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Prevention of phosphine-induced cytotoxicity by nutrients in HepG2 cells

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***Background & objectives*: Phosphides used as an insecticide and rodenticide, produce phosphine (PH_3) which causes accidental and intentional poisoning cases and deaths. There is no specific treatment or antidote available for PH_3 poisoning. It is suggested that PH_3-induced toxicity is associated with adenosine triphosphate (ATP) depletion; therefore, in this study the effect of some nutrients was evaluated on PH_3 cytotoxicity in a cell culture model.**

***Methods*: PH_3 was generated from reaction of zinc phosphide (10 mM) with water in the closed culture medium of HepG2 cells, and cytotoxicity was measured after one and three hours of incubation. ATP, glutathione (GSH) and lipid peroxidation were also assessed at one or three hours post-incubation. ATP suppliers including dihydroxyacetone, glyceraldehyde and fructose were added to the culture medium 10 min before PH_3 generation to prevent or reduce phosphine-induced cytotoxicity.**

***Results*: Phosphine caused about 30 and 66 per cent cell death at one and three hours of incubation, respectively. ATP content of the cells was depleted to 14.7 per cent of control at one hour of incubation. ATP suppliers were able to prevent cytotoxicity and ATP depletion induced by PH_3. Dihydroxyacetone, α-ketoglutarate, fructose and mannitol restored the ATP content of the cells from 14.7 per cent to about 40 , 34 , 32 and 30 per cent, respectively. Lipid peroxidation and GSH depletion were not significantly induced by zinc phosphide in this study.**

***Interpretation & conclusions*: The results supported the hypothesis that phosphine-induced cytotoxicity was due to decrease of ATP levels. ATP suppliers could prevent its toxicity by generating ATP through glycolysis. α-keto compounds such as dihydroxyacetone and α-ketoglutarate may bind to phosphine and restore mitochondrial respiration.**

Key words Dihydroxyacetone - fructose - glyceraldehyde - phosphine - α-ketoglutarate

Aluminium phosphide is used widely as a cost-effective and highly potent grain fumigant and rodenticide in developing countries. Easy availability of this fumigant insecticide makes it an important public health concern in Asian countries. Many cases of intentional or accidental poisoning by aluminium phosphide or zinc phosphide occur every year, specially reported from Iran and India[1-5]. It has extreme

toxic effects on humans because no effective and suitable treatment or antidote is available. Therefore, it is essential to find effective antidotes for phosphine poisoning. In contact with moisture, metal phosphides yield phosphine (PH_3) gas which is the ultimate pesticide or rodenticide[6,7]. Metal phosphides react with hydrochloric acid content of stomach and lead to release of phosphine vigorously[1,2].

Phosphine is a non-specific toxin and many organs of the body are affected by this toxin including gastrointestinal, cardiovascular, neurological, hepatic, pulmonary and musculoskeletal systems[8-12]. Clinically, poisoning with metal phosphides presents with nausea, vomiting, severe shock, hypoglycaemia, restlessness, abdominal pain, acute respiratory distress, pulmonary oedema, cyanosis, hypotension and cardiac arrhythmia. Various neurobehavioural changes such as ataxia, tremor, convulsion and central nervous system depression have also been observed[2,5,6,13].

Phosphine has been shown to have a strong inhibitory effect on cytochrome c oxidase, the complex IV of mitochondrial respiratory chain[14,15]. Generation of reactive oxygen species (ROS) and lipid peroxidation has also been reported to be involved in phosphine toxicity[16,17].

The management of acute intoxication with phosphine is mainly supportive, as there is no specific and effective antidote or treatment available for phosphine poisoning. Intravenous administration of magnesium salts has been reported to decrease the mortality rate possibly by preventing oxidative stress[18].

Because the main mechanism of toxicity of phosphine has been suggested to be inhibition of mitochondrial respiration and energy depletion, in the present study, the effect of some α-keto acid adenosine triphosphate (ATP) suppliers was evaluated on cytotoxicity of zinc phosphide in HepG2 cell cultures as a model for cytotoxicity. Because the liver is the main metabolic organ of the body and HepG2 cells are more similar to hepatocytes, this cell line was chosen for this study.

Material & Methods

This study was carried out during 2011-2013 at School of Pharmacy, Shiraz University of Medical Sciences, Iran.

Cell culture and viability measurement: Human liver cancer cell line $HepG_2$ (Pasteur Institute, Tehran, Iran) after thawing was cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (Gibco, USA), *p*H 7.3, containing 0.37 per cent sodium bicarbonate ($NaHCO_3$), five per cent foetal bovine serum albumin (BSA, Gibco, USA), 100 IU/ml penicillin and streptomycin 100 IU/ml (Biosera, Germany) at 37°C under an atmosphere of 95 per cent air/5 per cent CO_2 at five per cent humidity. After 18 h, the medium was aspirated and replaced with fresh RPMI medium. The cell suspension was centrifuged at 200×g for 5 min at 4°C (Sigma 2k15, Germany); the supernatant was replaced with one ml of the fresh medium. Equal volume of 0.4 per cent trypan blue (Merck, Germany) was added to the cell suspension and the viable/dead cells were counted using a light microscope (Leitz, Germany).

Treatment of cells: The cells were transferred to 6-well plates [2×10^6/ml for cytotoxicity studies, 3×10^6/ml for glutathione (GSH) or lipid peroxidation measurement and 5×10^6/ml for ATP measurement] and immediately were used for zinc phosphide toxicity studies. Zinc phosphide (Riedel-de Haen, Germany) was dissolved in RPMI and immediately added to the plates and sealed with Parafilm to prevent exit of phosphine gas. The cells were incubated for one or three hours with zinc phosphide in the presence or absence of nutrients: fructose, mannitol, DL-glyceraldehyde (GAD), α-ketoglutarate (α-KG), dihydroxyacetone (DHA) or N-acetylcysteine (NAC) (Sigma, Germany) which were added to the culture medium either 10 min before or simultaneous with zinc phosphide.

ATP measurement: ATP levels were measured using a Knauer high-performance liquid chromatography system, C-18 column, coupled with a dual absorbance ultraviolet (UV) detector (Model 2487) and ChromGate software program (Germany), as previously explained[19]. Briefly, culture (1 ml) containing 5×10^6 cells was treated with 1 ml of 0.35 perchloric acid plus 1 M Na-EDTA on ice. The mixture was centrifuged at 9000 g for 4 min at 5°C. The supernatant was neutralized with 2 M potassium hydroxide (KOH) and centrifuged after vortexing. The supernatant was used for measuring ATP. The mobile phase was 0.1 M ammonium dihydrogen phosphate (*p*H = 6.0) with a flow rate of 1 ml/min. The absorbance of nucleotides was recorded at 206 nm and the run time was 20 min.

Measurement of lipid peroxidation: Lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) as described previously[20]. Cells were washed with 2 ml of 0.5 M phosphate buffer and centrifuged. Two millilitres of a mixture of 20 per cent

trichloroacetic acid (TCA, Merck, Germany), 0.8 per cent thiobarbituric acid (Sigma, Germany) and 0.5 per cent HCl were added to the pellet and mixed by vortex. After boiling for 20 min in hot water, the absorbance of the supernatant was measured at 532 nm (Pharmacia Biotech, Ultrospec 2000 UV, Sweden).

Measurement of glutathione: The cells were treated with 0.2 ml TCA solution (20%) plus 1.8 ml phosphate-buffered saline (PBS). After centrifugation, GSH and oxidized glutathione (GSSG) were determined in the supernatant as previously described[21]. For GSH, 2 ml of 0.3 M Na_2HPO_4 buffer (*p*H 7.4) was added to 1 ml of the supernatant. Then, 0.5 ml of 0.1 M DTNB [5'5'-dithiobis (2- nitrobenzoic acid); Merck, Germany] solution was added to the mixture; after 20 min absorbance was measured at 412 nm. For GSSG, 1 ml of the supernatant was incubated with 1 ml of five per cent sodium borohydride ($NaBH_4$) solution at 45°C for one hour to reduce GSSG to GSH and the GSH content was measured as described above.

Phosphine binding assays: Phosphine trapping by dihydroxyacetone was determined by measuring the absorbance of free DHA at 271 nm[22], using a Shimadzu spectrophotometer (Japan). The compounds were dissolved in de-ionized water. After mixing equimolar amounts of zinc phosphide (10 mM) and dihydroxyacetone, the tubes were sealed, and after 5 min (to produce phosphine on hydrolysis) the reduction in the absorbance of free DHA was measured.

Statistical analysis: Significant differences between control and experimental groups were obtained using Student's t-test where two groups were compared and one-way ANOVA where more than two groups were compared and controlled by honestly significant difference test (Tukey's HSD test) and Dunnett's post-hoc test. The GraphPad InStat 3.0 (GraphPad Software Inc., La Jolla, California, USA) and SPSS 11.5 (SPSS Inc., Chicago, IL, USA) software were used for running the tests.

Results & Discussion

The exact mechanism of phosphide toxicity is not known, but it is generally believed that phosphine gas (PH_3) is produced upon the interaction of metal phosphides with water in biological or aqueous systems. PH_3 prevents ATP synthesis causing disruption of cell homoeostasis and finally cell death[23-25]. However, oxidative stress and lipid peroxidation and some other mechanisms have also been suggested to be involved in its toxicity and fatality[15,16,26]. Because of the similarity of the mechanism of cytotoxicity of phosphine with cyanide and antidotal effects of ATP supplying agents such as dihydroxyacetone, glyceraldehyde or fructose against cyanide-induced cytotoxicity[27], cytotoxicity of

Table I. Effect of zinc phosphide and nutrients on viability and adenosine triphosphate (ATP) content of HepG2 cells

Addition	Percentage of viability		ATP content (μmol/5×10⁶ cells) at 1 h
	1 h	3 h	
None (control)	92±5	89±6	44.9±6.1
ZnP (10 mM)	70±5*	34±5*	6.6±2.8*
ZnP (10 mM) + α-KG (5 mM)	84±4†	56±12*†	15.3±1.9 *†
ZnP (10 mM) + GAD (5 mM)	80±4*†	55±4*†	9.5±2.5*
ZnP (10 mM) + DHA (5 mM)	84±6†	37±3*	17.9±4.4*†
ZnP (10 mM) + fructose (10 mM)	82±5†	30±2*	14.6±2.8*†
ZnP (10 mM) + mannitol (10 mM)	80±6*	36±10*	13.5±4.6*†
ZnP (10 mM) + NAC (0.05 mM)	86±7†	52±9*†	10.6±5.3*
α-KG (5 mM)	89±8†	80±7†	33.9±6.3†
GAD (5 mM)	85±5†	78±3†	36.4±7.2†
DHA (5 mM)	89±5†	81±6†	29.3±8.1*†
Fructose (10 mM)	87±6†	84±2†	27.9±4.9*†
Mannitol (10 mM)	86±5†	82±5†	31.8±0.9*†
NAC (0.05 mM)	90±3†	89±3†	41.5±5.5†

Data are mean±SD, n=3. *P<0.05 compared to control untreated cells; †P<0.05 compared to ZnP treated cells. ZnP, zinc phosphide; α-KG, α-ketoglutarate; GAD, glyceraldehyde; DHA, dihydroxyacetone; NAC, N-acetylcysteine

zinc phosphide and antidotal effects of α-keto acids and ATP suppliers in HepG2 cell line were studied.

The addition of zinc phosphide to HepG2 cells resulted in ATP depletion followed by cytotoxicity in a concentration- and time-dependent manner. Zinc phosphide (10 mM) caused about 30 and 66 per cent cell death at one and three hours of incubation, respectively (22 and 55% more than control; Table I). At one hour after incubation with 10 mM zinc phosphide, the ATP content of the cells was depleted to about 14.7 per cent of the normal value (Table I), while the cell viability was only 22 per cent less than control untreated cells. Perhaps cytotoxicity, as measured by plasma membrane disruption, occurred when ATP depletion was extensive enough to cause irreversible cell injury. Zinc phosphide cytotoxicity was significantly prevented at one hour of incubation by all nutrients *i.e.*, α-KG (14%), GAD (10%), DHA (14%), fructose (12%) and mannitol (10%); however, at three hours of incubation, only α-KG (22%) and GAD (21%) were significantly cytoprotective (Table I).

Dihydroxyacetone prevented ATP depletion better than other compounds at the first hour of incubation (40%) followed by α-KG (34%) (Table I). Addition of nutrients without zinc phosphide also partially depleted ATP content of the cells, but these did not induce cell death (Table I). These are possibly cytoprotective because of supplying energy by protecting mitochondrial respiratory chain from inhibition by phosphine or by supplying ATP through glycolysis. All of these compounds except fructose have a carbonyl group in their structure which is known to reversibly react with nucleophiles such as cyanide both *in vitro* and *in vivo*[28]. Because phosphine also has a nucleophile nature, it is possible that the carbonyl group of α-KG, GAD and DHA reversibly bind to free phosphine and partially reduce the inhibitory effect on cytochrome c oxidase and restore mitochondrial respiration.

DHA showed a peak at 271 nm which was concentration-dependent, but addition of zinc phosphide immediately decreased the absorbance

Table II. Effect of zinc phosphide on the absorbance of dihydroxyacetone

Concentration	OD (λ_{max}=271 nm)
DHA (2.5 mM)	0.135
DHA (5 mM)	0.175
DHA (10 mM)	0.275
DHA (20 mM)	0.435
DHA (5 mM) + ZnP (10 mM)	0.032
DHA (10 mM) + ZnP (10 mM)	0.070
ZnP (10 mM)	0.064

DHA, dihydroxyacetone; ZnP, zinc phosphide

Table III. Effect of zinc phosphide and nutrients on thiobarbituric acid reactive substances production and glutathione/oxidized glutathione content of HepG2 cells

Addition At 3 h	MDA (pg/3×10^6 cells)	GSH (µmol/3×10^6 cells)	GSSG (µmol/3×10^6 cells)
None (control)	320±30	6.0±0.7	0.5±0.3
ZnP (10 mM)	205±20*	5.6±1.8	2.1±0.2*
ZnP (10 mM) + α-KG (5 mM)	420±30*†	1.8±0.8*†	4.3±0.4*
ZnP (10 mM) + GAD (5 mM)	200±10*	5.5±1.4	0.8±0.1*†
ZnP (10 mM) + DHA (5 mM)	200±10*	5.2±2.1	0.3±0.1*†
ZnP (10 mM) + fructose (10 mM)	400±50†	2.0±0.1*†	4.0±0.4*
ZnP (10 mM) + mannitol (10 mM)	530±50*†	1.4±0.1*†	5.0±0.2*
ZnP (10 mM) + NAC (0.05 mM)	430±50*†	11.5±2.2*†	0.9±0.1*†
α-KG (5 mM)	610±70*†	7.9±1.1	1.97±0.3*
GAD (5 mM)	280±7	5.9±0.2*†	0.19±0.1†
DHA (5 mM)	230±30	4.8±1.5	0.4±0.1*†
Fructose (10 mM)	470±40*†	4.3±1.9	0.39±0.1*†
Mannitol (10 mM)	420±30*†	5.7±2.2	1.38±0.1*
NAC (0.05 mM)	270±20	11.8±2.6*†	0.8±0.0*†

Data are mean±SD, n=3. $^*P$<0.05 compared to control untreated cells; $^\dagger P$<0.05 compared to ZnP treated cells. ZnP, zinc phosphide; α-KG, α-ketoglutarate; GAD, glyceraldehyde; DHA, dihydroxyacetone; NAC, N-acetylcysteine; MDA, malondialdehyde; GSH, glutathione; GSSG, oxidized glutathione; SD, standard deviation

(Table II). This suggests that DHA and possibly other α-keto nutrients bind phosphine. The protective effects of these compounds were less at three hours of incubation, which could be because of their metabolism by the cells during this period leading to a decrease in their concentration.

NAC, an important antioxidant to replenish the intracellular GSH, enhanced cell survival (16%) without significantly changing ATP content against phosphine cytotoxicity (Table I). NAC possibly protected cytotoxicity of phosphine by increasing GSH content of HepG2 cells and detoxifying the toxic metabolite(s) of oxidative damage and decreasing malondialdehyde (MDA) level. Phosphine toxicity could be the consequence of energy insufficiency as well as oxidative stress and also the depletion of GSH levels[3, 29].

In this study, significant lipid peroxidation or GSH depletion was not seen (Table III). Co-treatment of zinc phosphide with α-KG, fructose, mannitol and NAC increased MDA and GSSG concentration, but GAD and DHA decreased MDA and GSSG levels after three hours incubation. It has been reported that oxidative stress, lipid peroxidation and GSH depletion occur by aluminium phosphide[16,18,30]. The difference in our results could be because of the antioxidant effect of zinc which prevents cell against oxidative stress.

In conclusion, our study showed that the main mechanism of phosphine- induced cytotoxicity was by depletion of energy content of the cell. ATP suppliers and specially α-keto acids prevented its cytotoxicity by both supplying ATP in glycolysis process and trapping phosphine and restoring ATP production.

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***Conflicts of Interest*:** None.

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