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The Effect of Chlorpyrifos, an Organophosphorus Pesticide, on Glucose Uptake in Whole Blood

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Abstract

Background: The sub-chronic organophosphorous pesticide exposure increases insulin resistance and thereby the risk of developing Diabetes mellitus. Its acute exposure also leads to hyperglycemia. However, the mechanism is not clearly understood. Hence the present study explores the effect of low and high concentrations of chlorpyrifos, an organophosphorous pesticide on glucose uptake by whole blood and its possible mechanism.

Methods: Level of hemolysis was determined at 2 and 4 hours of 1% DMSO (Dimethyl sulfoxide) and chlorpyrifos (0.3 and 10 mg/ml) exposure. The whole blood was treated *in vitro* with vehicle i.e., 1% DMSO or high (10 mg/mL) or low (0.3 mg/mL) concentrations of chlorpyrifos dissolved in DMSO. Then plasma glucose was assayed by glucose oxidase method at 2 and 4 hours of DMSO/chlorpyrifos exposures.

Results: There was no effect of DMSO or 0.3 mg/ml chlorpyrifos treatment on hemolysis even after 4 hours. 10 mg/ml chlorpyrifos causes 2% and 14% hemolysis at 2 and 4 hours respectively. Plasma glucose level decreased in a time dependent manner after collection of blood (15.9% at 2 hours and 34.2% at 4 hour) in presence of DMSO. Chlorpyrifos did not influence glucose uptake by whole blood in low concentration but suppressed it in high concentration.

Conclusion: Chlorpyrifos does not impair glucose uptake at low concentration in whole blood probably because major glucose uptake in blood is insulin independent. The impaired glucose uptake at high concentration is mostly due to toxic metabolic impairment and partly due to its hemolytic effect.

Keywords: Organophosphorus compounds; Chlorpyrifos; Glucose uptake; Hemolysis

Introduction

Chlorpyrifos, an organophosphorus pesticide that acts by inhibiting cholinesterase, is widely used for agricultural purpose and in public health programs. This insecticide is also considered as a low toxic chemical for domestic use [1]. The use of pesticides leads to environmental contamination and the pesticide residues are found in food, water and air. As the pesticide residues are in the food chain, its sub-chronic exposure is practically unavoidable for human population.

The prevalence of diabetes mellitus is currently at epidemic proportion and is predicted to increase even further over the next decade. The genetic predisposition and unhealthy lifestyle are commonly accepted reasons for the occurrence of type 2 diabetes. In a number of recent studies, pesticide exposure has been claimed to induce insulin resistance. Thus sub-chronic exposure to these leads to development of diabetes [2-5]. Besides, there are reports that show that acute toxicity induces hyperglycemia [6]. Pesticides are found to impair insulin stimulated glucose uptake [7]. Chlorpyrifos, in similar way has been showed to be associated with diabetes. Studies on rat have demonstrated that its administration causes hyperglycemia [8,9].

Whole blood contains Red Blood Cells (RBCs), White Blood Cells (WBCs) and platelets amongst which glucose uptake by B lymphocytes and monocytes is only insulin dependent (GLUT 4 mediated) [10]. Whether insulin dependent or not these cells take up glucose in a time dependent manner and decrease in plasma glucose concentration by 5-7% per hour after collection [11]. The effect of pesticides on glucose uptake by whole blood is not known yet. Impairment in glucose uptake could explain pesticide-induced hyperglycemia. However, mechanism of this impairment is not clearly defined. The effect may be due to pesticide-induced cell lysis or their toxicity influencing cellular metabolism. Chlorpyrifos is metabolized to chlorpyrifos-oxon by Cytochrome P450 (CYP450) in liver, which has an anti-cholinesterase effect. This chlorpyrifos-oxon is subsequently metabolized by paraoxonase/chlorpyrifos-oxonase/non-enzymatic method to inactive 3,5,6-Trichloro-2-Pyridinol (TCP) or Diethyl Thiophosphoric Acids (DTPA) [12].

In view of the above, the present study is designed to determine the effect of high and low concentrations of chlorpyrifos on glucose uptake in whole blood and the possible mechanism of its effect.

Materials and Methods

This study was conducted in the Department of Biochemistry, Maulana Azad Medical College, New Delhi, India. The study protocol

was approved by the Institutional Ethics Committee at Maulana Azad Medical College, New Delhi, India.

Reagents and chemicals

Glucose Oxidase Kit was supplied by Randox (County Antrim, United Kingdom). Chlorpyrifos (99% pure) was obtained as free sample from Coromandel International Ltd. (Gujarat, India). Dimethyl Sulfoxide (DMSO) was procured from Sigma Aldrich (Saint Louis, M.O., USA).

Sample collection

After informed consent, 9 ml of venous blood was collected in heparin tubes from each volunteer (n=3). All the volunteers were healthy male subjects (Age: 30, 31 and 27 years). Plasma/serum fasting glucose, urea, creatinine, total bilirubin, ALT, AST, Alkaline Phosphatase and hsCRP were within reference range which was performed before recruiting them in the study.

Experimental design

It was an *in vitro* experiment done on whole blood. From 9 ml of blood collected from each volunteer, 2 ml was used for hemolysis assay. One ml of blood was used for baseline (time-zero) plasma glucose assay. Rest 6 ml of blood was divided into 3 aliquots of 2 ml each. To one aliquot DMSO was added (final conc. 1%). In other 2 aliquots, chlorpyrifos dissolved in DMSO (final conc. 1%) was added to make final concentration of the pesticide 0.3 mg/dl and 10 mg/dl respectively. The pesticide was mixed by gently shaking the aliquots and incubated at room temperature (25°C). One ml blood was taken after 2 hours from each aliquot and plasma glucose was measured. After 4 hours of incubation, measurement of the same was done in all the 3 aliquots.

Hemolysis assay

Hemolysis assay was done by method as described by Hu et al. [13]. Two ml of heparinized blood was added to 4 ml sterile Dulbecco's phosphate buffer saline (DPBS) and centrifuged at 3000 rpm for 5 min using clinical centrifuge (REMI R-8C) to wash Red Blood Cells (RBCs). The RBCs were further washed 3 times with 10 ml of DPBS and finally diluted to 20 ml with DPBS. Two hundred microlitre diluted RBC was transferred to 1.5 ml tubes. Positive control was prepared by adding 800 µl distilled water. Rest of the tubes was added with 800 µl of DPBS. One of these tubes was used as negative control and rest of them were added with 10 µl DMSO containing chlorpyrifos to make final concentration of the pesticide 0.3 mg/ml and 10 mg/ml. After incubating at room temperature for 2 hours, 500 µl samples were taken and centrifuged for 5 minutes at 3000 rpm. After 4 hours remaining samples were centrifuged similarly. One hundred microlitre of the supernatant of each sample was transferred to a 96 well plate and absorbance was measured by a microplate reader (Biorad 680 XR, CA, USA) at 570 nm with 655 nm as reference. The degree of hemolysis was calculated as hemolytic ratio using the following formula:

$$\text{Hemolysis ratio} = \{(OD_{test} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control})\} \times 100$$

Plasma glucose estimation

Plasma glucose level was determined using glucose oxidase kit adopted to random access automated clinical chemistry analyzer (DXC800, Beckman, CA, USA). The glucose level at time 0 was used as the reference (time-zero) glucose value and the subsequent values (at 2 and 4 hour) were expressed in percentage considering time-zero glucose level 100%.

Statistical analysis

Data were expressed as the average of three experiments ± SD. Comparison between variables was performed using one-way Analysis Of Variance (ANOVA) followed by Tukey's post hoc test using the SPSS PC version 17. The differences were considered statistically significant at P value <0.05.

Results

Plasma glucose level decreased in a time dependent manner after collection of blood when whole blood was kept without addition of any pesticide. However, DMSO (1%) was added as vehicle to the sample. The effect of different concentrations of chlorpyrifos on glucose uptake by whole blood is presented in Table 1.

	Glucose level at 0 hour	% reduction in plasma glucose	
		At 2 hour (Mean ± SD)	At 4 hour (Mean ± SD)
Control (vehicle 1% DMSO)	100%	15.6 ± 1.5	35.3 ± 2.5
Chlorpyrifos (0.3 mg/ml)		16.0 ± 3.0	36.3 ± 2.5
Chlorpyrifos (10 mg/ml)		9.7 ± 1.5 *	20.7 ± 3.0 *

Table 1: Effect of exposure to high and low concentration of chlorpyrifos on glucose uptake by whole blood (n=3). *P<0.05 in comparison to control by one way ANOVA followed by Tukey's Post hoc test.

Chlorpyrifos did not significantly alter glucose uptake at 0.3 mg/ml concentration but suppressed it significantly (p<0.05) at 10 mg/ml concentration in comparison to vehicle treated control. Table 2 shows the effect of chlorpyrifos on hemolysis. With 0.3 mg/ml concentration, the hemolysis was negligible even after 4 hours of exposure. With 10 mg/ml, hemolysis was 3% after 2 hours and 14% after 4 hours of exposure.

	% of hemolysis	
	At 2 hour	At 4 hour
Control (vehicle 1% DMSO)	0	0
Chlorpyrifos (0.3 mg/ml)	0	0.01 ± 0
Chlorpyrifos (10 mg/ml)	3 ± 0.5	14 ± 1

Table 2: Effect of exposure to high and low concentration of chlorpyrifos on hemolysis (n=3).

Discussion

In this study, the effect of chlorpyrifos treatment on glucose uptake was estimated at 0.3 mg/ml and 10 mg/ml concentrations. These concentrations were selected based on its effect on hemolysis performed with an increasing concentration of chlorpyrifos (data not shown). At 0.3 mg/dL concentration, level of hemolysis was negligible (0.01%) even after 4 hours of exposure. So this concentration was considered as non-hemolytic concentration. With 10 mg/ml concentration, hemolysis was nearly 2% and 14% after 2 and 4 hours of exposure respectively. So this dose was considered to have significant hemolytic effect. Hemolysis caused by 1% DMSO was 0%. All the tubes contained 1% DMSO including the vehicle-treated control. One percent DMSO per se, did not influence the glucose uptake by whole blood (data not shown).

So the effect observed in this experiment was not due to DMSO but due to the presence of chlorpyrifos.

Exposure to low concentration of chlorpyrifos did not show significant effect on glucose uptake by whole blood (Table 1). Glucose is the universal fuel in humans in the sense that literally every type of cell in the body possesses the glycolytic pathway in its cytosol and can therefore metabolize glucose at least to the level of pyruvate or lactate. Whole blood contains RBCs, WBCs and platelets. These cells take up glucose. The plasma glucose is decreased in time dependent manner because of uptake of glucose by these blood cells. Glucose in whole blood at room temperature can undergo glycolysis at a rate of approximately 5-7% per hour in normal un-centrifuged coagulated blood [9]. Red Blood Cells (RBCs) are entirely dependent on glucose for its energy. Glucose is permeable into erythrocytes. Although red blood cells and platelets are essentially freely permeable to glucose (glucose is taken up by facilitated transport), only B-lymphocytes and monocytes among the WBCs contains insulin-responsive Glucose Transporter (GLUT-4) similar to adipocytes and skeletal muscle cells. Total number of RBCs present is in millions/cmm, while B-cells and monocytes are usually less than 1000/cmm in whole blood. In previous studies on cell culture, sub-toxic concentrations of pesticides were found to impair insulin dependent glucose uptake [14,15]. In whole blood such effect was not seen probably because majority of blood cells take up glucose in an insulin independent manner and contribution of glucose uptake by B-cells and monocytes is so meager that its effect, if at all any, is not detectable on glucose uptake measured by glucose oxidase method in whole blood. Plasma level of chlorpyrifos was found to be 0-1,726 ng/ml in an agricultural community [16] and acceptable daily intake is 0.001 and 0.003 mg/kg/day as estimated by Joint FAO/WHO Meeting on Pesticide Residues (JMPR) and US EPA respectively and 'No Observed Effect Level (NOEL)' was estimated to be 0.1 mg/kg/day as assessed from RBC cholinesterase [12]. This level is lower than the lowest concentration used in the present study. Hence at the physiological level, acute exposures are not expected to influence glucose uptake by whole blood. We performed the study to explore the effect of chlorpyrifos exposure for upto 4 hours, so we cannot comment on the effect of chronic exposures at this level or at physiological level.

However, the glucose uptake in whole blood was significantly attenuated in presence of high concentration of chlorpyrifos (Table 1). Hemolysis at 0.3 mg/ml and 10 mg/ml concentration of chlorpyrifos exposure was nearly 0.01% and 14% at 4 hrs in whole blood respectively (Table 2). LD50 of chlorpyrifos in rats is >96 mg/kg [12]. At this dose the blood level is expected to reach nearly 1 mg/ml at its peak. If the consumption is higher, this blood level may be still higher. So the dose we have selected doesn't carry much physiological relevance but has toxicological importance. Vehicle treated control sample showed a reduction of plasma glucose by 15.9% and 34.2% at 2 and 4 hours respectively. If 14% cells are hemolysed by 10 mg/ml of chlorpyrifos, expected level of decrease in plasma glucose is around 29.4%, but the observed decrease was by 22.5% which is much lower than that can be explained by the effect of chlorpyrifos-induced hemolysis alone. We attribute this disproportionate attenuation of glucose uptake that cannot be explained by hemolysis to metabolic derangement induced by chlorpyrifos toxicity. Metabolic derangement is a known phenomenon associated with pesticide toxicity [17]. RBCs are also rich in cholinesterase which is inhibited by organophosphorus compounds [18]. The active metabolite chlorpyrifos-oxon has anti-cholinesterase activity which is produced by cytochrome P450. RBC has CYP450 like monooxygenase activity which is expected to convert chlorpyrifos into chlorpyrifos-oxon in whole blood in *in vitro* experiment even in absence of liver CYP450 [19]. The derangement in cholinesterase activity is known to adversely affect the metabolic activities of cells [20]. However, we did not estimate cholinesterase activity and chlorpyrifos-oxon in this study.

Decrease in glucose uptake by blood cells might contribute to increase in plasma glucose level in pesticide toxicity as seen in various reports [21,22]. However, decreased uptake by other tissues is also expected to contribute to pesticide-induced hyperglycemia. Exposure to low concentration of pesticide did not make any change to glucose uptake by whole blood as seen in the present study. This indicates that glucose uptake assay by whole blood cannot be a suitable system to assess insulin resistance inducing property of pesticide chemicals in sub-toxic doses.

Conclusion

We conclude that whole blood is not a suitable system to assess the insulin resistance inducing property of pesticides in sub-toxic doses and the decreased uptake of glucose by whole blood is one of the contributors of raised blood glucose in acute chlorpyrifos toxicity. The decrease in uptake by high concentration of chlorpyrifos is mostly due to metabolic derangement by the pesticide and is partly contributed by hemolytic effect of the same.

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