Haryana Vet. (Dec., 2014) **53 (2)**, 110-112

Research Article

LOW DOSES OF THIOUREA AND THIOMERSAL INDUCES HORMETIC CELL PROLIFERATION CYTOTOXICITY ASSAY

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Received: 07.08.2014; Accepted: 06.09.2014

ABSTRACT

A study was conducted to observe the cytotoxicity of thiourea and thiomersal on mouse splenocytes by 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Thiourea and thiomersal were two fold serially diluted to obtain concentrations (ppm) of 1.6421, 0.8211, 0.4105, 0.2053, 0.1026, 0.0513, 0.0257, 0.0128, 0.0064, 0.0032, 0.0 for thiourea and 25.0, 12.5, 6.25, 3.125, 1.5625, 0.78125, 0.39063, 0.19532, 0.09766, 0.04883, 0.0 for thiomersal in a microtitre plate. One hundred microlitre of splenocytes (1×10^7 cells/ml) were added to each concentration of thiomersal and thiourea followed by incubation at 37°C for 16 h in a CO_2 incubator. Later, the viability of the cells was quantified using MTT. The results of cell viability (%) showed that IC50 of thiourea and thiomersal was 0.08681 ppm and 0.069046 ppm, respectively. In both the compounds, hormetic cell proliferation was observed at lower doses. In conclusion, hormetic cell proliferation of splenocytes both by thiourea and thiomersal may account for erratic immune response due to splenocyte proliferation.

Key words: Cytotoxicity, thiourea, thiomersal, MTT assay, hormetic cell proliferation, splenocytes

Since last two decades, there has been increasing scientific concern and public debate regarding the adverse effects of chemical pollutants present in the environment (Verma and Rana, 2009). A large number of environmental chemicals including thiourea and thiomersal have been found to have cytotoxic effects.

Thiourea is commonly used in the production and modification of textile and dyeing auxiliaries, leaching of ores, production of pharmaceuticals and pesticides, as a vulcanization accelerator and as an auxiliary agent in diazo paper. Thiomersal is an organic compound that has been used as an antimicrobial agent, topical antiseptic solutions and ointments, nasal sprays, eye solutions, vaginal spermicides, diaper rash treatments and most importantly as a preservative in vaccines and other injectable biological products, despite evidence of it to be potentially hazardous to humans (Geier *et al*., 2007; Ng *et al*., 2007). The present study was carried out to access cytotoxicity of two different compounds *viz*. thiourea and thiomersal by 3-(4, 5-dimethylthiazole-2-yl)-2, 5-dipenyltetrazolium (MTT) assay using mouse splenocytes.

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MATERIALS AND METHODS

Chemicals: Rosewell park memorial institute medium (RPMI-1640), MTT, ammonium chloride, foetal calf serum and dimethyl sulphoxide (DMSO) were obtained from HiMedia, Pvt. Ltd., Mumbai and were of analytical grade. Microtitre plates (96-wells) were obtained from Tarsons, Pvt. Ltd.

Cytotoxicity study (MTT Assay): Prior to the conduct of experiment, Institutional Animal Ethics Committee approval was obtained for this study. The MTT assay was performed as per the protocol described by Mosmann (1983). Briefly, the spleen was collected from two euthanized mice and mashed in 5 ml of RPMI-1640. The cell suspension was centrifuged and cell pellet was re-suspended in 2 ml of RPMI and 10 ml of ammonium chloride. Later, the centrifugation was carried out, the pellet was washed two to three times with RPMI and again centrifuged. Viability of spleen cells was assessed using 0.4% trypan blue under a light microscope. Cell density was adjusted to 1×10^7 cells/ml in RPMI supplemented with 10% foetal bovine serum.

One hundred microlitre of RPMI was added to each well of the ELISA plate. 100 µL of test compound

was added to the first well and two-fold serial dilutions were made in subsequent wells. To each of the wells, 100 µL of splenocyte cell suspension was added. Cell control and media controls were maintained. The ELISA plate was incubated for 16 h at 37ºC in a CO_2 incubator. Later, 10 µL of MTT reagent (5mg/ml) was added to all the wells 4 h before completion of incubation time. After completion of incubation time, ELISA plate was centrifuged at 1200 g for 10 min and the supernatant was discarded. To each well, 100 µL of DMSO was added to dissolve the formazan formed. The absorbance was read at 530 nm with an ELISA reader (Readwell touch –Robonik India, Pvt. Ltd., Mumbai) within 10 min.

Calculation of half maximal inhibitory concentration (IC50): IC50 was calculated from the linear trend line (y=ax+b) of the cell viabilities of splenocytes for thiourea and thiomersal using the following formula: IC50 = (0.5-a)/b where a = slope and b= y-intercept.

RESULTS AND DISCUSSION

Cytotoxicity of thiourea (Fig. 1) and thiomersal (Fig. 2) showed IC50 of 0.08681 and 0.069046 ppm, respectively. Cytotoxicity of thiourea was higher at concentrations exceeding 0.05 ppm whereas for thiomersal it was at 0.39 ppm. At low doses, a hormetic response characterized by low doses stimulation and high doses inhibition of cell growth was observed. Similar findings of hormesis were reported for nanosilver particles against *Staphylococcus aureus* (Kumar *et al*., 2014), silver nanoparticles in human hepatoma derived cell line (Kawata *et al.,* 2009) and for silver nanoparticles in peripheral blood mononuclear cells (Shin *et al.,* 2007). Implications of having a wide stimulatory zone may be clinically significant. Stimulatory zone defines the therapeutic window and is important to recognize that the hormetic stimulatory zone is graphically contiguous with the pharmacologic/toxicologic threshold (Calabrese, 2005).The hormetic dose response might have occurred as a direct stimulatory response, after an initial disruption in homeostasis followed by the modest overcompensation response or as a response to an 'adapting' or 'pre-conditioning' dose that is followed by a more massive challenging dose (Davies *et al.,* 1995).

The metabolic transformation of thiourea to formamidine sulfinilic acid is catalysed by microsomal flavin containing monooxygenases and the toxicity of this metabolite leads to depletion of reduced glutathione

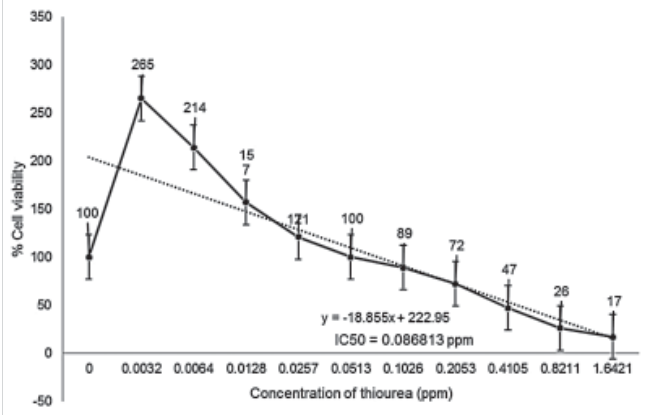

Fig 1. Cytotoxicity of thiourea on mouse splenocytes

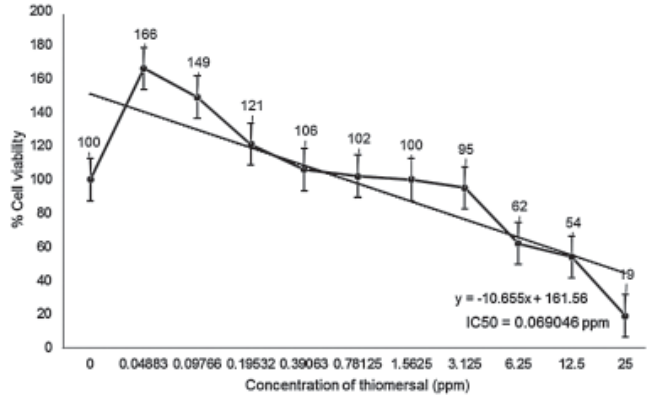

Fig 2. Cytotoxicity of thiomersal on mouse splenocytes

leading to cytotoxicity (WHO, 2003). At lower doses, a stimulatory effect was observed which may be due to interaction of antigen with cell fixed IgE antibodies which may trigger the release of histamine because of cytolysis leading to erratic immune response.

Thiomersal is an organic mercury compound and toxicity studies have implicated reactive oxygen species and depletion of intracellular glutathione as a major contributor to mercury induced cytotoxicity (Sanfeliu *et al*., 2001). Organic mercury has high affinity for the thiol (-SH) group on glutathione. Normally, the intracellular concentration of glutathione is extremely high (Meister, 1995), however, with its depletion, an excess of free mercury is available for binding to thiol group present in essential proteins leading to functional inactivation and cytotoxicity.

It has also been shown that thiomersal administration results in decreased production of pro-inflammatory cytokines, TNFα, IL-6 and IL-12 (Agrawal *et al*., 2007). One of the implications of thiomersal is development of autism. This is because of the fact that

undigested toxins may attack the central nervous system as intestinal wall is swollen and hyperplastic and the immune system in intestine is activated resulting in stimulatory effect at lower doses.

Hormetic dose response occurs in essentially all the species of plants, microbes, invertebrates and vertebrates in all organ systems and for a large number of endpoints, there is a generalized mechanistic strategy but no single mechanism. One such strategy is a single agonist that may bind to two receptor subtypes; one activating a stimulatory pathway whereas, other activating an inhibitory pathway. The receptor subtype with the greatest agonist affinity would typically have fewer receptors (i.e. lower capacity) and its pathway activation effects dominate at lower doses. Conversely, the second receptor subtype would have lower agonist affinity, greater capacity (i.e. more receptors) and become dominant at higher concentrations (Szabadi, 1977; Leff, 1994*;* Rovati and Nicosia, 1994*;* Jarv, 1995*).* It may also be directly applicable to situations in environmental toxicology in which the toxin induces dose-dependent changes in the concentrations of various endogenous agonists, a situation which is known to occur commonly.

The results of cell viability (%) showed a hormetic response. The hormetic cell proliferation of splenocytes by thiourea may account for erratic immune response and thiomersal may account for immunomodulation. Further *in vivo* validation is required as it has the potential to affect the design of pre-clinical studies and clinical trials as well as strategies for optimal dosing in the treatment of numerous diseases.

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