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RESEARCH ARTICLE

Chlorpyrifos Induced Dermal Toxicity in Albino Rabbits

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ABSTRACT

Organophosphates being acetylcholine esterase inhibitors are immoderately used as insecticides on a variety of agriculture crops. Toxicity of such compounds like chlorpyrifos (CPF) poses serious health issues to the individuals at risk. The concentrations below the probable occupational exposure were selected to ascertain the extent of dermal damage in experimentally exposed animals. To determine CPF induced dermal toxicity, a total of 24 adult albino rabbits of both genders and of the same age group was categorized as group A and B, respectively. Group A was further sub grouped into A1 (all males) and A2 (all females). Similarly, group B was divided into B1 (all males) and B2 (all females). Four different dosing sites of one square inch each were selected. For naked topical CPF application, each animal of sub group A1 and A2 topically dosed 500µl of 20%, 30% CPF ethanolic solution, its commercial formulation and ethanol (control) on four dosing sites separately. The dosage of CPF solutions (20% & 30%), commercial formulation and ethanol (control) were repeated every 24 hours for 72 hours and observed for 96 hours. Similar groups were made for occluded topical CPF. Treated animals were observed for gross dermal lesions (erythema scoring of varying grades). Skin tissues from treated sites were taken and processed for histopathological and morphometric changes. Erythema scoring was increased in animals treated with high doses of CPF both in different concentration and commercial preparations in a dose dependent response. The number of layers of erythema in chemically exposed skins of rabbits was also increased in dose dependent response both in naked and occluded groups. The dermal insult induced in both exposure concentrations at par with the commercial preparation indicated the level of toxicity of this compound and public health consequences might be predicted.

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INTRODUCTION

In developing countries, pesticides are frequently used over the last few decades in agriculture and veterinary practice to control different pests for optimum yields (Ahmad *et al*., 2012; Auon *et al*., 2014). Monitoring of such kind of chemicals is important and necessary to reduce the possible deleterious effects upon animals as well as public health. Among different groups of such chemicals (insecticides, pesticides and herbicides), organophosphate compounds are commonly employed in agro-production systems and house management (Hussain *et al*., 2014; Ghaffar *et al*., 2014). Organophosphates, being acetylcholinesterase inhibitors, are not only used as insecticides on varieties of cereal crops but also as chemical warfare agents. In developing countries, including Asia-Pacific region, approximately 3 million cases of acute deaths and severe cases of poisonings (0.3 million) due to organophosphate pesticides have been reported (Bertolote *et al*., 2006; Hussain *et al*., 2015). The organophosphate pesticides poisoning, occupational exposure as well as potential

adverse effects is of great public health concerns, where farmers do not follow the manufacturer's guidelines regarding its use. The prevailing scenario becomes important with regard to their acute and/or chronic health effects (Litchfield, 2005; Dharmani and Jaga, 2005; Recena *et al*., 2006; Rodriguez *et al*., 2006). Among the immediate, exposed populations, the agriculture spray workers and cotton pickers may be affected directly by the direct exposure of pesticides. Chlorpyrifos belongs to the organophosphate group of pesticides is highly neurotoxic. It irreversibly inhibits the acetylcholinesterase enzyme (Ahmad *et al*., 2015; Topal *et al*., 2016) in the brain, spinal cord, and the peripheral somatic and autonomic nervous system (Das *et al*., 2006). Chlorpyrifos causes irritation to the exposed skin, after systemic absorption, further responsible for a variety of systemic toxic insults both in human and animals (Krishnan *et al*., 2012; Shahzad *et al*., 2015). There are three possible routes by which this chemical may enter into the body, such as inhalation, oral and dermal. Dermal exposure is one of the common route through which the adverse effects of CPF are reported both in animals and human (Krishnan *et al*., 2012). Exposure of humans and animals to organo-chlorine pesticides like CPF also cause abnormalities and marked suppression of IFN-γ along with other health hazards (Daniel *et al*., 2001).

Chlopyrifos is lipophilic in nature and its dermal toxicity profiling has not yet been extensively explored. It is thought that some of the insecticides disrupt the uppermost layer of skin (*Stratum corneum* of the epidermis) and facilitate their absorption and hence induce toxicity to keratinocytes (So *et al*., 2004). The physicochemical nature and the vehicle are the two important factors that are responsible for toxic insult and dermal absorption. In comparison to jet fuel exposure, commercially available pesticides (having petrochemicals as a vehicle) induce relatively less dermal toxicity (Muhammad *et al*., 2005a; Muhammad *et al*., 2005b). To our best knowledge, no such report is available on the dermatotoxic effects and their exact mechanisms of different concentrations/ formulations of CPF in exposed animals. The current study is devised to elucidate the potential toxic effects of different concentrations of CPF as well as its commercially available formulation under different topical modes of application to the albino rabbits.

The New Zealand albino rabbit has historically been a model of choice for local as well as acute and sub-chronic toxicity. This is because of its body size, easy handling as well as the permeability of its skin. However, because of the more sensitivity of the its dermal insult, the findings of this model are considered over-predictive, but considered the preferred model currently by many regulatory models for dermal toxicity evaluations (Krishnan *et al*., 2012). Further, its more phylogenetic relevance to human than rodents also makes the best model (Fan and Watanabe, 2003).

MATERIALS AND METHODS

Chemical and Reagents: The chlorpyrifos (98.5%) was purchased from Sigma-Aldrich, India. The commercially available product Kimfast™ 40% EC (marketed by Four Brothers Agri Services, Pakistan) was procured from the local market. All other chemicals used were of the reagent grade and obtained from regular commercial sources. A stock solution of chlorpyrifos was prepared in 90% ethanol by dissolving the measured amount of chemical. The working solutions were prepared freshly before the application by dissolving the measured volume of stock solution in ethanol.

Experimental design: A total of 24 adult New Zealand white Albino rabbits of both gender and of the same age group (6-12 month) were procured from National Institute of Health, Islamabad and categorized as group A and B, respectively. They ranged in 1.5-2.0 kg in body weight. Group A was further sub grouped into A1 (all males) and A2 (all females). Similarly, group B was divided into B1 (all males) and B2 (all females). Each subgroup carried 6 animals.

After seven days of acclimatization (*ad libitum* access to water and feed) all the rabbits were shaved on flank region (03 inches^2) 24 hours prior to the application of respective pesticides and ethanol (control). Four different dosing sites of one square inch each were selected. For naked topical CPF application, each animal of sub group A1 and A2 topically applied 500µl of 20 and 30% CPF ethanolic solution, its commercial formulation and ethanol (control) on four dosing sites separately.

For occluded topical CPF application, each animal of sub group B1 and B2 was topically dosed with 500µl of 20 and 30% CPF ethanolic solution, its commercial preparation as well as ethanol (control) under the thick muslin cloth to mimic the occupational settings on four dosing sites, respectively. The dosage of CPF solutions (20 & 30%), commercial preparation and ethanol (control) was applied topically and under occluded condition for 72 hours and repeated after every 24 hours and observed till four days. Animals in both groups were examined for gross dermal lesions. The erythema scoring was counted as 0 (no significant change), 1 (very slight erythema), 2 (slight erythema), 3 (moderate to severe erythema) and 4 (severe erythema) (Muhammad *et al*., 2008).

After 96 hours, all the rabbits were euthanized with ketamine @ 10 mg/kg BW intramuscularly and 6 mm biopsies were preserved in 10% neutral buffered formalin for histological examinations. Formalin fixed skin biopsies were processed through a graded ethanol series and embedded in a paraffin block. 4-5 µm thick transverse sections perpendicular to the plane of section of the block were taken and stained with Hematoxylin and Eosin (H&E).

Data were analyzed by PROC MIXED procedure as appropriate using SAS statistical program (SAS Inc., USA). Within and between-group differences were tested by post hoc analyses. Dunnett's multiple comparison was done against a single control and differences were calculated by least square means procedures. The level of significance was set at $P<0.05$.

RESULTS

Parameters studied showed non-significant difference between male and female; therefore, the data are presented cumulatively. The average erythematic score in

the skin of rabbits exposed to low concentrations (20%) of CPF in both naked and occluded was 1.33±0.65 and 2.08±0.65, respectively. This parameter increased significantly with the increment of concentration as well as used in commercial preparation (Table 1). There was increasing trend in scoring of erythema in occluded animals, but this increase was not statistically significant from the corresponding ones in the naked group of animals except in commercial drug administered group where the erythema score was significantly increased from 2.83±0.65 to 3.58±0.65.

Like the erythematic score, dermal thickness also increased significantly (P<0.05) in all CPF exposed animals as compared to their respective control. The erythema thickness was also broader in all exposed concentrations in occluded formulation as compared to their respective naked formulations. The difference in thickness of erythema was highly significant in commercially available preparation exposed rabbits that increased from 104.26±1.41 to 120.59±1.41µm in naked and occluded rabbits, respectively (Table 1).

The number of layers of erythema in chemically exposed skins of rabbits was also increased in a dose dependent manner both in naked and occluded setup. The commercial preparation applied on the naked skin significantly (P<0.05) increased the number of layers of the erythema (3.16±0.17) when compared to that of control (1.33±0.17). However, in the individuals exposed to 20 and 30% CPF, the increase in the number of layers of erythema was also significant. Whereas, in occluded application, the erythema layers increased significantly in all exposure groups in applied commercial preparation group of rabbit skins. There was significantly more number of layers of erythema in all CPF exposed skins when applied on the occluded skin (Table 1).

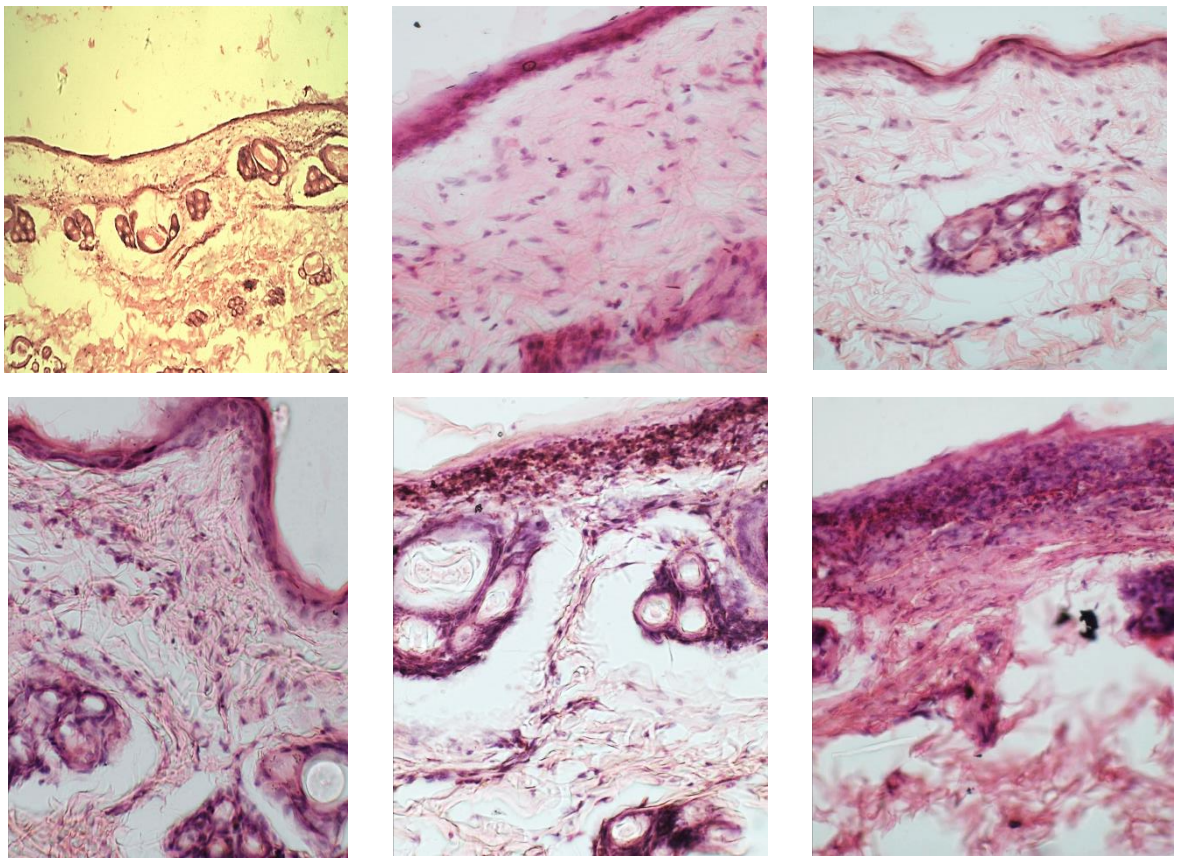

Fig. 1: Comparative microscopic changes in the skin of rabbits after topical application of different concentration of Chlorpyrifos (H&E; 40x). Note the normal thickness of epidermis in control rabbit (A). Epidermis thickness has been increased in naked (B) and occluded (C) group exposed to 20% CPF and naked group exposed to 30% CPF (D) along with subepidemal abscessation. CPF 30% commercially available preparations resulted in significantly increased epidermis thickness along with subepidemal abscessation in naked (E) and occluded (F) groups.

Table 1: Erythematic parameters in the skin of naked and occluded rabbits exposed to different concentrations of chlorpyrifos and its commercially available product

Groups	Naked			Occluded		
	Erythema-score (n)	Epidermal thickness (µm)	Epidermal layers (n)	Erythema-score (n)	Epidermal thickness (µm)	Epidermal layers (n)
Control	0.00±0.00	49.78±1.41	1.33±0.17	0.00±0.00	50.82±1.41	1.08±0.24
CPF 20%	1.33±0.65*	81.12±1.41*	2.16±0.17	2.08±0.65*	87.67±1.41*	2.50±0.24*‡
CPF 30%	2.58±0.65*	95.69±1.41*	2.75±0.17‡	2.75±0.65*	106.87±1.41*‡	3.41±0.24*‡
Commercial product	2.83±0.65*	104.26±1.41*	3.16±0.17*	3.58±0.65*‡	120.59±1.41*‡	4.08±0.24*‡

Data are expressed as Mean±SE. PROC MIXED analyses followed by Dunnet's post hoc test; *Chlorpyrifos (CPF) exposed versus control within naked or occluded (P<0.05). ‡CPF exposed between naked versus occluded within respective concentration (P<0.05). Commercial Product was Kimfast™ 40%EC.

Topical application of 20% CPF resulted in hyperkeratosis in naked (Fig. 1B) and occluded (Fig. 1C) exposed groups, whereas in 30% CPF concentration, hyperkeratosis and subepidemal abscessation were seen in naked exposed group (Fig.1D). CPF 30% commercially available preparations resulted in significantly increased epidermis thickness along with subepidemal abscessation in naked (Fig. 1E) and occluded (Fig. 1F) groups.

DISCUSSION

In the present study, rabbit's skin was exposed to different concentrations of chlorpyrifos and a commercially available product containing CPF. The exposure concentrations were comparatively evaluated for their potential toxic effects on epidermis in terms of erythema, in order to enhance the understanding of occupational toxicity of this pesticide. The chemical had produced epidermal injury in all exposed groups from both naked and occluded setups. However, the difference between the genders was not significant.

Pesticides and the environmental pollutants are the substances dispersed in the environment to affect our biological systems. Animals like human beings are highly exposed to various types of toxic substances throughout their life. The toxic substances, including pesticides as well as their commercial formulations are considered relatively less volatile and more adhesive to human and animal skin for longer durations (Muhammad *et al*., 2008). The rate of absorption of particularly the pesticides is related to their solubility, the presence of detergent in the formulation and integrity of the skin barrier (Nielsen, 2000; Brand and Mueller, 2002; Nielsen *et al*., 2004). Its uppermost layer, the *Stratum corneum* made up of tightly packed cells (keratinocytes), protects the skin naturally. It provides the maximum protection and resists the lipophobic solvents. The skin appendages like the opening of sweat glands, sebaceous glands and hair follicles facilitate a very minute fraction of these solvents to diffuse and thus become irrelevant for absorption. Contrary to this, the lipophilic moieties can easily cross the stratum corneum and deposit underneath the skin in the subcutaneous tissues and act as reservoirs. The mortar structure in which the keratinocytes are rested becomes important because hydrophobic substances (most pesticide formulations) disrupt the ceremide and estrone mortar. Hence, it not only increases the absorption, but also enhances the inflammatory risk many folds. However, it is an inevitable reality that a large fraction of pesticide diffuses transcellularly and also let the keratinocytes inflamed.

Organophosphate poisoning is graded as mild, moderate and severe (Khan *et al*., 2012; Peter *et al*., 2014; Pirsaheb *et al*., 2017). Chlorpyrifos is considered as a mild skin irritant (Babamir-Satehi *et al*., 2017), but its dermatotoxic effects have not been investigated thoroughly yet. The present *in vivo* study had explained the dermal damage of topically applied various CPF concentrations and commercial preparation in the rabbit's skin after repeated exposure for 4 days. Moderate to severe erythema (in terms of scoring) was observed after 4-day repeated exposures of CPF to the skin. However, the commercial formulations under occluded condition had induced relatively much pronounced erythema as compared to the same when applied topically on naked skin. The present findings were in accordance with our previous results (Muhammad *et al*., 2008) where the commercial preparation atrazine has also produced the enhanced erythema response in occluded skin.

The histopathalogical parameters considered for inflammatory response were epidermal thickness and number of epidermal stratifications. A statistically significant alteration was also recorded in both naked and occluded groups. Similarly, the most proliferative effect was observed in case of commercial formulation in both parameters. It may be interpreted that the presence of vehicles in commercially available products is responsible for exaggerated inflammatory and proliferative responses in the epidermis (Chandra *et al*., 2015; Weir *et al*., 2015). A wide variety of pesticides vehicles are being used by the pesticide industry, either compromise the dermal integrity of the skin or synergize in epidermal damage. In conclusion, we suggest further studies to explore the potential aspects of dermal toxicity associated with the vehicles as well as pesticides active substances for agricultural and other uses.

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Authors contribution: AR, AK, AY and FM conceived the idea and designed the project. AR and IAK executed the experiment. MU, and RH were involved in data analysis, interpretation and write up of the manuscript. All authors approved the manuscript.

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