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RESEARCH ARTICLE

Repeated exposure of pyriproxyfen to pregnant female mice causes developmental abnormalities in prenatal pups

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Abstract

The continuous exposure to conventional pesticides leads to severe health and environmental issues especially at prenatal stage during developmental period. Herein, we aimed to investigate the anomalies due to repeated exposure of pyriproxyfen in pregnant female mice and their neonates. Twenty-four pregnant female mice were repeatedly administered with pyriproxyfen at 30, 100, 300, and 1000 mg/kg by oral gauge from gestation day (GD) 7 to gestation day 17 and six females were given distilled water in the control group. All the live pups were euthanized at postnatal day (PND) 7 and their organs (heart, liver, kidney, and brain) were dissected out, weighed, and assessed for further histopathological examinations. The results exhibited a significant ($P < 0.001$) decrease in the body weight gain of all treated pregnant mice in comparison to the controls and a significant increase in the gestational length was observed in group IV ($P < 0.01$) and group V ($P < 0.001$). In addition, no live pups were born in groups IV and V and one pregnant female mouse was also found dead in both treatments. The body weights of the pups were significantly decreased in group II ($P < 0.05$) and group III ($P < 0.001$) and the relative organ (liver, heart, and kidney) weight of the pups was increased significantly ($P < 0.001$, $P < 0.01$, $P < 0.05$) due to prenatal exposure in group II as compared to group I. The relative brain weights of the pups were decreased significantly ($P < 0.001$) in groups II and III as compared to group I. The liver, kidney, heart, and brain sections exhibited various histological alterations in groups II and III by hematoxylin and eosin staining. Furthermore, immunohistochemical staining of the coronal sections of pup's brain showed significant ($P < 0.001$) reduction in cortical radial thickness and total neural count in group II and III as compared to group I. Therefore, the prenatal exposure to pyriproxyfen provoked the damage to various organs in mice offspring and an increase in fetal death at higher doses.

Keywords Pyriproxyfen · Repeated exposure · Deformities · Histopathology · Cortical radial thickness · NeuN+ cells

Introduction

Pesticides are widely used in agriculture, animal husbandry, and public health operations to kill the fungus, weeds, and insects, mainly *Aedes aegypti* larvae. These pesticides, if not properly used, may pose serious hazardous effects to people and environmental health (Mondal et al. 2012). Therefore, the main concern is with their minimal and proper use, so that they can be applied safely with proper instructions and guidance and have least effects on animals and human health (Mondal et al. 2009).

In the first instance, pesticides were linked to cancer after 20 years of their usage (UNEP 1993). In the World Health Organization (WHO) report, about 5.2 billion pounds of pesticides are used worldwide per year and keep on rising with the passage of time (Goldoni et al. 2014). In Pakistan, the same situation was observed after 1950s, when 250 metric tons of pesticides were imported for better crop production. Its usage increased by 2158.6% from 1952 to 2004 (Khan et al. 2010) and approximately 40,000 tons of pesticides were annually utilized in field crops (Hamid et al. 2012). In Pakistan, the prevalence of pesticide poisoning may even be more than reported due to lack of data, under-reporting, and misdiagnosis (Tariq 2005).

Systemic insect growth regulators (IGRs) were marketed during the 1980–1990s and include some selective compounds in comparison to their predecessors. Since 1990

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onwards, cartap, fipronil, and neonicotinoids are substituting old harmful chemicals in most developed and developing countries (Jeschke et al. 2010). To date, many classes of insecticides are used in combination and subjected to the target species in a repeated manner.

Pyriproxyfen (4-phenoxyphenyl (RS)-2-(2pyridyloxy) propyl ether) is an IGR, widely used in public and private sectors for the control of the broad spectrum of insect pests. In recent years, the use of pyriproxyfen leads to high stability in the environment and low solubility in water that may cause substantial health effects (Mehrnoush et al. 2013). It may cause anemia, impaired liver function, and tissue lesions. Pyriproxyfen is very toxic to aquatic life and may leave long-lasting effects on water-based plants and animals (Franc et al. 2012).

Pyriproxyfen is a juvenile hormone analog that can bind to mammalian retinoic acid (a metabolite of vitamin A) receptor (Palli et al. 1991; Harmon et al. 1995) in two possible ways, either acts as a blocker that prevents the normal retinoic acid from binding to the receptor when needed or activates the receptor at inappropriate times in development. During the developmental period, the retinoic acid receptor normally turns on gene expression, so either an inhibition or inappropriate activation at a sensitive period could be expected to lead to developmental abnormalities (Parens et al. 2017).

In early 2014, pyriproxyfen was widely used in high quantity than recommended levels in Brazilian drinking water to control the mosquito population (Truong et al. 2016). In a report from an Argentinian organization known as "Physicians in Crop-Sprayed Towns," it was documented that the continuous exposure of pyriproxyfen to the pregnant females and children and its extensive use in domestic and agricultural practices led to the development of microcephaly (REDUAS 2016). This report alleged that the pesticide was at fault for the rise in reported microcephalic cases (Freeman 2016; Russo 2016). Further toxicological studies proved by some experts tend to fill the significant gaps in evidence on teratogenic risks (SWETOX 2016).

In the previous study, the developmental toxicity of pyriproxyfen in zebrafish demonstrated the adverse morphological defects including behavioral effects, craniofacial defects, severe mortality, and teratogenicity at larval stages (Truong et al. 2016). In addition, the assessment of subacute toxicity due to diflubenzuron (an IGR) in adult male rats exhibited significant decrease in testis weight, daily sperm production, and number of sperms in treated rats (Barros et al. 2016).

Nowadays, insecticide exposure has become a large public health interest especially during pregnancy due to instability of the pregnant mother and the developing fetus (Stillerman et al. 2008). Contamination of soil, water, and vegetables with the application of pesticides may affect both animals and human health (Adjrah et al. 2013). Exposure to pesticides in occupational situations leads to risks of cancer, developmental defects, neurologic problems, abortion, and decreased fertility (Hanke and Jurewicz 2004; Meeker et al. 2006). Chronic exposures to organophosphorus and other pesticides during preconception and perinatal periods can increase the risk to a range of congenital anomalies in pregnancy outcome. In animal models and humans, maternal and in utero exposure to these pesticides have been reported to induce arhinencephaly (incomplete formation of the anterior cerebral hemispheres) and low brain mass (Parens et al. 2017), neural tube defects (Rull et al. 2006), anencephaly (Lacasana et al. 2006), cleft lip with or without cleft palate (Shaw et al. 1999), nervous system defects (Garcia et al. 1999), limb reductions (Kristensen et al. 1997; Engel et al. 2000), multiple anomalies (Garcia et al. 1999), and fetal death (Taha and Gray 1993; Ronda et al. 2005).

Therefore, the current study was designed to investigate the outcomes due to repeated exposure of pregnant mice which were already treated with the same doses of pyriproxyfen (30, 100, 300, and 1000 mg/kg) during their first pregnancies and also examined the developmental defects in prenatal pups. This project is the first, to our information, in which the repeated exposure of pyriproxyfen was evaluated in mice as animal model.

Materials and methods

Chemicals

The tested insecticide was Predator (pyriproxyfen 0.5% WDG, Evyol Chemicals Group Lahore, Pakistan). Ketamine and xylazine were purchased from Elite Pharmaceutical Company Ltd., Lahore, Pakistan. Alcohol, xylene, formaldehyde (37%), and hematoxylin and eosin (H&E) stains were obtained from Sigma Aldrich (Germany). The polyclonal antibody (NeuN RbFox3) was supplied by Elabsciences Biotechnology Inc. Lahore, Pakistan. Tris-EDTA buffer was obtained from British Drug Houses (BDH) Chemicals Ltd. Poole, England, and Distyrene Plasticizer Xylene (DPX) as mounting medium was supplied by Fluka™ (Germany). Disodium hydrogen phosphate anhydrous and sodium dihydrogen phosphate monohydrate were purchased from IMF Scientific Company Lahore, Pakistan.

Animals

A total of 30 Swiss albino female mice aged 9–11 weeks and average weight of 24 ± 2 g were used for this study.

Female mice procured from the Animal House at Department of Zoology, Government College University Lahore, Pakistan, were maintained in disinfected polyvinyl cages under optimized temperature (25 ± 2 °C), light cycle

(12:12 h light/dark cycle), and relative humidity (50 ± 5%). They had access to standard laboratory diet and drinking water. The handling and maintenance of animals were done according to the guidelines of "Ethical Committee for the Treatment with Animals", Government College University Lahore. The experimental protocols were approved by the Board of Studies Committee, Department of Zoology, Government College University Lahore, Pakistan.

Experimental design

Female mice were mated with male overnight and vaginal plug was detected in the next early morning for pregnancy identification, considered as gestation day (GD) 0. Five groups of pregnant females (six in each group) were housed in separate polyvinyl chloride cages. Group I was considered as control (given normal rodent diet and distilled water). Groups II–V were repeatedly exposed with pyriproxyfen at 30, 100, 300, and 1000 mg/kg respectively daily via oral gauge for 10 days from the seventh day till the seventeenth of gestation (previously, these females were already treated with same above doses of pyriproxyfen during their first pregnancies in order to check the neurodevelopmental abnormalities in neonates. The duration from the parturition of first pregnancy to the start of second pregnancy was 1 week). The body weights of all pregnant mice were monitored daily and their average weights were represented in respective gestational weeks, i.e., GW1 (GD1-GD7), GW2 (GD8-GD14), and GW3 (GD15 till parturition). After parturition, litter size and percentages of stillbirths in treated groups were observed in response of the double exposure of pyriproxyfen. The body weights of pups, behavioral changes, and their physiological deformities were also checked.

The pups were euthanized at PND 7 and different organs (brain, heart, kidney, and liver) were dissected out, weighed (using High-Precision Digital Balance) and incubated for 24 h in 10% buffered formalin at room temperature (Bishop et al. 2018). The relative weight of each organ was calculated as ratio of absolute organ weight (g) to body weight (g) × 100 (Sekhar et al. 2011).

Staining procedures

After preservation, the paraffin-embedded blocks were prepared by automatic tissue processor. Then, thin sections of the liver, kidney, heart, and brain (10 μm) were made and finally stained with hematoxylin and eosin (H&E), followed the process described by Suvarna et al. (2012). The sections were observed for pathological changes under the microscope (Olympus CX31 Binocular, Japan) at × 40. For immunohistochemical staining of brain tissues, coronal sections (4 μm) of the paraffin-embedded tissue blocks were cut and further processed according to the protocol defined by Suvarna et al. (2012). The prepared slides were observed at × 4 and × 40 for the measurement of cortical radial thickness and total neural cells count.

Statistical analysis

Statistical analysis was carried out by one-way ANOVA followed by Tukey's multiple comparison test as post hoc for inter-group pregnant female body weights, gestational length, and litter size. The body, organs and relative organ weights, stillbirths, neural cells count, and cortical radial thickness of pups' brain were also analyzed by one-way ANOVA, utilizing SPSS version 20. All the data were denoted as mean ± standard error mean (SEM). In all tests, the $P < 0.05$ was considered as significant.

Results

Initial body weights of pregnant female mice before mating

The body weights of pregnant female mice were recorded daily from the parturition of first pregnancy till the start of second pregnancy as 25.58 ± 0.83, 24.50 ± 0.81, 23.98 ± 0.64, and 22.79 ± 0.72 g in groups II, III, IV, and V as compared to 26.99 ± 0.88 g in group I. Significantly ($P < 0.05$), less body weight gain was observed in group V as compared to group I and no significant difference was observed among treated groups (See Table 1).

Net pregnant female body weights due to pyriproxyfen treatment

The weights of pregnant mice during pairing were monitored in all treated and control groups. There was significantly ($P < 0.05$) less body weight gain in groups III and IV versus controls and highly significant ($P < 0.001$) between group V and group I. The body weights of pregnant female mice were monitored daily during the gestation period, represented as GW1, GW2, and GW3.

During GW1, the pregnant females exhibited significantly less body weight gain in group III ($P < 0.01$), group IV, and V ($P < 0.001$) in comparison to group I and group II versus group V ($P < 0.05$). The pregnant mice showed less body weight gain in treated groups as compared to control group during GW2. All the treated groups showed significantly (group II $P < 0.05$ and group III, IV, and V $P < 0.001$) less body weight gain in comparison to group I, group II versus groups IV and V ($P < 0.05$). During GW3, significantly less body weight gain was observed between treated groups and

Table 1 Effect of pyriproxyfen on different parameters in mice

Parameters	Group I (control)	Group II (30 mg/kg)	Group III (100 mg/kg)	Group IV (300 mg/kg)	Group V (1000 mg/kg)	*P* values
Initial body weights of female mice before mating (g)	26.99 ± 0.88	25.58 ± 0.83	24.50 ± 0.81	23.98 ± 0.64	22.79 ± 0.72[a]	[a]$P_1 < 0.05$
Body weights of female mice during pairing (g)	28.03 ± 0.90.	26.12 ± 0.86	24.65 ± 0.88[a]	24.22 ± 0.73[a]	23.55 ± 0.63[b]	[a]$P_1 < 0.05$ [b]$P_1 < 0.01$
Body weights of pregnant female mice (g)	31.04 ± 0.82	28.25 ± 0.69	26.36 ± 0.75[b]	25.25 ± 0.78[c]	24.73 ± 0.61[c,a]	[b]$P_1 < 0.01$
GW1						[c]$P_1 < 0.001$ [a]$P_2 < 0.05$
GW2	35.31 ± 0.72	30.59 ± 0.59[a]	28.56 ± 0.61[c]	26.42 ± 0.95[c,a]	25.88 ± 0.75[c,a]	[a]$P_1 < 0.05$ [c]$P_1 < 0.001$ [a]$P_2 < 0.05$
GW3	40.03 ± 0.65	33.65 ± 0.99[c]	30.25 ± 0.63[c,a]	27.99 ± 0.92[c,a]	26.64 ± 0.92[c,c,a]	[c]$P_1 < 0.001$ [a]$P_2 < 0.05$ [c]$P_2 < 0.001$ [a]$P_3 < 0.05$
Net body weights of pregnant mice (g)	3.01 ± 0.35	2.12 ± 0.20	1.71 ± 0.23[a]	0.86 ± 0.27[c]	0.98 ± 0.25[a]	[a]$P_1 < 0.05$
GW1						[c]$P_1 < 0.001$
GW2	7.27 ± 0.30	4.47 ± 0.37[c]	3.90 ± 0.43[c]	1.83 ± 0.42[c,b,a]	1.93 ± 0.48[c,b,a]	[c]$P_1 < 0.001$ [b]$P_2 < 0.01$ [a]$P_3 < 0.05$
GW3	11.99 ± 0.31	7.53 ± 0.64[c]	5.59 ± 0.79[c]	3.14 ± 0.69[c,b]	2.57 ± 0.63[c,c,a]	[c]$P_1 < 0.001$ [b]$P_2 < 0.01$ [c]$P_2 < 0.001$ [a]$P_3 < 0.05$
Gestational period length (days)	20.0 ± 0.57	21.67 ± 0.55	22.17 ± 0.87	24.20 ± 0.86[b]	25.60 ± 0.81[c,b,a]	[b]$P_1 < 0.01$ [c]$P_1 < 0.001$ [b]$P_2 < 0.01$ [a]$P_3 < 0.05$
Litter size	7.67 ± 0.49	3.33 ± 0.80[c]	2.33 ± 0.76[c]	1.16 ± 0.30[c]	0.66 ± 1.42[c,a]	[c]$P_1 < 0.001$ [a]$P_2 < 0.05$
Still birth, *N* (%)	2/46 (4.32 ± 0.07)	9/20 (45.17 ± 0.70)[c]	8/14 (57.17 ± 1.24)[c,c,c]	7/7 (100 ± 0.00)[c,c,c]	4/4 (100 ± 0.00)[c,c,c]	[c]$P_1 < 0.001$ [c]$P_2 < 0.001$ [c]$P_3 < 0.001$
Pups' body weights						[c]$P_1 < 0.001$
PND1	1.57 ± 0.04	1.36 ± 0.08	1.01 ± 0.13[c]	-----	----	[a]$P_1 < 0.05$
PND7	3.76 ± 0.12	2.96 ± 0.16[a]	2.08 ± 0.16[c]	-----	----	[c]$P_1 < 0.001$
Organs weights (g)						[b]$P_1 < 0.01$
Liver	0.092 ± 0.003	0.075 ± 0.004	0.058 ± 0.005[b]	----	----	[b]$P_1 < 0.01$
Heart	0.015 ± 0.001	0.013 ± 0.001	0.009 ± 0.001[b]	----	----	[b]$P_1 < 0.01$
Kidney	0.032 ± 0.001	0.025 ± 0.002	0.019 ± 0.002[b]	----	----	[b]$P_1 < 0.01$
Brain	0.322 ± 0.012	0.237 ± 0.015[b]	0.149 ± 0.013[c]	----	----	[c]$P_1 < 0.001$
Relative organs weightsn						
Liver	2.45 ± 0.01	2.64 ± 0.02[c]	2.78 ± 0.03[c]	----	----	[c]$P_1 < 0.001$
Heart	0.408 ± 0.006	0.450 ± 0.006[b]	0.458 ± 0.008[b]	----	----	[b]$P_1 < 0.01$
Kidney	0.86 ± 0.011	0.90 ± 0.007	0.94 ± 0.014[a]	----	----	[a]$P_1 < 0.05$
Brain	8.52 ± 0.04	7.61 ± 0.09[c]	7.11 ± 0.08[c]	----	----	[c]$P_1 < 0.001$

Values are expressed as mean ± SEM of six mice in each group, P_1 represents significant difference between treated groups as compared to group I, P_2 indicates statistical difference between group II and other treated groups, P_3 represents significant results between group III and other treated groups, a = $P < 0.05$, b = $P < 0.01$, c = $P < 0.001$

group I ($P < 0.001$), group II versus group III, IV ($P < 0.05$) and group V ($P < 0.001$), group III versus group V ($P < 0.05$) as shown in Table 1.

The net body weights of pregnant mice during GW1 were significantly decreased in treated groups III, V ($P < 0.05$), and IV ($P < 0.001$) as compared to group I. A significant decrease

in net body weights of pregnant female was observed in all treated groups as compared to group I ($P < 0.001$), group II versus group IV and V ($P < 0.01$), group III versus group IV and V ($P < 0.05$). During GW3, the net body weights of pregnant females were significantly decreased in all treated groups in comparison to group I ($P < 0.001$), group III versus group V ($P < 0.05$) as represented in Table 1.

Prolonged gestation day length

Compared with the controls, pyriproxyfen treatment at different doses during pregnancy prolonged the length gestation period. The GD length in treated mice was 21.67 ± 0.55, 22.17 ± 0.87, 24.20 ± 0.86, and 25.60 ± 0.81 days in all the treated groups as compared to 20.0 ± 0.57 days in group I, showed a highly significant increase in groups IV ($P < 0.01$) and V ($P < 0.001$) as compared to group I, group II versus group V ($P < 0.01$), and group III versus group V ($P < 0.05$) as illustrated in Table 1.

Litter size and stillbirths

Litter size was also significantly ($P < 0.001$) decreased due to pyriproxyfen treatment such as mean number of pups in all treated groups (3.33 ± 0.80, 2.33 ± 0.76, 1.16 ± 0.30, and 0.66 ± 1.42 in groups II, III, IV, and V respectively) as compared to group I (7.67 ± 0.49), group II versus group V ($P < 0.05$) are shown in Table 1. Pyriproxyfen treatments caused an increase in fetal deaths in groups II and III and all born pups were dead in groups IV and V. The percentage of stillbirths in groups II and III was 45.17 ± 0.70 and 57.17 ± 1.24% respectively as compared to group I (4.30 ± 0.21%). The percentage of stillbirths in all treated groups showed highly significant ($P < 0.001$) increase as compared to group I, group II versus group III, groups IV and V ($P < 0.001$), group III versus groups IV and V ($P < 0.001$) are listed in Table 1.

Body weights of pups

Table 1 summarizes the body weights of live pups from PND1 to 7 in treated and control groups. Their weights were ranged from 1.36 ± 0.08 to 2.96 ± 0.16 g and 1.01 ± 0.13 to 2.08 ± 0.16 g in groups II and III respectively while in group I, the body weights of the pups were 1.57 ± 0.04 to 3.76 ± 0.12 g. During PND1, significantly less body weight gain was observed in group III as compared to group I ($P < 0.001$). The pups gain significantly less body weights in group II ($P < 0.05$) and group III ($P < 0.001$) as compared to group I on PND7 (See Table 1).

Organ and relative organ weights of pups

Liver

Liver weights were decreased in a dose-dependent manner in groups II and III (0.075 ± 0.004 and 0.058 ± 0.005 g) as compared to group I (0.092 ± 0.003 g), significantly ($P < 0.01$) between groups III and I. However, relative weights of the liver were significantly ($P < 0.001$) increased in groups II and III (2.64 ± 0.02 and 2.78 ± 0.03%) in comparison to group I (2.45 ± 0.01%) as shown in Table 1.

Heart

The heart weight was significantly ($P < 0.01$) decreased in group III (0.009 ± 0.001 g) as compared to group I (0.015 ± 0.001 g). Table 1 shows that the relative heart weight in groups II and III (0.450 ± 0.006 and 0.458 ± 0.008%) had a significant ($P < 0.01$) increase in comparison to that in group I (0.408 ± 0.006%) as listed in Table 1.

Kidney

Table 1 also represents a significant ($P < 0.01$) decrease in kidney weights in a dose-dependent manner as 0.019 ± 0.002 g in group III as compared to group I (0.032 ± 0.001 g) but the relative kidney weights were significantly ($P < 0.05$) increased in group III (0.94 ± 0.014%) as compared to group I (0.86 ± 0.01%).

Brain

The pyriproxyfen treatment caused a significant decrease in brain weights (0.237 ± 0.015 g in group II, $P < 0.01$) and (0.149 ± 0.013 g in group III, $P < 0.05$) as compared to group I (0.322 ± 0.012 g). The relative brain weights were decreased significantly ($P < 0.001$) in groups II and III (7.61 ± 0.09 and 7.11 ± 0.08% respectively) as compared to group I (8.52 ± 0.04%) as shown in Table 1.

Physical deformities

Figure 1 illustrates the repeated exposure of pyriproxyfen in pregnant female mice resulted physical deformities in prenatal pups. In group III, the dead limbless pup was born in a litter of female mice and this type of abnormal development was not observed in groups I and II. At PND 7, there was an obvious difference in the size, weight, and physical appearance in pups of the treated groups II and III as compared to group I. In addition, growth retardation and absence of fur were also observed in the treated pups.

Fig. 1 Physical deformities in prenatal pups due to pyriproxyfen treatments at PND 7 (left to right). **a** A dead pup was born with no limbs showed developmental defects in group III (a), showed retardation in growth of pup in group III (b), decrease in size and weight of pup in group II, **d** normal size, weight, and growth of pup in group I (c)

Histopathological changes

Liver

Histopathological studies of liver sections revealed the dilated sinusoids, degenerated hepatocytes, infiltration of lymphocytes, congestion, and necrosis in group II. Vacuolar and cytoplasmic degeneration of hepatocytes and severe congestion in central vein were more prominent in group III as compared to group I, having normal hepatocytes and Kupffer cells (See Fig. 2).

Kidney

In group I, the sections of kidney showed normal glomerular structure and cells of renal tubules. The histopathological examination of the kidney tissues showed atrophy in glomerular structure, vacuolization in tubules, and hemorrhage and infiltration of lymphocytes in group II. In group III, the kidney sections revealed more shrinkage and degeneration of glomerulus, scattered hemorrhages, inflammation, and vacuolization of epithelial lining of most renal tubules as shown in Fig. 3.

Heart

The heart sections showed normal muscle fibers and acidophilic cytoplasm. The muscle fibers were separated by interstitial connective tissues having flat nuclei in group I. The pyriproxyfen treatment exhibited degeneration and disorganization in myocardial fibers. The separation of myofibrils, vacuolization in cytoplasm of muscle fibers, presence of pyknotic nuclei, and congestion in arteries were also observed in group II. There were more vacuolization and congestion with extravasations of the blood in between the cardiac muscle fibers observed in group III (See Fig. 4).

Brain

Figure 5 represents the coronal sections of the brain showing normal histological features, illustrating distribution of normal neural cells, and glial cells in group I. No significant change was observed in the brain sections of control pups. In group II, the brain sections of prenatally exposed pups exhibited more distortion in the cellular structure with the presence of increased number of glial cells, vacuolization, and degenerated neurons. The coronal sections of the brain revealed a greater number of glial cells with more degeneration of neurons and vacuolization in group III.

Measurement of cortical radial thickness and neural cell count

In Fig. 6, our findings showed that the exposure to pyriproxyfen significantly reduced the cortical thickness of

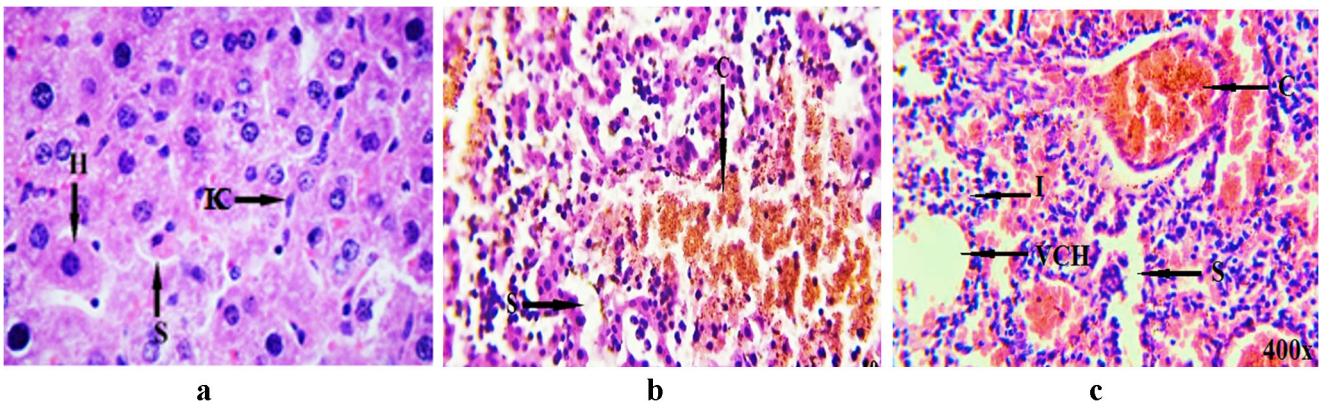

Fig. 2 Liver sections of group I showing normal hepatocytes (H), sinusoid (S), and activated Kupffer (K) cells (a). The dilated sinusoids (S), degenerated hepatocytes (H), infiltration of lymphocytes (I), congestion, and necrosis were observed in group II (b). In group III, the liver sections showed more vacuolar and cytoplasmic degeneration of hepatocytes (VCH) and severe congestion (C) in central vein (c)

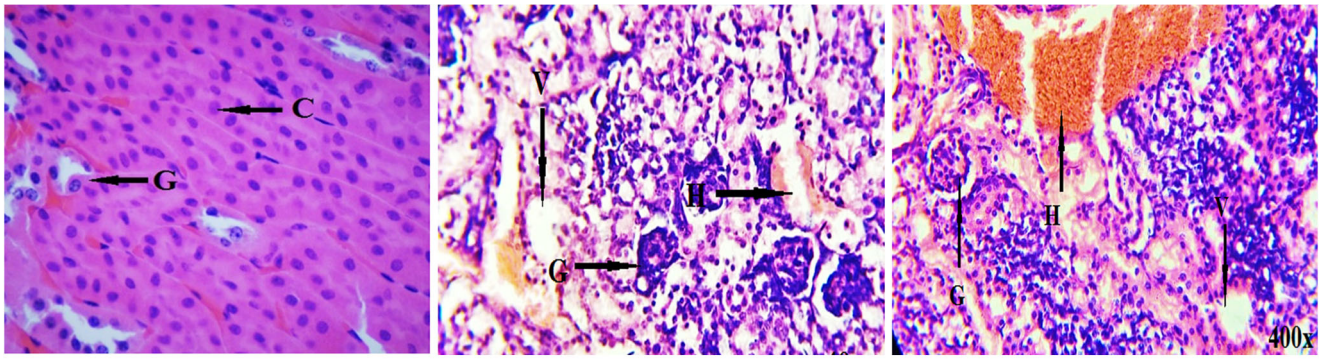

Fig. 3 In group I, kidney sections showed normal glomerular structure (G) and cells of renal tubules (C) (a). The degeneration of glomerular structure (G), vacuolization (V) in renal tubules, hemorrhage (H), and infiltration of lymphocytes were prominent in group II (b). In pups of group III, more shrinkage and degeneration of glomerulus (G), scattered hemorrhages (H), inflammation and vacuolization (V) of epithelial lining of most renal tubules, and necrosis were revealed (c)

pup's brain as 0.75 ± 0.01 and 0.526 ± 0.04 mm in groups II and III ($P < 0.001$) as compared to group I (0.872 ± 0.01 mm), group II versus group III ($P < 0.001$). Immunohistochemical results exhibited a significant decrease in NeuN+ cells per HPF (high power field) as 77.0 ± 1.50 and 55.5 ± 3.43 in treated groups (II and III) ($P < 0.001$) in comparison to group I (89.09 ± 1.02), group II versus group III ($P < 0.001$) as shown in Figs. 7 and 8.

Discussion

Recently, pyriproxyfen is highlighted due to its possible potential role in increased microcephalic cases while it is extensively used in water bodies in Brazil. The potential neurotoxicity due to pyriproxyfen was dismissed by some authorities and related the microcephalic cases with Zika Virus (ZIKV) infection but failed to explain the complete epidemiology of the microcephaly in infants (Albuquerque et al. 2016; Evans et al. 2016). Exposure to sublethal amount of insecticides during prenatal as well as postnatal period caused considerable maternal and developmental toxicity (Farag et al. 2003, 2006). We explored that the possible toxicity due to pyriproxyfen exposure may affect the maternal body weight and organogenesis of the neonates.

In the present study, there was a significantly ($P < 0.05$, $P < 0.001$) less body weight gain in repeatedly exposed pregnant female mice in all treated groups as compared to group I (control) during second and third gestational week. Consistent with the report by Uchendu et al. (2018), they documented significantly less mean body weight gain in Wistar rats treated with deltamethrin (DLT), chlorpyrifos (CPF), CPF + DLT, and alpha lipoic acid (ALA) + CPF + DLT as compared to S (Soya bean)/oil group. However, the CPF + DLT group showed the least mean body weight (only 28.31% increased from initial body weight) as compared to other treated groups. In addition,

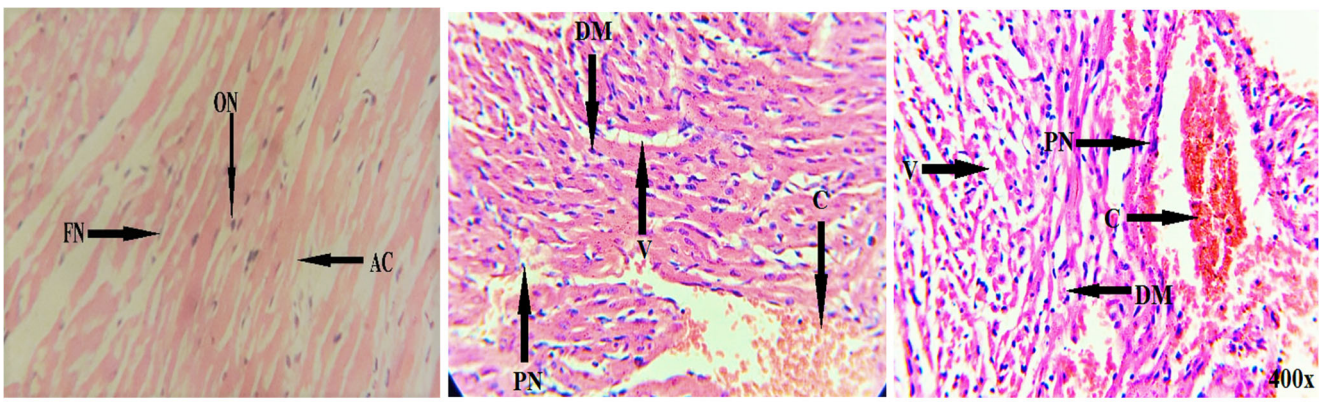

Fig. 4 Showing the normal heart muscle fibers with oval central nuclei (ON) and acidophilic cytoplasm (AC). The cardiac fibers were separated by interstitial connective tissues with flat nuclei (FN) in group I (a). The degeneration and disorganization in myocardial fibers (DM), vacuolization in cytoplasm of muscle fibers (V), pyknotic nuclei (PN), and congestion (C) were observed in group II (b). In group III, the heart sections showed more presence of pyknotic nuclei (PN), degeneration of myofibrils (DM), vacuolization (V), and congestion (C) with extravasations of the blood in between the cardiac muscle fibers (c)

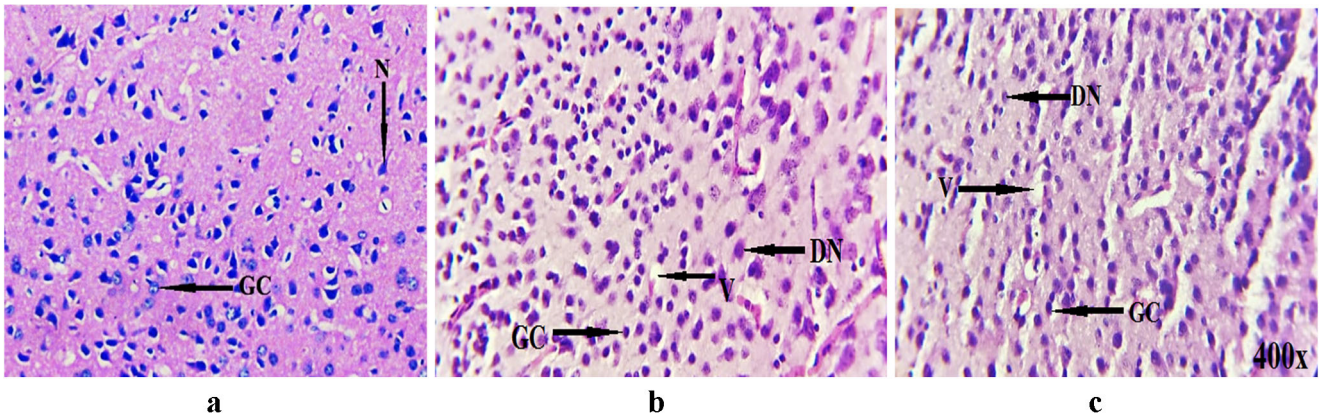

Fig. 5 A photomicrograph of coronal section of control mice brain showing full of cellular distribution with normal neural cells (N), glial cells (GC), and normal architecture in group I (a). A photomicrograph of coronal section of mice brain prenatally treated with pyriproxyfen showing highly distorted cellular structure with vacuolization (V), increased number of glial cell (GC) and degenerated neurons (DN) in group II (b). A photomicrograph of coronal section of mice brain showing fully degeneration of neurons (DN) with highly increased number of glial cells (GC), necrosis and vacuolization (V) in group III (c)

Mossa et al. (2011) also reported less body weight gain in Wistar rats treated with four organophosphate insecticides (chlorpyrifos, profenofos, diazinon, and malation) in combination (217.80 ± 20.17 g) as compared to the control group (276.80 ± 3.92 g).

In epidemiological survey, the use of pesticides has been linked with reduced fertility, menstrual disturbance, prolonged time to pregnancy, stillbirth, spontaneous abortions, and developmental defects (Bretveld et al. 2006). In our findings, the gestational period length was significantly ($P < 0.01$, $P < 0.001$) prolonged in groups IV and V as compared to group I due to the repeated exposure of pyriproxyfen in pregnant female mice. Previously, Verma and Mohanty (2009) documented GD lengths in Swiss pregnant female mice treated with organophosphate dimethoate at 4, 8, and 16 mg/kg (20.8 ± 0.34, 21.3 ± 0.98, and 23.6 ± 0.6 days respectively) were prolonged in comparison to controls (20.6 ± 0.87 days).

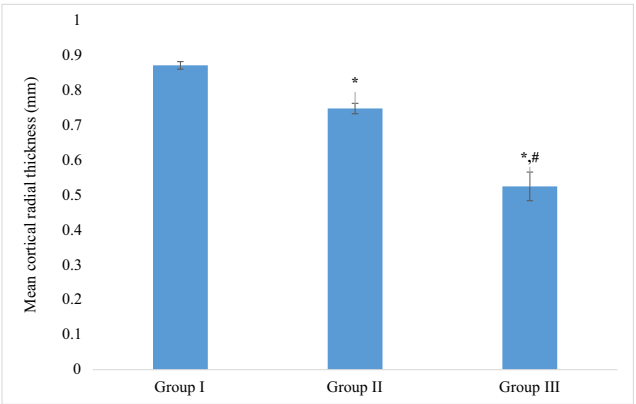

Fig. 6 Mean cortical radial thickness (mm) of pup's brain. Values are expressed as mean ± SEM of six mice in each group, * represents significant decrease in mean cortical radial thickness (mm) of pup's brain in treated groups (II and III) as compared to group I, # indicates significant decrease in mean cortical radial thickness (mm) of pup's brain in group II versus group III, $*,^{\#} = P < 0.001$

In the current study, the pyriproxyfen treatment also caused a significant ($P < 0.001$) reduction in the litter size of all treated pregnant female as compared to group I. The stillbirths of pups were significantly ($P < 0.001$) increased in groups II and III as compared to the controls. In accordance with present findings, Evans et al. (2016) demonstrated the effects of three doses of pyriproxyfen (100, 300, and 1000 mg/kg) in female rats during gestation period. The results revealed less number of pups (78) were obtained in the litter of female mice treated with 1000 mg/kg pyriproxyfen in comparison to expected number of pups (99). Consistent with the finding of present study, Verma and Mohanty (2009) reported organophosphate dimethoate treatment decreased the litter size (6.9 ± 0.65, 6.7 ± 0.18, and 6.4 ± 0.4 at 4, 8 and 16 mg/kg) as compared to the control (7.0 ± 0.91). The death rates of pups were also increased as 4.4 ± 0.82, 10.82 ± 0.6, and 15.6 ± 0.5% in the above treatments as compared to 3.6 ± 0.12% in the control group.

In the present findings, the prenatal exposure of pyriproxyfen showed a significant ($P < 0.05$, $P < 0.001$) less body weight gain in treated pups (groups II and III) as compared to group I at PND7. These findings were consistent with a previous study that the repeated postnatal exposure of chlorpyrifos-oxon (CPO) at 0.25 mg/kg/day showed a significant decrease in body weights of pups from PND 4 to 21 ($P = 0.029$), 8 and 3% decrease in body weight on PND 21 and PND 80 as compared to the controls (Cole et al. 2012).

In the current study, the relative liver and heart weights of prenatally exposed pups in relation to their body weights were significantly ($P < 0.001$, $P < 0.01$) increased in groups II and III, whereas the relative weights of the kidneys were significantly ($P < 0.05$) increased only in group III as compared to group I. In comparison to group I, the relative brain weights of

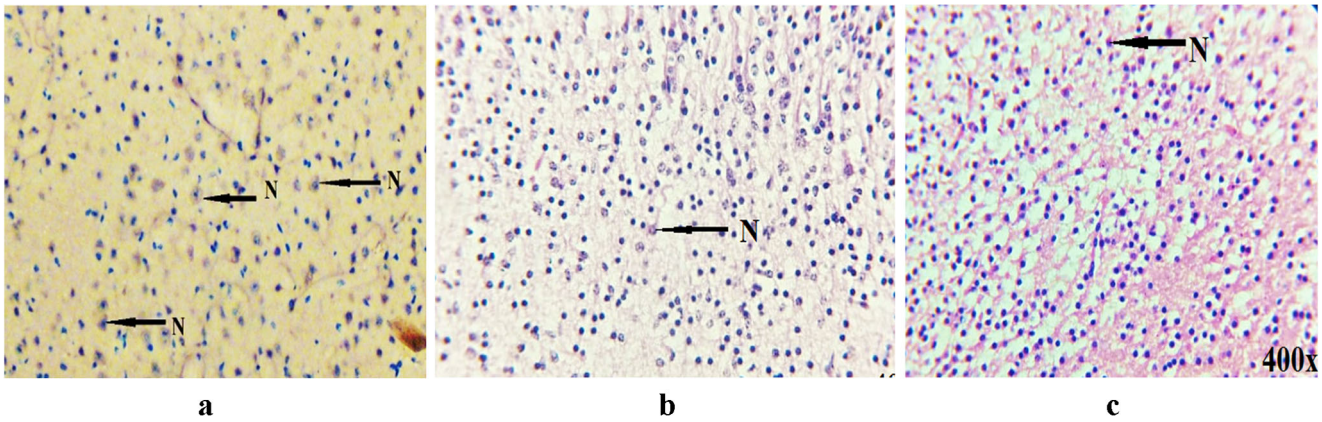

Fig. 7 A photomicrograph 89 NeuN+ cells per high power field (PHF) were recorded in group I (a), 77 NeuN + cells PHF in group II (b), and 58 NeuN+ cells PHF in group III (c)

pups were significantly decreased ($P < 0.001$) in group III. Our findings are in agreement with other reports that showed the effects of glyphosate-based herbicide (GBH) following acute, sub-chronic (6 weeks) and chronic (12 weeks) exposure (250 or 500 mg/kg/day) in mice treated from juvenile age until adulthood. The post hoc analysis showed that the treated groups, especially 500 mg/kg group, exhibited a significant decrease ($P < 0.05$) in organ's relative weight as compared to the control group (Bali et al. 2017). In another previous report, Mansour et al. (2008) evaluated the absolute liver and kidney weights (5.51 g and 1.79 g) and relative liver and kidney weights (2.85% and 0.93%) of control albino male rats respectively. In comparison to the control values of relative weight of the liver (2.85%), all the tested doses of five insecticides (abamectin, carbosulfan, fenpropathrin, methomyl, and profenofos) exhibited a significant increase and the mixture treatment showed highly significant increase in relative liver weight (4.54%). All the tested doses showed a significant decrease in the relative weight of the kidney in comparison to the control (0.93%), whereas the most obvious difference was found in the mixture treatment, recorded as 0.74%.

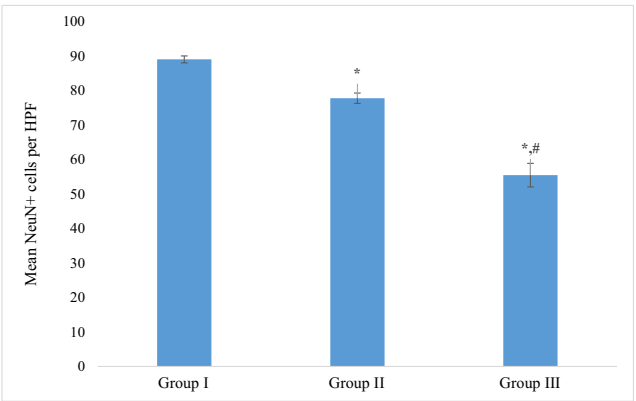

Fig. 8 Mean number of NeuN+ cells per high power field (PHF) in pup's brain. Values are expressed as mean ± SEM of six mice in each group, * represents significant decrease in mean number of NeuN+ cells (PHF) in pup's brain (groups II and III as compared to group I), # indicates significant decrease in mean number of NeuN+ cells (PHF) in pup's brain (group II versus group III), $*,^{\#} = P < 0.001$

In contrast to the present findings, Bonvallot et al. (2017) observed metabolome disruption due to the repeated exposure of a mixture of eight pesticides (acetochlor (246 μg/kg bw/d) + bromoxynil (12 μg/kg bw/d) + carbofuran (22.5 μg/kg bw/d) + chlormequat (35 μg/kg bw/d) + ethephon (22.5 μg/kg bw/d) + fenpropimorph (15.5 μg/kg bw/d) + glyphosate (12 μg/kg bw/d) + imidacloprid (12.5 μg/kg bw/d) in pregnant rats and their offspring. The mean liver weights of male fetuses in the exposed group were significantly higher than that of the non-exposed group ($P < 0.005$), whereas the mean brain weights of male and female fetuses were significantly higher ($P < 0.01$ for males, $P < 0.05$ for females) as compared to the controls.

Our work investigates the developmental defects such as a dead limbless fetus born to a female treated with 100 mg/kg and reduction in size and weight of pups also found in group II and III as compared to group I. The impact of three pesticides (glyphosate 500 mg/kg, mancozeb 500 mg/kg, and carbosulfan 10 mg/kg) on the fetuses of pregnant female rats has been reported by Abou-Amer et al. (2010), who demonstrated an increase in fetal death, growth retardation external hemorrhages, complete loss of ossification in digits and vertebrae (caudal), and less ossification of different parts of skull and legs in comparison to the controls.

In the present results, the histopathological examinations of liver sections revealed distorted hepatocytes, dilation of sinusoids, infiltration of lymphocytes, and hemorrhages in central vein in a dose-dependent manner. The kidney sections showed vacuolization in medullary tubules, shrinkage and degeneration of glomerulus, infiltration of inflammatory cells, renal cast, and hemorrhages in group III as compared to groups I and II. In addition, incorporation of blood cells in muscle fibers, vacuolization, appearance of pyknotic nuclei, and disorganization in the arrangement of cells occurred in heart tissues were dose-dependent. In group III, the brain sections of

pups revealed a slightly more distorted neuronal cellular structure with vacuolization, increased number of glial cells, and degeneration of neurons as compared to the controls. Similar results were observed by Mondal et al. (2014) who documented that the alterations in the liver, kidney, and heart tissues of Wistar female rats treated with multiple exposures to acetamiprid (ACP) in a dose-dependent manner (200, 100, and 25 mg/kg). In addition, Kaushal et al. (2007) demonstrated histopathological changes in albino rats administered with nitrosodiethylamine (NDEA). The results indicated slight granular to vascular degenerative changes and congestion in the liver, and necrosis and degeneration of renal tubules in the kidney.

Previously, there is scientific proof of the reproductive, carcinogenic, neurological, genotoxic, and immunological effects linked to non-persistent insecticides in adults (Koureas et al. 2012). However, limited data is available about the high risk of adverse reproductive dysfunction (Eskenazi et al. 2004; Rauh et al. 2012; Lacasana et al. 2006) and alterations in the neurobehavioral development and nervous system (Bouchard et al. 2010; Engel et al. 2011; Marks et al. 2010; Rauh et al. 2012; Eskenazi et al. 2007). In our findings, the development of the brain is highly affected by the repeated exposure of pyriproxyfen and had greater impact on the thickness of cortical region. In groups II and III, the mean cortical radial thickness of pup's brains was significantly ($P < 0.001$) decreased in comparison to the control. In agreement with our results, Rauh et al. (2012) demonstrated high exposure of chlorpyrifos in children (5.9 to 11.12 years) showed parietal and frontal thinning of cortical region exhibited inverse dose-response between cortical thickness and chlorpyrifos. In addition, early chlorpyrifos exposure in animals resulted in direct neurotoxic changes such as altered neuronal apoptosis, cell replication, and cortical lamination in a dose-dependent manner (Roy et al. 2005; Burke et al. 2017).

In the present findings, a significant ($P < 0.001$) reduction was observed in total neural cells count (NeuN+ cells per HPF) in groups II and III as compared to group I. These results agreed with the findings that the repeated exposure of diazinon in Sprague-Dawley rats exhibited a significantly high cell packing density in adolescence as well as in adulthood, resulting in reactive gliosis and neuronal loss. In addition, some regions of the brain as temporal/occipital, striatum, and cortex showed evident net cell loss resulting in more neurotoxic effects due to diazinon exposure (Slotkin et al. 2008).

Conclusion

The present findings reveal that the repeated exposure of pyriproxyfen causes a significantly less body weight gain in pregnant females during gestational weeks. The results showed that litter size and percentages of stillbirths were significantly altered in treated groups as compared to the controls. In addition, there were significant changes in the body and relative organ weights of pups, histopathology of organs, cortical radial thickness, and total neural count in brain sections observed due to pyriproxyfen treatments at prenatal stage.

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Compliance with ethical standards

Ethical approval The experimental protocols using animal model (mice) in the present study were approved by "Ethical Committee for the Treatment with Animals," Government College University Lahore.

Conflict of interest The authors declare that they have no conflict of interest.

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