Mutation Research 761 (2014) 10–17

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

In vitro studies on organophosphate pesticides induced oxidative DNA damage in rat lymphocytes

A. Ojha, N. Srivastava *

School of Studies in Biochemistry, Jiwaji University, Gwalior 474011, India

ARTICLE INFO

Article history:
Received 25 August 2012
Received in revised form 8 January 2014
Accepted 13 January 2014
Available online 24 January 2014

Keywords:
Chlorpyrifos
Malathion
Methyl parathion
Oxidative DNA damage
Superoxide anion generation
In vitro studies

ABSTRACT

Organophosphate (OP) pesticides are widely used for agricultural and household pest control. We studied the genotoxicity of the commonly used OP pesticides chlorpyrifos (CPF), methyl parathion (MPT), and malathion (MLT), individually and in combination, in Wistar rat peripheral blood lymphocytes in vitro. DNA single-strand and double-strand breaks were measured by single cell gel electrophoresis (SCGE; comet assay). To test whether the DNA lesions were caused by oxidative stress, the DNA repair enzymes formamidoaminopyrimidineglycosylase (Fpg) and endonuclease (Endo III), which convert base damages to strand breaks, were used. Significant increases in strand breaks and in levels of the reactive oxygen species (ROS) superoxide anion and hydrogen peroxide were observed in lymphocytes treated with pesticides. MPT exposure caused the greatest DNA damage and ROS production, followed by CPF and ML. Our results demonstrate genotoxic potential of these OP pesticides.

1. Introduction

Pesticides are chemical compounds used extensively in modern day agriculture and household for the control of pests. Pesticides also play a role in preventing the spread of infectious diseases. Despite the beneficial effects, many of these chemicals pose potential hazards to humans and to the nature. The widespread use of pesticides for preventing, destroying, repelling, or mitigating pests, had led to anxiety about the possible hazards to public health [1]. Since organochlorine pesticides such as DDT, BHC, HCH, *etc.* were banned or came in restricted use, because of their high persistence and greater toxicity, the second line of pesticides *i.e.* organophosphates (OPs) and pyrethroids have become the most common groups available to the world. Though there can be benefits using pesticides, inappropriate use can counter productively, increase pest resistance and kill the natural enemies of the pests. Some pesticides are found to be highly persistent in nature, thereby causing contamination of soil, ground and surface water. Their toxic effects are manifested in different ways such as bioaccumulation, biomagnification, chronic toxicity, acute immune response, allergic reaction, and mutagenic, teratogenic and carcinogenic effects [2,3]. Such contamination with low level of pesticides has resulted in serious environmental concern and some of the pesticides, though not showing an immediate effect *in vivo*, may pose long term health hazard to human beings. The environmental impact of pesticides is often greater than what is intended by those who use them. Over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species, including nontarget species, air, water, bottom sediments, and food [4,5].

The organophosphate (OP) pesticides like chlorpyrifos (CPF), methyl parathion (MPT), and malathion (MLT) are widely used in agriculture, in public health and in some countries, large quantities are applied for hygiene purposes. OP compounds including pesticides are anticholinesterase agents. Studies in animals have shown changes in neurotransmitter levels and alterations in neurobehavioral processes after exposure to OPs like monochrotophos, chlorpyrifos, methyl parathion, and malathion [6,7]. These pesticides are known to produce oxidative stress and extensive data suggest that oxygen free radicals are involved in the toxicity of pesticides, including OP, in animal studies [8], in *in vitro* experiments [9] and in pesticide manufacturing workers [10] or pesticide sprayers [11]. DNA damage and oxidative stress have been proposed as mechanism that could mechanistically link pesticide exposure to a number of health outcomes observed in epidemiological studies [12,13]. Diseases such as hepatitis, atopic dermatitis,

Abbreviations: CPF, chlorpyrifos; MPT, methyl parathion; MLT, malathion; ROS, reactive oxygen species; OP, organophosphorus; Fpg, formamidoaminopyrimidine glycosylase; Endo III, endonuclease III; SCGE, single cell gel electrophoresis; PM, pesticide mixture.

* Corresponding author. Tel.: +91 751 2442796; fax: +91 751 2341450.
E-mail addresses: nalinis21@yahoo.com, nalinisrivastavaju@gmail.com (N. Srivastava).

1383-5718/$ – see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.mrgentox.2014.01.007

autoimmune diseases (rheumatoid arthritis, systemic lupus erythematosus, *etc.*) which are associated with inflammatory response include the release of reactive oxygen species (ROS) [13,14]. ROS can play a critical role in defective sperm function and male infertility [15,16] and also cause aging by the gradual accumulation of free radical damage to biomolecules [17]. The high levels of oxidized bases present in patients infected with the human immunodeficiency virus (HIV) might influence the progression of the infection into acquired immunodeficiency syndrome (AIDS) [13]. DNA oxidative damage has been linked to other diseases, notably Alzheimer's disease, Huntington's disease and Parkinson's disease [14].

Reactive oxygen species (ROS), which include free radicals and other highly reactive forms of oxygen (*e.g.* hydrogen peroxide, superoxide anion radical, singlet oxygen, hydroxyl radical, *etc.*) cause DNA oxidation which is known to be one of the most common kinds of damage to DNA. Superoxide anion and H_2O_2 are known to be capable of inducing strand-breaks and base oxidation in intracellular DNA. The primary oxidant responsible for DNA damage is hydroxyl radicals ($OH^{\bullet}$), as it reacts directly with DNA molecule [18]. Oxidative DNA damage can be measured by employing endonucleases specific for oxidized bases, which recognize oxidized purines and pyrimidines [19–21]. The two enzymes in general use are formamidopyrimidine DNA glycosylase (Fpg), which detects primarily 8-oxo-7,8-dihydroguanine (8-oxoGua), and endonuclease III (Endo III) which recognizes oxidized pyrimidines [19] and introduce breaks in the cellular DNA that are then most commonly measured using the comet assay (alkaline single cell gel electrophoresis). The alkaline single cell gel electrophoresis (SCGE) or comet assay, is now a well-established genotoxicity test for the estimation of DNA damage at the individual cell level in both *in vivo* and *in vitro* studies [22]. Thus, very low, physiologically relevant levels of oxidative DNA damage can be investigated.

Besides being potent source of ROS, OP compounds also show alkylating properties and alkylating agents are known to cause DNA damage [23,24]. Among the studied pesticides most toxic OP is MPT (*O*,*O*-dimethyl-*O*-4-*p*-nitrophenyl phosphorothioate), that has been shown to induce genotoxic effects and sister chromatid exchange in human lymphocytes and demonstrated ability to interact directly with double-stranded DNA disturbing its stability and conformation [25,26]. Technical grade malathion appeared to have a potential to produce chromosomal changes including chromosomal aberrations and micronuclei in somatic and germ cells of mice [27]. The toxicity of malathion might be attributed to its metabolite maloxon, which unlike its parent compound damage DNA [28]. Chlorpyrifos has also been shown to induce DNA damage in rat brain and liver [29]. Malathion used as commercial product, *i.e.* containing malaoxon and isomalathion, can be considered as a genotoxic substance *in vitro* and may also produce DNA disturbances *in vivo*, such as DNA breakage at sites of oncogenes or tumor suppressor genes, thus playing a role in the induction of malignancies in exposed individuals. Therefore, malathion can be regarded as a potential mutagen/carcinogen [28].

As a range of insecticides is extensively used in pest management, the chances of exposure to multiple OP compounds simultaneously are high, especially among agricultural and public health workers. Furthermore, from dietary and other sources, there may be separate but closely timed exposures to such insecticides. Although health hazards of individual OP insecticides have been relatively well characterized, there is lesser information on the interactive toxicity of multiple OP insecticides. The data suggest that exposure to multiple OP-containing pesticide formulations may lead to synergistic neurotoxicity by a direct mechanism at the cellular level [30]. It is reported that the herbicide, terbutryn, had DNA damaging capability, as evaluated by the alkaline single-cell microgel-electrophoresis (comet assay) [31]. *In vitro* study of terbutryn was found to induce primary DNA damage, even though in the absence of a clear trend for dose-dependence and in the presence of a concomitant mild cytotoxic effect observed [32]. There is lack of reports which throw light on comparative and synergistic or antagonistic genotoxic potential of CPF, MPT, MLT and their mixture in *in vitro* systems. Literature survey suggests that exposure to mixture of pirimiphos methyl, chlorpyrifos, temephos and malathion may induce DNA damage, decrease in AChE activity, hepatotoxicity as well as nephrotoxicity in occupational workers [33].

Chlorpyrifos, methyl parathion and malathion are widely used pesticides, however, reports of cytogenetic and genotoxic potential of these pesticides either individually or in mixture, in *in vitro* system are lacking. Therefore, the present study is designed to assess the genotoxic potential of CPF, MPT and MLT individually, and in mixture, by measuring DNA single and double strand breaks in isolated rat lymphocytes incubated with CPF, MPT and MLT individually and in mixture for different period of time. The study was also aimed to unravel the mechanism of this DNA damage by standard comet assay in combination with Fpg–Endo III modified comet assay/SCGE to find out whether DNA lesions were caused due to oxidative stress. The levels of H_2O_2 and superoxide anions in cultured rat lymphocytes incubated with pesticides for different periods, were also measured to find out any correlation with the strand breaks.

2. Materials and methods

2.1. Chemicals

Normal melting agarose (NMA), low melting agarose (LMA), Triton X-100, ethylenediamine tetraacetic acid (EDTA), tris (hydroxymethyl)-aminomethane, ethidium bromide, endonuclease III (Endo III) and formamidopyrimidine-DNA glycosylase (Fpg), oxidized cytochrome *c*, phorbol 12– myristate-13- acetate (PMA), phenol red and hydrogen peroxidase were purchased from Sigma Chemicals Inc., St. Louis, MO. Sodium chloride, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate, sodium hydroxide, Hisep, dimethylsulfoxide, RPMI 1640, Trypan Blue, 4-(2-hydroxyethyl)-piperazine-1-ethanesulphonic acid (HEPES) buffer, potassium hydroxide, bovine serum albumin, and potassium chloride were purchased from Merck, Germany. Methyl parathion, chlorpyrifos and malathion were kind gift from Devidayal (Sales) Limited, Mumbai, India.

2.2. Experimental animals

Adult male albino rats of Wistar strain (*Rattus norvegicus*), weighing 120 ± 10 g were used in all experiments. Rats were obtained from Defence Research and Development Establishment, Gwalior, India, and were maintained in a light (light–dark cycle of 12 h each) and temperature (25 ± 2 °C) controlled animal room of our department on standard pellet diet and tap water *ad libitium*. Rats were acclimatized for one week prior to the start of experiment. The care and maintenance of animals were as per the approved guidelines of the 'Committee for the Purpose of Control and Supervision of Experiments on Animals' (CPCSEA, India).

2.3. Lymphocytes isolation

Peripheral blood was collected from healthy male Wistar rats (weighing about 120 ± 10 g) from eye orbital in vacutainer test tubes containing EDTA (Becton-Dickinson, Cedex, France). Blood samples were layered on the top of a Ficoll solution (1.077 g/ml) and the supernatant containing the leukocytes was removed after sedimentation of erythrocytes at 1000 g for 10 min at room temperature. Lymphocytes sedimented at the interface of the Ficoll layers were collected and washed twice with PBS, pH 7.4 at 20 °C. The cell viability was checked by Trypan Blue exclusion test [34] and was found to be about 95%. The final concentration of the lymphocytes was adjusted to $1–3 \times 10^5$ cells/ml by adding RPMI 1640 to the single cell suspension.

2.4. Pesticide treatment

Chlorpyrifos [4 h LC_{50} 0.2 mg/L, 35], methyl parathion [4 h LC_{50} 0.135 mg/L, 36], malathion [4 h LC_{50} >5.2 mg/L, 37] individually and in mixture were taken from DMSO stock solutions (LC_{50}) and added to the lymphocyte suspension to give final concentrations of 1/20, 1/10, 1/8, 1/6 and 1/4 LC_{50} individually and in mixture to perform time– and dose–response relationship study. The control cells were treated with DMSO without the organophosphates, which did not affect the processes under study. To examine modified bases, double strand DNA breaks, hydrogen peroxide, and superoxide anion production, the lymphocytes were incubated with pesticides for 2 h and 4 h at 37 °C in CO_2 incubator and results were compared with control lymphocyte cells. The positive control for DNA damage was hydrogen peroxide [38],