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Histomorphometric study on the effect of low dose deltamethrin on the developing cerebellar cortex

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Aim: Deltamethrin is a widely used type II pyrethroid-based insecticide. Studies have indicated that neonatal exposure to deltamethrin, even at relatively low doses, results in behavioral and neurological changes. In the present study, the effect of early neonatal exposure to low doses of deltamethrin on the histogenesis of the cerebellar cortex was investigated.

Materials and methods: Sprague Dawley male pups were exposed to deltamethrin daily at a dose of 1 mg/kg via intraperitoneal injection from the 2nd to the 5th postnatal day and sacrificed on postnatal days 6, 14, and 21. Following intracardiac perfusion, the cerebellum was removed, embedded with paraffin wax, and serially sectioned. The thickness of the different layers of the vermal cerebellar cortex was measured using Image-Pro Plus software.

Results: There were no significant differences in the measured thickness of the external granular, molecular, and internal granular layers between the deltamethrin-treated and the control animals in all age groups studied.

Conclusion: In contrast to a previous study, the present study showed that neonatal administration of low dose deltamethrin did not significantly affect the morphogenesis of the cerebellar cortex.

Key words: Pyrethroid, deltamethrin, neonatal, cerebellar cortex

Introduction

Deltamethrin, first discovered in 1974, is a widely used type II pyrethroid-based synthetic insecticide (1). As a group, pyrethroids are regarded as safer for humans than the other classes of insecticide (2). Deltamethrin is widely used in the agricultural sector, as it has high efficacy against a large number of insects. Its low toxicity to humans has made it one of the insecticides of choice in many countries (3). Deltamethrin acts on the nervous system via its interaction with various channels and receptors, although its primary target is the voltage-dependent sodium channels (4). Its neurotoxicity in adults is well characterized, although information regarding its developmental neurotoxicity is still limited (5).

Neonatal rats are 4-17 times more vulnerable to the toxic effects of pyrethroid than the adults (6). In rats, in terms of brain development, the neonatal period corresponds to the third trimester period in humans (7). With regard to the cerebellum, this is the active period of neuronal proliferation, migration, and synaptogenesis in the cerebellar cortex. Exposure to toxic substances during this vulnerable period may affect the cytogenesis, morphogenesis, and synaptic connectivity of the cerebellar cortex (8), which could lead to impairment in some of the brain functions (9). Delayed cerebellar morphogenesis and maturation have been observed after exposure to various substances such as alcohol (10), cyanide (11), propylthiouracil (12), and α-difluoromethylornithine

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(13). Therefore, the objective of this study was to examine the effect of deltamethrin exposure on early cerebellar cortex morphogenesis and maturation in neonatal rats. Only low doses of deltamethrin were tested, as previous studies have shown that low dose deltamethrin, 0.7-1.0 mg/kg of body weight, was able to affect certain brain receptors and alter the behavior and motor activity of the exposed animals (5,14,15). In addition, general population exposure to pyrethroids, including deltamethrin, occurs mostly at low dose levels (2).

Materials and methods

Animals

Virgin female Sprague Dawley rats were mated and housed singly in standard plastic cages. The pregnant rats were maintained on a standard pellet diet and water ad libitum, and they were monitored each morning and afternoon for delivery. In the present study, the day of delivery was designated as postnatal day (PD) 0. On the day following the delivery, the litters were culled to 8 pups containing at least 6 males using cross-fostering procedures as necessary. The pups remained with their respective dams throughout the experimental period, except during experimental procedures. The experimental protocol was reviewed and approved by the Animal Ethics Committee of the Universiti Sains Malaysia. All possible efforts were made to minimize any potential discomfort to the animals.

Experimental Protocol

Six male pups of each litter were equally divided into treatment and control groups. Pups assigned to the treatment group were given deltamethrin at a dose of 1 mg/kg body weight from PD 2 to PD 5 via intraperitoneal injection. The dose of 1 mg/kg deltamethrin represents only about 1.2% of the median lethal dose (LD_{50}) for adult rats (16). Deltamethrin (minimum 98% pure) was procured from Sigma (USA) and prepared by dissolving it in a corn oil vehicle immediately prior to the start of the experiment. In the control group, an equal volume of corn oil was administered via the same route during the corresponding period. The pups were sacrificed on PD 6, 14, or 21.

On the day of sacrifice, the animals were deeply anaesthetized with ether vapor and immediately perfused via the intracardiac route with normal saline followed by 10% buffered formalin. The brain was removed immediately after perfusion and weighed. The cerebellum, together with the attached brain stem, was separated from the cerebral hemispheres by cutting between the superior and inferior colliculi in the transverse fissure. The cerebellum was then separated from the brain stem via a cut through the cerebellar peduncles, and its weight was determined. The cerebellum was kept in the same fixative for 48 h before processing and embedding using an automatic tissue processor (Thermo Shandon, UK). Paraffin-impregnated cerebellum was then cut sagittally with sharp razor blades a few millimeters from and parallel to the vermis. This ensured that when a cerebellum was placed into the mold, it would lie on the same horizontal plane as other cerebella from other groups, hence minimizing the potential bias.

The cerebellum was then carefully trimmed with a Leica microtome until the first section of the vermis region was identified. The cerebellar vermis was identified by its having all 10 lobules in a section. The vermis was serially sectioned at 4 μm of thickness. During sectioning, the angle of cutting remained unchanged throughout the experiment. Five individual equally spaced sections (from 40 to 100 μm apart, depending on the age groups) were selected using systematic random sampling procedures, as described previously (23). The selected sections were used for Harris hematoxylin and eosin (H&E) staining using an automatic tissue stainer (Sakura Tissue Tek DRS). To minimize experimenter biases, each slide was given a secret code that was not revealed until after all slides had been analyzed.

Quantitative Histological Analysis of the Cerebellar Cortex

In the present study, cerebellar morphogenesis was assessed by measuring the thickness of various layers in the cortex of the vermis. Selected H&E stained slides were examined with a light microscope (Nikon Eclipse E600, Japan), which was connected to a computer (Dell) via a digital video camera (CoolSNAP-Pro, Media Cybernetics, USA). Through these connections, images from the microscope could be visualized on the color monitor with high

resolution. The computer was equipped with Image-Pro Plus software (Media Cybernetics) for quantitative analysis of the images.

Systematic random sampling procedures were employed to choose areas of the cerebellar cortex to be examined. Individual slides were first examined under low magnification (×2 objective); the projected image was then superimposed with a grid provided in the software and subsequently captured by the software. Guided by the image, every fifth area of the grid was systematically sampled with a random start between 1 and 5, beginning from the extreme upper left corner of the image. With this procedure, an average of 15-25 areas of cerebellar cortex was selected for analysis for each tissue section. The selected areas of the cerebellar cortex were then examined under higher magnification, where the actual measurements took place. Measurements of the external granular, molecular, and internal granular layers were taken using the linear measuring tool provided in the Image-Pro Plus software, performed perpendicular to the surface of the folia. For each cerebellar layer, 3 measurements were taken in each of the selected areas and the average length was recorded.

Statistical analysis

The data were analyzed using SPSS version 12 using an independent t-test after the homogeneity of variance between the groups was ascertained with Levene's test. The data were expressed as the mean ± standard of error (SEM), and $P < 0.05$ was accepted as statistically significant.

Results

Body, Brain, and Cerebellum Weights

The weights of the body, brain, and cerebellum of rats sacrificed on the various postnatal days are given in Table 1. Statistical analysis showed no significant differences between the body weights of the deltamethrin-treated rats and the control groups for all postnatal days. Similarly, the total brain and cerebellar weights of the deltamethrin-treated animals were not significantly different than those of the controls in all age groups.

Histogenesis of the Cerebellar Cortex

As predicted, the external granular layer became progressively thinner with age and completely disappeared by PD 21. This observation was true for both for the control and the deltamethrin-treated groups. Statistical analysis showed that the external granular layer thickness measured at PD 6 and PD 14 after deltamethrin treatment was not significantly different than that of the respective control groups (Table 2).

Analysis of the internal granular layer thickness also failed to show any differences between the deltamethrin-treated and the control groups at PD 6, 14, and 21. These findings are consistent with the above findings, since the migratory process of granule cells from the external granular layer contributes to the formation of the internal granular layer. Similar results were obtained for the molecular layer. The thickness of the molecular layer after treatment with deltamethrin was not significantly different than that of the control groups in all age groups (Table 2).

Table 1. Effect of deltamethrin administration on the body weight, brain weight, and cerebellar weight at postnatal day (PD) 6, 14, and 21.

Parameter	Control			Deltamethrin-treated		
	PD 6	PD 14	PD 21	PD 6	PD 14	PD 21
Body weight (g)	13.04 ± 0.18	28.55 ± 0.97	45.20 ± 1.23	12.60 ± 0.32	26.88 ± 1.27	42.93 ± 1.29
Brain weight (g)	0.518 ± 0.008	1.150 ± 0.013	1.290 ± 0.014	0.526 ± 0.006	1.129 ± 0.014	1.280 ± 0.019
Cerebellar weight (g)	0.0258 ± 0.0014	0.1264 ± 0.0026	0.1780 ± 0.0032	0.0266 ± 0.0016	0.1242 ± 0.0022	0.1756 ± 0.0043

Value represents mean ± SEM.

Table 2. The thickness (μm) of various layers of the vermal region of the cerebellar cortex measured at postnatal day (PD) 6, 14, and 21.

Layers	Control			Deltamethrin-exposed		
	PD 6	PD 14	PD 21	PD 6	PD 14	PD 21
EGL	33.3 ± 0.5	20.9 ± 0.5	0	32.8 ± 0.4	21.3 ± 0.5	0
ML	21.1 ± 0.3	82.2 ± 2.0	133.0 ± 2.5	20.0 ± 0.5	80.1 ± 2.2	132.3 ± 1.6
IGL	76.5 ± 1.6	101.1 ± 1.9	134.3 ± 2.4	73.5 ± 2.9	99. 3 ± 1.7	130.6 ± 3.1

Values are expressed as mean ± SEM.
EGL: external granular layer, ML: molecular layer, IGL: internal granular layer.

Observatory findings revealed that the alignment of the Purkinje cells into a single layer was achieved by PD 6 in both the deltamethrin-treated and control groups. Thus, administration of the deltamethrin did not seem to affect the monolayer formation of the Purkinje cells (Figure).

Discussion

The present study revealed that neonatal administration of 1 mg/kg of deltamethrin had no effect on the external granular layer or internal granular layer morphogenesis, as demonstrated by the complete disappearance of the external granular layer by PD 21 and the absence of significant differences in the measured external granular layer and internal granular layer thickness between the 2 groups. As migration and proliferation of progenitor granule cells of the external granular layer contributed to the formation of the definitive internal granular layer (17), the study suggests that deltamethrin at the current dosage does not seem to influence the granule cell migration or proliferation, at least not at a significant level. Similarly, the study demonstrates that deltamethrin administration has no influence on the molecular layer or Purkinje cell layer morphogenesis.

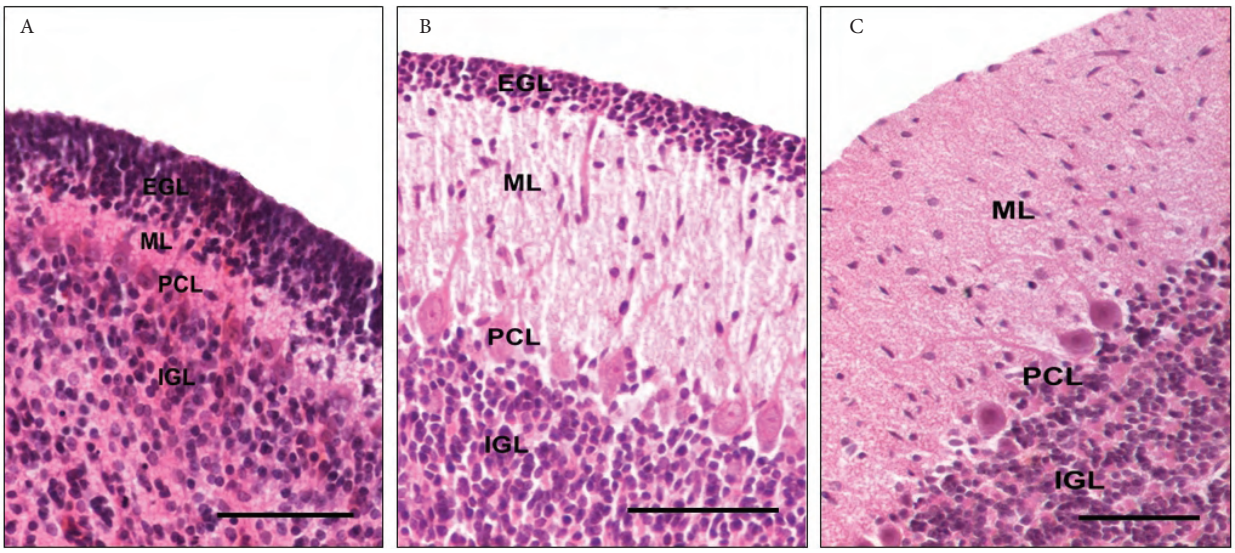

Figure. Sections of the cerebellar cortex stained with Harris hematoxylin and eosin from the deltamethrin-treated rats sacrificed at postnatal day 6 (A), 14 (B), and 21(C), showing various layers of the cerebellar cortex. EGL: external granular layer, ML: molecular layer, PCL: Purkinje cell layer, IGL: internal granular layer. Scale bars represent 50 μm.

These findings are in marked contrast to the findings by Patro et al. (18), who conducted the only morphological study so far on the effect of deltamethrin on the developing cerebellum. In that study, administration of 0.7 mg/kg of intraperitoneal deltamethrin (using propylene glycol as a vehicle) to PD 9 rats for 5 consecutive days was found to affect the cerebellar layer morphogenesis, although the effect was temporary, observed until PD 21 but not at PD 30. The authors suggested that the granular cells migrated at a much slower pace after deltamethrin exposure and the mechanism was revealed by subsequent research by the same authors. Bergmann cell processes, which act as the scaffold for the granule cell migration, were found to be disorganized, hypertrophic, and immature in the exposed animals (19).

Although the results of the study conducted by Patro et al. (18) seem convincing, details of the measuring methods, including the sampling procedures employed in the study, were not mentioned, giving ample space for speculation. Another curious feature of the study is the demonstration of deltamethrin-induced damage to the developing vasculature, leading to hemorrhage, thrombosis, and focal degeneration of the brain parenchyma. None of the stated pathology could be found in the present study. Nevertheless, the discrepancy of the results could be explained by the different method used in measuring the cerebellar cortex layers and the different type of vehicle used. It is known that the toxicological effects of deltamethrin are dependent on the route of administration and the vehicle used to dissolve the chemical (20). For example, through the oral route, deltamethrin is 100 times less toxic when dissolved in a gum arabic solution than when dissolved in oil or organic solvent (21).

Shafer et al. (5), in their detailed review, listed several limitations of the Patro et al. (18) study, including the suggestion that the observed effects were due to the decreased growth of the pups and not due to the direct neurotoxicity of deltamethrin. This is supported by other reviewers, Ray and Fry (4), who stated the possibility of nonspecific developmental delay contributing to the observed effects. During early postnatal development, the degree of the development of the cerebellar cortex is directly proportional to the body weight of the animals for that age period (22). In the Patro et al. study, the measured differences in body weight were enormous, reaching up to 40% and 30% when measured at PD 12 and 21, respectively (18). This is in contrast to the present study, in which no changes in body weight were observed after exposure to deltamethrin.

In conclusion, in contrast to a previous study, the results presented here show that exposure to low dose deltamethrin (1 mg/kg) does not seem to influence cerebellar cortex morphogenesis when administered during the early neonatal period.

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