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A preliminary study assessing the effect of isocyanate in neuroblastoma brain cells *in vitro*

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Isocyanate is an intermediate compound used in the manufacturing of a number of pesticides. The aim of this study is to understand the mechanism of isocyanate in SHSY-5Y neuroblastoma cells *in vitro*. Cells were treated with a chemical equivalent of isocyanate, i.e., N-succinimidyl N-methylcarbamate (NSNM). Cell cytotoxicity, as well as qualitative and quantitative alpha-synuclein protein levels were analyzed using different molecular techniques. NSNM at a concentration of 0.005 μM significantly increased cell death, in a time-dependent manner, as well as levels of alpha-synuclein protein in SH-SY5Y cells. These findings demonstrate the ability of low doses of isocyanate to increase neuronal vulnerability by inducing cell cytotoxicity and protein dysfunction *in vitro*.

Key words: pesticides, isocyanate, neurotoxic, N-succinimidyl N-methylcarbamate, exposure

INTRODUCTION

In today's world, understanding the effects on human health of occupational exposure to hazardous agents is a primary concern. A number of diseases such as cancer, diabetes, neurological impairment and lung diseases are associated with such exposures (Gangemi et al., 2016). There is a lack of human health risk assessments regarding exposure to natural and environmental hazards. A very limited number of studies have shown that isocyanates and their derivatives may have injurious health effects, while the molecular mechanisms behind such effects have yet to be addressed.

Isocyanate is an intermediate chemical used in the production of carbamate pesticides and includes methomyl, carbaryl, carbofuran and aldicarb. It is widely used in the formation of polyurethane forms, paints, insecticides, varnishes and elastomer industries (Hon et al., 2017). Methyl isocyanate has been found in the smoke from several types of cigarettes at 1–5 ppm. Screening for adverse effects of pesticides and their intermediates is very important from a health perspective, as these pesticides are used on a large scale and the primary mode of pesticide application is generally through aerosols, which can be inhaled easily and readily absorb into soils. To the best of our knowledge, no studies have been done to assess the effect of isocyanate on brain cells. In view of this, the aim of this study is to understand the molecular mechanisms induced by isocyanate using neuroblastoma cells *in vitro*. Isocyanate is highly toxic and not readily available in markets, thus N-succinimidyl N-methylcarbamate (NSNM; containing a functional isocyanate group) was used in this study to understand the mode of action of this compound in cultured SH-SY5Y neuroblastoma cells *in vitro*.

METHODS

Dulbecco's modified Eagle's medium (DMEM)/ Nutrient F-12Ham, fetal bovine serum (FBS), antibiotic-antimycotic solution, MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)], and NSNM were procured from Sigma Aldrich, and alpha-synuclein primary antibody and horseradish per-

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oxidase (HRP) conjugated secondary antibody from Santa Cruz Biotechnology.

Cell culture grade chemicals were procured from Himedia. Culture flasks, 96 ELISA plates and petri dishes were procured from BD falcon.

Cell culture condition and NSNM exposure

SH-SY5Y cells were gifted by Dr. Amit Deshpandey and were grown in DMEM/F-12Ham media with 10% FBS and 1% antibiotic/antimycotic solution in a humidified environment containing 5% CO_2 and 95% air at 37°C as per ATCC catalogue instructions. The study was designed in a time-dependent manner. Dimethyl sulfoxide (DMSO) was used to prepare a 1 M stock solution of NSNM. At 75% cell confluency, cells were treated with a 0.005 µM concentration of NSNM for 24, 48 and 72 h. For each parameter, untreated cells were used as a control.

Assessment of cytotoxicity

5×10^3 cells were seeded onto 24-well plates and kept overnight at 37°C in a 5% CO2 incubator.

The next day, cultured cells were treated with 0.005 µM NSNM and incubated for 24 h, 48 h and 72 h. After the appropriate exposure time, cells were harvested and washed with phosphate-buffered saline (PBS). An equal volume of trypan blue solution (0.4% in PBS) and cell suspension were mixed and incubated for 10 min. After incubation, cells were loaded on a Neubauer chamber and counted under a microscope. Cell death was calculated as the percentage of trypan blue positive cells in the total population of stained and unstained cells.

Cell cytotoxicity can be determined using yellow MTT dye. Living cells reduce the MTT to purple formazan with the help of mitochondrial dehydrogenase enzymes. Cells were plated on 96-well culture plates at a density of 5×10^3 cells/well. After overnight incubation, cells were treated with 0.005 µM NSNM. After the exposure time, cells were then incubated with MTT (0.5mg/mL) for 3–4 h. The formazan particles were then solubilized with 200 µl dimethyl sulfoxide and kept on an orbital shaker for 5 min. The absorbance was recorded at 570 nm through an ELISA reader. The results were expressed as viable cell percentage with respect to an untreated control.

Western blot for the detection of alpha-synuclein protein in SH-SY5Y cells

Western blot assay was performed to examine the alpha-synuclein protein expression level in SH-Y5Y cells. Cells were seeded and treated with 0.005 µM NSNM. After different time points of incubation, cells were washed with PBS. Lysis was carried out using ice cold cell lysis buffer containing 1% triton X-100 (prepared in 0.1 M sodium phosphate buffer), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) or 100 µl of protease inhibitor cocktail at 4°C for 30 min. The cell lysates were centrifuged at 1,000 × g for 10 min at 4°C. Concentration of protein was determined by the Bradford method. After determining the concentration of protein, extracted protein was loaded on to a 10% gel for SDS-PAGE. Blots were hybridized with antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH; dilution 1:1000) and against alpha-synuclein (1:2000) overnight at 4°C. The membranes were then washed and further hybridized with secondary antibody tagged with HRP (1:3000) for 1 h and washed twice with phosphate buffer saline-Tween 20 (PBST). The blots were developed using a suitable substrate and the expression of protein was visualized.

Indirect immunofluorescence for the detection of alpha-synuclein protein in SH-SY5Y cells

Through an indirect immunofluorescence assay, expression of alpha-synuclein protein was determined in SH-SY5Y cells. 2×10^4 cells were grown on glass cover slips for 24 h. After incubation, media was changed and 0.005 µM NSNM was added for 24, 48 and 72 h. After incubation, cells were washed with PBS, followed by fixation with 3% paraformaldehyde for 20 min and then permeabilized with 0.1% triton X-100 for 10 min. Cells were blocked with 10% BSA for 30 min. Primary antibody at a dilution of 1:1000 was added and the cells were incubated for 1 h. Cells were washed with 0.1% PBST. After washing, cells were incubated with fluorescein isothiocyanate (FITC) conjugated secondary antibody (dilution 1:40) for 2 h, preferentially in dark. Again, cells were washed with 0.1% PBST. The cover slip was mounted and viewed, followed by photographing using an inverted fluorescence microscope.

Quantification of alpha-synuclein in SH-SY5Y cells by cell based ELISA

5×10^3 cells/well were incubated in 96-well plates. After overnight incubation, cells were treated with NSNM for different time points. After the exposure time, cells were fixed with 0.1% formaldehyde in PBS. After removing the formaldehyde, the wells were gently washed with PBS three times. Methanol was added for 5 min. Blocking was carried out with 3% serum. Wells were washed with PBS. Alpha-synuclein primary

antibody was added (1:1000) for 2 h. Wells were washed with PBST 0.01% and further incubated with secondary antibody (1:5000) for 30 min. The cells were washed with PBST, as earlier, and TMB (3,3',5,5'-tetramethylbenzidine) was added to each well. 2N H2SO4 was used to stop the reaction and absorbance at 440–460 nm was taken using a plate reader (BMG Fluostar).

Statistical analysis

All experiments were done in duplicate. Statistical differences were analyzed using Student's t-test and a p-value ≤0.05 was considered statistically significant.

RESULTS

NSNM's effects on the viability of SH-SY5Y cells

When SHSY-5Y cells were treated with 0.005 µM NSNM, significant time-dependent effect on cell death was observed as compared to control ($p<0.05$) and measured by trypan blue exclusion assay (Fig. 1A). In addition, MTT assay results revealed that from 24 h to 72 h cell viability was significantly decreases as compared to control ($p<0.05$; Fig. 1B).

Qualitative analysis of alpha-synuclein protein expression in SH-SY5Y cells treated with NSNM

When SH-SY5Y cells were exposed to 0.005 µM NSNM for different time points, increased band intensity was observed with increasing incubation time as compared to control using the western immunoblotting technique (Fig. 2). GAPDH was used as a loading control. Indirect immunofluorescence results revealed elevated expression of alpha-synuclein protein under 40x magnification as compared to control. At 48 and 72 h the cell morphology of SHSY-5Y cells had also changed as compared to control (Fig. 3).

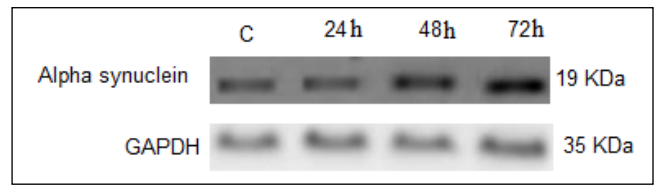

Fig. 2. Western blot analysis showing increased band intensity of alpha-synuclein protein with increasing incubation time.

Qualitative analysis of alpha-synuclein protein in SH-SY5Y cells after treatment with NSNM

To quantify the expression of alpha-synuclein protein, cell-based ELISA was performed and the results showed that, with an increasing incubation time for cells treated with 0.005 μM NSNM, the level of alpha-synuclein significantly increased as compared to control ($p<0.05$; Fig. 4).

DISCUSSION

Studies have reported that pesticides are associated with human health and environmental concerns.

Effects that have been linked with pesticides and herbicides in humans include skin, brain, lungs and

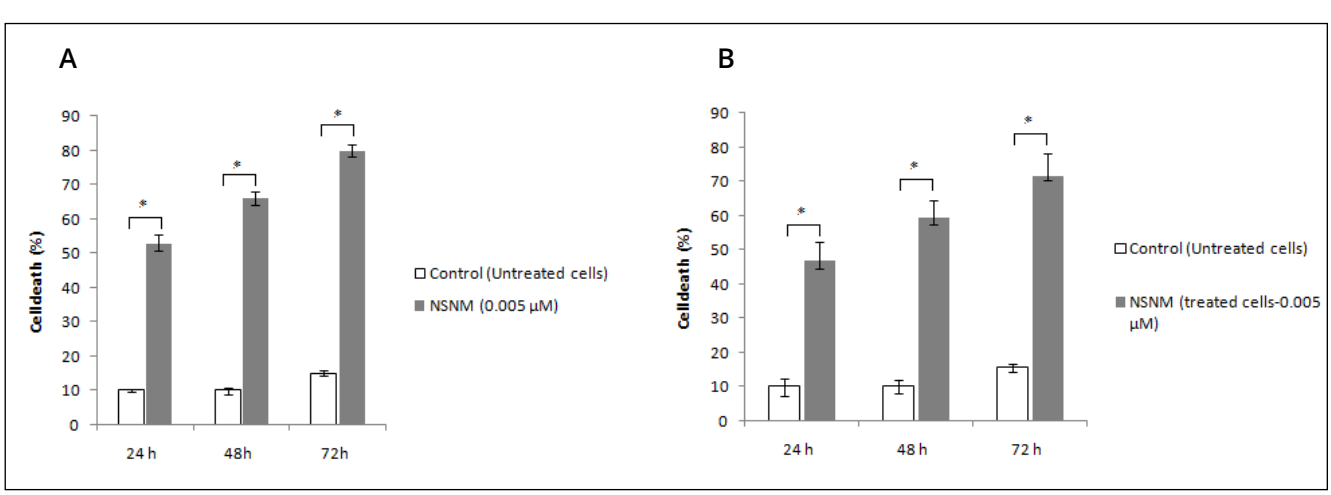

Fig. 1. (A) Cell death through trypan blue assay. Cell death significantly increased with increasing incubation time. *p<0.05. (B) Cell death determined by MTT assay. Cell death significantly increased with increasing incubation time. *p<0.05.

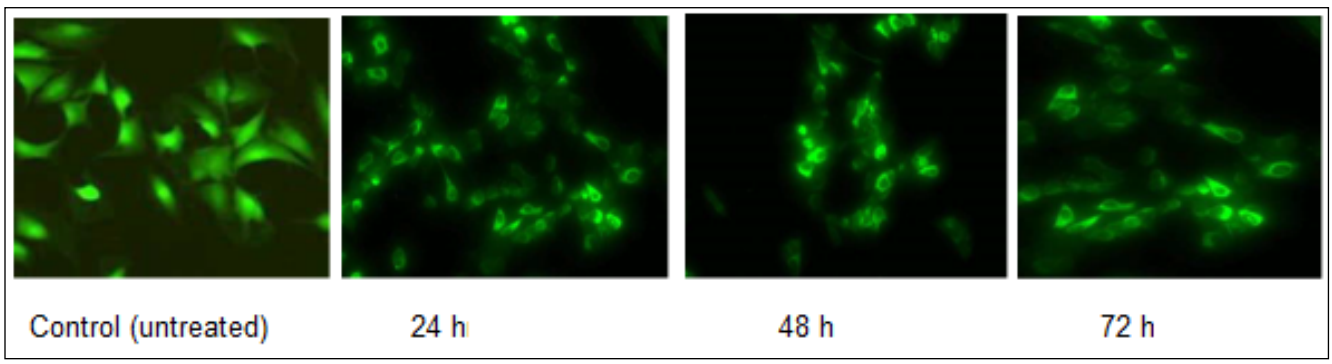

Fig. 3. Indirect immunofluorescence images showed elevated levels of alpha-synuclein protein from 24 h to 72 h as compared to control.

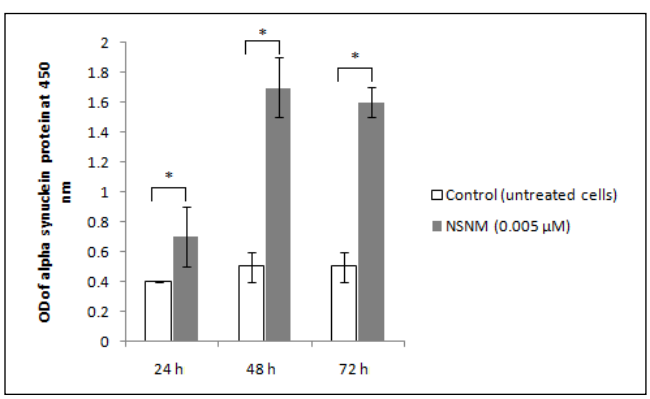

Fig. 4. Cell-based ELISA assays showed that with increasing incubation times of SH-SY5Y cells with NSNM, the levels of alpha-synuclein expression were also increased significantly as compared to control. *p<0.05.

stomach changes. Additionally, occupational or accidental exposures to pesticides can lead to urgent hospitalization and even death. Screening of such pesticides and understanding the underlying mechanisms causing illness is very important for intervention.

Isocyanate is an intermediate chemical used in the production of a number of pesticides. To our knowledge, isocyanate has not previously been studied to determine its effect in brain cells. In the present study we used cultured SH-SY5Y neuroblastoma cells to understand the mode of action of isocyanate. We used a chemical equivalent of isocyanate (NSNM) to study the effect in SH-SY5Y cells. We screened for cytotoxicity of NSNM as wells as levels of alpha-synuclein in cultured brain cells after exposure for different time periods. The cellular toxicity of NSNM on SH-SY5Y cells was assessed by the trypan blue and MTT assays. Our data showed that NSNM at the concentration of 0.005 µM induced cytotoxicity in a time-dependent manner. Previous studies reported that NSNM induces cell death in mouse-derived cell lines, as well as in yeast, and revealed that the apoptotic pathway and DNA damage proteins were involved in cell death mechanisms (Mishra et al., 2008). In addition, Azad et al. (2014) conducted an experiment on budding yeast and identified NSNM as an epigenetic modifier that forms an adduct with histone and leads to decreased histone acetylation.

Methyl isocyanate gas, which is known for the world's worst exposure disaster in Bhopal, India, also caused genotoxicity in rat brain cells *in vivo* in a dose-dependent manner (Anderson et al., 1990).

A case-control study conducted by Chuang et al. (2017) revealed that people exposed to carbamate pesticides were at a higher risk of developing Parkinson's disease.

In our study, immunofluorescence results demonstrated a change in the morphology of SHSY-5Y cells with increasing incubation time with NSNM. Our findings are in agreement with the finding of Chang and colleagues (2006), which found that 50 µM of aldicarb and carbaryl significantly decreased neurite length in neuroblastoma cells as compared to control. Our MTT assay results revealed cell cytotoxicity upon exposure to NSNM. Ruiz et al. (2006) conducted an experiment on Chinese hamster ovary cell lines and showed that mitochondrial integrity was disturbed upon exposure to seven different carbamate pesticides using the MTT assay.

In the present study, expression of alpha-synuclein protein was also studied in SH-SY5Y cells after exposure to NSNM. Many neurodegenerative diseases are characterized by the abnormal accumulation of aggregated proteins in the brain. Thus, we analyzed the expression of alpha-synuclein protein quantitatively, as well as qualitatively, by Western blot, indirect immunofluorescence and cell-based ELISA, respectively. Our results showed that with increasing incubation time the expression of alpha-synuclein protein also increased. Several studies (Viller-Pique et al., 2016; Xicoy et al., 2017) have been documented describing that certain pesticides increase the level of alpha-synuclein protein in SH-SY5Y cells. In this study, it was

noted that with increasing incubation time, increased cell death, as well as increased levels of alpha-synuclein protein, were observed. Several studies have reported that aggregation of alpha-synuclein makes neurons more prone to cell death (Yasuda et al., 2013; Vila and Przedborski, 2003). Our results are in agreement with other studies where cells treated with different classes of pesticides demonstrated an overexpression of alpha-synuclein, which led to mitochondrial dysfunction and cell death in various cultured cell lines (Norris et al., 2007; Chorfa et al., 2013). A study conducted by Ramalingam et al. (2019) demonstrated that complex and disrupted alpha-synuclein protein dynamics were involved in neurodegeneration in response to the pesticide rotenone through western blot analysis of alpha-synuclein protein. Another study explained that rotenone induced an upregulation of alpha-synuclein protein levels through the stimulation of their de novo synthesis rather than through a reduction of their chaperone-mediated autophagy degradation (Sala et al., 2013).

CONCLUSION

These findings represent the ability of low doses of isocyanate to increase the vulnerability of neurons by inducing cell cytotoxicity and protein dysfunction. The screening of environmental contaminants capable of causing deregulation of neuronal function and/or trigger brain-related disorders would be of significant benefit in controlling exposures and likely reducing the incidence of neurological impairments. Furthermore, additional studies are required to understand the internal signaling pathways associated with neural damage from exposure to isocyanate.

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