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Effects of Organophosphate on Cell Cycle, regulation, proliferation and viability in mitosis of neural progenitor stem cells

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ABSTRACT: The effect of organophosphates like paraoxon(POX) and parathion (PTH) in the sub cytotoxic level which alters the cell cycle or the deregulation caused by the low levels of toxins were detected. The sub cytotoxic levels of the toxins helps us to understand the pathological causes and the mechanism of toxicity in chronic exposure of the toxins. The methods used to detect the effect of these compounds on acetylcholinesterase which detects effect of the toxins inhibition on a standard concentration of enzyme with different concentrations of toxins. MTT assay which detects the cell metabolic activity and detect the number of viable cells present. Gel electrophoresis and western blotting is used to detect the amount of protein present in the sample of cell lysate used treated by the toxins. Cell imaging immunofluorescence is used to detect the cell and calculate the cell mass and neurite length which detected the effect of toxins. According to the results it was observed that the mitotic changes were observed at the sub-cytotoxic levels of the toxins. The study was to detect the changes in the morphology of the neurons and to find the changes in the cell cycle.

I. INTRODUCTION:

1.1 Importance of organophosphate compounds

They are generally used as insecticides in domestic conditions. Insecticides are used in both large scale as monoculture crops and small scale conditions like garden and orchard yields. Usage of organophosphate compounds also destroys the insects with aesthetic value of home landscapes (Japanese beetles, Gypsy moths), the structural stability of human residences (termites, carpenter ants), and the interior home insects (cockroaches, silverfish). They are used as antihelmintic, anti-flea and tick treatments(**Booth, et al. 1988**).

1.2 Medicinal uses of organophosphates

Organophosphates are also used as medicinal applications. Ecothiophate iodide and isofluorophate can relieve the intraocular pressure associated with glaucoma. They are used in application to the conjunctiva of eye, constriction of the ciliary and iris muscle decreases and allows intraocular pressure to decrease by allowing the reabsorption of intraocular liquid through the canal of Schlemm thus reduces the intraocular pressure (**Koelle 1994b**)

1.3 Toxic effects of organophosphates

Organophosphate pesticides inhibit the acetylcholinesterase(AChE), leads to the accumulation of acetylcholine(ACh) in the body, which is present in nerve synapse and receptors it acts by phosphorylating the serine hydroxyl residue on AChE, which inhibits AChE. AChE is critical for nerve function, thus the irreversible blockage of this enzyme, which causes accumulation of acetylcholine, results in overstimulation of muscles. In acute conditions the action on central nervous system causes seizures, convulsions, coma, respiratory failure. If the patient survives more than 36 hours' personality changes can occur, aggressive events, psychotic episodes, disturbances and deficits in memory and attention. If death occurs its due to the paralysis of respiratory muscle due to the depression of respiratory centre in the brain. In the chronic conditions neurotoxic effects in humans: cholinergic syndrome, intermediate syndrome, organophosphate-induced delayed polyneuropathy (OPIDP), and chronic organophosphate-induced neuropsychiatric disorder (COPIND)(**Moore 2009**). The intermediate syndrome (IMS) appears in period between the end of the cholinergic crisis and initial stage of OPIDP. Symptoms of IMSare found within 24-96 hours after exposure. OPIDP found in small number of cases, roughly two weeks after the exposure which

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causes temporary paralysis the symptoms worsen in 3-4 months. In most cases quadriplegia has been observed(**Jokanović and Kosanović 2010**). Organophosphates poisoning occurs through inhalation, ingestion, and dermal contact. The severity of toxicity depends on the specific compound, route of exposure, amount of toxin and its concentration (**Katz, et al. 2016**). Organophosphates and carbamate pesticide residues were found in cigarette tobacco and other food and agricultural commodities (**Mavrikou, et al. 2008)**. Commonly used organophosphates have included parathion, malathion, methyl parathion, chlorpyrifos, diazinon, dichlorvos, phosmet, fenitrothion, tetrachlorvinphos, azamethipphos, and azinphos-methyl. The two compounds used in the project were parathion (O-diethyl-O-(4-nitrophenyl) phosphorothioate) and paraoxon (Diethyl p-nitrophenyl phosphate). Parathion is mostly used organophosphate, it is highly toxic through all the routes of exposure dermal, ingestion and inhalation. On skin it may cause roughening and thickening effect but does not causes allergic reactions. On inhalation it may cause runny nose which may include blood, coughing, chest discomfort, and short breath. On ingestion causes muscular paralysis, visual impairment, breathing difficulty, sensory disturbance, restlessness, weakness, vomiting, convulsion, coma and death(**Peter, Sudarsan and Moran 2014**). The lowest toxic dose in humans is 240 µg/kg**(Hotchkiss, et al. 1989**).

1.4 Effects of organophosphates on the neuronal cells

Organophosphates causes the toxic effect in the sub cytotoxic concentrations chlorpyrifosnoncholinergic action which denotes the inhibition of brain development **(Crumpton, Seidler and Slotkin 2000a**). OP's also interact with muscarinic receptors inSH-SY5Y human neuroblastoma cells**(Ehrich, Intropido and Costa 1994**). Apoptosis and mitosis share morphological similarities if mitosis is inhibited it causes inappropriate activation of cell cycle enzymes which leads to cell death. Neuropathy target esterase (NTE) reacts to organophosphates which causes paralysis and degeneration of long nerves in legs and spinal cord. NTE is not required for dividing cells t is essential for post mitotic cell survival in placental secondary giant cell brain neurons (**Glynn 2006**). The effect of organophosphates decreases the mitochondrial enzymes like NADPH dehydrogenase, succinate dehydrogenase, and cytochrome oxidase. Thus it causes the death of mitochondria these functions were assessed by MTT reduction confirmed mitochondrial dysfunction and development of OPIDN (**Masoud, Kiran and Sandhir 2009)**. Chronic exposure to organophosphates was also a cause for neurological disorders like Alzheimer's, Parkinson's, and Amyotrophic Lateral Scleroosis diseases **(Sánchez-Santed, Colomina and Hernández 2016, Parrón, et al. 2011**). Prenatal exposure to the organophosphate insecticide chlorpyrifosenchances the brain oxidative stress and prostaglandin E2 synthesis in a mouse model of idiopathic autism **(De Felice, et al. 2016).** The morphological changes are observed in the neurons and the movement of axons the fundamental changes includes cytoskeleton and motor proteins involved in the axonal transport and mitosis of the cells **(Terry 2012).**

1.5 The organophosphates used in the study (Parathion and Paraoxon)

Parathion induces oxidative stress(**Beauvais, et al. 2000**) this compound has shown to have developmental neurotoxicity(**Adigun, et al. 2010**). Paraoxon cause long term neurotoxicity behavioural and pharmacological evidence(**Sarwara, Allwoodb and Innes-Kera 2014)**. In the low-level chronic exposure, which usually take place in occupational environment, can cause other neurotoxic effectsConditions of neurological and neurodegenerative diseases have been reported in epidemiologic studies performed with pesticide spreaders, greenhouse workers, agricultural workers and farmers occupationally exposed to pesticides. Low amount of chronic exposure is the cause of neuropathy and neuropsychiatric diseases found in British farmers **(Povey, et al. 2014**). Organophosphates have been the cause of disruption of cytoskeleton proteins (**Flaskos 2014**)and impairment of axonal growth in cell culture by inhibiting the enzyme neuropathy target esterase (**Sachana, et al. 2001**). Parathion decreases activity of either plasma pseudocholinesterase (ChE) or red blood cells acetylcholinesterase (AChE). Plasma ChE level appears to be a sensitive index of exposure and may be better correlated with clinical effects then blood concentrations. Another syndrome has been described after exposure to some Ops, it's also called as organophosphorus-induced delayed polyneuropathy. This is paralysing syndrome characteristized by the degeneration of nerve

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axons, which is clinically detectable 14 to 16 days' post-exposure. Conversely, this delayed neuropathy is triggered by the phosphorylation and further chemical modification of an esterase (other than AChE) called Neuropathy Target Esterase(NTE)(**Johnson and Glynn 1995**).

The aim of the study is to find the changes in the cultured neuroblastoma cells taking place during exposure to sub cytotoxic concentrations of the organophosphates like parathion and paraoxon and compare the changes in differentiating N2a cells. Neurotoxic effects depend on the developmental window in which the exposure takes place and can be particularly severely in early stages, when complex cellular-molecular process of neuroblastoma cell, proliferate and differentiate. The effects of toxins on these stages shows the chronic effect and we will be looking for the changes in the different phases of cell cycle on exposure to the sub cytotoxic level of toxin like mechanism of neurodegeneration. Adult Aims brains are well protected by the blood brain barrier (BBB) while the children's BBB is not fully differentiated till 6 months of the birth **(Adinolfi 1985, Kodavanti and Tilson 2000**) at the same time they have high absorption and diminished ability to detoxify the exogenous compounds like adults (**Meeker and Stapleton 2010**) . The two compounds at different concentrations at three different dilutions are exposed to cultured neuroblastoma cells. Extract of cells will be analysed by western blotting treated with parathion and paraoxon. Exposure to parathion and paraoxon at different concentrations may also alter the fluorescence of N2a cell body staining (**Pamies, et al. 2014)**. N2a (Neuro 2A cells) are the fast growing mouse neuroblastoma cell line **(Salto, et al. 2015**). N2a cells helps in the studies of cell differentiation, different properties of neurons, including neurofilaments. They also help in neurite outgrowth and neurotoxicity studies **(LePage, et al. 2005**). N2a cells are useful in differentiating cell line study of organophosphate **(Hargreaves, Sachana and Flaskos 2011**). To evaluate the minimum toxic dose and sub cytotoxic dose of organophosphates Parathion and Paraoxon on chronic exposure. This study will help to evaluate the effect of organophosphates bringing changes in different stages of cell cycle and the cause of cell death can be known. The different stages and the changes in the cell cycle shows the pathological changes on the chronic exposure of organophosphate to find the solution to neurological disorders.

II. MATERIALS

2.1 Materials used in Cell culture

N2a cells, DMEM 10% foetal bovine serum containing 2Mm L-glutamine, Penicillin 100 units/ml and Streptomycin 100 mu g/ml provides supplements to DMEM, Foetal bovine serum,T-25/T-75 Cell culture flask Sterile pipettes, Ethanol 90%, Haemocytometer, Confocal microscope, +4˚C Refrigerator, -20˚C Freezer, 37˚C CO2Incubator, Vortex mixture, pH meter, Centrifuge, Centrifuge tubes, Micropipette sterile tips.

2.2 Materials used for MTT assay

24 and 96 well plates are used, Toxins: Parathion and Paraoxon diluted in different concentrations, MTT (Thiazolyl Blue Tetrazolium Bromide), DMSO (Dimethyl Sulfoxide), Incubator ,Ethanol 90%.

2.3 Materials used for SDS-PAGE

Materials used for preparation of polyacrylamide gel, N-tetramethylethylenediamine TEMED, 10%w/v ammonium persulphate, Resolving buffer 1.5M Tris/HCl pH 8.9, Stacking buffer 0.5M Tris/HCl pH 6.7, Resolving Acryl mixture (per mixed solution) 29.2g Acryl, 0.8g Bisulphate, Running buffer pH 8.3(10× stock) 6.0g Tris 28.4g glycine dissolved in 1.0 litre + 0.1%(w/v) SDS, An electrophoresis chamber and power supply, Gel casting trays, Sample combs, Pipettes of 0.5-50 µl, 2-20 µl, 20-200 µl,100-1000µl, Sterile tips, Eppendorf tubes.

2.4 Materials used for western blotting

Blotting apparatus, Continuous transfer buffer (CTB): made by the mixture of SDS and methanol at ratio of 4:1 respectively, Tris buffer saline (TBS): 0.5M Tris pH 7.6 containing 9%(w/v) NaCl, PBS (pH 7.4), 12 pieces of 3MM filter paper cut slightly larger than the gel (8cm×9cm for mini gels), 1 piece of nitrocellulose membrane filter (6cm×9cm for mini gels), 3%(w/v) bovine serum albumin (BSA)in PBS, 0.5%(v/v) Tween 20 in PBS (PBS/Tween), Sodium Azide, Primary antibodies: α-anti tubulin, anti-phosphoserine, anti-PCNA, Secondary antibodies: Anti-mouse conjugated to HRP, Anti-mouse to FITC, Magnetic stirrer.

2.5 Software's used

Molecular (high content image processing software), Image Xpress(Metaxpress), Graph pad Prism 7, Microsoft word and Microsoft excel.

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III. METHODS

3.1 Growth medium

10% foetal bovine serum containing 2Mm L-glutamine, penicillin 100 units/ml and streptomycin 100 mu g/ml provides supplements to DMEM. Store at 4˚C.

3.2 Procedure for maintaining cell lines

Monolayers of N2a cell lines were maintained in T25 flask with growth medium and stored in an incubator maintaining 37˚C with atmosphere 5% Co_2 /95% of air. At regular time intervals the cell lines are passaged to maintain the healthy environment by maintaining the adequate growth medium and space for the growth of cells. When there is approximately 80% confluence. Soon after the cells are ready to sub culture. N2a cells are isolated by removing all except 1-2ml of growth medium. Then the growth medium was repeatedly blown for about 21 times using a sterile Pasteur pipette across the monolayer to detach the cells from the base and the cell containing medium is transferred to centrifuge tubes and made up to 10ml of growth medium. The cell containing growth medium was centrifuged at 300 rpm for 5 min to get a cell pellet. Through a Pasteur pipette the cell pellet will be re suspended in 1 ml of growth medium into the t25 flask containing 10ml of growth medium. After the time intervals of 3-4 days were readily to passage. Only cells below passage number 25 were used in the experiment. The flask was monitored on regular basis.

3.3 Cell passage and plating

After the cells are detached, centrifuged and when the cell pellet is obtained remove the supernatant growth medium and 2ml of fresh growth medium was added and mixed well using a pipette. Then take 10µl of suspension mix it with 90µl of fresh growth medium vortex for 10 seconds to mix well. Add 10µl of suspension to the chamber of haemocytometer for the cell count.

No of cells/ml = Average cell count × 10 × dilution factor (10)

Further the cells were plated by diluting suspension to a density of 50,000 cell/ml appropriate volume of diluted cell suspension will be added to the 24 well plate for MTT analysis or 8 well plates for cell cycle or to passage the cells to the next T75 flask. Then the flask or the well plates were allowed to incubate for 24hrs for the cells to grow normally in the wells. Then growth medium was removed and added with the toxin mixed growth medium and further incubate of 24hrs to have the exposure of toxin to the same period as growth medium.

3.4 MTT Analysis

MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) it is the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening. The MTT substrate is prepared in a physiologically balanced solution, added to cell culture, usually at the concentration of 0.5mg/ml, and incubated for 30 mins. Assays are performed to assess the metabolic activity of cell. Changes in the MTT assay denotes the changes in the metabolism of the cell. The product is insoluble in water should be dissolved in adequate solvent which gives purple solution spectrophotometrically. Dimethyl Sulfoxide (DMSO) is the solvent of choice for N2a. Among any cytotoxic assay some are trypan blue exclusion, ATP measurement, neutral red uptake or release shows membrane integrity, macromolecular synthesis, glutathione one depletion.

The method used to perform the assay was the toxin dilution of 1ml of 10mM concentration of paraoxon and parathion further from the 10mM toxin 0.5ml of toxin is transferred to other tube and diluted with 0.5ml of DMSO which makes the concentration of 5mM, from 5mM 0.5ml of toxin mixture was taken and diluted with 0.5ml of DMSO which makes concentration of 2.5mM. Further the dilution is made from 2.5mM by withdrawing 0.5ml of toxin mixture and dilute it with 0.5ml of DMSO which makes the concentration of 1.25mM further which is diluted and further concentration of toxin is prepared to 0.625mM. Control is done with DMSO. Then 2.5ml of growth medium is taken in 6 different tubes added with 12.5µl of 5 different concentration of toxins and in 1 tube with control of 12.5µl of DMSO. The toxin and control mixtures were added to the 24 well plates which were cultured with N2a cells for 24hours. Then let the cells get the same exposure time of toxins as it had with growth medium so it was further incubated for 24hours at 37˚c. After 24 hours all the solution from the wells were removed and added with 50µl of MTT solution and left for 30minutes incubation. Then all the MTT solution was removed and added with 0.5ml of DMSO and left on flat bed shaker for 10minutes. Then 200µl of medium was transferred to 96 well plate. Then the absorbance is taken at 570nm and the graph is plotted on absorbance verses concentration.

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3.5 Acetylcholinesterase Assay

The toxins are diluted in 6 different concentrations dilution are from the concentration of 10mMand further diluted with 50µl of 10mM toxin which is diluted with 250µl of DMSO which is2mM. 50µl of toxin is extracted from 2mm and diluted with 250µl of DMSO which makes 0.4mM. 50µl of toxin is extracted from 0.4mm and diluted with 250µl of DMSO which makes the concentration of 0.08mM. 50µl of toxin is extracted from 0.4mM and diluted with 250µl of DMSO which makes the concentration of 0.08Mm. 50µl of toxin is extracted from 0.08mM and diluted with 250µl of DMSO which makes the concentration of 0.016Mm. 50µl of toxin is extracted from 0.016mM and diluted with 250µl of DMSO which makes the concentration of 0.0032mM. Then acetylcholinesterase enzyme is diluted with phosphate buffer and made into 4 different dilutions. The stock of acetylcholinesterase is 10µl was taken concentration of 0.05mg/ml added with the 490µl of buffer further diluted by withdrawing 200µl from the stock solution and diluting with 800µl of buffer makes the concentration of 0.001mg/ml. Same way the further dilution is done with concentration of 0.001mg/ml by extracting 200µl from it and diluting with 800µl of buffer further diluted same and the next concentration was 0.004mg/ml. The concentration of 0.004mg/ml of acetylcholinesterase is used for the mixture with toxins for the experiment. Thus 0.004mg/ml AChE is used with 500µl added with 2.5µl of DMSO as control or the different concentration of toxins. The other important step performed for the AChE assay was DTNB and AI mixture with 1.5ml of each and mixed. Finally, 96 well plate is taken and 100µl of AI+DTNB mixture is added to the wells and added with 100µl of the AChE+ toxin mixture to each well with different replicates was done and the reading was taken at 570nm. The graph is plotted between the time and absorbance.

3.6 Acetylcholinesterase protein assay

The N2a cells are cultured and passaged in the T75 flask with the count of 50000/ml and left in the growth medium for 24 hours for the cells to have normal life cycle after 24hours the growth medium is removed and the toxin mixed growth medium is introduced into the flask the 20ml of growth medium was added with 100µl of toxin. The flask is then stored in the incubator to have normal environmental conditions to find the exact effect of toxin on the cells. After 24 hours the toxin mixed growth medium is removed and added with 10ml of ice cold PBS and cells are detached and centrifuged at 1000 rcf for 10minutes to pellet cells. Supernatant liquid was removed and re-suspended the cells in 10ml of ice cold PBS and centrifuged for 10 minutes for 1000 rcf. The supernatant liquid was removed and 2ml PBS was added to the pellet. Mixed well with pipette and transfer to the Eppendorf tube further centrifuged with 13000 rpm for 30 mins the pellet is obtained by removing the supernatant liquid. The lysis buffer was added to the pellet which is phosphate buffer and 0.2% Triton. Finally centrifuged for 30 minutes for 30000 rpm and the supernatant is the cell lysate to be used in the experiment. Then add 100µl of cell lysate to the wells and then add 100µl of DTNB+AI mixture. Finally, the absorbance is taken at 560nm. According to the graph plotted the amount of protein present in cell lysate treated with paraoxon was 1.9mg/dl and 1.68mg/dl for parathion.

3.7 Electrophoresis and western bolting

For performing immunoblotting technique, the N2a cells will be seeded in growth medium in T75 flask at density of 50,000cells/ml and left to differentiate in addition of the drugs parathion and paraoxon for 100µl of toxins to 20mlof suspended growth medium. After the exposure time of 24 hrs the growth medium was removed and cells were detached using the cooled PBS and centrifuged at 1000 rcf for 10 min. Then the pellet will be removed to Eppendorf tube and further centrifuged to 13000 rpm for 30 min, thus the cell pellet will be obtained and added to heated SDS to lysate the cells and obtain to proteins from the chromosomes and further centrifuged to 13000 rpm for 30 minutes to obtain the cell lysate substrate. This cell lysate substrate will be analysed to protein assay and amount of protein present will be found and further analysed by SDS-PAGE. The sample buffer will be used as the dye (Laemmli et al. 1970). By western blotting the separated proteins will be blotted onto nitro cellulose membrane. The western blotting technique will be performed to check the transfer of proteins of right sample. The blots containing monoclonal antibodies that recognize to α-tubulin and HSP mouse antibody with incubation. Antibody reactivity was recognized by increased chemiluminescence (ECL, Amersham, UK). Quantiscan image analysis system was performed on western blots interfaced to high resolution camera. Protein levels were calculated by directly related antibody activity (**Sachana et al. 2011**)

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3.8 Preparation of gel

It is prepared by resolving and stacking solution. These two prepared by resolving buffer reagent A (1.5M Tris/HClpH 8.9) and stacking buffer reagent B (0.5M Tris /HClpH6.7) Resolving acryl mixture reagent C 29.2g Acryl, 0.8g bisulphate.

To prepare 10% gel resolving 5 ml of reagent A was used. 5ml of reagent C was used. 0.1ml of 20% (w/v) SDS, 9.6ml of H2O, 60µl of 10%(w/v) ammonium per sulphate is used and 40µl of TEMED is used. The total mixture of 20ml is used as the resolving solution. To prepare the stacking solution 5ml of reagent B is used and 2.5ml of reagent C is used 20%(w/v) SDS 0.1ml, 12.3ml of H2O, 60µl 10%(w/v) ammonium per sulphate and 40µL of TEMED. Total volume makes about 20ml. After the two solutions are prepared the revolving solution is poured into the gel cast plate till 1cm below. Then make up with the stacking solution, and Teflon well forming comb is inserted into the gel cast. Overlay do butanol and allowed to polymerize. Remove comb from the cast and wash it with the distilled water. The gel is ready to run. The apparatus is prepared the gel was fitted into the tank and filled with running buffer PH 8.3 (10× stock) 6.0g Tris 28.4g glycine dissolved in 1.0 litre + 0.1%(w/v) SDS. Then connect the electrodes to the power supply, power supply was turned on and voltage was adjusted to 120volts. Finally, the gel is run for 30 minutes till the standard mark comes just above 1cm from the other end of the gel. Turn off the power supply, disconnect the electrode leads, and remove the chamber lid and removed the electrophoresis chamber from the tank. The gel is removed from the cast carefully without breaking and continued with western blotting.

3.9 Western blotting

After the electrophoresis the gel is removed from the glass cassette and soaked briefly in continuous transfer buffer (CTB) 1:4 ratio of 99% methanol with SDS. The surface of cathode and anode plates of western blotting apparatus were moistened with distilled water. Six pieces of blotting paper were moistened and 3 papers were placed on the anode plate and nitrocellulose membrane was placed followed by the gel and finally placing the 3 filter papers. Air bubbles were removed by rolling the filter papers with a glass rod. The cathode plate was positioned on the filter paper/gel sandwich. Power supply was connected and run the transfer at a current of 60Ma per gel for 60 minutes. After the process of blotting nitrocellulose membrane is incubated with a blocking agent to prevent the binding of primary antibody to the nitrocellulose membrane during probing. The blocking is done with 3% BSA in PBS which is incubated for 1 hour or overnight or can be left at this stage for days at 4˚C. Then primary antibody is added and incubated for 1 hour or for overnight at 4˚C, the different primary antibodies used were α-anti-tubulin, anti-phosphoserine. After the incubation period the washed are done with PBS/Tween for 6×10minutes washes to remove unbound primary antibody. Further the secondary antibody is added and incubated for 1hour or overnight and further washed with PBS/Tween for 5times and the last 1 time is washed with PBS. Finally, the blot is washed with ECL reagents which is made with 1:1 ratio mixture and spread evenly on the surface of the blot and left for 1 minute at room temperature. Excess reagent is removed with the blotting paper and exposed on ECL camera system immunofluorescence.

Quantification of antibody reactivity on western blots

Desitometric scanning of western blots was performed by using the Quantiscan image analysis system interfaced to a high-resolution CCD video camera. Blots were digitized and the protein levels were quantified by directly relating antibody to specific protein level. Using the quantiscan software program, individual lanes were superimposed over the region of interest, from which a peak profile can be constructed.

3.10 Immunofluorescence

This technique is used to detect the specific fluorescence antibodies to the antigen present in the sample to specify the biomolecule target within the cell. It determines whether exposure to organophosphate induced changes in the intracellular distribution of α-tubulin or GAP-43. The procedure is done by culturing the cells count at 50000/ml in 8 well plates and incubated for 24hours. Then the growth medium is removed and added with toxin mixed with growth medium toxin of concentration of 5mM, 2.5mM and 1.25mM which are mixed with growth medium and added to 8 well plate and exposed to 24hours. Carefully all the medium is removed and fixation is done with 300µl of formalin and incubated 10minutes at room temperature. Remove fixative and give monolayer 3 washes with PBS for 2minutes washes. Permeability is done with 300µl

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per well with PBS/Tween for 3 times for 10minutes interval. Then remove PBS/Tween and replace 3% BSA in PBS for blocking and incubated for 1 hour or stored with overnight. After the incubation the wells are washed with PBS/Tween for 3 times for 5minutes interval. Then added with DAPI and stored for overnight and imaging is done with immunofluorescence.

IV. RESULTS

4.1 Images of N2a cells at different concentrations of POX and PTH

In the above images the cellular structure length of dendrites, cell mass, cell density is observed in different concentration of toxins (Parathion and Paraoxon)

The normal healthy N2a cells are characterized by phase-contrast microscopy and immunocytochemistry in control (non-treated) and exposed to toxin cultures. Neurons with round cell bodies and neurites are presented with good confluence in well and with well-defined cell membrane and healthy neurites. These cells were inoculated with 50000cells/ml and incubated at 37˚C for 24 hours.

Fig.1. A. and Bin the 8 well plate the cells were exposed with DMSO as the control imaging was done in green and blue fluorescence. Green detects the neurite outgrowth whereas blue fluorescence detects the nucleus of the cells and there was no cell injury was observed, neuronal phenotype showing outgrowth of neurites.

Fig.2. A. and B in the 8 well plate the cells were exposed to with concentration of 0.1µM of Parathion for 24hours further undergoing the steps of fixation washing and permeabilization. Finally, imaging was done in green and blue fluorescence. Green detects the neurite outgrowth whereas blue fluorescence detects the nucleus of the cells and there was no significant cell injury was observed, neuronal phenotype showing outgrowth.

Fig.3. A. and B in the 8 well plate the cells were exposed to with concentration of 0.5µM of Parathion for 24hours further undergoing the steps of fixation washing and permeabilization. Finally, imaging was done in green and blue fluorescence. Green detects the neurite outgrowth whereas blue fluorescence detects the nucleus of the cells and there was low cell injury was observed, neuronal phenotype showing decreased size of neurites and bulging of cells shows stress

Fig.4. A. and B in the 8 well plate the cells were exposed to with concentration of 2.5µM of Parathion for 24hours further undergoing the steps of fixation washing and permeabilization. Finally, imaging was done in green and blue fluorescence. Green detects the neurite outgrowth whereas blue fluorescence detects the nucleus of the cells and there was no cell injury was observed more than the concentration of 0.5µM, neuronal phenotype showing very low size of neurites and bulging and shrinkage of cells was observed which shows the shrinkage of cells and damage to DNA.

Fig.5. A. and B in the 8 well plate the cells were exposed to with concentration of 0.1µM of Paraoxon for 24hours further undergoing the steps of fixation washing and permeabilization. Finally, imaging was done in green and blue fluorescence. Green detects the neurite outgrowth whereas blue fluorescence detects the nucleus of the cells and there was no significant cell injury was observed, neuronal phenotype showing outgrowth of neurites.

Fig.6. A. and B in the 8 well plate the cells were exposed to with concentration of 0.5µM of Paraoxon for 24hours further undergoing the steps of fixation washing and permeabilization. Finally, imaging was done in green and blue fluorescence. Green detects the neurite outgrowth whereas blue fluorescence

detects the nucleus of the cells and there was low cell injury was observed, neuronal phenotype showing decreased size of neurites and bulging of cells shows stress

Fig.7. A. and B in the 8 well plate the cells were exposed to with concentration of 2.5µM of Paraoxon for 24hours further undergoing the steps of fixation washing and permeabilization. Finally, imaging was done in green and blue fluorescence. Green detects the neurite outgrowth whereas blue fluorescence detects the nucleus of the cells and there was no cell injury was observed more than the concentration of 0.5µM, neuronal phenotype showing very low size of neurites and bulging and shrinkage of cells was observed which shows the shrinkage of cells and damage to DNA.

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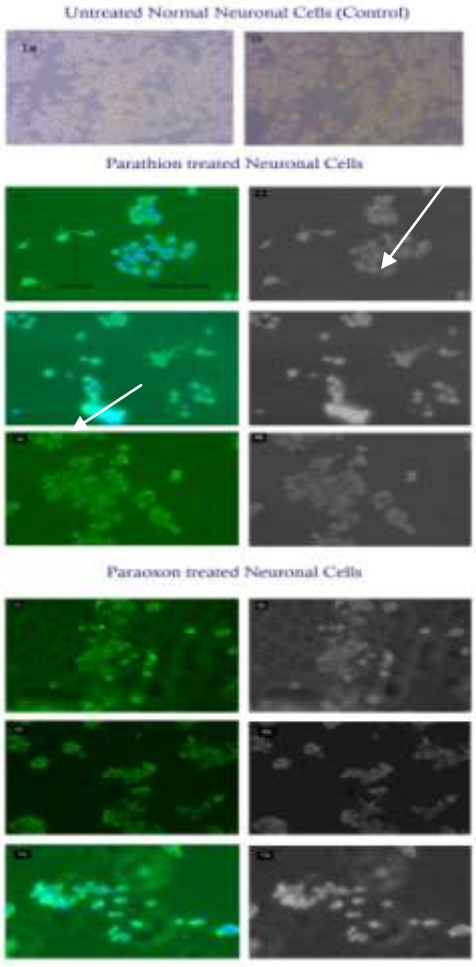

4.2 MTT assay

To determine cytotoxic effect of paraoxon on mitotic N2a cells using MTT Assay method

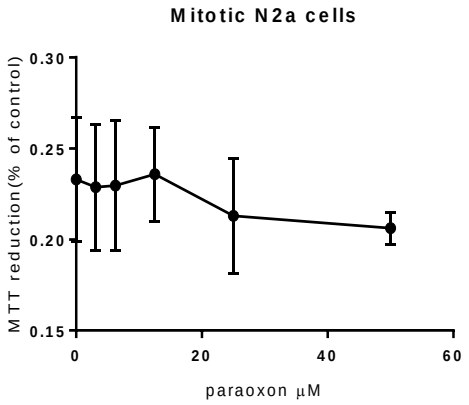

Fig.8: Cytotoxic effect induced by Paraoxon on N2a cells

The above experiment enables us to distinguish the mitotic changes in sub cytotoxic levels byusing different concentration of paraoxon.

The experiment was based on MTT viability assay in which DMSO is solubilized to form formazan crystals measuring at 570nm. 50,000 cells/ml were seeded in 24 well plate in triplicates and incubated at 37˚C for 24hours MTT assay was carried out at assigned incubation. Mitotic change and cell death after incubation with paraoxon was expressed as percentage of control (control represents cell treated with DMSO instead paraoxon). Concentration of 0 to 10mM induced significant mitotic changes and cell decline N2a cells. From above experiment 5mM induced a significant difference in N2a cells compared to control, 10mM drug induced greater decline in N2a cells. By conducting above experiment deduce optimum changes in N2a were attained. To determine the cytotoxic effect of parathion on mitotic N2a cells using MTT assay method

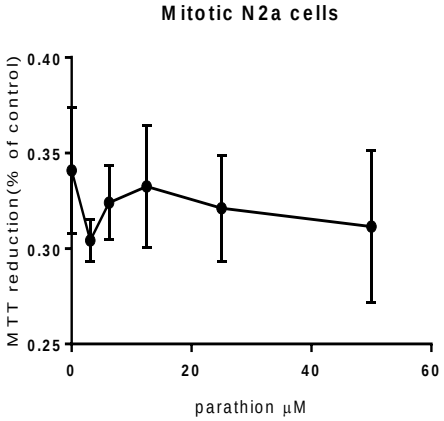

Fig.9: Cytotoxic effect induced by Parathion on N2a cell

The above experiment enables us to distinguish the mitotic changes in the sub cytotoxic levels by using different concentrations of parathion.

The experiment was based on MTT viability assay in which DMSO is solubilized to form formazan crystals measuring at 570nm. 50,000 cells/ml were seeded in 24 well plate in triplicates and incubated at 37˚C for 24hours MTT assay was carried out at assigned incubation. Mitotic change and cell death after incubation with parathion was expressed as percentage of control (control represents cell treated with DMSO instead parathion). Concentrations of 1.25mM and 0.625mM showed a significant change but it could

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be less seeding of cells or high amount of toxins pipetted. Concentration of 0 to 10mM induced significant mitotic changes and cell decline N2a cells. From above experiment 5mM induced a significant difference in N2a cells compared to control, 10mM drug induced greater decline in N2a cells. By conducting above experiment deduce optimum changes in N2a were attained.

4.3 Acetylcholinesterase assay

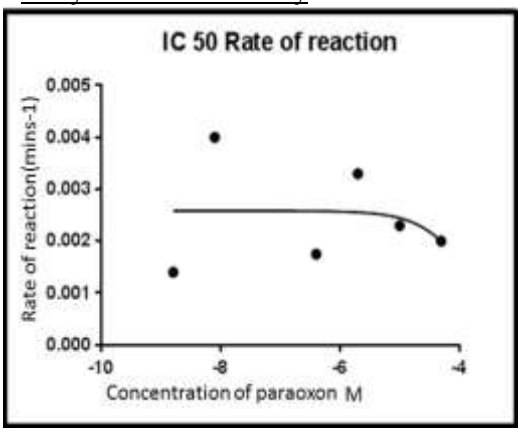

Fig 10: Acetylcholinesterase assay was performed to obtain the value of IC50 the value of half maximal inhibitory concentration. The IC50 of a drug can be determined by constructing a dose response curve and examining the effect of different concentrations of antagonist on reversing antagonist activity. It is used to calculate the effectiveness of a Paraoxon inhibiting the acetylcholinesterase. It represents the molar concentration of paraoxon required for obtaining 50% of acetylcholinesterase inhibition. Thus the value is IC50:0.000227

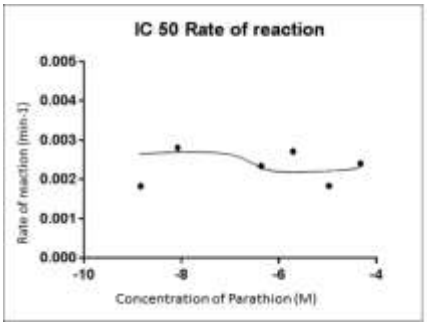

Fig.11: Acetylcholinesterase assay was performed to obtain the value of IC50 the value of half maximal inhibitory concentration. The IC50 of a drug can be determined by constructing a dose response curve and examining the effect of different concentrations of antagonist on reversing antagonist activity. It is used to calculate the effectiveness of a Parathion inhibiting the acetylcholinesterase. It represents the molar concentration of parathion required for obtaining 50% of acetylcholinesterase inhibition. Thus the value is IC50:0.003304. IC50 values are also used to compare the potency of two drugs paraoxon and parathion. According to the values paraoxon proved to be more potent than parathion by comparing the IC50 values.

4.4 Western blotting

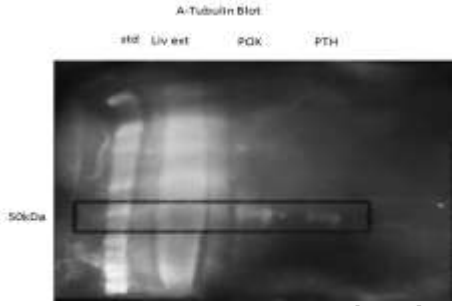

Fig.12: POX -Paraoxon, PTH – Parathion, liv ext-guinea pig liver extract.

Western blotting analysis is done to detect the cytoskeleton proteins. Cells were incubated and allowed to differentiate for 24 hours in a normal ecological conditions without the treatment of toxins. Then the toxins paraoxon and parathion were added to the growth medium and the concentration of toxin was 2mM. The cell lysate was prepared by the toxin exposed cells, then PAGE and western blot technique was used. Blocking of the blot was done with 3% BSA in PBS. Blots were probed with primary antibody α-tubulin, HRP secondary antibody and finally developed with ECL reagent. The comparison of blot is sample is done with the standard. The bands travelled a long distance which was about 50Kd and the other cell lysate samples of POX and PTH.

4.5 Morphological changes on N2a cells

Molecular high content image processing software

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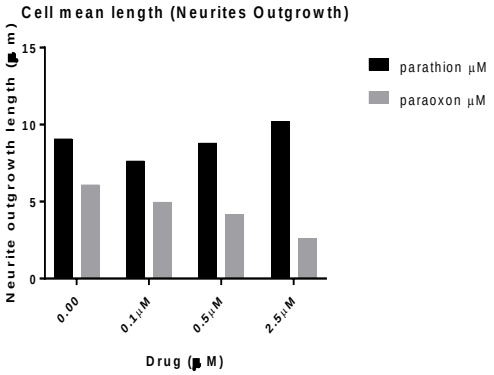

Fig.13: Morphological changes on the cell mean length of neurite outgrowth.

The above experiment enables us to distinguish the morphological mitotic changes in the sub cytotoxic levels by using different concentrations of parathion and paraoxon. Molecular high content image processing software was used to measure the cellular morphological changes. 8 well plate for cell cycle immunofluorescence study was used.Paraoxon showed the proportional decrease with the increase of toxin concentration which shows it was cytotoxic at sub cytotoxic concentration. Parathion showed the significant increase of mean length of neurons proportionally to the concentration of toxins which shows there was no significant toxic effect of parathion. In the overall data the range of increase of length of neurites on exposure of parathion was 9.10µm - 10.23µm whereas the decrease of length of neurites on exposure of paraoxon was 6.11µm – 2.62µm. Thus the result shows the overall decrease of length of the neurites was significant than the increase.

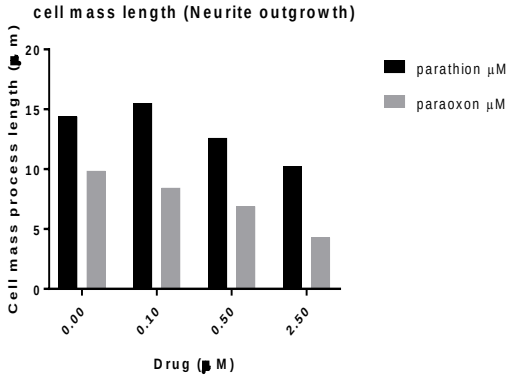

Fig.14: Morphological changes on the cell mass process of neurite outgrowth

The above experiment enables us to distinguish the morphological mitotic changes in the sub cytotoxic levels by using different concentrations of parathion and paraoxon. Molecular high content image processing software was used to measure the cellular morphological changes. 8 well plate for cell cycle immunofluorescence study was used.Paraoxon showed the proportional decrease with the increase of toxin concentration which shows it was cytotoxic at sub cytotoxic concentration. Parathion also showed the significant decrease of mean length of neurons proportionally to the concentration of toxins which shows there was significant toxic effect of parathion. In the overall data the range of decrease of cell mass on exposure of parathion was 14.39µm - 10.23µm whereas the decrease of length of neurites on exposure of paraoxon was 9.85µm – 4.28µm. Thus the result shows the overall decrease of cell mass process of the neurites.

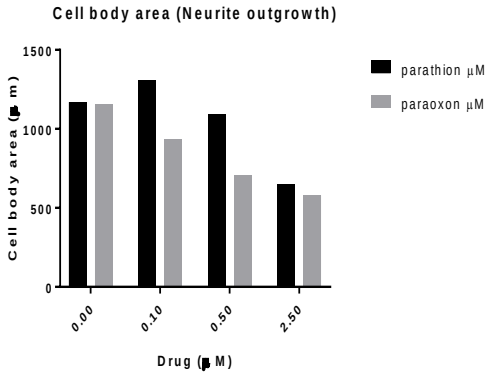

Fig.15: Morphological changes on the cell body area of neurite outgrowth

The above experiment enables us to distinguish the morphological mitotic changes in the sub cytotoxic levels by using different concentrations of Parathion and Paraoxon. Molecular high content image processing software was used to measure the cellular morphological changes. 8 well plate for cell cycle immunofluorescence study was used.Paraoxon showed the proportional decrease with the increase of toxin concentration which shows it was cytotoxic at sub cytotoxic concentration. Parathion also showed the significant decrease of cell mass of neurons proportionally to the concentration of toxins which shows there was significant toxic effect of parathion. In the overall data the range of decrease of cell body area on exposure of parathion was 1169.43µm - 651.18µm whereas the decrease

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of length of neurites on exposure of paraoxon was 1155.03µm – 580.22µm. Thus the result shows the overall decrease of cell body area of the neurites.

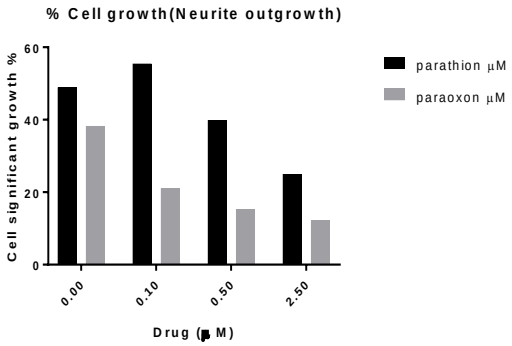

Fig.16: Morphological changes on the cell significant % neurite growth

The above experiment enables us to distinguish the morphological mitotic changes in the sub cytotoxic levels by using different concentrations of parathion and paraoxon. Molecular high content image processing software was used to measure the cellular morphological changes. 8 well plate for cell cycle immunofluorescence study was used.Paraoxon showed the proportional decrease with the increase of toxin concentration which shows it was cytotoxic at sub cytotoxic concentration. Parathion also showed the significant decrease of % Cell significant growth of neurons proportionally to the concentration of toxins which shows there was significant toxic effect of parathion. In the overall data the range of decrease of % cell significant growth on exposure of parathion was 48.91% - 25% whereas the decrease of length of neurites on exposure of paraoxon was 38.29% – 12.16%. Thus the result shows the overall decrease of % cell significant growth process of the neurites.

V. DISCUSSION

The main mechanism for acute effect of organophosphate is the inhibition of acetylcholinesterase in the nervous system. Other deleterious effects have been eported for these compounds such as inhibition of other esterase like neuropathy target esterase (NTE), synthesis of RNA and DNA is damaged**(Crumpton, Seidler and Slotkin 2000b)**dysregulation of signal transduction pathways, oxidative stress and astrological proliferation (Ehrich M et al 1994).

In this study we used a neuroblastoma cells of undifferentiated N2a cells, to study the effects of paraoxon and parathion (Organophosphates which can affect the neuronal cells). Paraoxon alters the different genes and signalling pathways related to chromatin assembly and nucleosome integrity at 1µM which is the non-cytotoxic concentration. The data presented on neurite outgrowth demonstrated that both POX and PTH caused neurite retraction in a dose and time dependent manner when pre-differentiated N2a cells in both axon and dendrite like process. These changes were found and compared by MTT analysis and cell cycle fluorescence imaging. MTT analysis showed a change in cell cycle with shows the effect of toxins according to the data obtained. Acetylcholinesterase assay showed the minimum inhibition comparing the IC50 data paraoxon was detected to be slightly more potent than parathion in inhibition of the enzyme. Western blot technique showed the better response to the antibody and the results showed the drug response to the cytoskeleton proteins.

5.1 Assessment of cell viability using MTT Assay

To find the mitotic changes and the intracellular changes in the cell cycle caused by the sub cytotoxic level of POX and PTH was evaluated. (Hogberg H.T,2011) (Camon E.,2004). Cells were seeded at the density of 50000 cells/cm^2 in 24 well plates incubated for 24 hours next the growth medium was removed and growth medium mixed with toxin was of 2.5µl of toxin in 12.5ml of growth medium. Experiment was performed with 4 replicates and 5 different concentrations of 2 toxins (10mM,5mM, 2.5mM,1.25mM,0.625mM) then the medium was removed and added with 50µl of MTT and incubated for 30mins at 37˚C. Then the MTT was removed and added with 200µl of DMSO and left on shaker for 10 mins at room temperature, and the medium of DMSO is removed and transferred to the 96 well plate and the absorbance was calculated at 540nm in a microplate reader and the cell viability after exposure was calculated assuming 100% of viability for the absorbance recorded in control (DMSO treated) .through the data obtained its concluded that the two concentrations of 5Mm and 10Mm of both toxins were potent to cause changes in the cells. Being the parent compound POX had potent effect compared to PTH.

5.2 Acetylcholinesterase Assay

Effect of substrate concentration on rate of reaction it was found that the rate increased with the concentration of substrate up to 10mM, the range of concentrations were between 0.0032Mm

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to 10Mm. As the range of concentration increased the acetylcholinesterase inhibition increased. The study showed that the inhibition of AChE takes place at sub-cytotoxic levels which alters the metabolic and morphological changes. The effect of enzyme concentration on rate of reaction when the concentration of enzyme was increased the activity declines but in this experiment we used a single concentration of the enzyme 0.0004mg/ml to detect the rate of reaction of the substrate according to the concentration IC50 graph is plotted, it's the concentration of an paraoxon where the AChE inhibition is reduced to half. The IC50 of a drug can be determined by constructing a dose response curve and examining the effect of different concentrations of antagonist on reversing antagonist activity. It is used to calculate the effectiveness of a Parathion inhibiting the acetylcholinesterase. It represents the molar concentration of parathion required for obtaining 50% of acetylcholinesterase inhibition. Thus the value is IC50:0.003304. IC50 values are also used to compare the potency of two drugs paraoxon and parathion. According to the values paraoxon proved to be more potent than parathion by comparing the IC50 values.

5.3 Cell imaging

In the cell imaging the normal growth medium the N2a neuroblastoma cells were seen with good cellular structure of neurites and dendrites. The different concentration of toxins POX and PTH were used at 0.1Mm concentration there was no changes observed in both the toxins. At the concentration of 2.5Mm there were changes observed in the cell membrane and neurites which shows the effect of both the toxins. At the concentration of 5Mm there was high cell injury was observed with the shrinkage of cells short neurites and less number of cells are the cause of neurodegeneration.

5.4 Western blotting

Western blot technique is used to detect specific proteins in cell lysate sample in this experiment. The proteins are then transferred to a nitrocellulose membrane **(Burnette 1981**)through western blotting and blocked with 3% BSA in PBS then they were probed with primary antibody α-tubulin and secondary antibody of HRP-mouse specific to target proteins**(Towbin, Staehelin and Gordon 1979**). And finally the blots were developed with ECL reagent with 1:1 ratio. The comparison of blot is sample is done with the standard. The bands travelled a long distance which was about 50Kd and the other cell lysate samples of POX and PTH.

VI. CONCLUSION

The results obtained from the study suggest that N2a cell line is suitable for the study of mitotic changes in the cells on exposure of sub-cytotoxic level of toxins like POX and PTH. Paraoxon alters the initial in vitro differentiation process of neuroblastoma cells. Further studies are needed to clarify whether these effects might cause some of the neurodevelopmental toxic effects attributed to organophosphates. Being the parent compound POX showed potent effect than PTH on the N2a differentiating cells. In the cell cycle images, the proportional effects of the toxins according to their concentrations shows the morphological changes in the cell stages. We found alterations in the neurotransmitters pathways known to be highly targeted by organophosphates in the study (**Slotkin 2004)** in brain development, by acetylcholinesterase assay. Further study should be done on different time period exposure of the N2a cells to the toxins and know the effect of the chronic effect and changes taking place in the cell cycle. Future study can be done by inhibiting NTE and the changes should be observed by the parent drug paraoxon.

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