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Original Research

GESDAV

Genotoxic potential of pirimiphos-methyl organophosphate pesticide using the mouse bone marrow erythrocyte micronucleus and the sperm morphology assay

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ABSTRACT

Background: Pirimiphos-methyl is a potent organophosphate (OP) pesticide used worldwide for the control of pests in stored grains and various insects in domestic, public, agricultural, commercial and industrial settings. However, its pervasive use and broad-spectrum nature could have adverse effects on non-target organisms and the environment which require constant monitoring and assessment. **Materials and Methods:** In this study, the acute toxicity of pirimiphos-methyl, injected intraperitoneally at concentrations of 833.33, 416.67, 166.67 and 83.33 mg/kg was evaluated. Also, the genotoxic and mutagenic potentials of pirimiphos-methyl were assessed using two eukaryotic assays: The micronucleus and sperm morphology assays in mice, at concentrations of 83.33, 41.67, 20.83, and 8.33 mg/kg. **Results:** Administration of 416.67 mg/kg pirimiphos-methyl caused 100% mortality in exposed mice under 24 h. Fifty percent mortality resulted from the administration of 166.67 mg/kg dose. There were dose dependent significant increases ($P < 0.05$) in bone-marrow micronucleated polychromatic and normochromatic erythrocytes as well as aberrant sperms (wrong angle hook, amorphous, banana, no-hook heads, double-tailed and folded sperms) equivalent to the cyclophosphamide induced aberrations. **Conclusion:** These results indicate that pirimiphos-methyl induced genotoxic damage in exposed mice with consequences for environmental health and safety. These erythropoietic and reproductive changes could be used as biomarkers for monitoring OP pesticide exposed environment.

KEY WORDS: Biomonitoring, mice, micronucleus, pesticide, pirimiphos-methyl, sperm

INTRODUCTION

Agricultural mechanization has shifted from the use of natural pest control to that of synthetic pesticides due to rapidity and potency of their actions. Environmental hygiene and safety had benefited from pesticide use but also suffered adverse effects on non-target organisms including human due to uncontrolled use.

Pirimiphos-methyl is an organophosphate (OP) insecticide for the control of pests in stored grains. It controls a wide range of pests including those resistant to synthetic pyrethroids, organochlorine and some OP compounds [1,2]. Pirimiphos-methyl has also been used for the control of cockroaches, ants, fleas, spiders, bed bugs, flies, mosquito larvae in and around domestic, public service areas, agricultural buildings, commercial and industrial areas and refuse tips [3]. Pirimiphos-

methyl is a cheap pesticide widely used worldwide and especially in Africa to protect food against pests. It has gained widespread acceptance as a substitute to malathion [4]. Though very effective as insecticide, there is a possibility that this compound could affect non-target organisms and wildlife in the ecosystem and ultimately human health. Inhalation, dermal absorption and ingestion are the common route of exposure of pirimiphos-methyl [5]. However, inhalation is the most common route of exposure because of its volatility [6]. Hematologic effects including aplastic anemia were associated with children's exposure to household insecticide products containing mixture of insecticide that includes pirimiphos-methyl.

The primary mechanism of action of OP pesticides is inhibition of carboxyl ester hydrolases, particularly acetylcholinesterases (AChE) in the brain, plasma and the red blood cells. Once an OP binds to AChE, the enzyme can undergo endogenous hydrolysis of the phosphorylated enzyme by esterases or paraoxonases; reactivation by a strong nucleophile such as pralidoxime (2-pyridine aldoxime methyl chloride) or by irreversible binding and permanent enzyme inactivation (aging) [7,8].

There have been conflicting reports on the effects of different OP pesticides on the male reproductive organ at different concentrations. For example, administration of pirimiphos-methyl (125 mg/kg) resulted in an increase in the relative weights of testes and epididymis of rats [9] while there was a decrease in the relative weights of testes from rats treated with 12.5 to 25 mg/kg of bromophenophos [10]. Thus further studies are required to reach a conclusion.

Studies have also shown the clastogenic and aneugenic potential of some OP pesticides. Dichlorvos (DDVP) has been shown to induce a significant percentage of polychromatic erythrocyte (PCE) with micronuclei [11], chromosome aberrations and sister chromatid exchange in bone-marrow, as well as, chromosome aberrations in cultured spleen cells of the mouse [12]. However, there is paucity of information on the possible clastogenic and aneugenic effects of pirimiphos-methyl.

Consequently, this study reports on the effects of OP pesticides on the male reproductive organ and the genotoxic effects of pirimiphos-methyl using two mammalian eukaryotic assays: The mouse bone-marrow micronucleus (MN) assay and the sperm morphology assay.

MATERIALS AND METHODS

Test Animal and Laboratory Cultures

Young 6-12 week male Swiss-albino mice (average weight of 30 g) were obtained from the Nigeria Institute of Medical Research, Lagos, March 2010 and 2011. They were acclimatized in a pathogen-free, well ventilated animal lab under standard conditions of 28 ± 2 °C temperature and 12 h dark/light cycle at the Department of Biosciences and Biotechnology, Babcock University, Ogun State, Nigeria for 2 weeks. They were fed *ad libitum* on rat chow and uninterrupted water supply. Eight week old mice were used for MN assay while 12 week mice were used for sperm morphology assay. The experiments were conducted in April 2010 and 2011.

Test Chemical

An OP pesticide, pirimiphos-methyl (Emulsifiable concentrate, 250 g/L, Actellic, Batch no: CHL5G11-06, Syngenta, UK) with the chemical name, O-2-dimethylamino-6-methyl-4-pyrmidinyl O-O-dimethylphosphorothioate, $C_{11}H_{20}N_3O_3PS$, was utilized for the bioassay.

General Bioassay Techniques

Acute toxicity test

Concentrations of 833 mg/kg body weight (bwt), 416.67 mg/kg bwt, 166.67 mg/kg bwt and 83.33 mg/kg bwt of pirimiphos-methyl were used for the acute toxicity studies. Five mice per concentration were randomly selected from the holding tank and introduced into the bioassay cages. Animals were injected intraperitoneally (IP) with 0.5 ml of each concentration. Another set of five mice was injected IP with 0.5 ml of distilled water for negative control setup while 0.5 ml of cyclophosphamide (20 mg/kg) was used for positive control. Mortality and symptoms were observed every 3 h for 24 h.

Sublethal test

MN assay
A similar experiment as above was set up based on $^1/_2$, $^1/_4$,$^1/_8$ and $^1/_{20}$ fractions of the derived 50% mortality (LD_{50}) values. Four concentrations, 83.33 mg/kg bwt, 41.67 mg/kg bwt, 20.83 mg/kg bwt, and 8.33 mg/kg bwt of pirimiphos-methyl were administered IP (0.5 ml for four consecutive days) to the test animals. This route of administration was employed to assess the effect of this pesticide because IP route is the fastest route of delivery of the test sample into the system of the organism. Distilled water and 20 mg/kg bwt cyclophosphamide were used as negative and positive controls respectively.

MN assay was performed as previously described [13]. The femurs were surgically removed from animals that were sacrificed by cervical dislocation. The bone-marrow was flushed from the femurs with foetal bovine serum (Sigma Aldrich Cheme GmbH, Germany). The cells were centrifuged for 5 min at 2000 rpm. Prepared slides were subsequently stained with May-Grunwald and Giemsa stains respectively. About 1000 cells/animal were scored for micronuclei in PCE (MNPCE).

Sperm morphology assay
Same concentrations, number of animals, controls and route of administration as in MN assay was utilized. The animals were exposed to 0.5 ml daily injection for each concentration for 5 consecutive days. Thirty-five day exposure period was considered as spermatogenesis takes about 34.5 days to complete in mice. Sperms were sampled from the caudal epididymes after the animals had been sacrificed by cervical dislocation [14,15]. Two

sperm suspensions were prepared from the caudal of each testis by mincing the caudal in physiological saline. The prepared slides were stained with 1% eosine Y for 45 min after which the slides were air dried. Eight hundred sperm cells per mouse were assessed for morphological abnormalities under oil immersion at ×1000 [16].

Statistical Analysis

The SPSS® 15.0 statistical package (SPSS Inc., Chicago, IL, USA) was used for data analysis. Significance at the different dose-level of each assay was tested by using the Dennett *t*-test. Differences between the negative control-group and individual dose-groups were analyzed at 0.05 probability level.

RESULTS

Acute Toxicity Studies

Pirimiphos-methyl concentration of 416.67 mg/kg bwt caused 100% mortality of exposed mice within 24 h. Symptoms accompanying mortality were shrinking of pupils, twitching tremor, muscular weakness, isolation and loss of appetite. Administration of 166.67 mg/kg bwt resulted in LD_{50} of exposed mice.

Sublethal Toxicity Studies

MN assay

The number of MNPCE increased significantly ($P < 0.05$) at all concentrations. The values obtained were thrice those of the negative control and greater than the positive control even at the lowest dose (8.33 mg/kg bwt). Furthermore, there was dose-dependent significant increase in normochromatic erythrocyte (NCE) and MNNCE at all concentrations compared to the negative control. The ratio of PCE to NCE decreased significantly ($P < 0.05$) from 5.27 (negative control) to 1.52 (83.33 mg/kg bwt pirimiphos-methyl) and 1.01 (positive control) [Table 1 and Figure 1].

Sperm morphology assay

The results showed that pirimiphos-methyl caused various abnormalities in sperm morphology. It impaired sperm structure (10.6%) as much as cyclophosphamide (positive control 11.3%) compared to the negative control [2.4%; Table 1]. Morphologically, abnormal sperms observed in this study included wrong angle hook, amorphous, banana and no-hook heads, double-tailed and folded sperms [Figure 2]. Folded sperm cells were the most prominent (45.94%) while sperm cells with double-tail had the least occurrence (0.40%) [Figure 3].

DISCUSSION

Owing to great economic loss recorded annually from agricultural produce due to livestock and crop pests, synthetic pesticides have gained worldwide acceptance and use [17]. Pirimiphos-methyl, an OP, is a broad-spectrum pesticide for combating a wide range of insects. Its effectiveness, affordability, wide range use and broad-spectrum activities predisposed non-target organisms and human to its toxicity risks. In this study, the mutagenic and genotoxic effects of pirimiphos-methyl were assessed using two mammalian assays of albino mice. The results of the acute toxicity studies showed that pirimiphos-methyl is toxic above 83.33 mg/kg bwt resulting in various levels of mortality within 24 h of exposure. Generally OPs have been found to cause headaches,

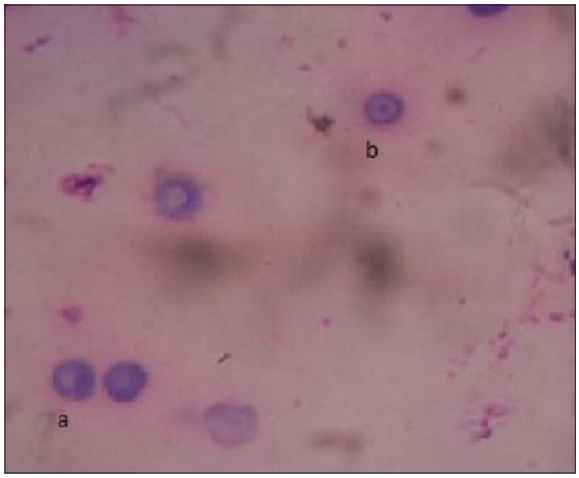

Figure 1: Normal polychromatic erythrocyte (PCE) (a) and micronuclei in PCEs (b) observed in bone-marrow of mice exposed to pirimiphos-methyl (May-Grunwald and Giemsa stain, ×1000)

Table 1: Frequency of MNPCEs and MNNCEs and aberrant sperms induced by sublethal doses of pirimiphos-methyl

Treatment	PCE/NCE	†MNPCE Mean±SD	†MNNCE Mean±SD	§Aberrant sperms	Frequency of aberration (%)
Distilled water	5.27	13.00±7.21[a]	7.00±1.00[a]	96±3.52[a]	2.4
Pirimiphos-methyl (mg/kg bwt)					
8.33	1.89	46.00±3.61[b]	19.00±6.66[b]	211±3.06[b]	5.28
20.83	2.06	56.00±11.14[b]	24.33±12.86[b]	271±12.12[b]	6.78
46.67	1.87	58.00±13.58[b]	27.33±7.64[b]	380±15.04[b]	9.50
83.33	1.52	87.00±4.36[c]	49.67±13.00[c]	425±36.51[c]	10.63
Cyclophosphamide (20 mg/kg b.w.)	1.01	38.67±15.95[b]	21.33±13.11[b]	453±1.0[c]	11.33

†1000 cells in five mice were considered per treatment. §4000 sperms in five mice were considered per treatment. [a,b,c]Different alphabet indicates significance at $P<0.05$. PCE: Polychromatic erythrocyte, NCE: Normochromatic erythrocyte, MNPCE: Micronuclei in polychromatic erythrocytes, MNNCE: Micronuclei in normochromatic erythrocyte, SD: Standard deviation

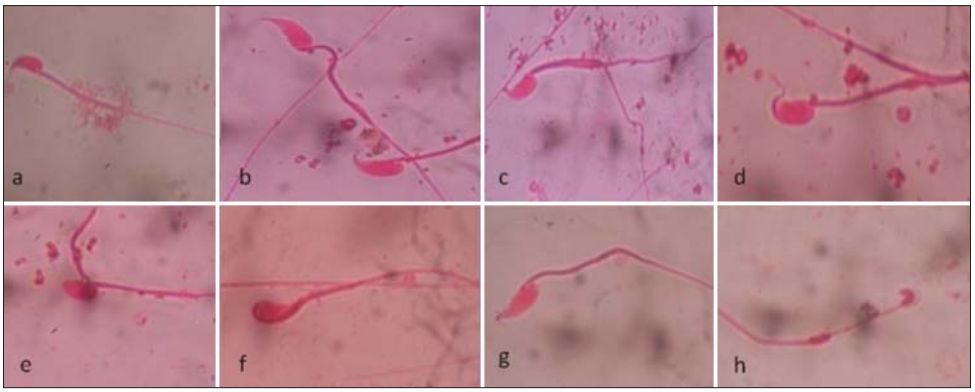

Figure 2: Abnormal sperm cells induced in mice exposed to different concentrations of pirimiphos-methyl (a) normal sperm cell, (b) wrong-angled hook, (c) double-tailed sperm, (d) knobbed hook, (e) no hook, (f) amorphous head, (g) hook at wrong angle, (h) pin head, ×800

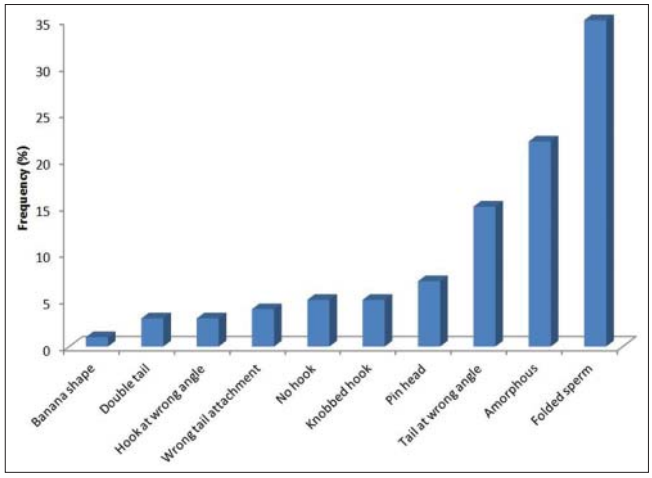

Figure 3: Frequency of different sperm abnormalities induced in mice exposed to different concentrations of pirimiphos-methyl

dizziness, extreme weakness, ataxia, tiny pupils, twitching, tremor, nausea, slow heartbeat, pulmonary edema and sweating among others in animals and humans [18,19]. Studies have shown that pirimiphos-methyl caused delayed neuropathy and acute toxicity in hen [20], macrocytic anemia, decrease in hemoglobin and erythrocyte count in fish [21], dermal toxicity in female rats and severe poisoning leading to coma, inability to breath and death in humans [22]. Mortality was accompanied by such biomarkers as loss of appetite, shrinking of pupils, twitching tremor, muscular weakness and isolation in this study. These behavioural biomarkers could be used on the field as evidence of OPs pollution and hence isolation of such ecosystem before decontamination and treatment of the organisms.

In the sublethal studies, pirimiphos-methyl induced a high frequency of MNPCE and MNNCE in the tested animals. This shows that the tested compound is capable of chemical induction of DNA damage in the exposed animals. The process of erythropoiesis in the hematopoietic organs (bone-marrow and spleen) involves proliferation and maturation of stem cells. Administration of a given substance during cell proliferation may cause chromosome damage and also act on the macromolecules related to the function of chromatid disjunction (e.g. tubulin) causing spindle dysfunction, depending on the mechanism of action [23]. Although the specific mechanism of action of induction of MN by pirimiphos-methyl is not clear, possible mechanisms of OPs toxicity may include: Inhibition of AChE within the CNS [24], effects on the cardiovascular system [25] or the immune system [26,27], modification of cellular ultrastructure [28], tissue damage through pesticide induced free radical generation [29,30] and alteration of proteolytic enzymes activities in major organs [31,32]. Previous reports also indicated increase in MN frequency in cultured rat hepatocytes treated with pirimiphos-methyl [33] and benomyl separately and in mixture [34]. Other OPs and chemicals reported to have similar effects include DDVP and its residues [35], adriamycin [36], benzinidazole [37], servin [38] and Hinosan [39]. This study further showed that there is a dose-dependent significant increase in the NCE/MNNCE levels at all concentrations. The PCE to NCE ratio decreased significantly in the pirimiphos-methyl treated mice as in the cyclophosphamide treated mice unlike the control. The PCE-to-NCE ratio between test agent-treated animals and vehicle-control animals, therefore, provides a cytotoxicity index [23]. This is a an indication that there is an increase in the rate of aging of these erythrocytes from PCE to NCE, thereby decreasing their normal life span and increasing the risk of genotoxicity.

There was significant increase in abnormal sperm morphology with increasing concentration of pirimiphos-methyl compared to the negative control ($P < 0.05$). Aberration in sperm morphology may result from alterations in sperm chromatin compaction possibly due to protamine deficiency or incomplete protamine sulfhydryl oxidation [40-42]. It has also been shown that chemical samples can alter spermatogenesis leading to *in vivo* increase in abnormal sperm cells in male mice germ cells [14]. Furthermore, formation of abnormal sperm head may result from mistakes in the packaging of the genetic material due to an agent that interferes with the integrity of the DNA itself or with the expression of the genetic material [16]. Therefore, pirimiphos-methyl is mutagenic with capacity for genetic damage in the mouse germ cells leading to the observed aberrant sperms.

Induction of abnormal sperm cells had also been observed for other OPs such as methyl parathion [43], benomyl [44,45], adriamycin [36], carbendazin [46] and lannate [47].

CONCLUSION

The findings of this study are important as chemically induced genetic damage has been implicated in the etiology of many genetic diseases. The increased genetic damage caused by pirimiphos-methyl indicates a potential genetic hazard. This is of importance to public health since this chemical is widely used as pesticides worldwide.

Frequencies of micronuclei and NCE, and aberrant sperms (wrong angle hook, amorphous, banana and no-hook heads, double-tailed and folded sperms) are tested biomarkers of sublethal concentration of pirimihos-methyl for containment of contamination before they reached large scale mortality.

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