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Role of folic acid in chlorpyrifos induced teratogenicity in mice

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Folic acid is known to reduce the incidence of neural tube defects, in animal experiments however, it has not been effective in reducing the congenital anomalies caused by antiepileptics and many other chemicals. Pesticides of organophosphate group such as chlorpyrifos are widely used in agriculture and household, and are shown to be teratogenic in animal studies. This study was designed to explore the effect of folic acid on chlorpyrifos induced teratogenicity. Pregnant mice were divided into groups of 8 animals each, and were exposed to oral dose of chlorpyrifos and/or folic acid on gestation days (GD) 6 and 7 or GD 1 to 15. Fetuses were recovered on 15th day of gestation. On morphological and morphometeric examination it was found that chlorpyrifos is teratogenic to mice fetuses in an oral dose of 100 ug/gbw when given on GD 6 and 7. It was embryo toxic and caused growth retardation and morphological anomalies of skull, body curvature, skin, limbs, eyes and ears. Folic acid in a dose of 2 ug/gbw, when given at the time of chlorpyrifos exposure (GD 6 and 7), lead to significant improvement in crown-rump (CR) length and weight of fetuses, reduction in the number of resorptions and frequency of anomalies. However the CR length and weight of fetuses given folic acid and chlorpyrifos were significantly less than the control. There was no additional benefit of giving folic acid in the same dose from GD1 to 15. Thus this study was aimed at confirming the teratogenic potential of cholorpyrifos in mice and assessing the role of folic acid in the prevention of teratogenesis induced by chlorpyrifos.

Key words: Folic acid, organophosphates, teratogenesis, chlorpyrifos.

INTRODUCTION

Based on animal studies, interventional trials and epidemiological studies, maternal folic acid is known to be protective in neural tube defects (Blencowe et al., 2010; Hamner et al., 2009). Consumption of folic acid by women of childbearing age is an important public health goal in US (Tinker et al., 2010). Role of folic acid in the prevention of other congenital anomalies is still controversial. Folic acid has shown to reduce the incidence of teratogenesis induced by ethanol, arsenic, retinoic acid, valproic acid and ochratoxin A, in animal experiments, but has failed to decrease the congenital anomalies caused by antiepileptics and many other chemicals

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Abbreviations**: GD,** Gestational day; **gbw,** gram body weight; **CR,** crown-rump.

Table 1. Dose schedule of chlorpyrifos and folic acid.

Group	Chemical given	Dose (ug/gbw)	Time of administration
Group A (Experimental Group)	Chlorpyrifos	100	GD 6 and 7
Group B (Experimental Group)	Chlorpyrifos and folic acid	100 and 2	GD 6 and 7, GD 6 and 7
Group C (Experimental Group)	Chlorpyrifos and folic acid	100 and 2	GD 6 and 7, GD 1 to 15
Group D (Folic acid control)	Folic acid	2	GD 1 to 15
Group E (Plain Control)	Food and Water	*ad libitum*	--

(Yanaguita et al., 2008; Katagiri et al., 2007). Pesticides of organophosphate group are widely used in agriculture and household. Human population, including the pregnant women is exposed to its harmful effects (Eskenazi et al., 2004). Organophosphates, such as chlorpyrifos have been shown to be highly teratogenic in animal studies (Tian et al., 2005; Chanda et al., 1995; Ahmad and Asmatullah, 2007). The present study was designed to explore, whether folic acid has any protective role in teratogenesis induced by chlorpyrifos.

MATERIALS AND METHODS

The present research was conducted on Swiss Webster strain of albino mice *Mus musculus*. Animals were reared in the animal house of the Department of Zoology, University of the Punjab, Lahore. They were kept under optimum conditions and fed commercially prepared chick feed No.3 and water *ad libitum*. Stage of estrous cycle was determined. The females found to be in late pro-estrous and estrous were kept over- night with males in separate cage in 2:1 female to male ratio. Presence of vaginal plug in the morning or sperms in vaginal smear confirmed successful mating and that day was taken as day one of pregnancy.

Pregnant female mice were separated from males and divided into various groups of eight animals each. They were labeled and given oral doses of Chlorpyrifos and/or Folic acid with the help of a plastic syringe, according to the schedule in Table 1. Pregnant female mice were sacrificed on the 15th day of gestation, under ether anesthesia. The two horns of gravid uteri were dissected out. Implantation sites were carefully observed and resorptions noted. Number of live fetuses in each horn of uterus was noted. The uteri were cut open and the fetuses were carefully recovered. These fetuses were then put in Bouin's fixative for 48 h, and washed in 70% alcohol to remove the fixative. The separated fetuses were preserved in 80% alcohol.

Morphometeric and morphological study of 50 fetuses per group was done. Crown-rump length of fetuses was measured with a millimeter scale and the weight was determined in milligrams on an electric balance. Detailed morphological study was carried out under dissecting microscope using a magnification of 10 x. Head, ear, eye, trunk, tail and skin characteristics were carefully examined and compared in different experimental and control groups. Anteroposterior and transverse diameter of skull and length of tail was taken with a digital vernier caliper. The head circumference was calculated from skull dimensions. Head circumference to CR length ratio and tail length to CR length ratio was calculated and compared. Any abnormality noticed in morphological examination, was recorded. Selected fetuses were photographed with a digital camera.

RESULTS

Control groups

Group E (plain control)

The fetuses of control group E had a semi curved body which was distinctly divisible into head, trunk and tail regions. Skull had acquired a rounded smooth contour and suture lines were faintly visible under the dissecting microscope. Eyes were well formed showing an elliptical aperture and bulging rounded lens in the middle. Upper and lower eyelids had also reached a considerable state of development but still were unable to completely close the eyes.

External ears were also advanced in development. An external auditory meatus could be clearly seen almost completely hidden by a large well developed pinna. The snout had also taken its typical protruding shape, with two nostrils located quite close to each other at its anterior tip.

Upper and lower lips were also clearly defined. Multiple rows of vibrissae were visible at the sides of the two nostrils. Trunk part of the fetus was divisible into a relatively flat thorax and a protuberant abdomen. The outline of the liver could be made out easily. Tail was quite long and reached till the snout area. It showed the typical curve towards the cranium which is quite peculiar to this stage of development (Figure 1).

Group D (folic acid control)

The control fetuses of group D (given only folic acid on GD 6 and 7) were similar to group E control fetuses in morphological appearance. The morphometric results are shown in Tables 2 and 3.

Experimental groups

Group A (given 100 ug/gbw chlorpyrifos on GD 6 and 7)

The general morphological appearance of fetuses of this group varied considerably. The small fetuses appeared to

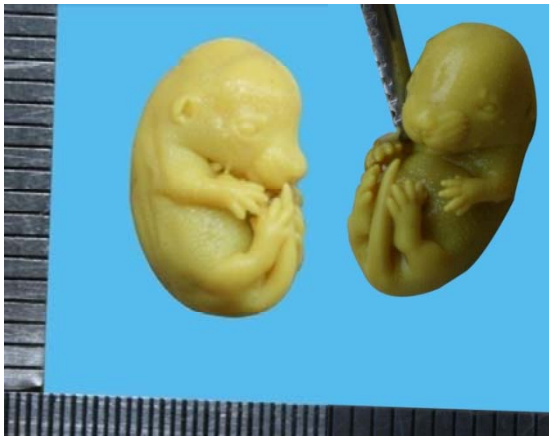

Figure 1. Photograph of lateral and anterior view of fetus of control Group showing normal development of skull, eye, pinna, snout, upper limb, lower limb, trunk and tail.

Table 2. Quantitative data of morphological features of fetuses of various groups.

Group	Number of fetuses recovered	Number of resorbtions	Variation in litter size	Mean CR length(mm)	Mean Wt. in mg	Mean head circum (mm)	Head circ.: CR length (mm)
Group A	30	All fetuses resorbed in two mice	2 to 9	8.84	133.47	13.88	1.57:1
Group B	47	15	3 to 10	11.22	247.51	16.17	1.45:1
Group C	42	17	6 to 8	11.32	249.4	16.01	1.42:1
Group D	66	1	8 to 10	13.63	377.10	19.60	1.44:1
Group E	61	0	8 to 12	14.32	399.68	20.26	1.42:1

Table 3. Morphological features of fetuses of various groups.

Group	Skull	Wide Fontanellae (%)	Hges. (%)	Disturb body axis (%)	Eyes absent (%)	Rounded eyes (%)	Small eyelids (%)	Small pinna (%)	Low set ears (%)
Group A	Small	43.3	16.6	3.3	30	66.6	56.6	80	33.3
Group B	Small in all	23.4	17	8.5	2.1	59.5	23.4	68.1	4.2
Group C	Small in 50%	28.5	2.3	2.3	0	26.2	19	40	7.8
Group D	Round smooth	0	Nil	Nil	Nil	Nil	nil	nil	Nil
Group E	Round smooth	0	Nil	Nil	Nil	Nil	Nil	Nil	Nil

be a curved mass of tissue in which head and tail ends could be made out with difficulty. Lower half of the body was almost as thick as the cranial end and tail was not distinguishable from the rest of the body (Figure 2). There were absolutely no impressions in the head area indicative of ear and eye differentiation. Limb buds had just started forming. These buds were very small and not discernable into parts. In one fetus only one upper limb bud had formed. Various levels of developmental arrest and malformations were observed in fetuses of this group. Body axis was abnormal in 4 fetuses, showing scoliosis, a twist in the spine or increased curvature (Figure 3).

Tail was very variable in size and appearance. Mean length of the tail was 4.79 mm. It was so long in few fetuses that it was extending beyond the snout. In few fetuses it was twisted (Figure 4a). In small fetuses the tail appeared almost as thick as the rest of the body. 16.6%

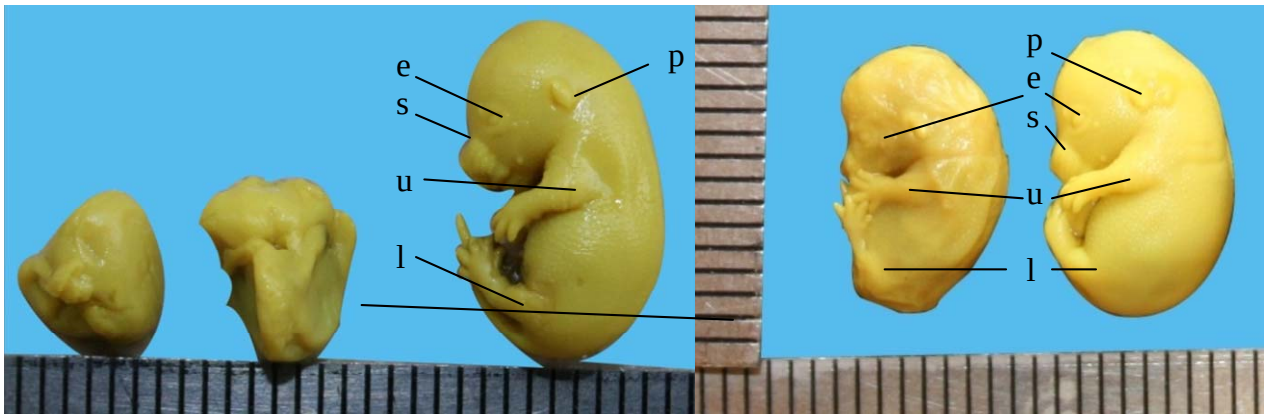

Figure 2. Photographs of group A fetuses (given 100 ug/gbw chlorpyrifos on GD 6 and 7) with different sizes and developmental status. e, Eye; s, snout; p, pinna; u, upper limb; l, lower limb.

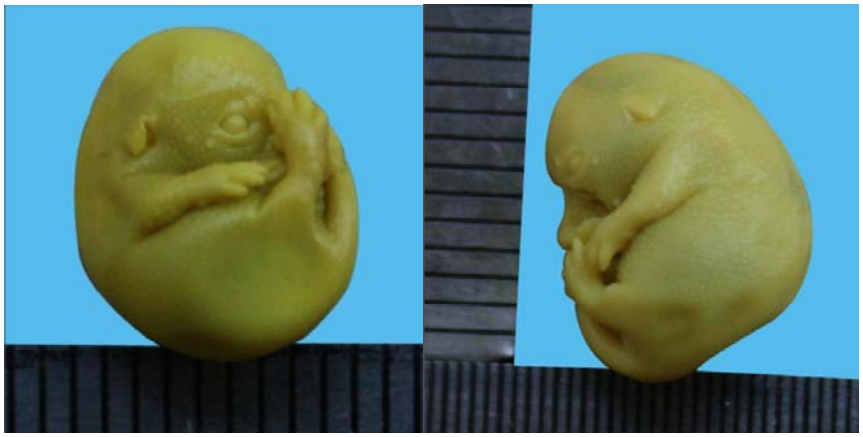

Figure 3. Photograph of fetuses with abnormal body axis. Note scoliosis.

fetuses showed hemorrhagic spots in skin at different regions (Figure 4b). Tail: CR length ratio was 0.54:1 in group A fetuses, which was not significantly different from control. Skull of almost all fetuses was found to be small and in 43.3% fetuses, fontanellae were wide. The contour of the skull was not round and smooth as in control. Two fetuses showed high degree of convexity in the cervical region whereas another two showed a raised skull in parietal region. (Figures 4 to 5).

In 20 fetuses, eyes were round indicating immaturity in the stage of development. In 17 fetuses eyes were very small in size in comparison to control, whereas there were faint impressions in the region of eyes showing the initiation of formation of eyes in five fetuses. In another four fetuses, eyes were not formed at all. In 80% fetuses, pinna was formed but was quite small as compared to control fetuses. In three fetuses there was just a faint pit indicating the ear region and no indication of pinna formation. In 33.3% fetuses, pinna was located at a lower level in comparison to control (Figures 4 to 6).

Group B (given chlorpyrifos and folic acid on GD 6 and 7)

In most of the fetuses, eyes were normal in position. In 23.4% fetuses eyes were small. In one of the fetus, right eye was smaller than the left. In another fetus eyes were not visible at all. In 59.5% fetuses the shape of the eyes was round indicating immaturity in development. Pinna was quite well formed in 31.9% fetuses however in 20 fetuses it was smaller in size in comparison to control. In one fetus the size of pinna was different on two sides, the left one being smaller. The location of the pinna was normal in majority of the fetuses of this group (Figure 7).

Group C (given chlorpyrifos on GD 6 and 7 and folic acid on GD 1 to 15)

The skull showed protuberance in parietal region in two fetuses, whereas another one showed a bulge in occipital

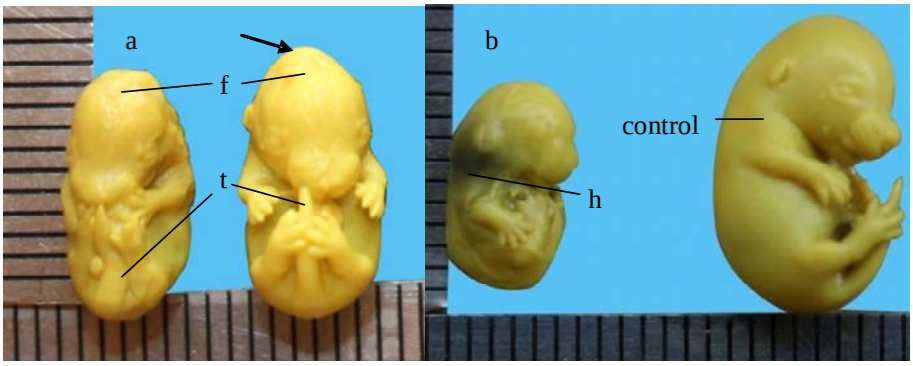

Figure 4. (a). Group A fetuses Left- with f. prominent fontanella and t. twisted tail, Right- with f. prominent fontanella and bulge in the parietal area of the skull (arrow); t, tail extending straight upto snout. **(b).** Lt. Deformed group A fetus with h. hemorrhagic spots. Rt, Control fetus.

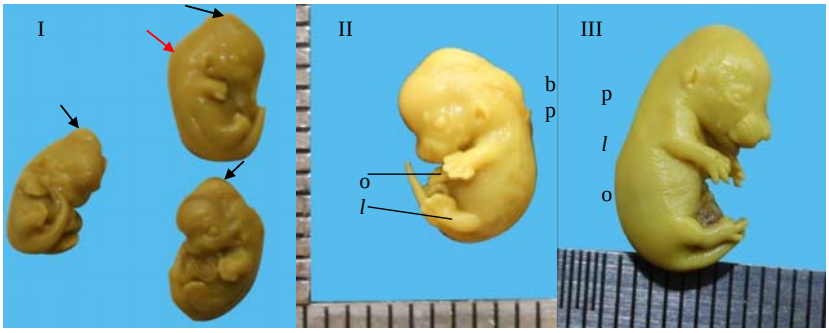

Figure 5. Photographs of deformed fetuses. **(I)** Parietal bulge (black arrow), cervical bulge (red arrow). **(II)** *l,* Paddle shaped limb buds; b, occipital and cervical bulges; p, small pinna and o, omphalocoele. **(III)** p, small pinna; *l*, straight limb bud; o, omphalocoele.

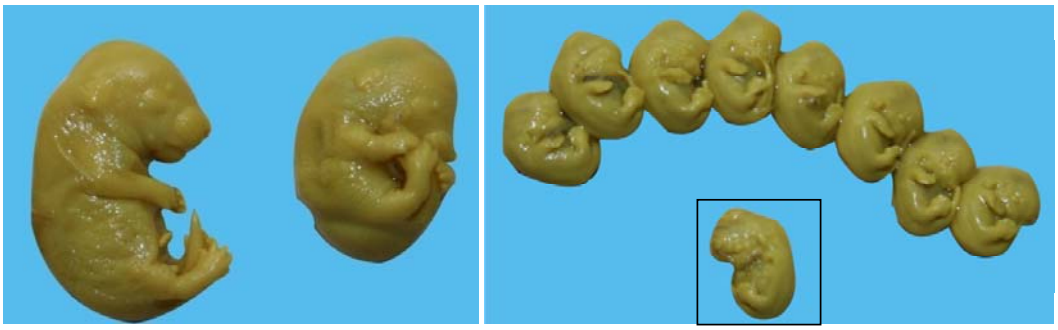

Figure 6. Photograph of affected fetuses. **(I)** The small size of pinna, faint impressions of closed eyes, variation in tail, limb buds and body curvature. **(II)** Photograph of affected fetuses of group A. Note the level of pinna, variation in length of tail and paddle shaped limb buds. Boxed fetus shows amelia of the upper limb.

region. In most of the fetuses, eyes were normal in position, but the shape and size revealed variation. The eyes were round in 11 out of 42 fetuses whereas the size was quite small in comparison to control in 8 out of 42 fetuses. Pinna formation was apparently normal in most of the fetuses. It was quite small in comparison to control

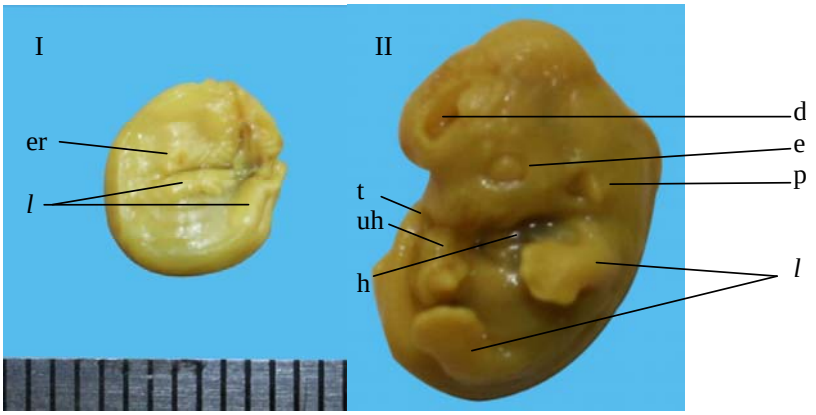

Figure 7. Photographs of malformed fetuses of group B (given 100 ug/gbw chlorpyrifos and 2 ug/gbw folic acid on GD 6 and7) showing: (I) er, ear pit; *l*, malrotated limb buds; (II) p, very small pinna; *l*, Paddle shaped limb buds; e, rounded eyes; d, deficient skull bones; t, long tail uh. umbilical hernia; h, thin skin with hemorrhages.

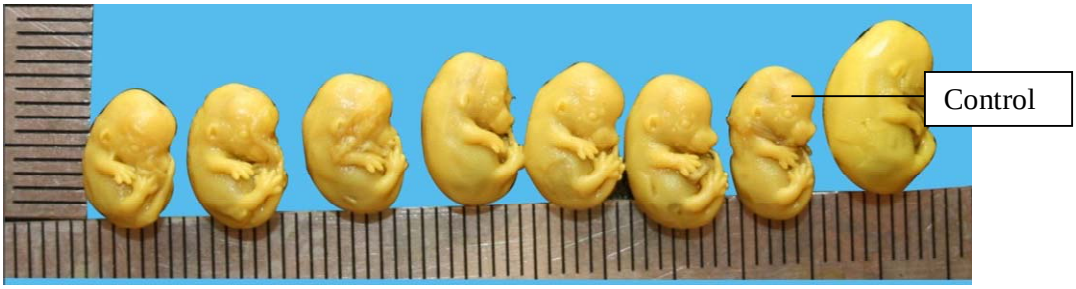

Figure 8. Photograph of a litter recovered from group C mouse (given 100 ug/gbw chlorpyrifos on GD 6 and 7 and 2 ug/gbw folic acid on GD 1-15). The fetus on extreme right is from the control group. Note the difference in size and level of development.

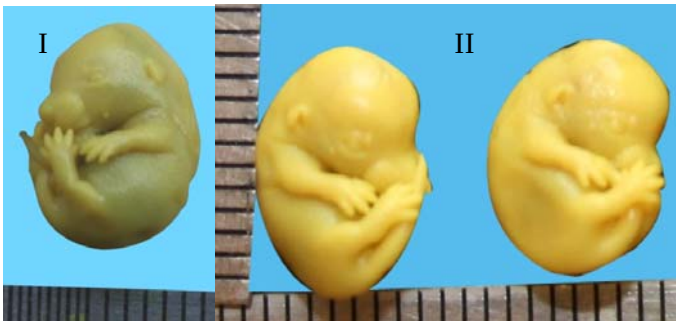

Figure 9. Photographs of group C fetuses (given 100 ug/gbw chlorpyrifos on GD 6 and 7 and 2 ug/gbw folic acid on GD 1-15). **I.** Increased curvature and absent elbow bend. **II.** Increased curvature and long tail extending beyond snout.

in 13 out of 42 fetuses. In three fetuses pinna was located at a lower level. (Figures 8 to 9).

Histograms showing the comparison of mean CR length, mean weight of fetus and mean head circumference to CR length ratio are shown in Figures 10 to 12.

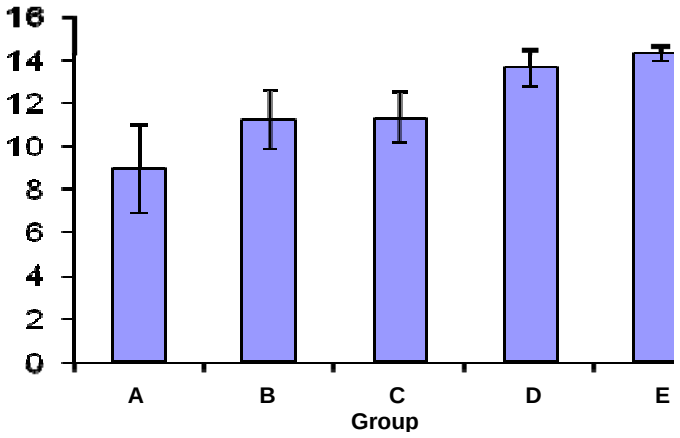

Figure 10. Histogram showing comparison of mean CR length.

Table 4. Statistical analysis of data for variations in skin features and body axis of fetuses.

Group	Skin					Body Axis		
	Normal	Hge	Wrinkles	others	total	Normal	Distorted	Total
A	24	5	1	0	30	26	4	30
B	38	8	0	1	47	43	4	47
C	41	1	0	0	42	41	1	42
D	46	4	0	0	50	50	0	50
E	50	0	0	0	50	50	0	50
Total	199	18	1	1	219	209	9	218

Chi-square likelihood ratio 24.679 and 14.622, P-value 0.006.

Statistical analysis

Morphometeric parameters

ANOVA test revealed significant difference between experimental and control groups. For comparison between different groups the data was further subjected to Bonferroni analysis. Group A fetuses, (given chlorpyrifos on GD 6 and 7) when compared with other experimental and control groups revealed significant difference (p-value <0.001). The difference between the values from fetuses of group B (given chlorpyrifos and Folic acid on GD 6 and 7) and C (given chlorpyrifos on GD 6 and 7 and Folic acid on GD 1 to 15) was not significantly statistically. (P-value of 1.0). Fetuses of groups B and C however were significantly different from those of control groups (D and E), with p-value <0.001. The fetuses of two control groups (D and E) were quite similar with insignificant statistical difference. Tukey test revealed that fetuses of group A were affected the most.

The difference between control group (E) and experimental groups (A, B and C) was statistically significant in following parameters (Tables 4 to 7): 1. CR length, weight, head circumference, tail length, size and shape of eyes; 2) The difference in head circumference: CR length ratio was statistically significant only between group A and all other groups including control; 3) As regards skin hemorrhages, body axis and presence of protuberance in different regions of skull, the occurrence of these abnormalities was statistically significant in fetuses of group A and B, and 4) A statistically significant difference of group A and C fetuses from control was noted in location of eyes and ears at lower level and a small size of pinna.

DISCUSSION

It has become quite clear from this study, that chlorpyrifos is teratogenic to mice fetuses in a single oral dose of 100 ug/gbw when given on 6^{th} and 7^{th} day of gestation. On giving chlorpyrifos, there was decrease in the litter size and increase in the number of resorptions and malformations. The CR length and weight of chlorpyrifos treated fetuses was significantly reduced in comparison to control.

When 2 ug/gbw dose of folic acid is given at exposure to chlorpyrifos, it leads to reduction in the number of

Table 5. Statistical analysis of data for variations in fontanellae and skull bulge in fetuses.

Group	Fontanella				Skull Bulge		
	Approx (normal)	Open (Prominent)	Wide (v.prominent)	No comments	parietal region	cervical region	occipital region
A	17	5	7	1	2	2	0
B	29	11	6	1	10	0	0
C	27	12	3	0	2	0	1
D	50	0	0	0	0	0	0
E	50	0	0	0	0	0	0
Total	172	28	16	2	14	2	1

Chi-square likelihood ratio 72.282 and 35.851, P-value<0.001.

Table 6. Statistical analysis of data for variations in position, size and shape of eyes of fetuses.

Group	Position of eyes			Size of eyes				Shape of eyes		
	normal	lower	No comments	Normal	small	Very small	Not formed	elliptical	Round	No comments
A	20	6	4	0	9	17	4	0	20	10
B	46	0	1	34	11	1	1	18	28	1
C	38	4	0	34	6	2	0	31	11	0
D	50	0	0	50	0	0	0	50	0	0
E	50	0	0	50	0	0	0	50	0	0
total	204	10	5	168	26	20	5	149	59	11

Chi-square likelihood ratio 40.585, 156.517 and 176.811, P-value <0.001.

Table 7. Statistical analysis of data for variations in size and position of pinna of fetuses.

Group	Size of pinna						Position of pinna		
	Very well formed	Well formed	small	Very small	Not formed	Different on two sides	normal	low	No comments
A	0	1	2	24	3	0	17	10	3
B	15	10	20	0	1	1	45	1	1
C	17	12	11	2	0	0	39	3	0
D	50	0	0	0	0	0	50	0	0
E	50	0	0	0	0	0	50	0	0
total	132	23	33	26	4	1	201	14	4

Chi-square likelihood Ratio =255.401 and 47.301, P-value < 0.001.

resorptions and frequency of anomalies. There was also significant improvement in CR length and weight of fetuses on giving folic acid along with chlorpyrifos on GD 6 and 7. However the CR length and weight of fetuses given folic acid and chlorpyrifos were significantly less than the control. When folic acid was continued for longer period, from GD 1 to 15 in the same dose, there was no additional benefit.

Tian et al. (2005) evaluated the potential teratogenicity and developmental toxicity of chlorpyrifos in mice. A single intraperitoneal injection of 80 mg/kg on GD10 resulted in significant reduction in number of live fetuses and increase in resorptions, as compared to control fetuses. They also observed, external and skeletal malformations and cleft palate with this dose of chlorpyrifos, however there was no indication of maternal toxicity. Chanda et al. (1999) and Ahmad and Asmatullah (2007) have also reported chlorpyrifos to be teratogenic to mice embryos. The results of present study are quite similar to these studies. In the present study a high dose

of chlorpyrifos, proved to be embryotoxic and teratogenic in mice when given during the period of organogenesis. The number of resorptions was significantly increased (19 resorptions) as compared to control (1 resorption). External malformations of eyes and ears were noticed.

A dose of 100 ug/gbw on GD 6 and 7 did not produce any overt toxicity in mothers in the present study. Two mothers showed transient hyper salivation and restlessness after the second dose, which was completely reversible. Ahmad and Asmatullah (2007) calculated the LD 50 of chlorpyrifos in the range of 144 ug/gbw. A dose lower than LD50, was given to mothers in the present study. This may be the reason why no significant toxic effects were observed in mothers.

Calvert et al. (2007) reported the birth of three infants with congenital anomalies, in Collier County, Florida. They were born within 8 weeks of one another and their mothers worked for the same tomato grower. All three mothers worked during the period of organogenesis in fields recently treated with several pesticides. Although it was difficult to pin point which particular pesticide was responsible, but this incident definitely highlights the need to reduce the pesticide exposure to the pregnant population. There is also a further need for epidemiological studies to examine the role of pesticide exposure in the etiology of birth defects. At the same time the use of nutrients which may reduce the incidence of congenital anomalies must be encouraged. Folic acid is one of such nutrients.

Role of folic acid in the prevention of neural tube defects is a well established fact and is backed by many experimental and epidemiological studies. Folic acid has been shown to reduce the incidence of neural tube defects caused by valproic acid (Dawson et al., 2006; Padmanabhan and Shafiullah, 2003) retinoic acid (Reynolds et al., 2003; Firat et al., 2005), ochratoxin A (Katagiri et al. 2007), Fumonisin B1 (Sadler et al., 2002), ethanol (Yanaguita et al., 2008), arsenic (Gefrides et al., 2002) and hyperthermia (Shin and Shiota, 1999; Li et al., 2003). Its role in the prevention of other congenital defects has not yet been fully explored. Since chlorpyrifos has been found to be teratogenic to many organs and systems. Thus, the role of folic acid in the prevention of chlorpyrifos induced teratogenicity becomes quite important.

In the present study, oral dose of 2 ug/gbw folic acid was given with an intention to reverse or decrease the teratogenic effects of chlorpyrifos. When given on GD 6 and 7, folic acid was able to decrease the frequency of congenital malformations induced by chlorpyrifos. When once daily dose of folic acid was continued over a period of 15 days, there was no further reduction in the frequency and severity of birth defects. Moreover it is interesting to note that in the present study there are indications that folic acid therapy from GD1 till the exposure to chlorpyrifos on GD 6 and 7, was unable to prevent the teratogenic damage. Once the damage was done, further doses of folic acid were unable to reverse the damage.

In an attempt to observe the effects of ethanol and folic acid deficiency on outcome, the pregnant mice with different folate status when exposed to different doses of ethanol from 7 to 9th GD. The animals were sacrificed on 18th GD. The results of this experiment revealed that in animals receiving a commercial diet, a high dose of ethanol was deleterious to pregnancy, including congenital anomalies, intrauterine growth restriction, reduction of the placenta and increased late fetal deaths and resorptions, events that did not occur with the low dose of ethanol. However, with a folate free diet, a low ethanol dose was as deleterious as a high dose.

Yanaguita et al. (2008) concluded that supplementation with the recommended dose of folic acid (2 mg/kg), was not effective in preventing the deleterious teratogenic effects induced by ethanol, indicating the need for an increased dose. Firat et al. (2005) in their experiment on rats, administered Retinoic acid in different doses, orally on GD8. Folic acid of 4.0 mg/kg was injected intra-peritoneally on 7th to 9th GD. Folic acid administration prevented the decrease in mean fetal weight and height of the embryos treated with 40 mg/kg retinoic acid. In addition there was a marked decrease in the number of degenerated chondrocytes and an improvement in the structure of granular endoplasmic reticulum along with intact nuclei.

Folic acid of 4.0 mg/kg used in this study was reported to be in the therapeutic range since there was no pathological alteration neither in body weight and height nor in histological appearance of Meckel's cartilage of the embryos born to mothers treated with Folic acid alone (Firat et al., 2005). We have seen that even 2 ug/gbw (equal to 2 mg/kg) folic acid has been effective in improving the fetal weight and height of chlorpyrifos treated fetuses. In the present study, a dose of 2 mg/kg folic acid did not produce any toxic effects neither in mothers nor in fetuses. This dose was also not teratogenic to the fetus. The CR length and weight of fetuses given folic acid was more than the mean values of control fetuses but the difference was statistically insignificant. Thus there was no harmful effect of giving folic acid throughout pregnancy, on the fetal development. It should be noted that the handling of mothers for giving the daily doses of folic acid did not adversely affect the fetal development either or folic acid therapy was able to counteract the effect of stress of experimentation.

It has been confirmed at multiple levels that folic acid consumption decreases the incidence of neonatal deaths mainly by decreasing neural tube defects (NTDs) (Blencowe et al., 2010; Hamner et al., 2009). In countries like US, in order to prevent NTDs, folic acid fortified food items are made available to general population and there are various public awareness programs emphasizing the usefulness of folic acid consumptionn in women of child-

bearing age. In spite of all the efforts, it was found out in a study conducted in Atlanta from 2003 to 2006, that only 24% of non-pregnant U.S women of childbearing age consumed the recommended dose of folic acid (Tinker et al., 2010).

Pakistan is an agricultural community where organophosphate pesticides are widely used. People are generally indifferent to instructions due to illiteracy and pregnant population is exposed to harmful effects of chemicals including pesticides. Lassi and Butta (2012) have recently discussed the possibility of adding folic acid to oral contraceptives in this country. An evaluation of present health strategies needs to be done and measures must be employed to make women aware of importance of avoiding exposure to pesticides and promote the use of folic acid.

Conclusion

Chlorpyrifos is teratogenic to mice fetuses when given on GD 6 and 7 of gestation. Administration of folic acid on GD 6 and 7 does ameliorate the adverse effects of this pesticide to a considerable extent however, administration of folic acid for a longer duration does not appear to have an additional benefit in alleviating the adverse effects of chlorpyrifos. It is further concluded that folic acid administration to pregnant mice in a dose of 2μg/gbw from gestational day 1 to 15 does not have any toxic or teratogenic effect.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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