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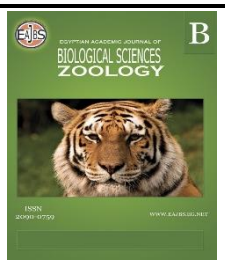


# Pyriproxyfen-Induced Developmental Neurotoxicity and Genotoxicity Among the Developing Chick Embryos, (*Gallus domesticus*)

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**Keywords**: Larvicide; Brain derived-neurotrophic factor **(**BDNF); Heat shock proteins; Tuj1; Sox10.

**ABSTRACT**

The present study was designed to evaluate the effects of pyriproxyfen (PPF) larvicide on ovo-chick neurodevelopment and genotoxicity in embryonic days (EDs) 7 and 14. Fertilized chicken eggs were divided into three groups (G1, G2, and G3) injected with three sub-lethal doses 15, 30, and 45ug PPF/egg, respectively. In both EDs, PPF induced significant levels of mortality, whereas hematoma formation and microcephaly were the common disorders in the head region of all treated groups. The injected groups demonstrated histological neuronal damage in the pallial ventricular zone with distributions of chromatolysis and vacuolated neurons and decreased BDNF expression with restorative expression in ED 7 embryos. The levels of Hsps 70, and 90 of embryos on ED 14 expressed a higher level of gene expression than those of ED 7 with different significant levels. Additionally, the Tuj1 and Sox10 gene expressions revealed a significant decrease during ED 7 with the highest decrease among the G2 embryos. On the other hand, Tuj1 expression also decreased during ED 14 in G2, but Sox10 gene expression seemed to be upregulated with regenerative ability. In conclusion, ED 7 younger embryos were more affected by PPF and exhibited significant teratogenic brain disorders in developing chick embryos owing to its direct effect on neurogenesis and downregulation of regenerative process proteins.

## INTRODUCTION

Pesticide use causes environmental contamination, health issues, and decreased fertility during crop production (Andreska *et al.*, 2020). As insecticides remain in the environment and accumulate in living species, their widespread use has come under fire in recent years. It is categorized as "moderately toxic, " based on animal research (Hussein and Singh, 2016).

Pyriproxyfen (PPF) is an insect growth regulator. It is commonly formulated as a larvicidal as it inhibits the sexual maturity of larvae and stops their reproduction. Therefore, Therefore, in many countries, PPF is extensively used to control mosquito larvae (malaria vectors) (Sullivan and Goh, 2008; WHO, 2008). PPF was recently brought into the public interest following an intense debate involving claims that its widespread use in Brazil may

be the cause or enhancing the increased incidence of newborn microcephaly cases reported in Brazil. This is due to its use in drinking water supplies to fight the proliferation of mosquito larvae since 2014 (Parens *et al*., 2017). Several previous studies have documented the developmental toxicity of PPF on vertebrates including zebrafish (Truong *et al*., 2016; Dzieciolowska *et al*., 2017; Horie *et al*., 2017; Maharajan *et al.*, 2018; Horie *et al*., 2023), amphibians (Lajmanovich *et al*., 2019), mammals (Shahid and Saher, 2020), and chicken embryos (Luckmann *et al*., 2021).

Brain-derived neurotrophic factor (BDNF) is a powerful activator of neuronal growth and survival of neurons (Rauskolb *et al*., 2010). Moreover, BDNF regulates subventricular zone (SVZ) neurogenesis, and treatment increases the long-term survival of rat *in vitro* neuroblasts ( Kirschenbaum and Goldman, 1995; Bartkowska *et al.*, 2010). Also, disruption of its signaling during embryonic development resulted in morphological disorders with cortical expansion and narrowing of the SVZ (Linnarsson *et al.*, 2000; Tiunova *et al*., 2018). In addition, Linnarsson *et al.* revealed that BDNF null mice take at least two weeks after birth to show alterations in SVZ neurogenesis (Linnarsson *et al*., 2000).

Early exposure to pesticides and insecticides disrupted gene regulatory signals, causing craniofacial dysmorphism in domestic chicks (Sharma *et al*., 2019). Homozygous Sox10 mutants caused embryonic lethality, neural crest defects, and cell-autonomous defects(C Paratore *et al*., 2001; Potterf *et al*., 2001). Additionally, normal protein homeostasis and stress responses occur through the gene expression of heat shock proteins (Hsps), which are expressed intermittently in all tissues and serve as chaperones to refold proteins that are folded incorrectly. However, the expression of these genes was noticeably elevated under specific physiological conditions (Das *et al.*, 2015).

The present study proposed that the loss of trophic support caused cells to experience stress because it is crucial for promoting neuronal survival. The survival of a neuron may then depend on its capacity to initiate such a reaction. Therefore, the present study was undertaken to investigate the effect of PPF on the embryonic development of domestic chickens in EDs 7 and 14. This study was designed to investigate the neural defects in the pallium and SVZ through the immunohistochemical expression of BDNF and gene expression of Tuj1, Sox10, Hsps 70, and 90 in the olfactory bulb.

## MATERIALS AND METHODS

### 1-Chemicals and Reagents:

Experiments were performed using a commercially available pyriproxyfen-containing formula traded as Proximo10% EC (comprised of 10% w/v active ingredient, pyriproxyfen), which is marketed by Agrimatco Company and manufactured by Afraza Company, Spain.

### 2-Fertile Eggs and Experimental Groups:

Freshly fertilized chicken eggs (average weight of approximately 66 g) were kindly provided by the Ismailia-Miser Poultry Company, Egypt. The eggs were weighed, cleaned with a cotton pad moistened with 70% ethanol solution to eliminate any external contamination, and incubated at 37°C and 55%-60% relative humidity. The eggs were divided into five groups (40 eggs per group) and marked according to their groups. Group CN was the (-ve) control group, Group CP was the vehicle group that was injected with corn oil (+ve) control group, and groups G1, G2, and G3 were PPF-injected according to selected dose concentrations.

### 3-Experimental Design and Pyriproxyfen Exposure:

First, the viability and embryotoxicity of PPF were assessed by a preliminary dose range study using a series of various dilutions of the insecticide as follows (10, 20, 30, 40, 50, 60 ug PPF/egg). Dilutions were prepared using corn oil. Based on the survival rate of

the embryos, the lethal median dose $LD_{50}$ and the three doses (15, 30, and 45 ug/egg) were calculated and determined to be less than the $LD_{50}$.

PPF was injected into the air space of the eggs after 24 h of incubation using an insulin syringe. Immediately after the injection, the hole was sealed with melted paraffin wax, and the eggs were re-incubated until EDs 7 and 14 were reached. Twenty eggs were opened for each embryonic stage in each group. Embryos were anaesthetized by cooling for 15 min at 4◦C, removed from their extra-embryonic membranes, washed with saline solution, and decapitated according to the AVMA guidelines (AVMA, 2020; Leland *et al.*, 2019).

**4-Histological Study of The Forebrain During EDs 7 and 14:**

Embryos were carefully examined for head and brain abnormalities in EDs 7 and 14. Bouin's fixative was used for the excised brains, which were embedded in paraffin wax. Coronal serial slices were cut, mounted, deparaffinized, and dipped in alcohol series to prepare for staining with hematoxylin-eosin, and toluidine blue staining according to the protocol of Sridharan and Shankar (2012). The sections were dehydrated in an ascending ethanol series, cleared in xylene, and mounted on slides using the DPX mounting medium.

**5-Brain-Derived-Neurotrophic Factor (**BDNF**) Immunohistochemistry Staining:**

The mounted coronal sections were treated for 30 min with 10% goat serum in phosphate-buffered saline with Tween 20 (PBST) and 0.3% $H_2O_2$ in Phosphate-buffered saline (PBS, PH 7.4). The sections were then incubated overnight at 4 °C with the primary antibody anti-BDNF (BDNF-9/2-5 ug/ml, DSHB, The University of Iowa, USA). At room temperature, the sections were incubated for two hours with biotinylated goat anti-mouse IgG [1:200] and streptavidin-peroxidase complex [1:200] secondary antibodies. The sections were treated with normal serum from the species from which the secondary antibodies were collected for an hour at room temperature before incubation with the secondary antibody mixtures. Various sections were incubated alternately with primary antiserum, normal serum, or antiserum against several antigens in the immunohistochemical control tests with positive results. No signs of lingering immunostaining were observed.

**6-Quantitative Real-time (qPCR) Estimation in Embryo Olfactory Bulb Homogenate:**

Reverse transcriptase was used to extract RNA from the olfactory bulb tissue homogenate, and quantitative real-time PCR was used to generate cDNA (RNA kits-Thermo Scientific, USA, CAT # 12183018A). At 260 nm, the concentration of isolated total RNA was measured using a spectrophotometer with 1 g of total RNA and a high-capacity reverse transcription cDNA kit for synthesis of cDNA (Thermo Fisher Scientific, USA, CAT #K4374966). Applied Biosystem version 3.1 software was used for real-time qPCR amplification with SYBR Green I (Step One™, USA).

The qPCR assay using the specific primer sets for Tuj1, Sox10, Hsp70, and Hsp90 (Table 1) according to Jevans *et al.* (2018), control housekeeping gene β-actin gene expression, and water samples were used to verify that the reaction mixture was free of DNA contamination and was amplified in triplicate. The PCR data sheet contained the Ct values of the genes assessed for expression and the housekeeping gene β-actin. The correlation between the β-actin gene and each target gene expression was compared and calculated according to the following equation:

$\Delta Ct = Ct_{\text{assessed gene}} - Ct_{\text{reference gene}}$. $\Delta\Delta Ct = \Delta Ct_{\text{sample}} - Ct_{\text{internal control gene}}$. $RQ = 2^{-[\Delta\Delta Ct]}$.

**Table 1:** Primers sequence of studied genes:

| Genes | Forward Primer Sequences | Reverse Primer Sequences |
|---|---|---|
| **TuJ1** | 5'GCCCCGACAACTTCATTTT3' | 5'GCAGTCGCAGTTCTCACACT3' |
| **Sox10** | 5'-GCCTTCACAGGGTTTGCT-3' | 5'GAGAGGCAGTGGTGGTCTTC3' |
| **HSP70** | 5'ATTCTTGCGTGGGTGTCTTC3' | 5'-GATGGTGTTGGTGGGGTTC-3' |
| **HSP90** | 5'TGAAACACTGAGGCAGAAGG3' | 5'AAAGCCAGAGGACAGGAGAG3' |
| **β-Actin** | 5'-TCACCAACTGGGACGACA-3' | 5'-GCATACAGGGACAGCACA-3' |

**7-Image Analysis Quantification and Statistical Analysis:**

Brain-derived neurotrophic factor protein expression by immunohistochemistry in the pallium ventricular zone area and the variation differences in the quantitative gene expression of Tuj1, Sox10, Hsps70, and 90 were examined and compared during both EDs 7 and 14 using Image J software and statistically analyzed using Student's t-test and one-way ANOVA with Prism 7 software (GraphPad, San Diego, CA, United States). The distribution of each protein in the pallium area of the brain regions of the two EDs is shown as the standard error (SE) of the mean for all data. Fisher's exact test was used to determine the significance of death and malformation rates among the various groups.

## RESULTS

**1-Mortality Rate and Malformations Of Embryos**:

Pyriproxyfen induced variable malformations and mortality rates among the injected groups. The malformation was in the dorsal head length, microcephaly, and exencephaly (Fig. 1A, B), and mortality percentages, as shown in Figure 1C. PPF induced significant ($P<0.0001$) mortality differences among the different groups and the CN group on EDs 7 and 14. On embryonic day 7, embryos showed significant differences between G1 and G2 and between G1 and G3 ($P<0.01$ and $P<0.001$, respectively). Moreover, there were no significant differences in mortality rates between G2 and G3 (Fig. 1C). Comparing the mortality rates of the treated groups, ED 14 displayed no significant differences between G1 and G2 and G1 G3, and a significant ($P<0.05$) difference between G2 and G3 (Fig. 1C).

On one side, on ED 7 the highest rate of mortality was recorded in G3 representing 46.87% followed by G2 showed 43.75% and the lowest rate of mortality was in G1 which displayed 34.37% (Fig. 1C I). On the other hand, ED 14 embryos showed higher levels of mortality than those of ED 7, with significant values in descending order as follows: G3, G1, and G2 with 62.50%, 53.12%, and 43.75%, respectively (Fig. 1C II). In ED7, the different doses of PPF injected into the chicken eggs did not reveal significant changes in the morphometric analysis of the skull in the anterior-posterior dorsal head length, however, in ED14, there was a significant reduction($P<0.0001$) in the anterior-posterior dorsal head length in all PPF- injected group (Fig. 1B).

In addition, PPF induced different types of malformations in the surviving embryos in both EDs. During EDs 7 and 14, hematoma formation with damaged blood vessels was the most significant ($P<0.0001$, *versus control*) apparent malformation. In ED 7, these malformations displayed complete occurrences in G3 (100%) and slightly decreased occurrences in G2 and G1 (94.44% and 84.21%, respectively). Additionally, all PPF-injected 14-day-old embryos in both G3 and G2 showed 100% hematoma formation, and in G1 represented 86.67%. Furthermore, in ED 7 PPF-injected eggs, microcephaly was the second most common disorder in the head region, represented by 44.4%, 43.75%, and 26.32% for G2, G3, and G1 embryos, respectively. On the other hand, on ED 14, all individual embryos of G3 displayed microcephaly at 100%, followed by G2 and G1 at 88.89% and 73.33%, respectively.

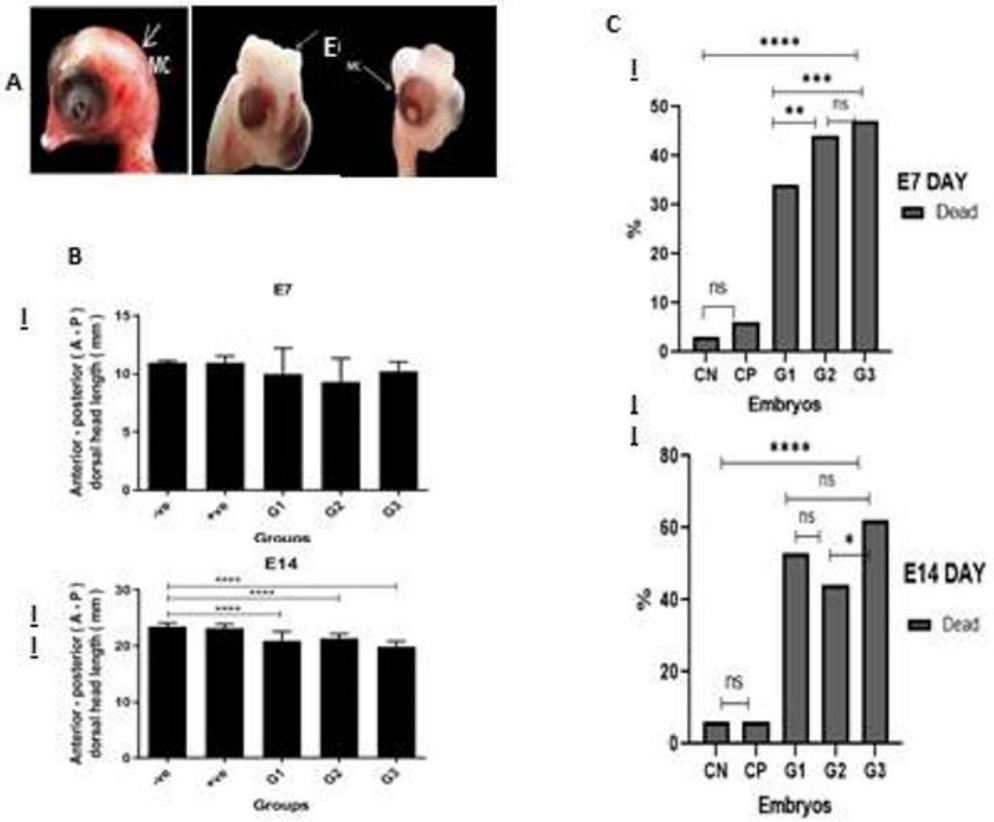


**Fig. 1:** The effect of the PPF on chick embryo heads morphology, brain wet weight, and mortality rate.
A: Brain malformations, MC: microcephaly and EC, exencephaly; B, dorsal head length during ED 7 [I] and ED 14 [II]; C: Mortality rate percent during ED 7 [I] and ED 14 [II].

**2-Histological Analysis and Immunohistochemistry of BDNF**:

Histological examination of the lateral ventricles of chick embryos of EDs 7 and 14 was carried out independently by two different investigators. Examination of the pallium area of the control brain on ED 7 revealed a normal distribution of cellular structures with normal size in both control groups, and normal ependymal cells were distributed in the lining layer of the lateral ventricles. Additionally, the negative control (CN) group showed a small number of degenerated cells (Fig. 2D). The medial pallium area (Mpall) and developed embryonic striatum were shown to have typical neural cells with compact morphology when stained with toluidine blue.

In G1 injected with the lowest dose (15ug PPF/egg), there were multiple distributed degenerated cells in the compacted area lining the ventricles and in the dorsal pallium at the apical part of the lateral ventricles, and apparent degenerated ependymal cells (Fig. 2 G1/A). Moreover, it revealed distributed glial cells with multinucleated cells in the dorsal pallium area (DP). In the G2 group (30ug PPF/egg), there were distributed hyperchromatic cells, cellular atrophy, shrinkage with cellular necrosis, and neuronal swelling with chromatolysis (Figs. 2 G2/C and D). On the other hand, a distributed multinucleated cell near the ventricles may be an inflammatory response consisting of aggregations of macrophages, lymphocytes, and neutrophils (Fig. 2 G2/C). The G3 (45ug PPF/egg) injected group revealed highly destructive forms in the lining area of ependymal cells with uncharacterized features (Fig. 2 G3/B). The most degenerative forms were detected in the SVZ regions immediately surrounding the lateral ventricle of the parenchyma adjacent to the lateral ventricle (Fig. 2 G3/ A, B). Additionally, degenerated cells were distributed in the medial pallium, striatum, and cortical neuronal cells (Fig. 2 G3/ C-F).

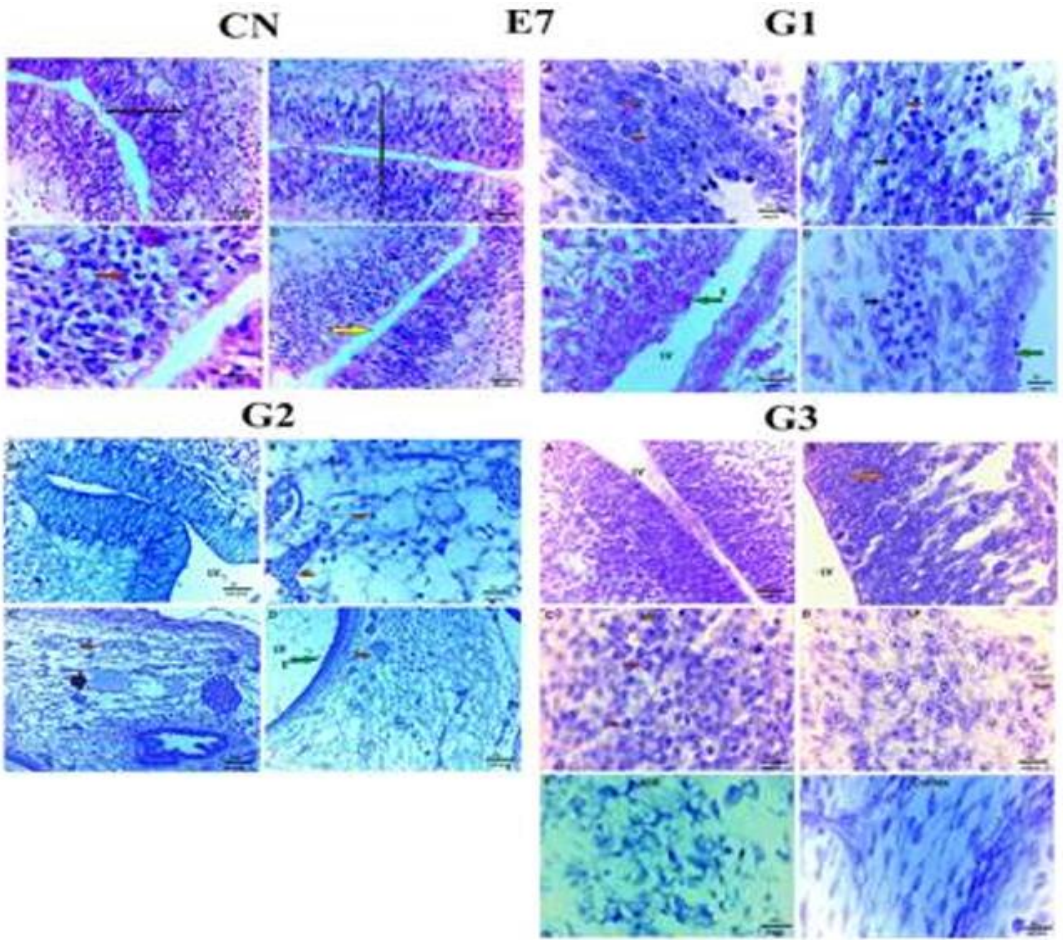


**Fig. 2: P**hotomicrographs of 7-day-old embryos of the brain pallial-ventricular area. CN, negative control; G1,15 µg PPF/egg; G2,30 µg PPF/egg; G3,45 µg PPF/egg. G1: Dorsal pallium (red arrows) chromatolytic cells, B [black arrow] glial cells, C [green arrow] degenerated ependymal cells, D (black arrow)] inflammatory aggregations. G2: A dorsal pallium [DP], B [red arrow] chromatolysis and inflammasomes, C (dark blue arrow) swollen neurons, and D (red arrow) inflammatory aggregates. G3: Degenerated dorsal pallium cells with narrow ventricles; B, C medial pallium MP; D, lateral pallium LP; E, striatum STR; and F cortex (red arrows) indicate severely degenerated cells and distributed inflammatory aggregates.

Microscopic examination of ED 14 revealed multiple and variable forms of degenerations, G1 revealed degenerated cells in the dorsal pallium in the form of chromatolysis (Fig. 3 G1/A and B) and necrotic neurons undergoing phagocytosis in the ventral pallium (Fig. 3 G1/C). Within G2, the ventral pallium was the most affected area in the embryo brain, with multiple degenerated cells in the form of necrotic cells (Figs. 3 G2/B and C). Multiple degenerated ependymal cells (Fig. 3 G2/D), the medial pallium, and the lateral pallium showed vacuolated and Wallerian degeneration (Fig. 3 G3/E and F).

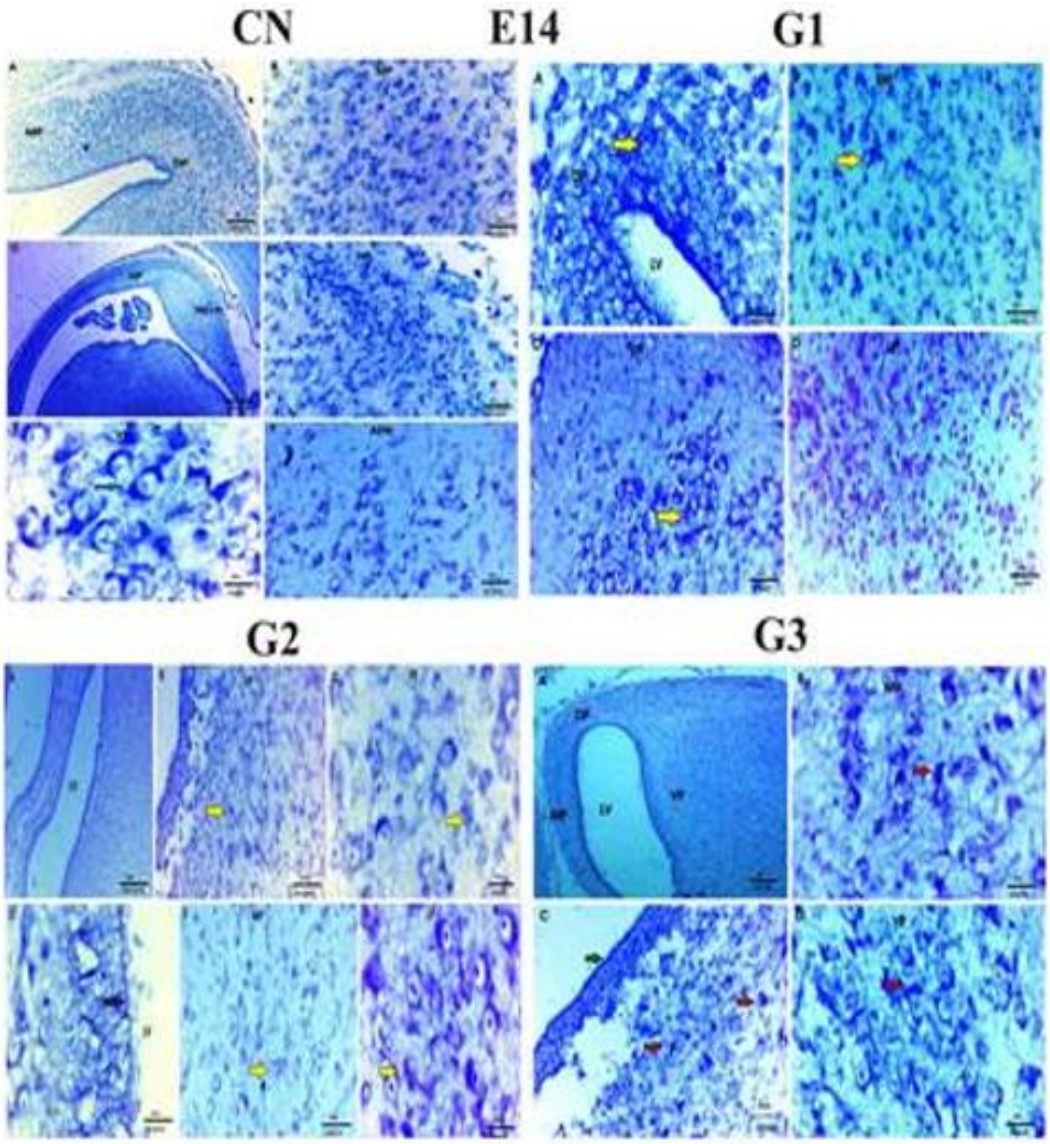


**Fig. 3:** Photomicrographs of 14-day-old embryos of the brain pallial ventricular area. **CN:** normal distributed cells in MP, DP, hippocampus [Hp], hippocampus media [Hcm], parahippocampal [APH]; G1: A/DP, B/MP, C/VP, D/LP yellow arrows in A, B, and C vacuolated cells with chromatolysis; G2: B/VP [yellow arrows degenerate Wallerian cells in axon ends]; G3: B/MP and D/VP [red arrow] are swollen degenerated cells and Wallerian neuron end injury; C [green arrow] degenerated ependymal cells.

Immunostaining for BDNF revealed a specific distribution throughout the pallium area. Following Kuenzel and Masson (1988), BDNF nomenclature used throughout this description was visible as a diffuse scattered reaction product that was dispersed throughout the cytoplasm of perikaryal cells and their closest processes without nuclear occupation (Fig. 4). The quantitative assessment of staining was performed based on low- and high-magnification examinations of the tissue in the pallium and ventricular zones. Additionally, there were no distinguished diffused punctate fibers, and cells with a neuronal appearance were the only cells on which BDNF immunostaining was observed. The number of BDNF-expressing cells increased among ED 14 individual embryos injected with PPF. BDNF expression was not significantly different between the -ve (CN) and oil-injected (CP) +ve control groups. The PPF-injected groups with the three different doses G1, G2, and G3 revealed BDNF expression to be concentrated in the SVZ and parenchymal area along the rostro-caudal area of the forebrain. Moreover, densitometry analysis of BDNF expression

in the brain pallial-ventricular zone of individual embryos from eggs injected with PPF doses revealed that the G2 group exhibited the lowest expression protein on both EDs. Embryonic day 7 of G2 expression was lower than that of G1 and G3 groups, which showed significant (P<0.01 and P<0.05, respectively) expression (Fig. 4B).

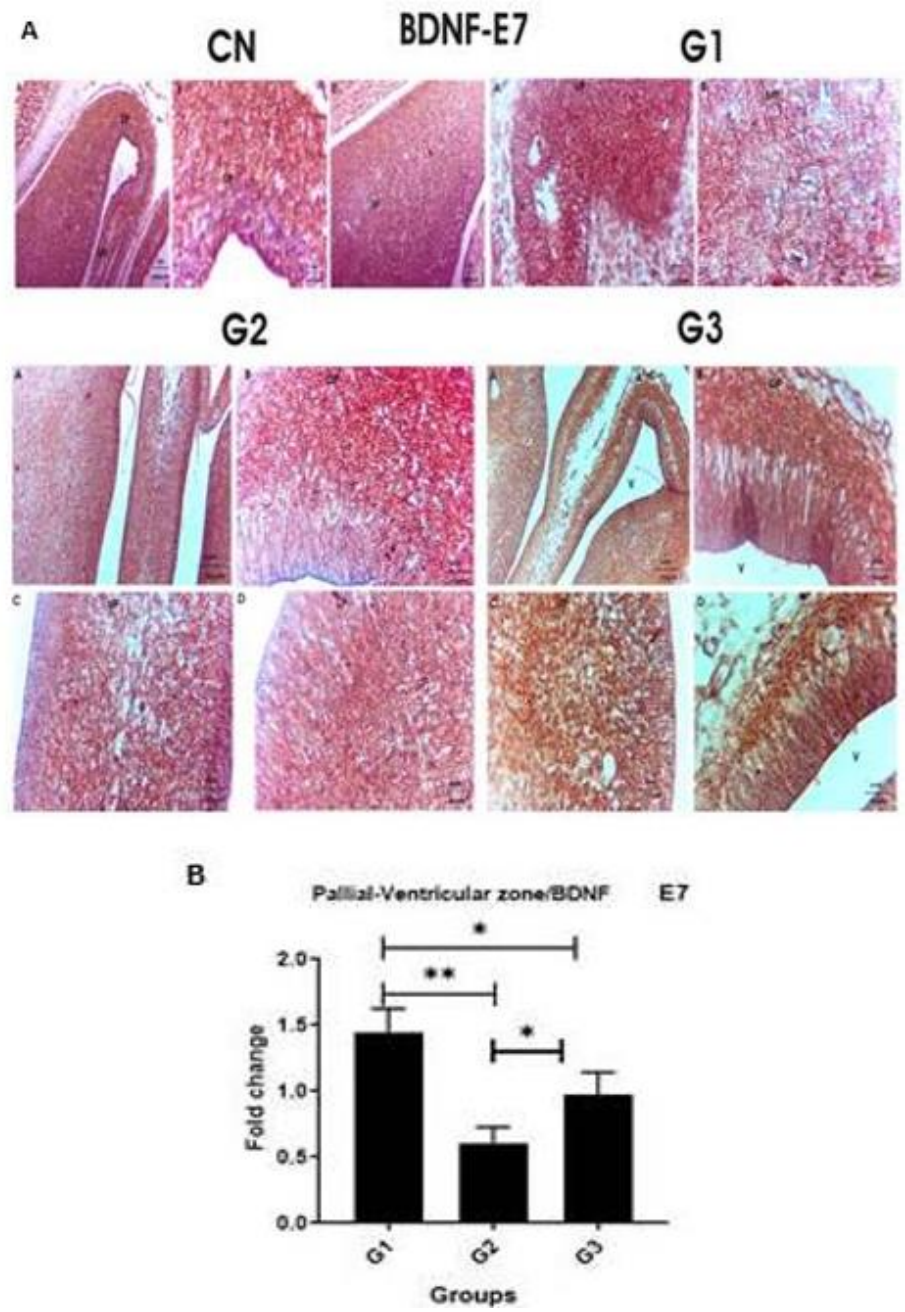


**Fig. 4:** Photomicrographs of brain-derived neurotrophic factor immunohistochemistry expression in the pallial-ventricular zone of 7- day-old chick embryos. A: BDNF images; B: Densitometry expression analysis of BDNF expression among the PPF injected groups. **p*<0.05, ***p*<0.01, ****p*<0.001.

Additionally, BDNF expression among embryos on ED 14 showed that G2 was less significant than G1, which showed a highly significant (p<0.0001) expression and no significant difference compared to G3 (Fig. 5B). The protein expression differences among the different PPF groups revealed a consistent decrease in ED 7 compared to ED 14 in all groups with P<0.05 and P<0.01, respectively (Fig. 5C).

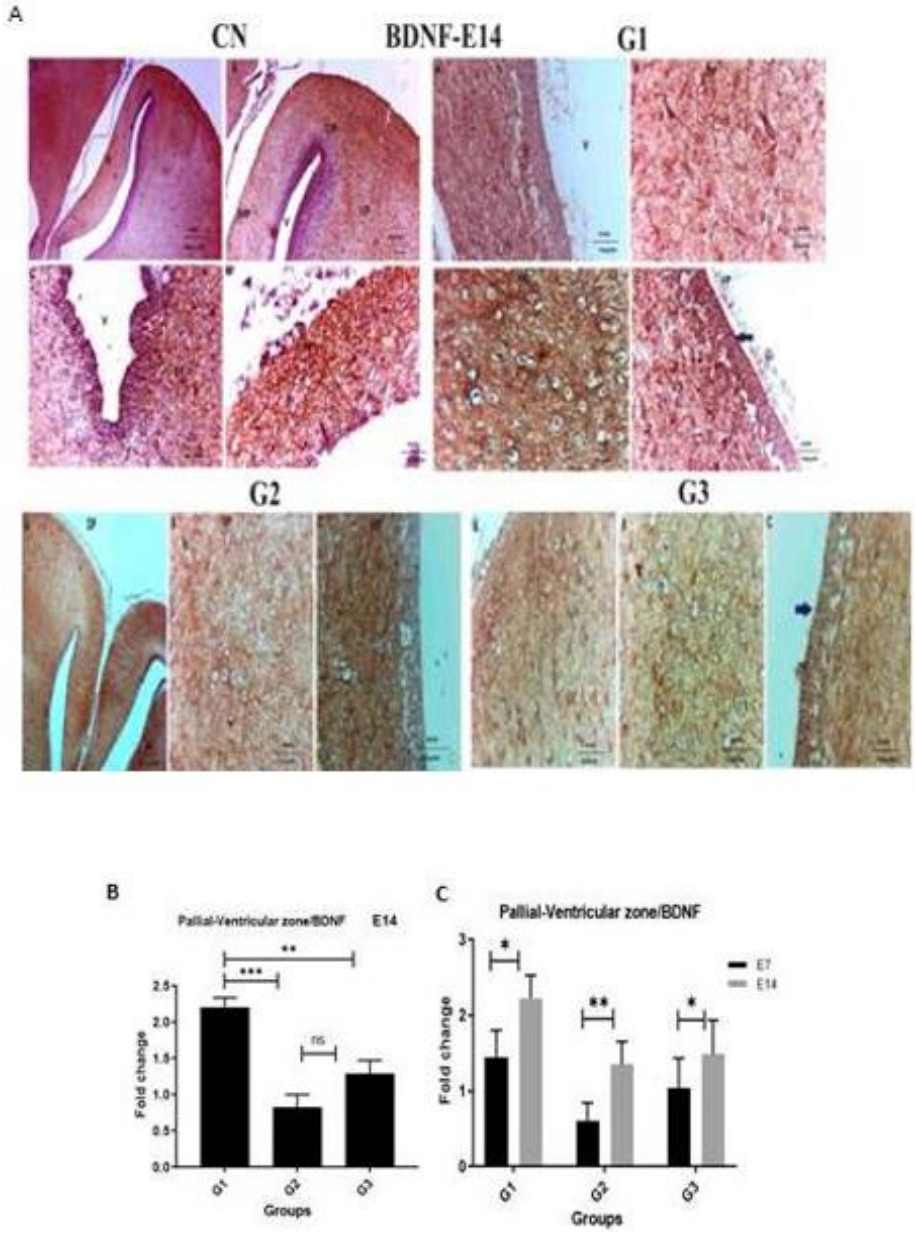


**Fig. 5:** Photomicrographs of brain-derived neurotrophic factor immunohistochemistry expression in the pallial-ventricular zone of 14-day-old14-day-old chick embryos. A: BDNF images; B: Densitometry expression analysis of BDNF expression among the PPF injected groups; C: EDs 7 and 14 expression differences. *$p$<0.05, **$p$<0.01, ***$p$<0.001.

**3-Quantitative Expression of Genes In The Olfactory Bulb of EDs 7 and 14 of the Developing Chick:**

The estimation of Tuj1, Sox10, Hsps70, and 90 gene expression was used to evaluate the damaging effects of PPF on the developmental activity of the embryo's brain on ED 7 and ED 14.

First, the expression on ED 7 of olfactory bulb class III beta-tubulin (Tuj1) revealed a significant decrease among the PPF-injected groups compared to the negative and positive controls (CN and CP). G1 (15 ug), G2 (30 ug), and G3 (45 ug) were highly significant (P<0.0001, 0.001, and 0.05 Versus control, respectively). Moreover, no significant effects of CN and CP were observed. Additionally, it was evident that the most affected embryos were in G2 **(**Fig. 6A).

Second, Sox10 gene expression is considered an important gene for neural progenitor cells, such as neural crest derivatives, and their defects in Schwann cells and melanocytes, migration, differentiation, and multipotency. The Sox10 expression activity among ED 7 embryos was significantly (P<0.01, p<0.001, *versus control*) decreased for G1, for both G2 and G3. However, no significant differences were observed between the PPF-injected groups **(**Fig. 6B).

Third, to evaluate the effect of PPF on homeostatic temperature level disturbances between the olfactory bulb of 7- and 14-day-old embryos, we investigated the expression levels of both Hsps70 and 90. Hsp70 gene expression revealed a significant (P<0.01, *versus -ve control*) decrease in G1 embryos and P<0.0001 for both G2 and G3. The most affected embryos that showed a significant decrease in their gene expression levels were G2 embryos (Fig. 6C). Hsp90 expression was significantly (P<0.001) decreased in G1 and (P<0.0001) in G2 and G3, with a significant (P<0.05) difference compared to G1 embryos (Fig. 6D).

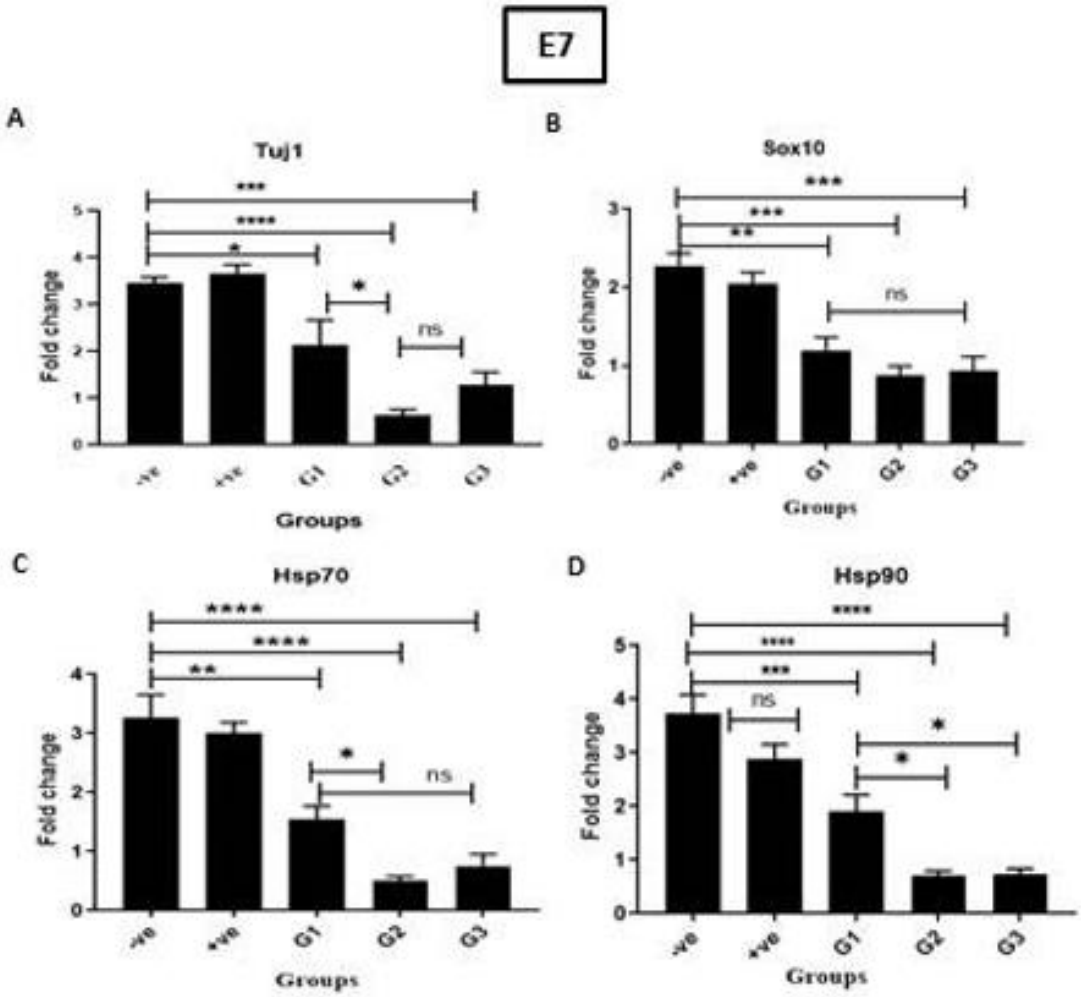


**Fig. 6:** The statistical differences of fold changes of gene expression of 7-day-old chick embryo's olfactory bulb. A: Tuj1, B: Sox10, C: Hsp 70, D: Hsp, 90. *p<0.05, **p<0.01, ***p<0.001.

Moreover, damaging embryological effects of the PPF-injected eggs were confirmed on ED14. The most significant decrease in Tuj1 expression was observed in G2. The decrease in the gene expression of Tuj1 in G1 (P<0.05), G2, and G3 (P<0.001) *versus -ve control* **(**Fig. 7A). Sox10 gene expression decreased in the PPF-treated 14-day-old embryos, significantly with low levels (P<0.01, *versus -ve control*)] for G1 and G2 and high levels (P<0.001, *versus -ve control*) for G3, which was the lowest gene expression level

among groups (Fig. 7B). Additionally, with more interest in Hsp70 gene expression in the olfactory bulb, G3 showed the lowest expression level (P<0.01, *versus -ve control*), while G1 and G2 showed non-significant differences. Furthermore, G3 was significantly (P<0.01 and P<0.05, *versus -ve control*) lower than G1 and G2, respectively (Fig. 7C). HSP90 gene expression among G1 embryos was significantly (P<0.01, *versus -ve control*), while G2 and G3 exhibited significantly higher levels (P<0.0001, *versus -ve control*) (Fig. 7D).

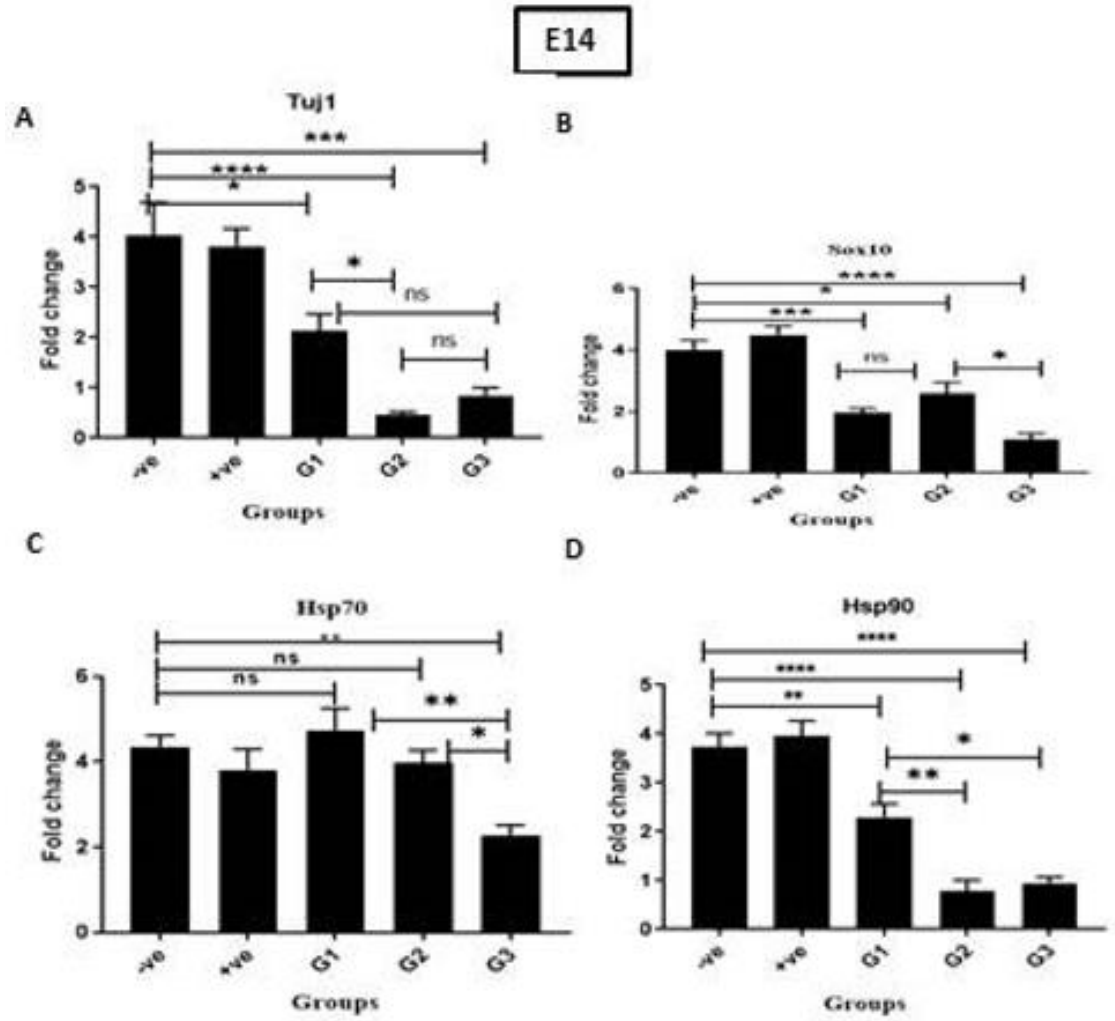


**Fig. 7:** The statistical differences of fold changes of gene expression of 14-day-old chick embryo's olfactory bulb**.** A: Tuj1, Sox10, Hsp 70, Hsp, 90. **p*<0.05, ***p*<0.01, ****p*<0.001.

Moreover, we assessed the expression levels of genes in the PPF-injected groups (G1, G2, and G3) to evaluate which genes were most affected in the olfactory bulb. The study revealed that G1 and G2 did not reveal significant differences in the expression level of Tuj1 between ED7 and ED14, whereas G3 showed a slight significant (P<0.05) decrease between the two embryonic stages (Fig. 8A). Sox10 gene expression in the G1 group revealed a significant (P<0.05) difference with up-regulation among ED14 and (P<0.0001) for G2, but no significance for G3 (Fig. 8B). For Hsp70, there were significant (P<0.0001) differences among the three groups G1, G2, and G3 (P<0.01 on EDs 7 and 14, with continuous up-regulation on ED 14 (Fig. 8C). Hsp90 did not reveal any significant differences among the groups during either embryonic day (Fig. 8D).

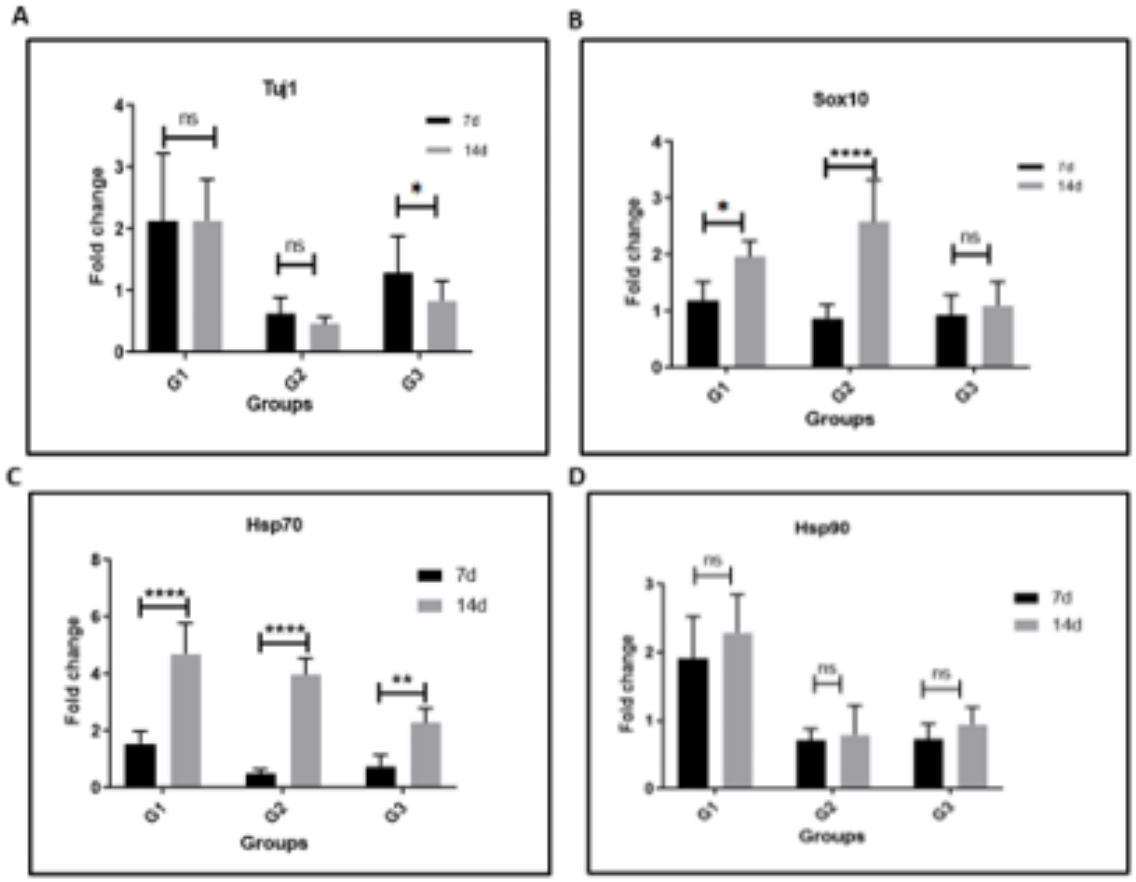


**Fig. 8:** The statistical differences of fold changes of gene expression among PPF injected groups during ED7 and ED14 of chick embryo's olfactory bulb. A: Tuj1, Sox10, Hsp 70, Hsp, 90. *$p$<0.05, **$p$<0.01, ***$p$<0.001.

## DISCUSSION

Pyriproxyfen has been found to be a partial endocrine stressor, posing nutritional risks and teratogenic effects, and being hazardous to aquatic life with the capacity to boost viral mass production (Linton *et al*., 2009; Bayoumi *et al.*, 2003). Concerns have also been expressed regarding a possible link between PPF and human microcephaly (Parens *et al.*, 2017). PPF interacts with retinoic acid during CNS development and induces physiological disorders (Kuadkitkan et al., 2020; Shahid and Saher, 2020) and microcephaly (Brookes *et al.*, 2023). PPF induced multiple disorders among chick embryos with different degenerative forms, such as chromatolysis and necrosis in the neuronal cells in the pallium area and near the lining layer of ventricles of the ependymal cells, with a significant effect on BDNF expression. The current study is regarded as the first to assess the impact of three different PPF doses and the manner in which the brain's regeneration mechanism responded to each dose. The BDNF gene encodes neurotrophins that are crucial for neuronal survival, differentiation, and long-term potentiation in adulthood (Park and Poo, 2013). Li and Pozzo-Miller (2014) and Liu *et al.* (2015) reported that neurodevelopmental diseases, such as Rett syndrome and ADD/ADHD, have been linked to decreased BDNF expression. In contrast, environmental enrichment exercise has been shown to have neuroprotective benefits via increased BDNF expression (Dandi *et al*., 2018). Therefore, the present study revealed that the mid-dose of PPF was the most effective among the two embryological stages studied (days 7 and 14). This may indicate the ability of chick embryos on ED 14 to restore their activity to compensate for the shortage and start to rebuild regeneration mechanisms more

than the affected damaging area among younger embryos on ED 7.

Additionally, BDNF expression was dedicated to the area of the dorsal pallium and parenchymal lining cells of the ventricles, along with the ependymal cells. Park *et al*. (2014) reported that BDNF is conditionally depleted postnatally, affects the plasticity of striatal neurons, causes severe neurodegenerative changes (Vondran *et al*., 2010), and its upregulation may regulate myelin development (Audouze *et al*., 2018; Xiao *et al.*, 2010). Moreover, its consistent expression in certain layers and pathways facilitates motor learning throughout development and is maintained in adulthood. Andreska *et al*. (2020) stated that in human embryos, BDNF protein levels in the anterior and motor cortices reach their highest expression around postnatal week 21, and thereafter decline throughout adulthood.

The PPF side impact appears because the receptors of these substances are identical to one another and bind to the retinoic acid receptor, influencing the development of vertebrate embryos as well as insect embryos (Rhinn and Dollé, 2012). Retinoic acid activity at retinoic acid receptors may be inappropriately activated or inhibited by PPF, which could disrupt gene expression cascades and cause congenital defects (Sonnenberg-Riethmacher *et al*., 2001; Parens *et al.*, 2017).

Additionally, regarding the passive effects and resulting disorders due to PPF injection, we investigated some neurodegenerative factors in the olfactory bulbs. The defects and the decrease in expression among embryos of ED 7 were more severe than those of ED 14, which may indicate some brain anatomical disorders, such as microcephaly and exencephaly. Homozygous mutants of Sox10 trigger dominant defects in Schwann cell and melanocyte development accompanied by a lack of migration and differentiation of neural crest derivatives that lead to embryonic mortality (Morshead and Kooy, 1992; Potterf *et al.*, 2001). The increased BDNF expression on ED 14 may reflect its role during the developmental stages against conditional cellular stress to confer PPF toxicity. Moreover, BDNF stimulates cell survival and differentiation. BDNF can prevent the death of freshly generated cells during adult forebrain development (Chiasson *et al*., 1999; Suzuki and Goldman, 2003). This study shows the importance of BDNF in the early development of the forebrain by showing how it encourages cell growth in the lateral ventricles and pallial-ventricular regions and is widely expressed in the ventricular zone.

Additionally, *in vitro* studies have shown that BDNF can support the survival of SVZ cells in both young and old rats ( Kishimoto *et al.*, 2012; Cacialli *et al.*, 2018). In addition, they explained that the enhanced BDNF expression in zebrafish brains was due to mature neurons that triggered BDNF translation after the lesion, rather than freshly formed neurons. The migration of neuronal precursor cells from the ventricular zone to the damage site was too short, causing a maximal increase in BDNF mRNA-expressing neurons shortly after the lesion (Cheung and Briscoe, 2003).

The gene is necessary for glial differentiation, according to studies on neural crest cells produced from *Sox10*$^{-/-}$ mouse embryos ( Paratore *et al*., 2001). Cheung and Briscoe (2003) and Cheung *et al*. (2005) stated that mice without Sox9 had severe malformations in the cranial and cardiac neural crests (NC), while mice lacking Sox10 exhibited problems in numerous lineages of the trunk NC, demonstrating the crucial roles that these two genes play in NC development (Kelsh, 2006). Moreover, the increased mortality and malformations may be due to the sox10 decreased gene expression, which is crucial for vertebrate development in the NC (Stolt *et al*., 2002; Kim *et al*., 2003). In addition, Stolt *et al.* proved that Sox10 plays a crucial role in NC stem cell survival, pluripotency preservation, melanocyte growth, and peripheral glial development, ultimately influencing oligodendrocyte differentiation in the central nervous system (Gabai *et al*., 1997).

However, the drawbacks and resulting disorders following PPF injections are their ability to deregulate the temperature homeostatic mechanism during developmental stages and their potential value in preventing organ failure by fixing misfolded proteins and

boosting cellular survival (Pirkkala *et al*., 2001). The present study revealed that the 7-day-old embryos were more affected in their expression of heat shock proteins Hsps70 and 90 more than those in ED 14 embryos, suggesting that the ability of larger embryos (ED 14) to resist various stressors may induce higher mortality and malformation rates. Increased synthesis of Hsps is the result of heat-shock-induced factors binding to the promoters of $hsp_s$ (Hasan Siddiqui *et al.*, 2020). Hasan-Siddiqui *et al*. reported that acute heat exposure alters Hsps70, 60, and 47 protein and mRNA expression in the duodenum, jejunum, and ileum of chicken. The small intestine increases Hsp protein and gene expression but subsides once heat tolerance develops. The hen liver and small intestine are structurally harmed (Loones *et al*., 2000).

In addition, the deregulated heat shock proteins Hsp90 and Hsp70 expression levels on ED 7 and ED 14 reflected the highly toxic effect of PPF as they are associated with the cell cycle, with Hsp90 levels being highest between G0-G1 and Hsp70 levels being highest between G2-M. As these proteins are differentially expressed between control and heat-stressed olfactory bulb cells, we can infer that they play unique functions in this developmental process. Subsequent studies have expanded on this work by investigating the regulation of HSPs in later embryonic stages and their role in protecting the misfolding of specific proteins for cellular protection (Miller and Fort, 2018).

**Conclusions**

This study demonstrated that pyriproxyfen significantly influenced developmental disorders in domestic chick embryos between embryonic days 7 and 14. There was a marked decrease in BDNF expression in the pallium, SVZ, and ependymal cells as well as in the expression of Tuj1, Sox10, and Hsps 70 and 90 in the olfactory bulb. Deteriorated cells were observed in the compacted area lining the ventricles and the dorsal pallium at the apical section of the lateral ventricles, with some ependymal cells in G1 at the lowest dose [15 µg PPF/egg] also showing degeneration. BDNF immune staining was confined to cells with neuronal morphology, showing reduced expression in embryos at ED 7 compared to those at ED 14. The gene expression disorders of Tuj1, Sox10, and hsp70 in the olfactory bulb indicated susceptibility to PPF, with younger embryos more affected due to PPF's impact of PPF on neural regenerated cells and high-stress conditions. Therefore, the future necessitates the re-evaluation of PPF use, especially in combination with other pesticides, with regulatory bodies enforcing stricter guidelines and thorough safety assessments. Implementing alternatives with varied mechanisms in integrated pest management can reduce reliance on a single insecticide and curb resistance. Researchers are exploring nanoparticles for precise pesticide delivery to target specific plant parts, minimize usage, and reduce environmental impacts. Governments and regulatory bodies should promote sustainable pesticide use, set reduction targets, and endorse safer alternatives.

**Declarations:**

**Ethical Approval:** All embryos were treated according to the regulations and ethical approval committee (no/281122) of Suez University, Egypt.

**Competing interests:** The authors have declared that no competing interests exist.

**Authors Contributions.** The authors have no conflicts of interest to disclose or confirm their approval for submission. Prof. Mobarak and Prof. Hani S Hafez designed the experimental hypothesis and were responsible for the analysis of the brain histology and molecular data gene expression in addition to the ideas, formulation, or evolution of overarching research goals and aims. Verification, whether as a part of the activity or separate, of the overall replication/reproducibility of results/experiments and other research outputs was the responsibility of all the authors. All authors were responsible for management activities to annotate (produce metadata), scrub data, and maintain research data (including software code, where it is necessary to interpret the data) for initial use and later reuse. The methods of execution, data collection, and dissection were performed by

Sedeek. The manuscript review was performed by Prof. Hani S Hafez and was revised by Prof. Yomn M. Mobarak, Prof. Nour E. Sherif, and Prof. Mahmoud E. Mohallal.
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## ARABIC SUMMARY

**السمية العصبية والجينية المستحثة بالبيربروكسيفين بين الأجنة النامية للكتكوت (*جالاس دومستيكا*)**

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هدفت الدراسة الحالية إلى تقييم تأثيرات المبيد اليرقي بيربروكسيفين على النمو العصبي لجنين الكتكوت في اليومين 7 و14 من النمو الجنيني. وتم تقسيم البيض المخصب إلى ثلاث مجموعات (مج1 ومج 2 ومج 3) وتم حقن البيض بثلاث جرعات تحت المميتة من البيروبروكسيفين (15, 30, 45 ميكروجرام/ بيضة) وقد وجد أن في يومى النمو الجنيني (7 و 14) كان للبيربروكسيفين تأثيرات ذات دلالة إحصائية على حيوية الأجنة، وكانت أكثر التأثيرات هو ظهور التجمعات الدموية في منطقة الرأس المتزامن مع صغر حجم الرأس في كل المجموعات المعاملة بالبيربروكسيفين. أظهرت المجموعة الأولى التى تم حقنها ب 15 ميكروجراماً\بيضة انخفاض ذو دلالة إحصائية في أوزان المخ لجميع الأجنة النامية فى اليوم السابع والرابع عشر.بالاضافة الى ذلك فقد أظهرت أجنة اليوم السابع المعاملة بالجرعات المختلفة بالبيربروكسيفين تلف في المنطقة البطنية للقشرة المخية مصحوبا بظهور انحلال لمادة الكروماتين فى النواة مع تكون فجوات في الخلايا العصبية. ايضا قد أظهرت الأجنة إنخفاض في مستوى التعبير البروتينى لعامل التغذية العصبية المستمد من المخ (BDNF) . كما سجلت مستويات التعبير الجينى الخاصة بانتاج بروتينات الصدمة الحرارية (Hsps) 70 و90 ارتفاعا بين أجنة اليوم الرابع عشر عن أجنة اليوم السابع وكانت ذو دلاله إحصائيه. بالإضافة ذلك، قد كشف التعبير الجيني لجينات التيبيولين بيتاIII (Tuj1) والسوكس 10 (Sox 10) إنخفاض ذو دلالة إحصائية في أجنة اليوم السابع، وكان ذلك الانخفاض أكثر ارتفاعا بين أجنة مج 2. من ناحية أخرى، قد كان التعبير الجيني للتيبيولين بيتا III منخفضا في أجنة مج2 لليوم 14، ولكن بدا التعبير الجيني للسوكس 10 مرتفعا وذلك مصحوبا بالقدرة على التجدد . الخلاصة، وجد أن الأجنة ذات الأعمار الأصغر كانت أكثر تأثرا بالبيربروكسيفين وأظهرت اضطرابات في مخ الأجنة النامية للكتكوت والتي عزيت إلى التأثير المباشر للبيربروكسيفين على تكوين ونمو الخلايا العصبية والانخفاض في مستويات البروتينات المساعدة في عملية التجدد للأنسجة التالفة.