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Scanning electron microscopic study of the effect of chlorpyrifos on the developing neural tube in comparison with Arsenic in mouse embryo

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KEYWORDS

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Abstract *Background:* Arsenic is an important environmental toxicant which is usually found in drinking water in inorganic form. Arsenic exposure in pregnant mice causes neural tube defects (NTDs). Chlorpyrifos, an organophosphorus insecticide, recommended universally and in Egypt to control various pests, was evaluated for its potential developmental toxicity. Studies have shown increasing evidence to suggest an association between environmental exposure to this agricultural pesticides and adverse reproductive outcomes. The hypothesis tested in this investigation is chlorpyrifos causes significant defects on the developing central nervous system compared to the proven Arsenic.

Objectives: The aim of this work was to assess congenital malformations induced by the organophosphorus insecticide chlorpyrifos on the neural tube and brain development in comparison with the positive control Arsenic.

Methods: Virgin female ICR (CD-1) mice, approximately 10 weeks old were mated with adult males. The day the vaginal plug was found was considered day 0 of gestation. It consisted of 320 mice. They were subdivided into four groups of 80 bred mice each. Each group was divided into 4 subgroups, and 20 mice per each were treated by gavage as follows: 30 mg/kg/day chlorpyrifos (tested group), 40 mg/kg/day sodium Arsenite (positive control group), and corn oil and distilled water (negative control groups) on days 6–15 of gestation. Maternal observations throughout gestation were reported. In each subgroup the mice proved to be pregnant were sacrificed on gestational days; GD 10, 11, 12 and 16. The day of scarification was determined according to the neural tube developmental stages. The conceptus extraction was done and their number reported to be subjected to the SEM study.

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After mice scarification, the uteri were opened and a total of 30 embryos and fetuses, randomly selected from each subgroup were processed for scanning electron microscopy investigating the neural tube developmental defects.

Results: CPF ingested by gravid mice at dose of 30 mg/kg/day started from 6th day of gestation proved to produce NTDs as compared to Arsenite.

Conclusion: Neural tube defects are due to chlorpyrifos that may directly influence brain cell replication and differentiation.

1. Introduction

Despite restriction on production for home use, chlorpyrifos (CPF), [O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl)-phosphoro thioate], remains one of the most widely used insecticides, and there is concern over the potential consequences of fetal and childhood exposure.[1–5] Uses of CPF, as indoor pests, were restricted in June 2000.[5,6] Despite this restriction CPF is still widely used as indoor pesticide and pet collars in Egypt. Immature organisms are more susceptible to CPF induced toxicity than adults.[7,8] The presence of CPF residue in the umbilical cord and its association with decreases in fetal growth among women during pregnancy raises question about the sensitivity of residue parameter of CPF.[8] Furthermore, it has been emphasized that perinatal exposure of women to CPF would lead to shortening of the gestational period.[9] In addition, several studies have reported that repeated exposure to CPF during gestation can cause fetotoxicity and marked neurochemical changes in the developing brain.[10–13] Chlorpyrifos exposure during the perinatal period is known to evoke deficits in neuritic outgrowth, specifically including the target of cholinergic projections.[14,15] Exposure of pregnant female mice to 30 mg/kg/day CPF through 6–15 days of gestation resulted in increase of gestation length and post implantation loss, decreased litter size and weight, and survivability.[16,17] It is questionable whether malformations produced by a dose of 30 mg/kg/day, particularly neural tube defects, could be increased. Therefore, the present study was conducted to assess the ability of CPF to cause brain and neural tube defects at a dose of 30 mg/kg/day CPF compared to Sodium Arsenite which was used as a positive control and confirmed to produce developmental toxicity and neural tube defects.[18–20]

2. Material and methods

320 female ICR (CD-1) mice, approximately 10 weeks old, were mated. The gravid mice were subdivided into four groups of 80 bred mice each. Each group was divided into 4 subgroups, and 20 mice per each were treated by gavage as follows: 30 mg/kg/ day chlorpyrifos (tested group), 40 mg/kg/day sodium arsenite (positive control group), and corn oil and distilled water (negative control groups) on days 6–15 of gestation. On GD 10, 11, 12 and 16, after mice scarification, the uteri were opened and a total of 30 embryos and fetuses, randomly selected from each subgroup were processed for scanning electron microscopy investigation.[21]

3. Results of the scanning electron microscopic (SEM) study

Study of the neural fold fusion with SEM was done in the chlorpyrifos and sodium arsenite treated groups in mice embryos on gestational days GD10, 11, 12 and in mice fetuses on day 16 of development and compared to the controls (Table 1, Figs. 1–4).

On day 10 of development, examination of embryo specimens showed that the neural folds in the control groups approached each other and the neural groove became concave for the completion of closure of the neural tube. At the same

Table 1 The number of mice embryos and fetuses with neural fold fusion disruption as evident by SEM examination on days 10, 11, 12 and 16 of development randomly selected from each of the 4 groups of the study.

Study groups	Number (%)			
	Negative control (1)[a]	Negative control (2)[b]	Positive control[c]	Chlorpyrifos 30 mg/kg/d
Number of embryos examined in each subgroup	30	30	30	30
Day 10 of development	0 (0)	0 (0)	6 (16)*	9 (30)**
Day 11 of development	0 (0)	0 (0)	7 (23)*	10 (33)**
Day 12 of development	0 (0)	0 (0)	8 (26)*	11 (37)**
Day 16 of development	0 (0)	0 (0)	9 (30)**	12 (40)**

[a] Corn oil as a vehicle of chlorpyrifos.
[b] Distilled water as a vehicle of Arsenic.
[c] Arsenic as Sodium Arsenite (40 mg/kg/day).
* Significantly different from control at $P \leqslant 0.05$.
** Significantly different from control at $P \leqslant 0.01$.

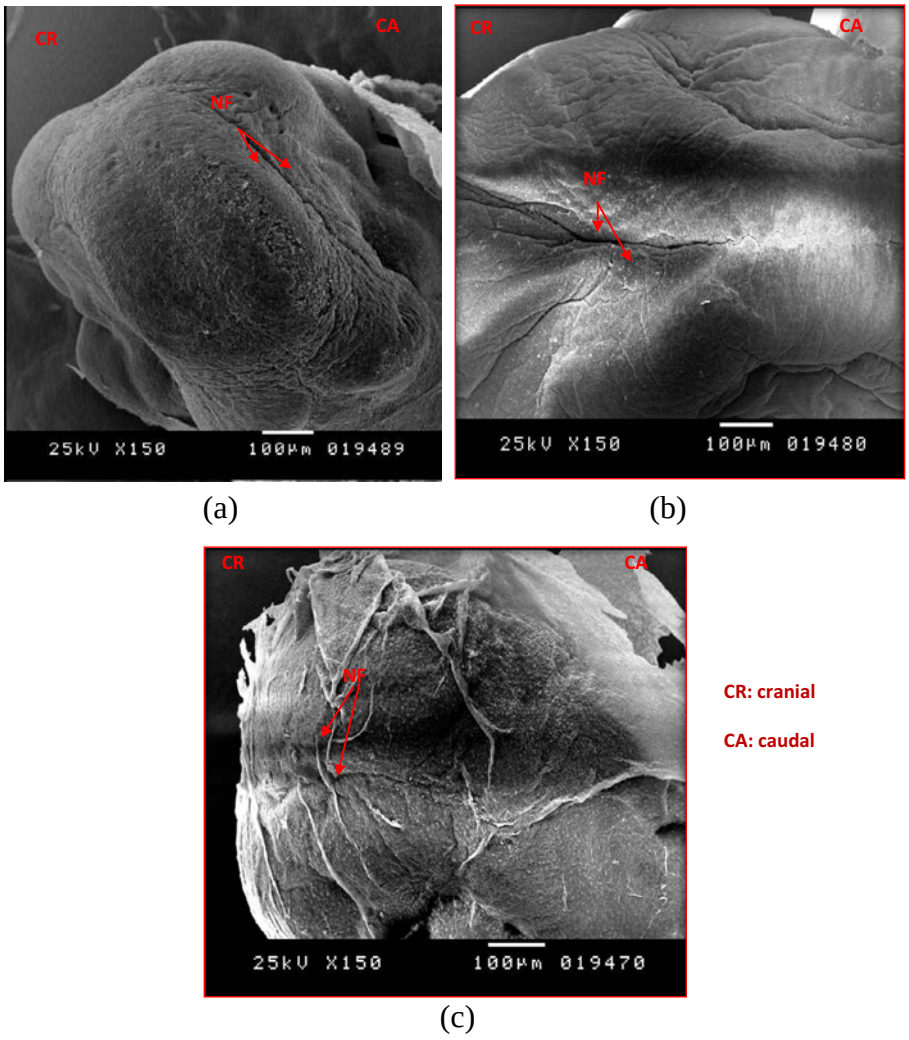

Figure 1 Scanning electron micrograph of the dorsal view of cranial end of the neural tube of mouse embryo on day 10 of development showing: (a) Normal apposition of the neural folds (NF) delineating narrow linear groove in the negative control. SEM 150×. (b) Separated edges of the neural folds (NF) delineating a wide linear groove in the sodium arsenite group. SEM 150×. (c) More separated edges of the neural folds (NF) delineating a wider linear groove in the chlorpyrifos group. SEM 150×.

stage in the chlorpyrifos treated group, the neural folds were still away from each other and had an irregular crumpled appearance in 9 out of 30 (30% of) embryos, showing a significant delay in initiation of neural tube closure. Also the positive control sodium Arsenite mice group showed delay in neural tube fusion compared to its negative control in only 6 (16%) of embryos (Table 1, Fig. 1a–c).

On day 11 of development, in both of the two control groups, the neural folds showed progressive opposition of each other along their full length and the neural tube was almost closed in all embryos. At the same stage in the chlorpyrifos treated group the neural tube was still widely opened in ten (33%) of mouse embryos compared to its corresponding negative control group. Similarly in the Sodium Arsenite group, the neural tube was opened in only 7 (23%) of mouse embryos compared to its corresponding negative control group indicating a delay in neural tube development (Table 1, Fig. 2a–c).

On day 12 of development, mice embryos of the control groups had a completely closed neural tube. At the same stage the neural tube was still opened in 11 (40%) and 8 (26%) of mice embryos in the chlorpyrifos treated group and the Sodium Arsenite treated group respectively compared to their corresponding negative controls indicating a significant increase in neural tube closure defect compared to the negative controls (Table 1, Fig. 3a–c).

On day 16 of development of the mouse fetus, an opening in the head was observed in 12 (40%) in the chlorpyrifos group and 9 (30%) in the Sodium Arsenite group compared to their corresponding negative controls (Table 1).

The lateral view of mice embryos on day 11 of development showed an overall delay of neural development in both chlorpyrifos and Sodium Arsenite treated groups to the two negative control groups. A less distinct optic vesicle and otic placode, hypoplasia and a general retardation of development

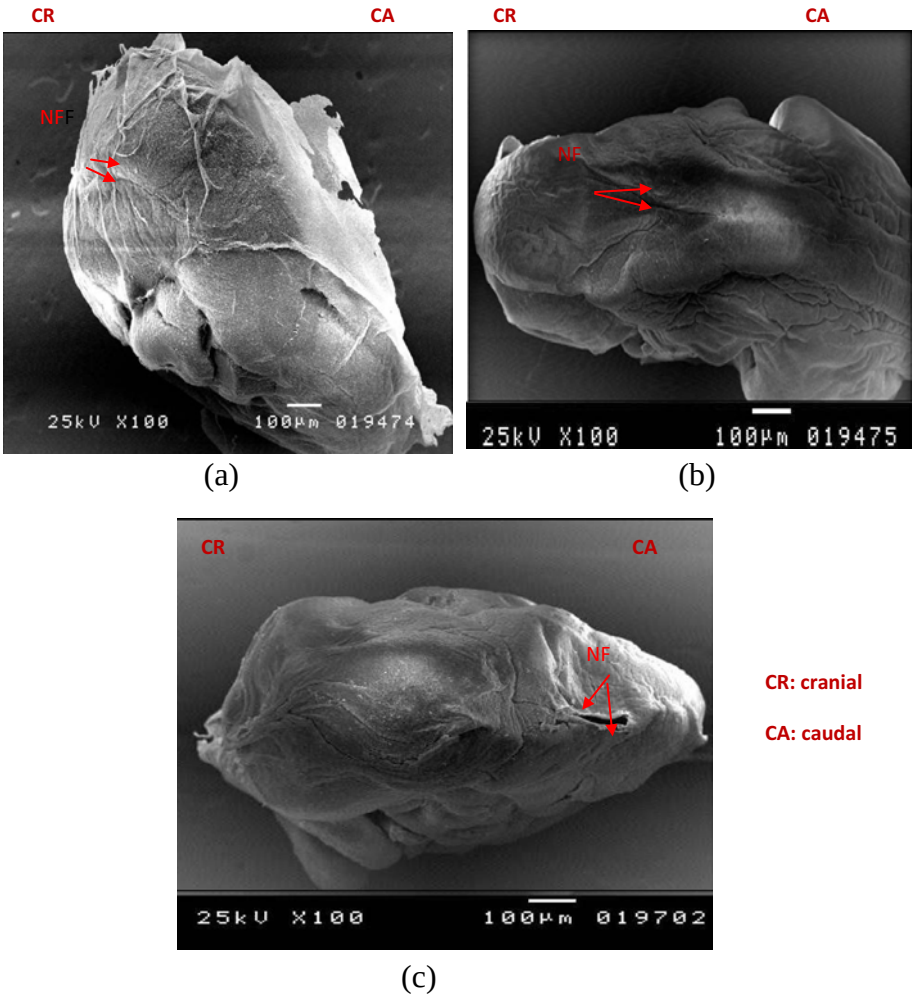

Figure 2 Scanning electron micrograph of dorsal view of mouse embryo on day 11 of development showing: (a) Almost closed neural tube through complete apposition of the neural folds (NF) in the negative control group. SEM 100×. (b) Partially opened neural tube through separated neural folds (NF) in the sodium arsenite group. SEM 100×. (c) Partially opened neural tube through separated and averted neural folds (NF) in the chlorpyrifos group. SEM 100×.

of the head and limb buds were noted in both chlorpyrifos and Sodium Arsenite treated groups compared to their corresponding negative control groups (Fig. 4a–c).

The lateral view of mice embryos on day 12 of development showed more developmental delay of different nervous tissues. The head was not well developed in the chlorpyrifos treated group with exencephaly, and in the Sodium Arsenite group compared to the negative controls. The nasal pits showed delayed development in both treated groups compared to the negative controls. Also the limb plates did not show digital rays and circular constrictions in both treated groups compared to the negative controls (Fig. 5a–c).

4. Discussion

Chlorpyrifos itself is a developmental neurotoxicant due to the ability of its metabolite chlorpyrifos-oxon to inhibit cholinesterase. Although biologic effects of chlorpyrifos are attributed to the biotransformation to chlorpyrifos-oxon and other metabolites that act to inhibit a cholinesterase, CPF disrupted maturation of sea urchin embryos during the specific period during which development is regulated by neurotrophic factors.[23] Recent evidence suggests that chlorpyrifos itself may directly influence brain cell replication and differentiation.[11–13] Chlorpyrifos has immediate direct inhibitory actions on DNA synthesis and hence on neural cell replication, with preferential targeting of gliotypic cells.[22] Therefore, chlorpyrifos may induce damage by both noncholinergic and cholinergic mechanisms extending from early stages of neural cell replication through late stages of axonogenesis and terminal differentiation.[24]

CPF itself is a developmental neurotoxicant above inhibition of cholinesterase by its metabolite, chlorpyrifos-oxon.[11] The most thoroughly investigated hypothesis is that fetal brain cholinesterase is able to recover more quickly between chlor-

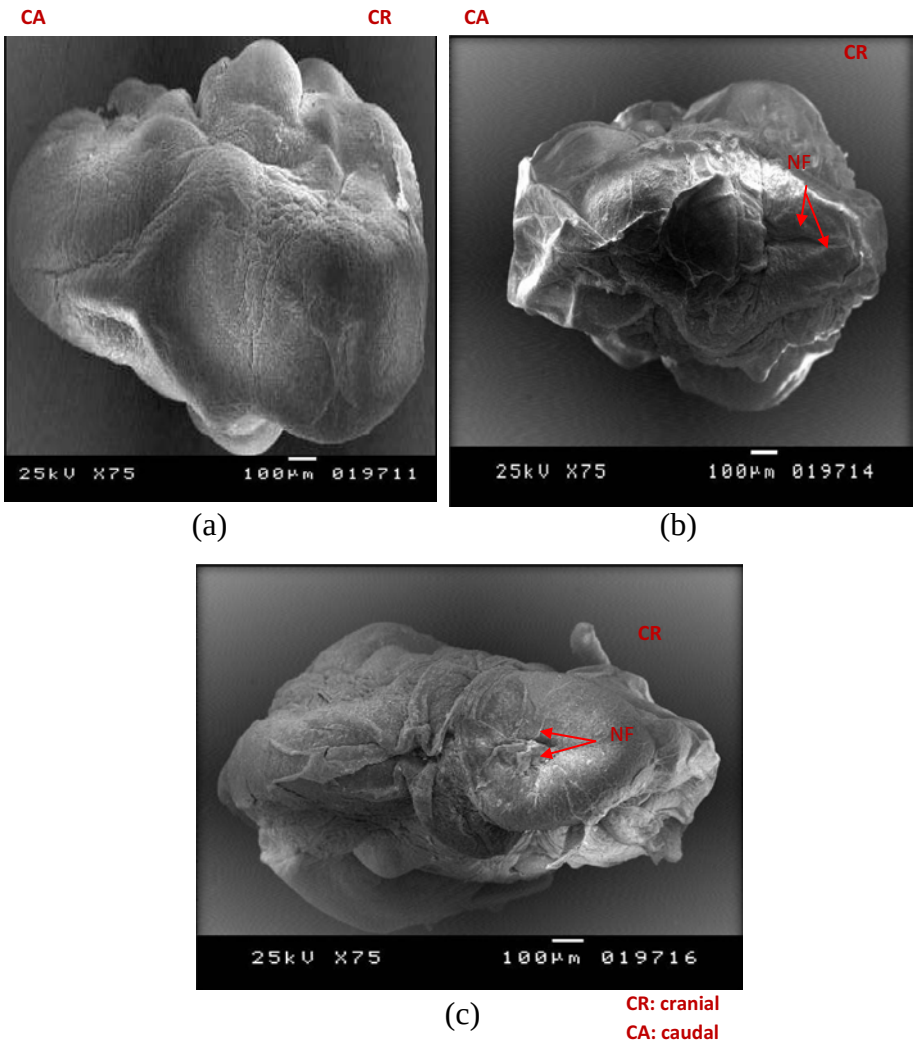

Figure 3 Scanning electron micrograph of dorsal view of mouse embryo on day 12 of development showing: (a) The neural folds completely fused along their full length in the negative control group. SEM 75×. (b) Partially opened neural tube through separated neural folds (NF) in the sodium arsenite group. SEM 75×. (c) Partially opened neural tube through separated neural folds (NF) in the chlorpyrifos. SEM 75×.

pyrifos dosages. The exceptional recovery capacity of fetal brain cholinesterase may represent a form of protection, but ultimately the pesticide still inhibits cholinesterase activity in the fetal brain. The inhibition of fetal brain cholinesterase, even intermittently, may be deleterious to the coordinated development of the brain given the postulated novel role for the cholinesterases in nervous system development. The inhibition of AChE caused by CPF is more persistent than that caused by other organophosphates. This result may be attributed to the high lipophilicity of chlorpyrifos.[22]

In the electron microscopic study of the present work, cranial neural fold development of the control embryos and fetuses showed a morphogenetic pattern generally similar to those described in previous studies. NTDs were concentrated on the fusion process of the neural folds in mouse embryos as a basis for a better understanding of closure defects and were able to show the precise steps of fusion in rat, hamster and mouse embryos.[25] The delay in timing of neural fold fusion in this study for both chlorpyrifos and Arsenic may be a reflection of hypoplasia and a general retardation of development of the neural tube when compared with negative control embryos on the same days of gestation. Moreover a delay in development in the different developing systems and exencephaly was observed. These findings are similar to those previously reported.[25,26] Putz and Morriss-Kay also reported an abnormal neural fold development with exencephaly in mouse embryos with triosomy 12 and triosomy 14.[26]

Chlorpyrifos may directly influence brain cell replication and differentiation and has immediate direct inhibitory actions on DNA synthesis and hence on neural cell replication. Therefore, chlorpyrifos may induce damage by both noncholinergic and cholinergic mechanisms extending from early stages of

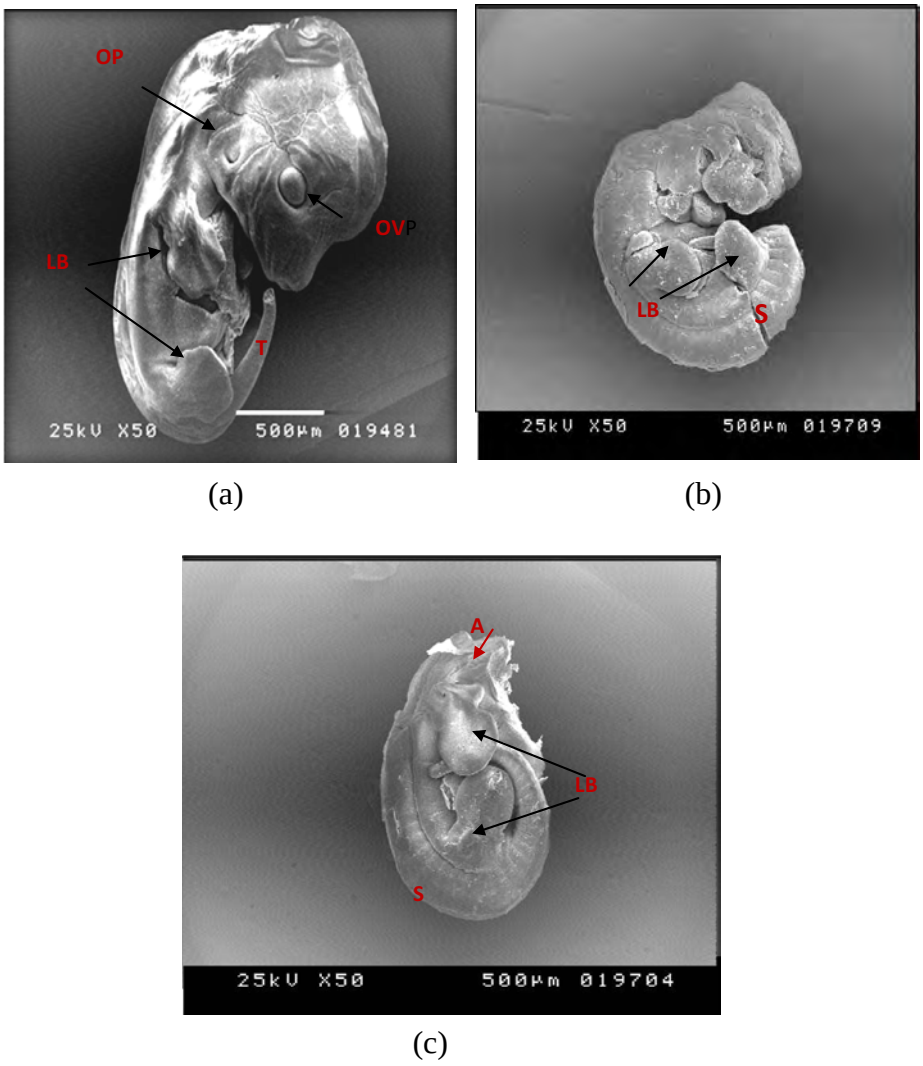

Figure 4 Scanning electron micrograph of lateral view of mouse embryo on day 11 of development showing: (a) The head folded with a large size of the head, optic vesicle (OV) and otic placode (OP) and with the limb buds (LB) and a tail (T) in the negative control group. SEM 50×. (b). Small and malformed folded head with optic vesicle and otic placode not apparent and with the limb buds (LB) not well developed and somites (S) in the sodium arsenite group. SEM 50×. (c) Cranial malformation as anencephaly (A) with the limb buds (LB) not well developed and somites (S) in the chlorpyrifos group. SEM 50×.

neural cell replication through late stages of axonogenesis and terminal differentiation.[12,13,24]

Several recent studies suggest that chlorpyrifos affects relatively early and late events in brain development. These studies focused around the proliferation, differentiation, and functioning of glial cell, the cells that provide metabolic support for neurons and that guide axons to their proper targets within the developing central nervous system.[27] In addition, Lassiter et al. (1998) and White et al. (2002) found that prenatal chlorpyrifos exposure can disrupt architectural organization of specific subregions, including apoptosis and changes in cell migration.[24,28] In turn, these findings raise the issue of identifying the critical window for adverse effects of chlorpyrifos on neurodevelopment. If late-occurring processes are involved, then vulnerability will extend into childhood, a period in which exposures may be particularly high.[29–31]

5. Conclusion

Chlorpyrifos showed fetotoxicity at a maternal dose of 30 mg/kg per day in the form of neural tube defects. This is due to chlorpyrifos that may directly influence brain cell replication and differentiation. Although restriction of indoor use has been recommended, CPF is still used at least as pesticide with hazardous environmental pollution. Females living in areas with high risk of this pollution are more susceptible to give births with neurological deficits so it is highly recommended to restrict the use of CPF also as pesticide and as anti-flea in pet collars.

Conflict of interest

No conflict of interest.

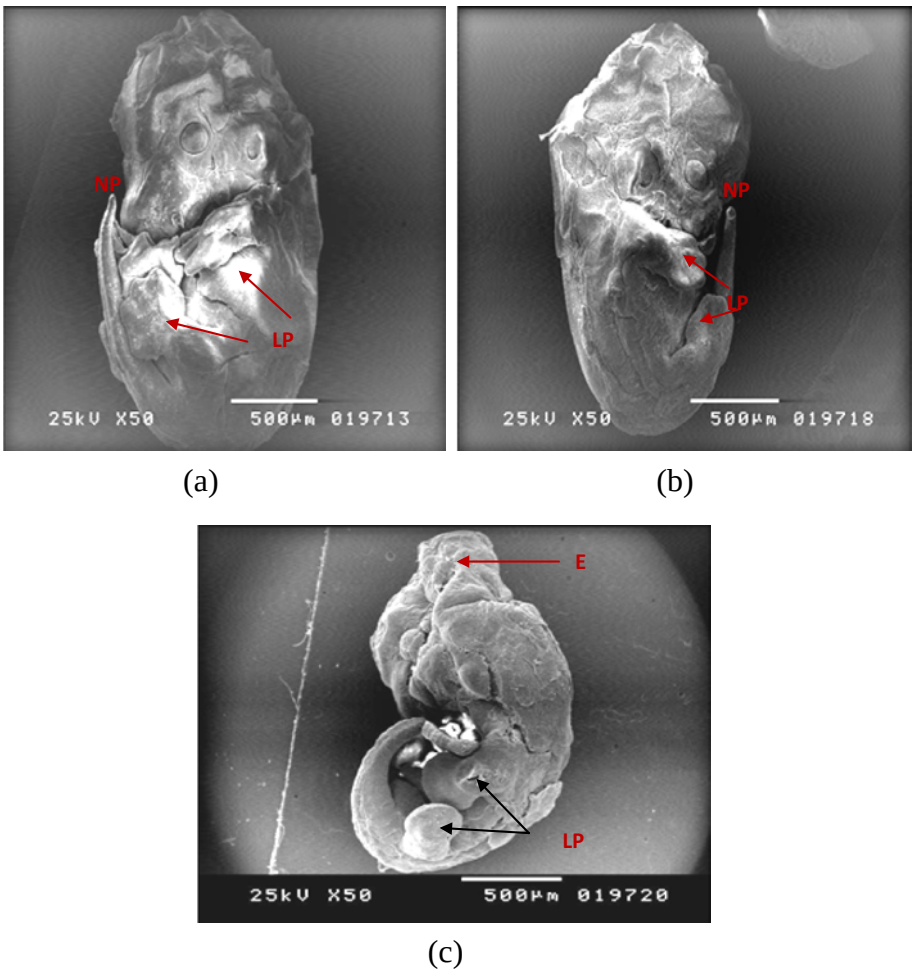

Figure 5 Scanning electron micrograph of lateral view mouse embryo on day 12 of development showing: (a) The embryo tightly C shaped, has nasal pit (NP) and the fore- and hindlimb plates (LP) in the negative control group. SEM 50×. (b) Small sized and partially undifferentiated head, not well apparent nasal pit (NP) and the limb plates (LP) are not well developed in the sodium arsenite group. SEM 50×. (c) Exencephaly (E), not well apparent nasal pit and the limb plates (LP) are not developed in the chlorpyrifos group. SEM 50×.

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