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Original Scientific Article

EFFECTS OF ORGANOPHOSPHATE CHLORMEPHOS ON DEVELOPMENT AND FUNCTION OF TESTES AND BRAIN

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Summary: Several chemicals, known as endocrine disrupting chemicals have the capacity to interfere with hormone action in the mammalian body. The aim of our study was to establish whether long term exposure to low doses of organophosphorus compound Chlormephos affects the development and function of reproductive tract and endocrine parts of the brain. Adult male and female mice were exposed to 3.5µg/ml and 0.35µg/ml of Chlormephos in the drinking water during mating, pregnancy and lactation until weaning of the offspring. Testes development was studied in the offspring of exposed mice by monitoring daily sperm production, number of apoptotic cells and spermatogenesis. Immunoexpression of antimüllerian hormone and 3ß-hydroxysteroid dehydrogenase was assessed qualitatively in testes from 9 and 19 days old offspring, respectively. No statistically significant differences (or qualitative differences in assessment of immunoexpression) were found in any of the observed parameters, suggesting that low dose of Chlormephos does not act as an endocrine disruptor in reproductive tract. To examine whether exposure to low doses of Chlormephos affect brain development, offspring of the exposed mice were tested for anxiety like behaviour in the elevated plus maze. Mice exposed neonatally to the higher dose of Chlormephos spent significantly less time in the open arms of the elevated plus maze in comparison to mice from control group, suggesting an increase in the anxiety like behaviour. Ultrastructural analyses did not reveal any changes in brain ultrastructure, in particular in blood-brain barrier which has been reported before to be a target for organophosphorous compounds.

Key words: mice; testis; brain; organophosphorus; daily sperm production; electron microscopy; apoptosis; anxiety-like behavior

Introduction

Endocrine disruptor chemicals (EDCs) in the environment could possibly interfere with the endocrine system and mimic or depress the function of endogenous hormones. EDCs usually interfere with the endocrine system through hormone receptors such as estrogen and androgen receptors. In recent years, several studies have shown that environmental contaminants such as isomers of dichlorodiphenyltrichloroethane (DDT), some polychlorinated biphenyls (PCBs), the plasticizer bis-phenol A, and some detergents such as p-nonylphenol, can exhibit estrogenic or anti-androgenic activity (1).

Organophosphates (OPs) are a large class of chemicals, which act as irreversible inhibitors of acetylcholinesterase (AChE). OPs cause accumulation of neurotransmitter acetylcholine and they are often used in farming as insecticides and antiparasitics (2, 3). Chlormephos (*S*-chloromethyl *O,O*-diethyl phosphorodithioate) is an organophosphorous (OP), insecticide that was introduced to the market in 1973 to control soil-dwelling pests (4). Chlormephos was in use until 2006 when it was withdrawn from all EU markets.

OP could act as endocrine disruptors in reproductive system, brain development and behavior. *Sarkar et al.* reported that OP pesticide quinalphos

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disrupts hypothalamo-pituitary-gonadal axis. Sublethal chronic administration of quinalphos resulted in decreased testicular mass and AChE activity in central and peripheral organs; increased serum LH, FSH, prolactin and testosterone concentrations; decreased pituitary and increased testicular activity and severe disruption of spermatogenesis with increasing doses of pesticide (5). *Verma and Mohanthy* (6) showed that *in utero* and lactational exposure of mice to low doses of dimethoate caused reduced testis weight, reduced sperm count, histopathological changes in testes and epididymidis, lower plasma LH and testosterone and reproductive dysfunction of adult mice. Study by *Umzucu et al.* (7) demonstrated that early postnatal exposure of rats to metoxyclor resulted in reduced ovarian weight, inhibition of folliculogenesis and stimulation of anti-müllerian hormone production.

Several studies investigated effects of low-level exposure to OPs pesticides during pregnancy on neurodevelopment and behavior of newborns. Prenatal exposure of children to higher doses of chlorpiryfos resulted in Psychomotor Development Index and Mental Development Index delays, attention problems, attention-deficit/hyperactivity disorder problems, and pervasive developmental disorder problems at 3 years of age (8). Developing blood-brain barrier is highly vulnerable to single or repeated exposure to low doses of certain pesticides such as organophosphate quinalphos, pyrethroid cypermethrin and organochlorine lindane although there seems to be some species differences in vulnerability of blood-brain barrier to the pesticides (9, 10).

The aim of our study was to determine whether long term exposure to low levels of organophosphorus compound Chlormephos can affect the development and functioning of testes and brain in mice. Although there are no data about human exposure to Chlormephos, the data for exposure to other organophosphorous pesticides show that human dietary or environmental exposure is similar to doses used in our study (11, 12).

Material and methods

Animals

Sexually mature (60 – 70 days old) BALB/c male and female mice (all together 18 control females, 16 females exposed to 3.5 μg/ml and 12 females exposed to 0.35 μg/ml Chlormephos) were breed in standard conditions at 20 - 25° C, humidity 55–65%, dark : light cycle 12:12, with chow without phytoestrogens (2916, Harlan Tekland, England) ad libitum. All animal experiments were approved by the Veterinary commission of Slovenia (approval number 3440-176/2006) and were done according to the EU directive and NIH guidelines.

Mice were supplied either with drinking water containing 3.5 μg or 0.35 μg of Chlormephos per ml (10 and 100 times lower dose of LD50 for mammals) (13). As Chlormephos is insoluble in water, it was first dissolved in ethanol at concentration 1 mg/ml, and this stock solution was used for preparing final drinking water solution. Final concentration of ethanol in the drinking water was 3.5 μl per ml in group exposed to 3.5 μg of Chlormephos per ml and 0.35 μl per ml in group exposed to 0.35 μg of Chlormephos per ml. Control group received drinking water without Chlormephos, containing 3.5 μl of ethanol per ml (0.35 %, vol/vol).

The effects of Chlormephos were monitored in the offspring of exposed animals. Sexually mature male and female mice were randomly divided into control or treated groups; weighted and exposed to Chlormephos/Ethanol for seven days. After seven days, one male and one female mouse were joined in a single cage. Mice were exposed to Chlormephos/Ethanol throughout pregnancy and lactation period until offspring weaning at 21 days. Litter size was carefully monitored; pups were always counted on the day of delivery. Male pups from different litters were randomly assigned to groups for sacrifice at different postnatal age (in each age group of mice, pups from at least three different litters were included). Pups were sacrificed at 9, 19, 48 and 70 days of age, 48 and 70 days old mice were exposed to Chlormephos/Ethanol only until weaning on day 21. Nine and 70 days old mice were sacrificed by CO_2 exposure followed by cervical dislocation. Mice 19 and 48 days old were anaesthetized by a mixture of ketamine (Vetoquinol Biowet, Gorzowie, Poland; 100 μg/g BW), xylazine (Chanelle Pharmaceuticals Ltd., Loughrea, Ireland; 10 μg/g BW) and acepromazine (Fort Dodge Animal Health, Fort Dodge, IA, USA; 2 μg/g BW) and perfused with Bouin's solution. Testes were isolated and postfixed in Bouin's solution for 4 to 20 hours depending on size. Subsequently, testes were processed into paraffin wax using standard procedures. At the time of sacrifice, whole mice as

well as testes and seminal vesicles were weighted. Testis weight (average of left and right testis) was expressed as a percentage of total body weight (= relative testis weight). Some offspring of exposed animals were breed in adulthood, number of pups was monitored. Anxiety tests were performed on offspring of exposed mice.

Effects of Chlormephos on daily sperm production (DSP)

Daily sperm production was examined in 70 days old male offspring of treated and control mice as described before (14). Briefly, testes were dissected, weighed and homogenized in 5ml of physiological saline containing 0.05% Triton X-100. Spermatozoa were counted in Bürker – Türk chamber under 400x magnification. Ten visual fields per sample were counted and daily sperm production was calculated using formula N x 25 x 5000 (N = number of counted cells, 25 and 5000 dilution factors), divided by testes weight and this result was divided by 4.48, thus obtaining average DSP.

Immunohistochemistry

Immunohistochemistry was performed as described before (15) using antibodies against antimüllerian hormone (AMH) in concentration 1: 200 and 3β-hydroxysteroid dehydrogenase (3β-HSD) in concentration 1:1000 using peroxidase anti-peroxidase system. Specific rabbit antibodies against AMH were gift from Natalie Josso (France), polyclonal rabbit antibodies against 3β-HSD from dr. Ian Mason (United Kingdom).

Apoptosis detection

Apoptotic cells were detected by ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA)) following manufacturer's protocol. Apoptotic cells were counted in testicular sections in 40 visual fields for each sample in testes from 48 old male mice exposed neonatally to Chlormephos. Results are presented as average number of apoptotic cells in one visual field.

Anxiety-like behavior test

In 70 to 80 days old mice of both sexes, standardized test for anxiety-like behavior using elevated plus maze was performed (16). Tests lasted 5 minutes during the beginning of the dark cycle with dim red light. Time in opened and closed arms of the maze as well as latency to enter closed or opened arms was recorded using Stopwatch+ program (version 1.5.1, Center for behavioral neuroscience, Atlanta, USA).

Electron microscopy

For electron microscopy, offspring from control and higher concentration Chlormephos treated group were sacrificed by carbon dioxide at 70 days of age. Part of the brain cortex from the frontal lobe was immediately fixed in 4% of glutaraldehyde for 2 hours. Samples were prepared by a standard procedure for electron microscopy with Durcupan (Fluka, Electron Microscopy Sciences, Hatfield, Pennsylvania, USA) embedding medium. Briefly, the brain tissue fixed in glutaraldehyde was rinsed several times in a 0.05M phosphate buffer (pH=7.1-7.4, 800 mOsm), postfixed with 1% OsO_4 and dehydrated in a series of ascending alcohols. Tissue was then incubated in a mixture of absolute alcohol and acetone (1:1, 30 min.), absolute acetone (15 min.) and mixture of absolute acetone and Durcupan (Fluka). This step was followed by embedding the brain tissue into 100% Durcupan. After a polymerisation of the embedding medium in a thermostat (at 60°C, 72 hours), tissue blocks were trimmed and prepared for sectioning. Semithin sections (section thickness = 0.9 μm) were made by Power-Tome XL ultramicrotome (Bal Tec/RMC) and stained with 1% toluidine blue. The sections were analysed by Nikon binocular microscope (Eclipse E200). Based on the bright filed microscopic analysis of semi-thin sections, ultra-thin sections were made (section thickness = 70 nm), placed on copper grids, and contrasted with lead citrate and uranyl acetate. The sections were then examined by transmission electron microscope Zeiss 902A (Centre for Electron Microscopy, Medical School University of Zagreb).

Statistical analyses

Differences between groups were analyzed by two-way analysis of variance with sex and treatment as independent variables, followed by Bonferroni posthoc test to determine differences between groups. Statistical significance was determined at $p < 0.05$.

Results

Litter size, body size and testis size in control and treated groups

We have reported before in our report of preliminary data (17) that there were no differences in the litter size between control and Chlormephos exposed groups (mice that received Chlormephos through drinking water). There were also no differences in body size, absolute testis weight, relative testis weight and seminal vesicle weight between control mice and mice exposed to either concentration of Chlormephos during neonatal period through mothers (17). Similarly, when adult mice, exposed to Chlormpehos in neonatal period were mated, no statistical differences in litter size were observed with average 4.5 ± 0.9 (n=8 litters) pups in control group, 5.5 ± 0.8 (n= 4 litters) and 4.8 ± 1.2 (n=4 litters) in groups exposed neonatally to 0.35 and 3.5μg per ml Chlormephos, respectively.

Daily sperm production (DSP)

Similarly to our previously reported preliminary data (17), analysis of daily sperm production at 70 days old mice did not show any statistical differences between control and treated groups. In control group, DSP was 32,259.999 ± 1,602.126 (n=8) while it was 36,207.131 ± 539.750 (n=4) and 30,876.956 ± 5,151.737 (n=5) in groups exposed to high or low concentration of Chlormephos, respectively.

Testes histology, number of apoptotic cells and 3β-HSD and antimüllerian hormone expression

Tissue sections stained with haematoxylin and eosin from testes of all age groups of offspring were examined to determine any irregularity in spermatogenesis. No differences between control and treated groups were noted. Similarly, statistical analysis of apoptotic cells labeled with TUNEL method in 48 days old testes did not reveal any differences in the number of apoptotic cells between groups (n was 5 for each group).

Qualitative assessment of immunohystochemical staining for antimüllerian hormone (testes from 9 days old mice) and 3β-hydroxysteroid dehydrogenase (19 days old offspring) did not reveal any differences in the immunoexpression of these two proteins (Figure 1).

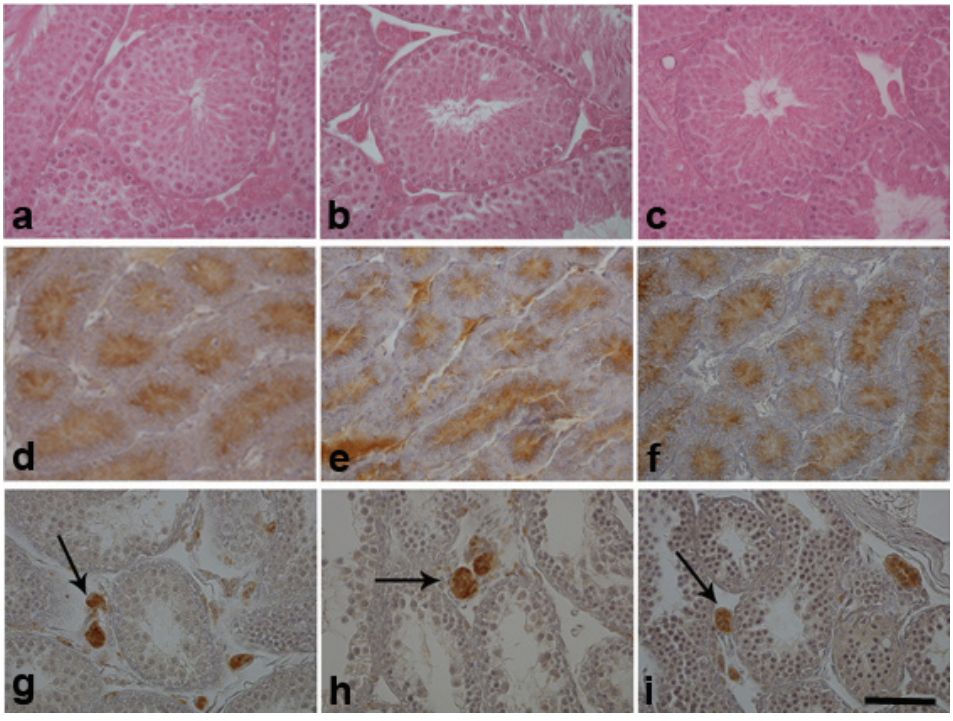


Figure 1: Histological analyses did not reveal any differences in general testis histology at 48 days of age between control (a) and groups exposed to Chlormephos through their mothers (b – 0.35 μg/ml Chlormephos; c – 3.5 μg/ml Chlormephos). Similarly, there were no obvious differences in intensity or pattern of antimüllerian hormone (d, e, f) immunoexpression in Sertoli cells in testes from 9 days old pups or 3β-HSD immunoexpression (g, h, i) in Leydig cells from 19 days old pups between control (d, g) and Chlormephos exposed mice (e, h – 0.35 μg/ml Chlormephos; f, i – 3.5 μg/ml Chlormephos). Arrows in panels g, h and i represent newly developed adult Leydig cells. Bar = 50 μm

Anxiety-like behavior test

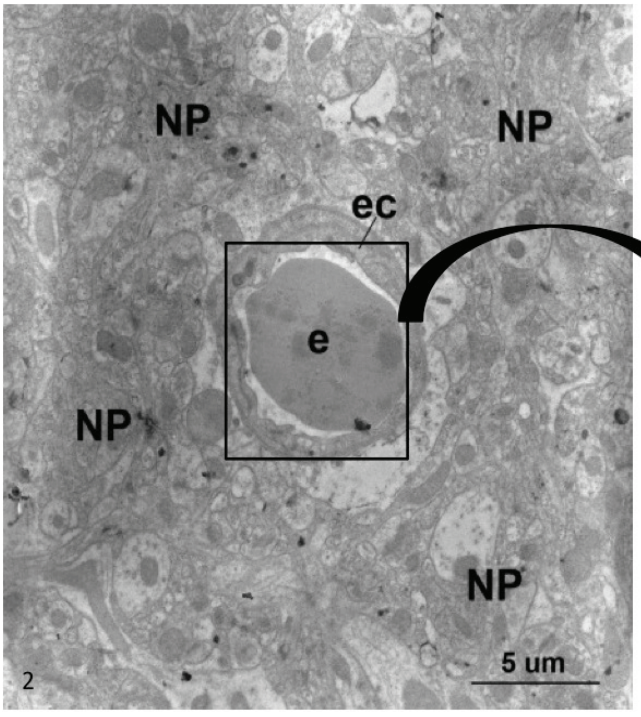
Elevated-plus maze tests at 70 days of age results revealed statistically significant differences between control mice and mice neonatally exposed to higher dose of Chlormephos. There was a significant effect of treatment in both time spent in open arms ($p < 0.05$) and time spent in closed arms ($p < 0.05$). Posthoc bonferroni test revealed significant difference between control groups and group exposed neonatally to 3.5μg per ml of water, but no difference between control group and group exposed neonatally to lower dose of Chlormephos. However, there was no significant difference between control and both Chlormephos exposed groups in latency to enter open arms or number of entrances into the open arm of elevated plus maze (Table 1).

Table 1: Statistical analysis of behavior of mice in elevated plus maze revealed significant difference (* = $p < 0.05$) between control group and group exposed to 3.5 μg/ml Chlormephos in both time spent in open arms and time spent in closed arms. There was no significant difference between groups in latency to enter open arms or number of entries to open arms, and no difference in any of parameters between control group and group exposed to 0.35 μg/ml Chlormephos. All data are presented as mean ± SEM

	Time spent in closed arms (s)	Time spent in open arms (s)	Number of entries into open arms	Latency to enter open arms (s)
Male control (n=10)	73.12 + 40.18	177.34 + 38.63	4.11 + 1.38	28.16 + 21.75
Female control (n=13)	113.19 + 22.31	124.16 + 23.18	5.13 + 1.06	53.53 + 20.84
Male Cl0.35 (n=8)	71.50 + 21.36	170.37 + 38.79	7.67 + 2.11	22.92 + 7.26
Female Cl0.35 (n=8)	113.08 + 29.87	148.65 + 37.50	5.83 + 1.54	53.68 + 32.03
Male Cl3.5 (n=10)	96.08 + 22.66*	105.19 + 29.25*	5.31 + 1.62	47.98 + 32.83
Female Cl3.5 (n=13)	162.44 + 16.88*	50.66 + 14.16*	3.45 + 0.88	84.10 + 30.67

Electron microscopy

Detailed analysis of brain – blood barrier in the cortex region from offspring of control mice and mice treated with higher concentration of Chlormephos (3 females and 3 males in each group) did not reveal any structural changes. In brains from control and treated mice, brain capillaries displayed normal morphology: endothelial cells had a flattened nucleus and an elongated cytoplasm. The euchromatin was located in the central region of the nucleus, whereas a



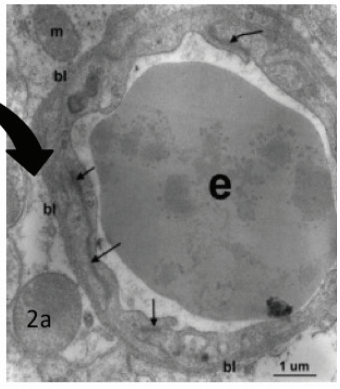


Figure 2: Blood vessel ultrastructure appeared to be intact in the brain from 70 days old mice exposed neonatally to 3.5 μg/ml Chlormephos.

NP – neuropil; e – erythrocyte; ec – endothelial cell; bl – basal lamina; m – mitochondria; arrows – junctional complexes (2 – magnification 3000x; 2a – magnification 7000x)

thick area of heterochromatin was arranged along the nuclear membrane. Within endothelial cells, one could occasionally observe a number of endocytotic vacuoles that were located either in the cell cytoplasm or associated with the cell membrane. Neighboring endothelial cells were joined by well-developed junctional complexes and laid on a continuous basement lamina. There was no damage or disruption of the basement membrane and/or endothelial cell cytoplasm. Capillaries displayed a normal perivascular space in all investigated animals. Neurons and the accompanying glial cells and neuropil preserved normal fine structure in the Chlormephos treated animals when compared to controls (Figure 2).

Discussion

Endocrine disrupting chemicals (EDC) are a group of chemicals that could potentially interfere with an endogenous hormonal system. Most EDCs could interfere with endocrine system by binding to estrogen or androgen receptors although other mechanisms such as interference with expression of activity of steroidogenic enzymes or interactions with aryl-hydrocarbon receptor were described (18, 19). Exposure of animals and human to EDCs could disturb development and function of reproductive system, and possibly of central nervous system (20). The aim of our study was to determine if Chlormephos, an organophosphorous compound, could acts as endocrine disruptor.

Several studies suggested that different organophosphorus compounds can act as EDCs. Narayana et al. (2005) reported, that treatment with metyl parathion in doses relevant for human exposure (0.5-1 mg/kg) resulted in decreased number of spermatozoa and higher number of morphological defective spermatozoa in treated mice, although this treatment did not affect the number of offspring in treated rats. Several other studies also reported effects of certain organophosphorus compounds can affect endocrine system in humans (21, 22) and animals (23, 24, 25, 26). Recio et al. evaluated relationship between OP metabolites in urine and serum levels of pituitary and sex hormones of agricultural workers exposed to mixture of different OP (methylparathion, metamidophos, endosulfan, dimethoate and diazinon) and reported that OP disrupted FSH and LH levels (22).

Okahashi et al. (2005) studied effects of organophosphorus insecticide fenithrotion in rats. Fenithrotion in high doses decreased the activity of brain cholinesterase in parental generation, but did not affect reproduction, organ weight, histopathology and semen parameters in rats treated with sub-lethal doses of fenithrotion. Furthermore, offspring of exposed mice did not differ from control groups in anogenital distance, beginning of puberty, organ weights and histopathology suggesting that fenithrotion did not act as endocrine disruptor (27). These results are similar to the results from our study where we could not find any differences between control and treated groups in any reproductive parameters studied. Our results therefore suggest, that Chlormephos does not act as endocrine disruptor and therefore does not pose a risk for endocrine system even if it would be released into the environment.

In addition to effects on reproductive organs, some endocrine disruptors could also affect central nervous system development and/or function. Some studies have suggested that different chemicals such as bisphenol A and metoxychlor, can affect sexual and non-sexual behaviors (28, 29, 30, 31). Exposure to malathion, chlorpyrifos and other OP during development can result in long-term impacts on intellectual function and delayed effects on the functioning of the central nervous system (8, 32, 33). Blood cholinesterase was also decreased in Wistar rats exposed to low doses of dichlorvos, relevant for human exposure (24).

Animals exposed to higher dose of Chlormephos in our study showed increased anxiety-like behavior in comparison to the control group. Previous studies have shown that organophosphates could negatively affect growth and terminal differentiation of nerve axons in the developing brain, thus causing long lasting changes in the brain that could be expressed only in the adult life (34, 35, 36). Organophosphates are liposoluble, so they can pass across the blood-brain barrier and come into direct contact with nerve cells, causing lasting damage to these cells. Several studies also revealed that low doses of organophosphates could have deleterious, long lasting effect on the blood-brain barrier at least in rodents (9, 10, 37). Interestingly, Song et al. (37) have shown structural damage in the blood-brain barrier in adult rats, exposed neonatally to paroxon, however, when paroxon was given to adult rats, no effect on blood-brain barrier was observed, suggesting that blood-brain barrier might be especially sensitive to paroxon during neonatal period. We exam-

ined cerebral cortex in control and treated mice using electron microscopy, but could not detect any defects in the structure of the brain tissue at the ultrastructural level. In particular, there were no differences in capillaries' walls that are part of blood-brain barrier. However, only small part of the brain was examined by electron microscopy and therefore, it is not possible to firmly conclude that blood-brain barrier was not affected perhaps in some other specific area.

In conclusion, our results showed that Chlormephos treatment of parents did not disturb development of testes and reproductive tract in the offspring of treated mice. Therefore, our study suggest that Chlormephos does not act as an endocrine disruptor in the reproductive system. However, treatment with higher dose of Chlormephos (3.5 µg/ml) produced long lasting effects on behavior as evident by increased anxiety like behavior in mice exposed to Chlormephos only in neonatal period through mothers. This study, therefore, suggests that Chlormephos as a representative organophosphorous compound could affect brain development in experimental animals and further studies are needed to examine possible long lasting effects of organophosphorous compounds on brain development in humans.

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VPLIV ORGANOFOSFATA KLORMEFOSA NA RAZVOJ IN DELOVANJE MOD IN MOŽGANOV

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Povzetek: Različne kemične snovi, pogosto imenovane hormonski motilci, lahko motijo delovanje hormonskega sistema v sesalskem telesu. Namen naše raziskave je bil ugotoviti, ali dolgotrajna izpostavljenost organofosfornemu insekticidu klormefosu lahko vpliva na normalen razvoj spolnega sistema in endokrinega dela možganov. Odraslim mišim obeh spolov smo preko pitne vode dodajali klormefos v koncentracijah 3,5 µg/ml in 0,35 µg/ml. Pari miši so bili izpostavljeni klormefosu 2 tedna pred paritvijo, ves čas brejosti in nato do odstavitve mladičev pri 3 tednih starosti. Razvoj mod pri potomcih izpostavljenih miši smo proučevali s spremljanjem teže mod, dnevne proizvodnje semenčic, apoptoze v modih ter izraženosti beljakovin antimülerjevega hormona in 3-beta hidroksisteroidne dehidrogenaze. Pri nobenem od preiskovanih parametrov nismo ugotovili statistično zanesljivih razlik med kontrolno skupino in skupinama, izpostavljenima klormefosu, kar kaže, da klormefos ne deluje kot hormonski motilec spolnega sistema.
Za ugotavljanje morebitnih škodljivih učinkov klormefosa na možgane v času razvoja, smo pri potomcih izpostavljenih miši ugotavljali obnašanje, podobno anskioznemu s testiranjem v dvignjenem labirintu. Miši, ki so bile med razvojem izpostavljene višji koncentraciji klormefosa, so kazale statistično značilno poudarjeno obnašanje, podobno anksioznemu (več časa so preživele v zaprtih krakih labirinta), v primerjavi s kontrolno skupino. Preiskava možganov z elektronskim mikroskopom pa ni pokazala nikakršnih razlik v ultrastrukturi možganov in krvno-možganske pregrade, čeprav nekatera poročila kažejo, da naj bi bila prav krvno-možganska pregrada prizadeta ob zgodnji izpostavljenosti organofosfornim snovem.

Ključne besede: miš; modo; organofosforne snovi; dnevna proizvodnja semenčic; apoptoza; elektronska mikroskopija; anksioznemu podobno obnašanje.