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Pesticide induced insulin resistance in Whole Blood

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ABSTRACT

Organochlorine (OC) pesticides act as persistent organic pollutants (POPs) and their sub-chronic exposures cause insulin resistance. Different concentrations of DDT and lindane were added to whole blood collected from healthy subjects in heparin vials to develop an in vitro test for screening insulin resistance inducing properties of OCs,. Hemolysis and glucose uptake by blood cells were measured at 0, 2 and 4 hrs. The glucose uptake by blood cells did not differ on addition of either 1 mg/L of DDT or 7 mg/L of lindane from that of controls which was nearly 7% and 13.5% at 2 and 4 hrs. But DDT at 10mg/L or lindane at 70mg/L concentration further reduced the glucose uptake to nearly 6% and 12% at 2 and 4hrs respectively. At low concentration of OCs, hemolysis was negligible but at 10mg/L concentration of DDT or 70 mg/L of lindane, hemolysis was 5-6% and 12% at 2 and 4hrs respectively. As the decrease in glucose uptake by blood cells was proportional to the extent of hemolysis, this in vitro test failed to measure insulin resistance and hence cannot be used as a screening tool for insulin-resistance inducing property of POPs.

Keywords : *Organochlorine, DDT, lindane, insulin-resistance, type 2 diabetes mellitus and glucose.*

1. Introduction

Organo-chlorine (OC) pesticides are in use for many decades in agriculture and for domestic pest control. Dichlorodiphenyltrichloroethane (DDT) and gamma-hexa chloro cyclohexane (lindane) are two widely used OCs. Intrinsically being resistant to natural degradation process, these pesticides tend to accumulate in water, soil, air and food. In this regard, these compounds are termed as persistent organic pollutants (POPs). Exposure to these toxic compounds is literally unavoidable. Moreover, indiscriminate use of pesticides over the last few decades has raised alarm because of their deleterious effects on non-target organisms including human beings [1]. Recent studies have incriminated sub-toxic exposures to these compounds for pathogenesis of different chronic metabolic disorders including type 2 diabetes mellitus (T2DM) [2].

DDT and lindane, being lipophilic, have potential to enter into bloodstream easily from various sources through gastrointestinal tract, respiratory tract and skin. These molecules are known to affect human

health on chronic exposure even at very low doses. Infertility, abnormal prenatal and childhood development, cancers etc have been correlated with exposure to these pesticides [3-4].

T2DM, a metabolic disorder associated with insulin resistance, has become a major global epidemic in recent years. According to World health organization (WHO), T2DM will be the leading cause of death by 2030 [5]. Rapid urbanization, unhealthy lifestyle, genetic susceptibility, obesity, hypertension etc. are considered as etiologic factors of T2DM. In addition, a number of recent studies report that exposure to pesticides can induce insulin resistance state and thereby T2DM [6,7,8]. Studies have shown that pesticides induce insulin resistance and lead to development of T2DM in different experimental animal models [9,10].

Development of suitable, sensitive, cost effective and easy to use tool to screen insulin resistance inducing property of pesticides is very essential. Whole blood is easily available. It contains Red Blood Cells (RBCs), White Blood Cells (WBCs) and platelets. These blood cells take up glucose to fulfill their energy need. Even after collection if blood is kept *in vitro* without inhibiting their glucose utilization, these blood cell keep on consuming glucose from plasma/serum and reduce plasma/serum glucose level by 3-10% per hour from its basal level [11]. As insulin resistance is imparted in many cells by exposures to pesticides, addition of pesticides is expected to decrease glucose uptake by blood cells if insulin resistance is induced in these blood cells by pesticides. However, pesticides being toxic might cause hemolysis also. Hemolysed cells cannot uptake glucose. Therefore, reduction of glucose uptake can be contributed by hemolysis too [12,13]. In one of our previous studies, we found that chlorpyrifos, an organophosphorus pesticide caused impaired glucose uptake in blood and caused hemolysis [14]. Keeping these in view, the present study was designed to explore (a) if addition of DDT and lindane, the two prototype OC pesticides can alter glucose uptake by blood cells and inflict hemolysis and (b) if glucose uptake by whole blood can be utilized to develop a screening tool to detect insulin resistance inducing property of OC pesticides.

2. 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.

2.1 Reagents and chemicals

DDT (99% pure) was obtained as free sample from Hindustan Insecticide Ltd (Delhi, India). Lindane and Dimethyl Sulfoxide (DMSO) was purchased from Sigma Aldrich (Saint Louis, M.O., USA).Glucose Oxidase Kit was procured from Randox (County Antrim, United Kingdom).

2.2 Experimental design

It was an in vitro study done on whole blood. Three healthy male volunteers of age 30, 32 and 28 years were recruited for the study. They were having plasma/ serum levels of fasting glucose, urea, creatinine, total bilirubin, ALT, AST, Alkaline phosphatase and hs-CRP within reference range. Blood sample of 9 ml was collected in heparin tubes by venipuncture from each volunteer. Whole blood of 2 ml was used to perform hemolysis assay. Blood of 1 ml was used for baseline (time-zero) plasma glucose assay. Remaining 6 ml of blood was used for subsequent glucose estimation after OC administration.

2.3 Plasma glucose estimation

Blood sample of 6 ml was transferred to five aliquots of 1.2 ml each. DMSO that was used as vehicle to administer OCs to blood was added at final concentration of 1% to one aliquot. The remaining 4 aliquots, DDT (1mg/L and 10mg/L) and lindane (7mg/L and 70mg/L) were added after dissolving in DMSO (final concentration $\leq$1%). These pesticides were added, mixed gently by shaking the aliquots and then the tubes were incubated at 25°C. After 2 hours

of incubation with pesticides, 0.6 ml blood was aspirated from each aliquot, centrifuged immediately and plasma glucose was measured. Similarly, after completion of 4 hours of incubation with pesticides or DMSO, remaining 0.6ml blood was centrifuged and plasma glucose was measured. Plasma glucose level was determined using glucose oxidase and peroxidase kit adopted to random access automated clinical chemistry analyzer (DXC800, Beckman, CA, USA). The glucose level at time zero was used as the basal (time-zero) glucose value and the subsequent values (after 2 hour and 4 hour) were expressed in percentage considering basal glucose level as 100%.

2.4 Hemolysis assay

Red blood cells (RBC) hemolysis assay was performed by method as described by Hu et al. [15]. The quantity of 2 ml of heparinized blood was washed with 4 ml sterile Dulbecco's phosphate buffer saline (DPBS) and mixed gently by shaking and centrifuged at 3000 rpm for 5min for The RBC separations. The clear supernatant was discarded gently without disturbing RBC pellet. Then 10 ml DPBS was added, mixed gently by shaking and then centrifuged at 3000 rpm for 5 min. Washing of RBC was repeated 3 times with 10 ml of DPBS and finally the RBC pellet was diluted to 20 ml with DPBS. Quantity of 500µl of diluted RBC was transferred to 6 micro-centrifuge tubes. A 500µl distilled water was added to one tube for preparing positive control. Rest of the tubes was filled with 500µl of DPBS each. One of these tubes was used as negative control and rests were added with 10µl DMSO containing DDT (final conc. 1 mg/ml and 10 mg/ml) or lindane (final conc. 7mg/ml and 70mg/ml). After incubating at room temperature for two hours, 500µl sample was taken from each tube and centrifuged at 3000 rpm for 5 minutes. Similarly, after four hours remaining samples were centrifuged similarly. Quantity of 100µl of the supernatant of each sample was transferred to a 96 well (flat bottom microtiter) plate and absorbance was measured by a microplate reader (Biorad 680 XR, CA, USA) at 570 nm with 655 nm as reference. The percentage of hemolysis was assessed as hemolytic ratio using the following formula:

Hemolysis percentage ratio
= {(OD test - OD negative control) ÷
(OD positive control - OD negative control)}
× 100

2.5 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 HSD post hoc test using the SPSS PC version 17. The differences were considered statistically significant at P value <0.05.

3. Results

Plasma glucose level decreased in a time dependent manner in whole blood containing vehicle (1% DMSO). The decrease was around 7% at the end of two hours and 13.5% at four hours after blood collection.The effect of different concentrations of DDT and lindane on glucose uptakeby whole blood is depicted in Table 1.

DDT at 1 mg/L concentration did not significantly alter glucose uptake but suppressed it significantly ($p<0.05$) at 10 mg/L concentration in comparison to vehicle treated control. Similarly, lindane at 7 mg/L concentration did not alter the rate of reduction in plasma glucose level but at 70 mg/L concentration, glucose uptake by whole blood was significantly decreased at two hours as well as at four hours of incubation with lindane.

Table 2 shows the effect of DDT and lindane on hemolysis. With 1 mg/L concentration of DDT, the hemolysis was 0.5% and 0.8% at 2 and 4 hours respectively. With concentration of 10mg/L, hemolysis was 6% and 12% respectively. Lindane at 7 mg/L concentration caused hemolysis by 0.1% and 1.2% at two and four hours of incubation respectively. However, at concentration of 70 mg/L, hemolysis levels were increased nearly to 5% and 12% respectively.

Table 1. Effect of exposures to high and low concentrations of DDT and lindane on glucose uptake by whole blood (n=3)

	Glucose level at zero hour	% of reduction in plasma glucose	
		At 2 hr (Mean ± SD)	At 4 hr (Mean ± SD)
Control (Vehicle 1% DMSO)	100%	7.1 ± 0.21	13.49 ± 0.36
DDT (1mg/L)		7.3 ± 0.21	13.45 ± 0.31
DDT (10mg/L)		6.5 ± 0.31*	11.61 ± 0.47*
Lindane (7mg/L)		6.9 ± 0.46	12.51 ± 0.44
Lindane (70mg/L)		6.5 ± 0.35*	11.70 ± 0.50*

where, *P<0.05 in comparison to corresponding basal (zero hour) level by One-Way ANOVA followed by Tukey's post-hoc test.

Table 2. Effect of exposure to high and low concentrations of DDT and lindane on hemolysis (n=3).

	% of RBC hemolysis	
	At 2 hr (Mean ± SD)	At 4 hr (Mean ± SD)
Control (Vehicle 1% DMSO)	0.2 ± 0.01	0.4 ± 0.02
DDT (1mg/L)	0.5 ± 0.04	0.8 ± 0.02
DDT (10mg/L)	6.2 ± 0.45	12.02 ± 0.38
Lindane (7mg/L)	0.1 ± 0.62	1.21 ± 0.32
Lindane (70mg/L)	5.21 ± 0.85	12.10 ± 0.70

4. Discussion

In present study, we observed a time dependant reduction in plasma glucose concentration when blood was kept after collection at 25°C. This reduction of plasma glucose is a measure of glucose uptake by cellular components of blood. Low concentration of DDT (i.e. 1mg/L) and lindane (7mg/L) did not affect glucose uptake by whole blood significantly. However, high concentration of DDT (10mg/L) and lindane (70mg/L) attenuated glucose uptake in comparison to vehicle treated controls. At lower concentrations of above organochlorine exposure, hemolysis level was relatively less (upto 0.8%) than that (12%) caused by their concentrations at 4hrs of incubation. The extent of decrease in glucose uptake in presence of DDT and lindane was in proportion to the extent of hemolysis caused by those pesticides. So, we infer that hemolysis was the cause of decreased glucose uptake in presence of these pesticides and insulin resistance did not contribute to this decreased glucose uptake or else, the assay method failed to measure alteration in glucose uptake due to insulin resistance induced by these OCs. This is probably because this pesticide could not induce significant insulin resistance in blood cells. Among the blood cells, RBCs and platelets which constitute major part of packed cell volume take up glucose in an insulin independent manner, because they do not contain GLUT4 [16]. Only B-cells and monocytes among the WBCs have GLUT4 glucose transporter [17]. Transport of glucose in these cells may be affected by insulin resistance induced by DDT and lindane. But these cells constitute so less portion of the packed cell volume that their contribution to whole blood glucose uptake is negligible. The measurement of glucose by GOD/POD method is not enough sensitive to pick up the difference in glucose uptake caused by induction of insulin resistance by the pesticides in meager number of B-cells and

monocytes present in whole blood. Hence, probably hemolysis was the only contributor of measurable reduction in glucose uptake by whole cell in presence of DDT and lindane.

In a previously published article, we showed that chlorpyrifos, an organophosphate pesticide also caused reduction in glucose uptake by whole blood [18]. The extent of hemolysis could not explain the extent of reduction in glucose uptake by this pesticide in blood. We attributed the reduction in glucose uptake by blood to metabolic derangement and hemolysis as well, induced by chlorpyrifos. Although DDT and lindane are also toxic substances and are expected to exert their toxicity at the concentration used in the present study but that has not happened with reference to glucose uptake in the present study. This might be because of difference in mechanism of action of organochlorines pesticides and organophosphates. Organophosphate pesticides exert their toxicity by inhibiting cholinesterase enzyme which is known to influence metabolism in RBCs [19]. DDT exerts its toxicity through neuronal sodium channels [20]. Probably these channels are absent in RBCs and other blood cells. Hence, metabolism of glucose in blood cells is not impaired in presence of DDT. Lindane exerts its toxicity through GABA receptor [21]. There is no available report till date that detected GABA receptors in RBCs. Hence, probably glucose uptake by RBCs remains unaffected in presence of lindane. Hence, we conclude that glucose uptake by whole blood cannot be used for detecting insulin resistance inducing property of organochlorine pesticides.

The concentration of pesticide that is used in the study has toxicological relevance. In pesticide toxicity, hyperglycaemia is observed (22). The hemolysis-induced reduction in glucose uptake in blood might be contributing to this hyperglycaemia observed in pesticide toxicity.

But exposure due to environmental contamination cannot bring the blood level of OCs to the concentration used in this in vitro study. We may consider the blood level of OCs due to these environmental exposure as physiological levels. So we conclude that at physiological concentrations acute exposure does not cause any hemolysis or impairment of glucose uptake in whole blood. Effect of sub-chronic exposure on glucose uptake at physiological concentration is not possible to be evaluated in such in vitro experiment but is worth investigating to explore the mechanism of environmental pesticide exposure induced diabetes mellitus.

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