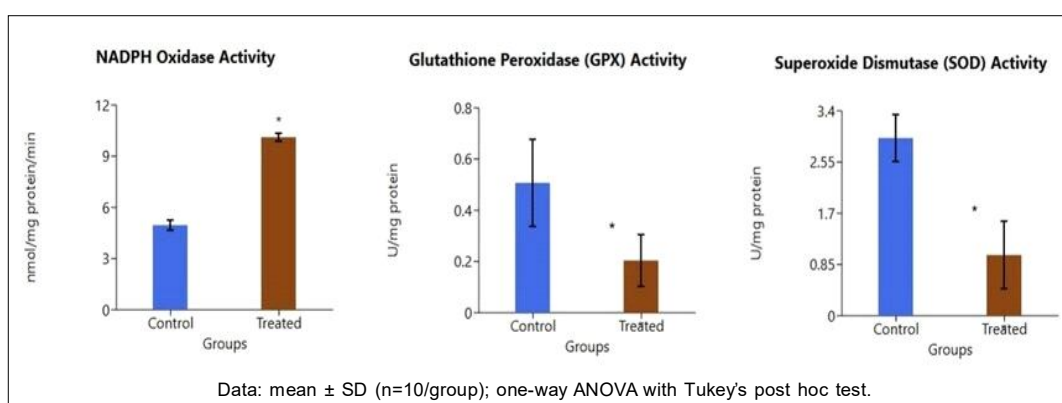


Fig 2: Oxidative stress markers in cerebellar tissue: NADPH oxidase, GPX and SOD activities in control (blue) versus treated (brown) groups.

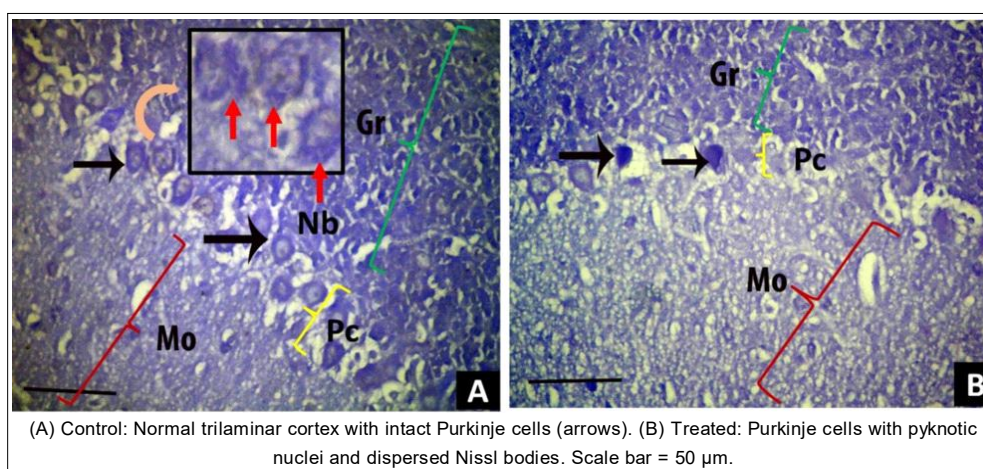


Fig 3: Cresyl violet-stained cerebellar sections.

across all cerebellar layers. In contrast, the treated group exhibited a significant decrease in ER β immunoreactivity. This reduction was apparent across all layers of the cerebellar cortex, with particularly diminished expression observed in the Purkinje cell population (Fig 4). The decreased ER β expression suggests a potential disruption of estrogen-mediated signaling pathways in the cerebellum following progesterin treatment.

Ultrastructural damage observed in purkinje cells by electron microscopy

To confirm cellular damage at the ultrastructural level, The Purkinje cells were examined using transmission electron microscopy. Under the electron microscope, Purkinje cells in treated rats had damaged nuclei, swollen mitochondria and vacuolated cytoplasm. These changes were not seen in controls. Ultrastructural examination using TEM revealed detailed cellular changes between the groups. In the control group, Purkinje cells displayed normal ultrastructural features, characterized by large cell bodies with euchromatic nuclei. The cytoplasm was rich in small electron-dense mitochondria and rough endoplasmic reticulum. Supporting

neuroglial cells showed appropriate relationships with blood capillaries and normal nuclear chromatin margination (Fig 5). The treated group demonstrated severe ultrastructural alterations. Purkinje cells appeared with smaller, ill-defined nuclei containing irregular patches of clumped chromatin. The perikaryon showed evident degeneration, characterized by multiple pathological features: presence of autophagosomes, enlarged organelles, distinctive whorled structures and numerous cytoplasmic vacuoles (Fig 5). These ultrastructural changes indicate significant cellular stress and potential dysfunction of cellular organelles in the treated group, providing detailed evidence of progesterin-induced cellular damage at the subcellular level.

The rising global prevalence of ASD necessitates improved understanding of its etiology. While early diagnosis and intervention before age three can lead to better outcomes (Okoye *et al.*, 2023) the pathophysiology remains poorly understood, with no established biomarkers or effective pharmaceutical treatments targeting core symptoms (Farmer *et al.*, 2013). Early diagnosis enhances understanding of fundamental pathophysiological processes and unknown causative factors (Ruggeri *et al.*, 2014).

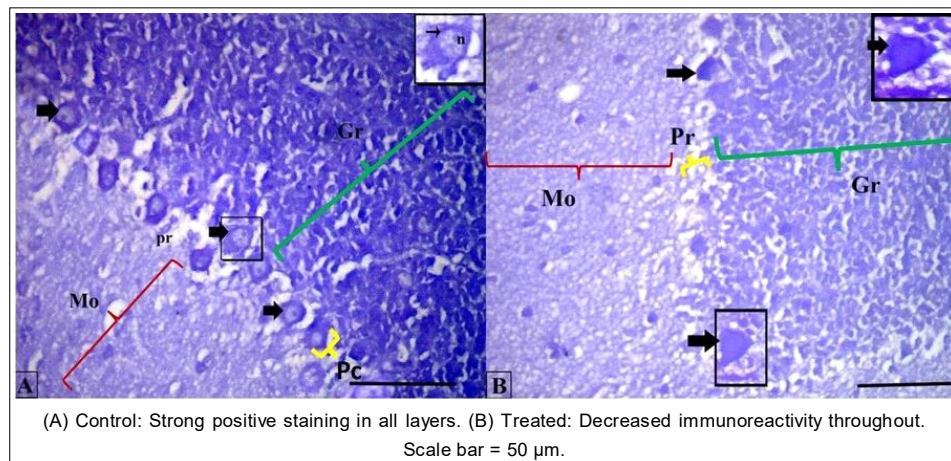


Fig 4: ER β immunohistochemistry.

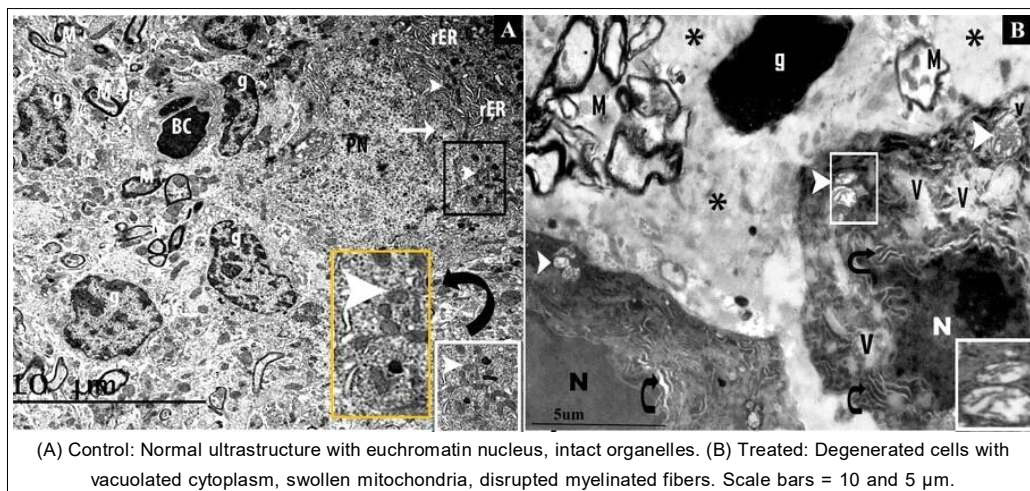


Fig 5: Electron micrographs of Purkinje cells.

Prenatal drug exposure prevalence is increasing globally (Thomason, 2020). Fetal brain development occurs throughout gestation, particularly during the first trimester when critical milestones are achieved. Early-life drug exposure can have lasting adverse effects on brain structure and function (Ross *et al.*, 2014). Several factors associate with prenatal progesterin exposure, including its use for threatened abortion prevention, which links to preterm delivery and maternal abortion history (Wahabi *et al.*, 2018). Additionally, maternal age under 20 correlates with progesterin contraceptive use and hormonal imbalances from elevated stress. Li *et al.* (2018) identified seafood as a primary source of progesterin contamination in Hainan province, China, potentially impacting ASD prevalence. Exogenous progesterone during pregnancy negatively affects fetal growth, skeletal development and sex organ formation (Tag *et al.*, 2021).

This study investigated the link between prenatal progesterin exposure and neurodevelopmental alterations in rat offspring, focusing on ASD-associated repetitive behaviors. The administered progesterone dosage falls within typical clinical ranges prescribed to pregnant women (Challa *et al.*, 2008). The marble burying and nestlet shredding tests are validated models for assessing compulsive-like behaviors characteristic of psychiatric conditions including OCD and ASD. In marble burying tests, buried marble quantity indicates repetitive digging behavior extent. Animals predisposed to OCD or ASD-related behaviors bury more marbles at accelerated rates. Enhanced marble burying has been documented in various autism mouse models (Burgett *et al.*, 2024).

Our histological analysis revealed cerebellar Purkinje and granular neuron degeneration in treated offspring. Cresyl violet staining identified damaged neurons across all cerebellar layers, with reduced Nissl stain affinity indicating ribosomal disconnection from rough endoplasmic reticulum. Similar Purkinje cell alterations occur in autistic individuals' cerebella in postmortem studies (Fatemi *et al.*, 2012). D'Mello (2015) found cerebellar structural atypicalities in autism.

Wang *et al.* (2014) demonstrated that cerebellar neurodevelopmental changes lead to cognitive atypicalities and disturbed cerebello-thalamo-cortical pathways, proposing that cerebellar insults during development causally impact cortical structures. The cerebellum contains over 50% of brain neurons and participates extensively in cognitive capacities including social communication and cognition. Elandalousi *et al.* (2022) documented structural cerebellar changes in autistic individuals, supporting complex relationships between cerebellar structure, social performance and IQ. Inhibitory Purkinje cells and excitatory granule cells are crucial for proper cerebellar development. Disturbances in these cell types significantly interfere with cerebellar growth. Recent theories propose ASD etiology partly involves extensive but subtle ongoing Purkinje cell system changes (van der

Heijden *et al.*, 2021), indicating irregular cerebellar-cortical pathway development significantly impacts social cognitive abilities frequently impaired in ASD. Early cerebellar development investigation could enhance understanding of pathway malfunction origins (Bloomer *et al.*, 2022).

The observed cerebellar structural alterations correlated with diminished ER β immunohistochemical expression. Li *et al.* (2018) reported similar observations following prenatal progesterin-contaminated seafood exposure. During normal development, ER β expresses in molecular and granular cerebellar layers. In Purkinje cells, ER β first appears on postnatal day 6, with peak immunostaining coinciding with axonal and dendritic growth between postnatal days 7-8, remaining elevated throughout Purkinje dendritic maturation. ER β participates in growth-related processes during cerebellar neuron and glial cell differentiation (Jakab *et al.*, 2001). Both *in vitro* and *in vivo* studies demonstrate progesterin exposure reduces amygdalar ER β levels (Zou *et al.*, 2017), suggesting ER β significantly contributes to social deficits in offspring following prenatal progesterin exposure. This aligns with studies indicating maternal hormone exposure as an ASD risk factor (Zou *et al.*, 2017).

Our study highlighted cerebellar oxidative stress in prenatal progesterin-exposed offspring, with significantly increased NADPH oxidase activity alongside decreased SOD and GPX activities. Recent research established connections between ASD and heightened oxidative stress manifesting through various mechanisms including protein post-translational modifications, abnormal metabolic processes and reactive oxygen species accumulation. Xie *et al.* (2018) demonstrated prenatal progesterone exposure induces offspring ASD by promoting oxidative stress linked to ER β suppression. ER β regulates superoxide dismutase expression, modulating cellular oxidative stress (Tamir *et al.*, 2002).

Oxidative stress is recognized as an ASD pathogenesis contributing factor. These detrimental influences cause neurodevelopmental impairments in developing fetal brain, ultimately producing behavioral symptoms. Oxidative stress represents adverse oxidative reaction effects where ROS interacts with DNA, lipids, proteins and enzymes, causing lipid peroxidation, genetic mutations, protein denaturation and enzyme inactivation (Uzefovsky *et al.*, 2019). Increased oxidative stress elevates molecular-level biological oxidative damage, leading to various diseases and accelerated aging (Usui *et al.*, 2023).

Our findings indicate prenatal progesterin exposure induces autism-like behaviors through significant cerebellar developmental effects, including structural alterations, Purkinje and granular cell degeneration, oxidative stress and diminished ER β expression. These results suggest progesterin exposure contributes to ASD-related behavioral traits, particularly repetitive behaviors. However, comprehensive assessment of other core ASD domains requires further investigation. The study emphasizes careful evaluation of

progesterin use during pregnancy and highlights potential neurodevelopmental risks associated with hormonal interventions during critical developmental windows.

CONCLUSION

Prenatal exposure to therapeutic doses of progesterin induced significant neurodevelopmental alterations in offspring. Exposed rats exhibited increased repetitive behaviors characteristic of autism spectrum disorder. These behavioral changes correlated with cerebellar pathology: Purkinje cell degeneration, oxidative stress (elevated NADPH oxidase, reduced antioxidant enzymes) and decreased ER β expression, suggesting a pathway linking progesterin exposure to autism-like behaviors through disrupted hormone signaling and redox imbalance.

These findings emphasize careful evaluation of progesterin use during pregnancy. Future research should establish safe dosage guidelines, develop screening protocols and investigate protective interventions when treatment is necessary. The study highlights environmental factors in neurodevelopmental disorders and the importance of cautious hormonal intervention during pregnancy.

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Author contributions

Amer, Albrakati and Mohamed contributed to methodology, investigation and histology. Amer handled conceptualization and project management; Albrakati performed animal and biochemical work; Mohamed managed statistics and visualization. All authors approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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