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P53 Alteration Studies and Tumor Suppressor Gene in Human Bladder Cancer of Subjects Exposed to High Levels of Chlordane

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ABSTRACT

The main objective of this work is to study the alterations that can occur in p53 in subjects exposed to chlordane. To study this various chlordane exposed subjects blood samples as well as bladder tissues are procured. Total blood protein is extracted from collected blood as well as from media from cultured cells. The main agenda behind such study is to identify for any mal formations in the p53 gene products in the extracted samples which do not have specificity to Anti p53 Antibody which in turn produces negative results in the immunological assays on the other hand those subjects which do not have any structural deformations in p53 protein produce a positive result in immunological assays using the same antibody. PCR is performed to the whole genomic DNA isolated from the subjects for amplification of p53 coding gene for cross referring the presence of the same in the amplified products. Various mixed results are procured out of which certain subjects have shown specific results which can conclude that p53 protein present in these subjects have nearly zero specificity to the Anti p53 Antibody which is used in the immunological assays for analyzing the same.

Keywords: blood protein, chlordane, DNA, p53 Antibody.

INTRODUCTION

Chlordane is a man-made chemical that was used as a pesticide in the United States from 1948 to 1988. It is sometimes referred to by the trade names Octachlor and Velsicol 1068. It is a thick liquid whose color ranges from colorless to amber, depending on its purity. It may have no smell or a mild, irritating smell. We do not know what it tastes like. Chlordane is not a single chemical, but is a mixture of many related chemicals, of which about 10 are major components. Some of the major

components are trans-chlordane, cis-chlordane, ß-chlordene, heptachlor, and trans-nonachlor. Chlordane does not dissolve in water. Therefore, before it can be used as a spray, it must be placed in water with emulsifiers (soaplike substances), which results in a milky-looking mixture. From 1983 until 1988, chlordane's only approved use was to control termites in homes. The pesticide was applied underground around the foundation of homes. When chlordane is used in the soil around a house, it kills termites that come into contact with it. Before 1978, chlordane was also used as a pesticide on agricultural crops, lawns, and gardens and as a fumigating agent. Because of concerns over cancer risk, evidence of human exposure and build up in body fat, persistence in the environment, and danger to wildlife, the EPA canceled the use of chlordane on food crops and phased out other above-ground uses over the next 5 years.

When used as a pesticide on crops, on lawns and gardens, and to control termites in houses, chlordane enters the environment. Although it is no longer used in the United States, it may be used in other countries. In soil, it attaches strongly to particles in the upper layers of soil and is unlikely to enter into groundwater. It is not known whether chlordane breaks down in most soils. If breakdown occurs, it is very slow. Chlordane is known to remain in some soils for over 20 years. Persistence is greater in heavy, clayey or organic soil than in sandy soil. Most chlordane is lost from soil by evaporation. Evaporation is more rapid from light, sandy soils than from heavy soils. Half of the chlordane applied to the soil surface may evaporate in 2 to 3 days. Evaporation is much slower after chlordane penetrates into the soil.

In water, some chlordane attaches strongly to sediment and particles in the water column and some is lost by evaporation. It is not known whether much breakdown of chlordane occurs in water or in sediment. Chlordane breaks down in the atmosphere by reacting with light and with some chemicals in the atmosphere. However, it is sufficiently long lived that it may travel long distances and be deposited on land or in water far from its source. Chlordane or the chemicals that chlordane changes into accumulate in

fish, birds, and mammals. Chlordane stays in the environment for many years and is still found in food, air, water, and soil.

Chlordane is still commonly found in some form in the fat of fish, birds, mammals, and almost all humans. Chlordane can enter the body through the skin if skin contact occurs with contaminated soils, through the lungs if breathed in with contaminated air, and through the digestive tract if swallowed. Uptake through the skin and digestive tract increases if chlordane is in an oily mixture, which might occur at hazardous waste sites. The importance of each of these ways for chlordane to enter the body depends on the kind of exposure.

For example, people living in houses that have been treated with chlordane will be exposed mostly by breathing the vapor in the air. Workers who sprayed chlordane as a pesticide were exposed mostly by breathing the compound in the air and by contact with the skin. Other people may be exposed to small quantities by eating food or drinking water that contains chlordane. People at or near waste sites may be exposed by touching chlordane in the soil, by breathing chlordane that evaporates into the air, by drinking water that contains chlordane or by eating contaminated fish or crops. The amount of chlordane that enters the body depends on the amount in air, food, or water, and the length of time a person is exposed to it. Most chlordane that enters the body leaves in a few days, mostly in the feces, and a much smaller amount leaves in the urine. Chlordane and its breakdown products may be stored in body fat, where they cause no bad effects, unless released from body fat in large amounts. It may take months or years before the chlordane and the breakdown products that are stored in fat are able to leave the body.

Tumour suppressor genes

p53 is the most commonly mutated gene in human cancers and more than 50% of human cancers contain p53 mutations Arnold Levine, David Lane and William Old discovered the p53 gene in 1979. It was first thought to be an oncogene, but10 years later team lead by Bert Vogelstein and Ray White, then studying colon cancer, showed p53 to be a tumor suppressor gene. In the past decade, the roles of p53

in human cancers have been investigated extensively in many aspects and intervention to restore wild-type p53 activities is an attractive approach for cancer therapy.

p53 gene is not reactive in cells where DNA is undamaged. When there is DNA damage, the gene suspends the cell cycle until the damage can be repaired. If there is a mutation in p53, the cell cycle continues unrestrained and reproduces the damaged DNA, leading to uncontrolled cell proliferation and cancer tumors. Cancer results as the cell with damaged DNA divides, the damaged DNA is replicated and each daughter cell's cycle is also unrestrained. Lu,et al(2008) elaborated the DNA damage checkpoint and p53 signaling pathways in human tumorigenesis. Moore et al.. Revealed aging-associated truncated form of p53 which interacts with wildtype p53 and alters p53 stability, localization, and activity. All cancer cells contain mutations in combinations of tumor suppressors and oncogenes.

The removal of functional p53, from a cell allows for the accumulation of even more DNA damage and the division of cells that contain damaged DNA. The mutation of p53 is one of the most frequent genetic changes seen in cancer cells. In addition to mutations that arise during the growth and development of individuals (sporadic mutations), there are forms of cancer associated with the inheritance of a damaged version of p53. In addition, several viruses have evolved ways of inactivating the p53 protein.

LITERATURE REVIEW

Retrospective cohort mortality studies of workers in chlordane and other organ chlorine manufacturing plants reported no increase in mortality rate and no increase in any specific cause of death attributed to chlordane exposure. Wang and MacMahon reported no increase in mortality rate in a prospective study of pesticide applicators. However, in a retrospective mortality study of 1,403 men employed for 23 months at two plants, a significantly increased risk of death from cerebrovascular disease was found, but the authors could not definitively attribute this excess to chlordane exposure.

In another retrospective mortality study of four cohorts (305-1,155 workers/plant exposed for ≥6 months) from four manufacturing plants, there was a significantly increased risk of death from noncancer respiratory disease, and a slight excess risk of cancer of the esophagus, rectum, liver, and hematopoietic system at plant 3, and a slightly greater risk of stomach cancer at plant 1.

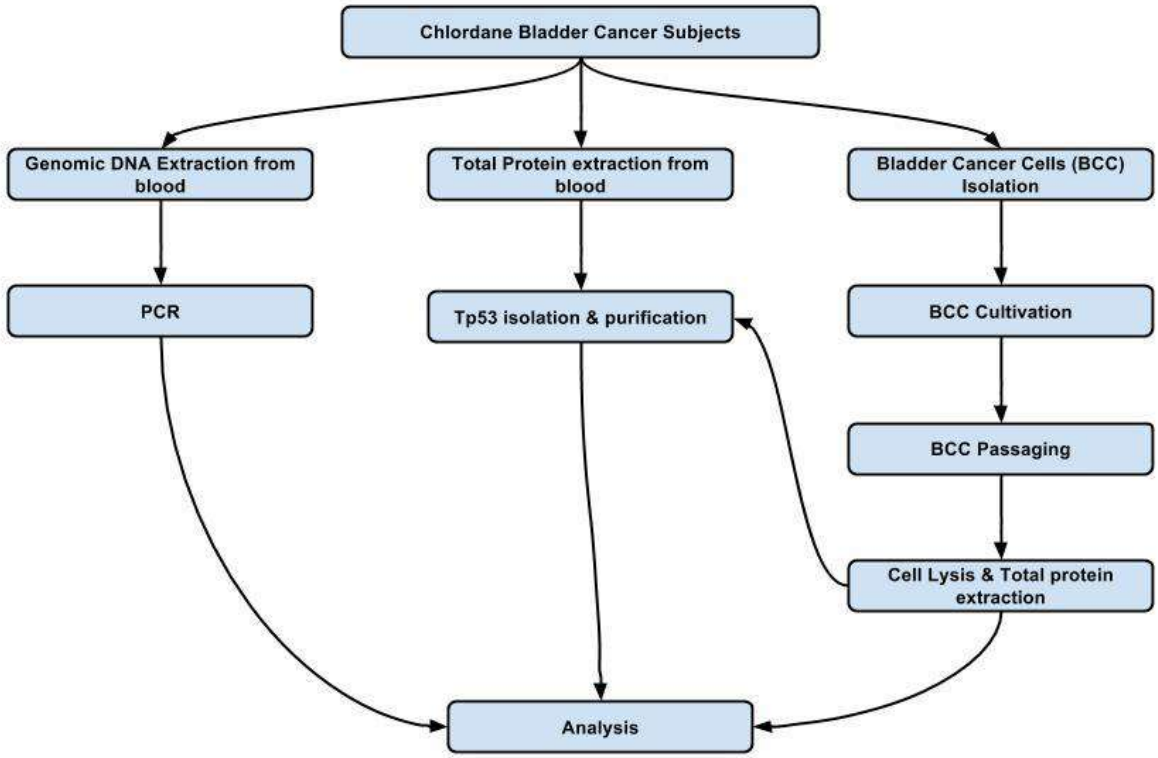
Chlordane was the only pesticide manufactured at plant 1, while aldrin, dieldrin, endrin, and dichlorodiphenyltrichloroethane were manufactured at plant 3. The statistical power did not allow for a conclusion that no association existed between cause-specific mortality and employment at the plants. However, in a follow-up study of these cohorts, which added 11 years of follow-up, no statistically significant excess risk of death from any cause was found (Brown 1992). All of these studies had serious limitations, including unquantified exposure concentrations and exposure to several pesticides.

An early study by Frings and O'Tousa (1950) reported mortality in mice exposed to chlordane. This study will not be discussed further because it has been established that the mortality and other toxic effects observed were due to the use of ―early‖ production chlordane (Ingle 1953, 1965). Early production chlordane was frequently contaminated with hexachlorocyclopentadiene, a highly volatile and toxic reaction intermediate that Ingle (1953, 1965) concluded was largely responsible for the observed effects. In another inhalation study (Ingle 1953), none of the mice died after 14-25 days of continuous exposure to ―later‖ production (more highly purified) chlordane at estimated concentrations of 25-50% saturation (estimated levels in air not reported).

In rats exposed to an unspecified concentration of ―refined technical grade‖ of chlordane for l-8 hour More recently, rats exposed for 2 days (8 hours/day) to 413 mg technical chlordane/m3 or similarly for 5 days to 154 mg/m3 died; rats similarly exposed to 28.2 mg/m3 for 28 days survived (Khasawinah et al. 1989). The female rats were more sensitive than the males. There was no mortality in rats or monkeys exposed intermittently to

technical chlordane at 10 mg/m3 8 hours/day, 5 days/week for 90 days (Khasawinah et al. 1989; Velsicol Chemical Co. 1984).

METHODOLOGY



MATERIALS AND METHODS:

Primer Designing

A primer is short synthetic oligonucleotide which is used is used in many molecular techniques from PCR to DNA sequencing. These primers are designed to have a sequence which the primer to anneal. When designing primers for PCR, sequencing or mutagenesis it is often necessary to make predictions about these primers, for example melting temperature (Tm) and propensity to form dimmers with itself or other primers in the reaction.

Primer length: it is generally accepted that the optimal length of PCR primers is 18-22bp. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature. Pairs of primers should have similar melting temperatures since annealing in a PCR occurs for both simultaneously. A primer with a Tm significancantly higher than

the reaction's annealing temperature may mishybridize and extend at an incorrect location along the DNA sequence, while Tm significantly lower than the annealing temperature may fail to anneal and extend at all.

Dialysis:

Dialysis is a physical process in which the precipitated sample is taken into the bag and it is tied tight on the both the ends with sterile thread so that nothing can not ooze out. Here in this study the dialysis bag used has a cut off value between 12000 to 14000 Daltons. Hence the main agenda behind performing this is to get rid of unwanted proteins and excess of PEG which now in this step called as a contaminant. A minimum amount of the precipitated sample is taken into the dialysis bag and then it is kept in a beaker containing Phosphate buffer. This mixture is now mixed for whole night. On the next day the buffer present in the beaker is drained and then fresh buffer is filled in it. Now this is mixed for another whole night. Such Buffer changes should be given at least 3 to 5 times for a better dialysis process. A visual change can experienced by observing the sample present in the dialysis bag before the buffer washes and after the washes

Protein extraction from tissue:

- A piece of tissue (about 1 mm3) is added to the extraction urea-containing buffer and glass beads. Buffer must cover the sample completely.
- Tissue is disrupted first by vortexing.
- To disintegrate the tissue further, perform sonication for 30 min, tube with sample must be kept on ice during procedure to avoid heating of the sample.
- Centrifugation at 16000 rpm for 15 min at +4 °C is performed to clarify sample and to provide better compression of the pellet
- Supernatant is collected which has the desired proteins.

PROCEDURE FOR CASTING THE GEL

- The glass plate sandwich of the electrophoresis apparatus is assembled using two clean glass plates and there 1 mm acrylic spacers. The sandwich is locked in the positioned and hen sealed along the three sides using molten 1% agar.

- The separating gel solution is prepared by mixing the reagents in correct proportions. Care should be taken that the freshly prepared APS and TEMED are added last, as they are responsible for the polymerization. Acryl amide being neurotoxin should be handled with care. The solution is mixed thoroughly and filled into the sandwich to the required height. A small amount of carbon tetrachloride is poured on top in order to level the top layer and also to cut on the oxygen supply. This arrangement is left for 15 minutes. So that the gel solidifies. The top layer of carbon tetrachloride is then poured off and the surface is washed with distilled water.
- Now the stacking gel solution is prepared by mixing the reagents in correct proportions. On pouring into the plates Teflon comb is slowly inserted into the plates so that no air bubbles get trapped between the two gels. This arrangement is also left for 15 minutes. After the gel solidifies the comb is removed and the wells are washed thoroughly with the tank buffer in order to remove any unpolymerized Acryl amide.
- The sandwich is fitted onto the vertical slab unit after removing the bottom spacer. Care should be taken to see that there are air bubbles between the bottom of the sandwich and the tank buffer, which is filled in the tank of the vertical slab unit. The protein samples are mixed with treatment buffer in the ratio 2:1 and boiled for 5 minutes. Appropriates amount of sample is then loaded into the wells using a microlitre syringe. The entire unit is connected to a powerpack and a current of 12mA is made to pass through the gel by applying potential difference between the two electrodes. The electrophoresis is preferably run at 4^0 C. This is to counter the heat that is produced during the electrophoresis, which might cause the gel to melt. When the proteins enter the separating gel the current is raised to 13mA. When the electrophoresis is completed the gel is stained.

Passaging:

Principle: In cell culture, passaging is the process of sub-culturing

cells in order to produce large number of cells from pre-existing ones. Passaging (also known as subculture or splitting cells) involves splitting the cells and transferring a small number into each new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence associated with prolonged high cell density. Suspension cultures are easily passage with a small amount of culture containing a few cells diluted in a larger volume of fresh media. For adherent cultures, cells first need to be detached, commonly done with a mixture of trypsin-EDTA. A small number of detached cells can then be used to seed a new culture, while the rest is discarded. Also, the amount of cultured cells can easily be enlarged by distributing all cells to fresh flasks.

In the average lab, adherent cells are grown in petri dishes, multi-wells plates or culture flasks, with FCS containing culture media at 37 °C with 5 % CO2. In the case of more or less fast growing cells like HeLa, a freshly seeded plate will reach confluences in two or three days. If nothing is done, the food will run out and the cells will die shortly thereafter, so passaging is required. Thus, the media is removed, the cells are washed with phosphate buffered saline (PBS), and then 1 ml of trypsin is added to make the cells detach from the bottom of the plate. Trypsin works best in a warm surrounding, so the plate is incubated for five minutes in 37 °C. In order to stop the reaction, 1 ml of trypsin inhibitor stock solution is added (1mg/ml in water or PBS). Then, 8 ml of PBS is added and the cells are resuspended. An appropriate number of cells in suspension is then transferred new plates, fresh media is added to each plate, and the new plates are incubated for the next growth phase. For best results, cells are kept less than 100 % (log phase of growth) but more than 10 % confluent. Cells die if they get too lonely (—apoptotic death of neglect||) or much too crowded (death by metabolic decay).

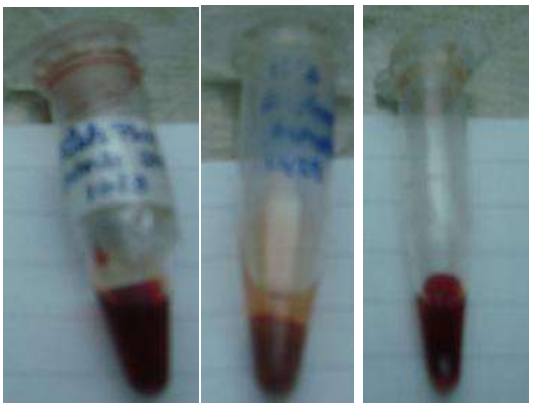
Trypsin is an end peptidase produced by the gastro-intestines of mammals, and has an optimal operating pH of about 8 and an optimal operating temperature of about 37 °C. In vivo, trypsin is produced in the pancreas in the form of inactive zymogen, trypsinogen. After secretion into the duodenum, the

enzyme enter peptidase activates a small number of the enzymes into trypsin by proteolysis cleavage, followed by autocatalysis of trypsins to activate the whole secreted mass.

Then, trypsin acts to hydrolyse pepsin-digested peptides by hydrolysis of peptide bonds. The aspirate residue (Asp 189) located in the catalytic pocket (S1) of trypsins is responsible for attracting and stabilizing positively-charged lysine and/or arginine. Thus, trypsin predominantly cleaves proteins C-terminally of the amino acids lysine and arginine, except when either is followed by proline. Trypsins should be stored at very cold temperatures (between −20 °C and −80 °C) or at a pH of 3 in order to prevent autolysis.

RESULTS

DNA Extraction from Human Blood

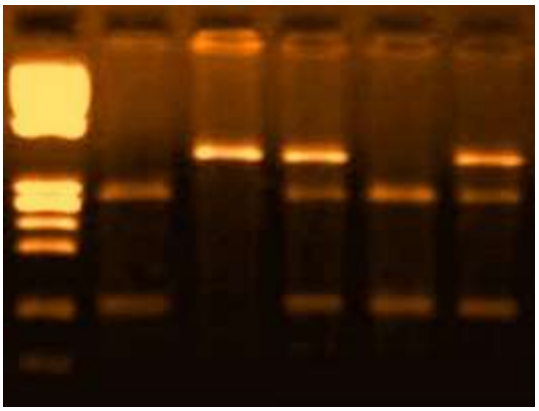


Blood samples are procured from the approved diagnostic laboratories who procure the blood samples for testing the levels of carcinogens for the carcinogenic study from bladder cancers. the subjects selected were people who have probable exposure to chlordane as reported in the medical reports that they are working in certain pesticide companies. Details regarding the subject as well as the entity are encrypted as per rules and regulations. fresh blood samples are used for this purpose to confirm the intracellular protein which are going to be used for analysis will be in their active form without crossing their half life period especially TP53 which is basically a intracellular protein in various cells is the major target in the protein isolation studies for that particular reason fresh blood blood samples which are drawn +- 2 hours are used for this study.

PCR:device which use to amplify of P53 gene:

Total human genome is extracted from the collected blood samples and preserverd at -20 degrees centigrade until the purification of the specific selected gene is performed. PCR is performed to amplify the p53 coding gene inorder to find out whether the gene is active in the selected cell or whether there is any kind of alterations in the selected gene. the main reason is to find out whether the molecular weight of Tp53 gene is intact or altered. Due to the restrictions of the objectives of this study gene sequences are not been performed. as the main project is concentrating on the specificity of the extraction of Tp53 gene molecular study is restricted only to identify whether the specific genes integrity is maintained in the cells or not.

Agarose Gel Electrophoresis:



1-first one from left strands mean the whole genome.

2- two bands dna-non mutated p53.

3- 3 bands dna is mutated p53.

Various batches done under this project

Hemolyzed plasma used for the precipitation

Pellets after precipitation with PEG

Partially Hemolyzed plasma used for the precipitation

The process of Dialysis
Precipitated plasma samples in Dialysis bag dialyzing against Phosphate buffer

Bulk Dialysis process performed for the production protocols

Total protein extraction is performed on this blood samples which are collected from the bladder cancer subjects in order to procure the intracellular Tp53 protein. as soon as the blood is drawn it is brought to the laboratory and then exposed to 4 degrees centigrade to maintain the minimum metabolic activity after that precipitation techniques are performed to extract proteins which are present in the plasma. further procedures are performed to break down the cells and extract intracellular proteins too. as the

combinational process all the proteins are collected into dialysis bags and then purification is performed.

Dialysis bags of the cut off values of 12 - 13 thousand Daltons are selected in order to remove all impurities(proteins) which are below 13,000 Da. all major proteins like 53Da serum albumin are ingnored as they are neutral in threir specificity and they donot pose any threat in immunological specificity assays like RID and DID. a few batches are run at 10 - 20 ml level and dialysis are performed for the same to procure ample amount of protein for a detail analysis.

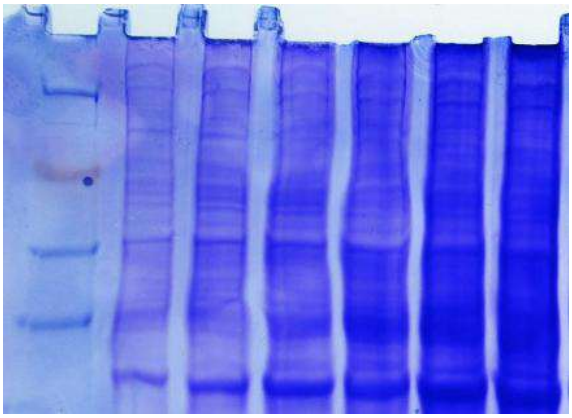
DID:

various batches of the protein extracted as well as the various batches of the purified and ultra purified samples are loaded in the outer wells as the antibody against P53 is loaded in the inner well in order to see the cross reactivity of this particular blood and cultivated cell lysate. the sample exhibiting the arc is showing the specificity to the antibody. The functional protein is missing in those samples

RID:

the sample which has shown the positive result in is selects in RID to see its precipitating capacity against the antibody. the ring was formed near to the well indicating the less binding capacity of the antibody to the sample. sample has specify as the others did not show specificity.

SDS - PAGE:

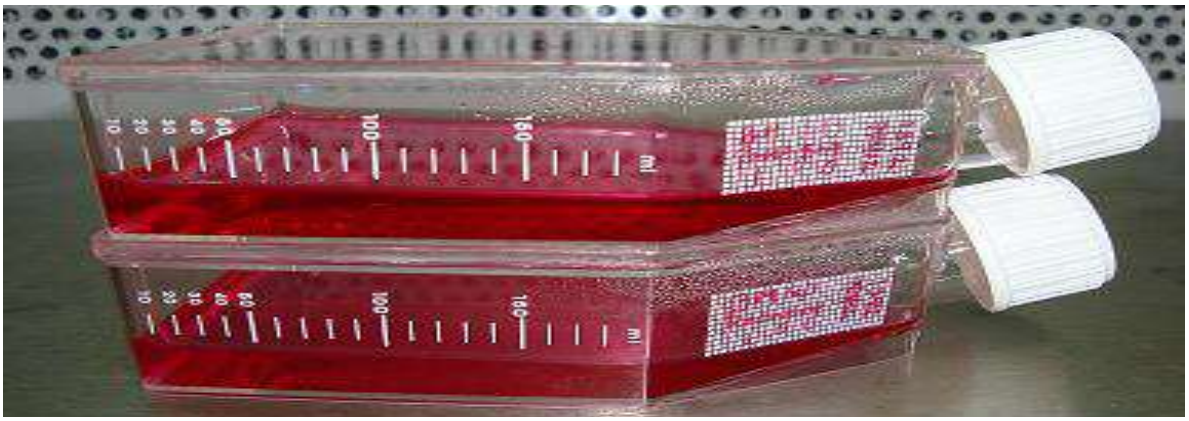


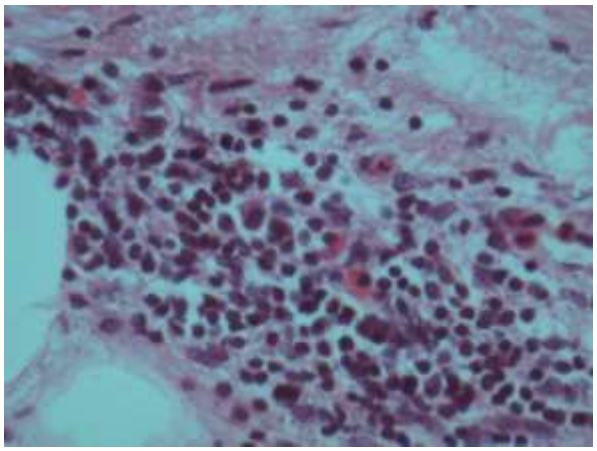
When incubated immunopurified human p53 with 0.01% or 0.1% glutaraldehyde and then subjected the mixtures to SDS/PAGE and silver staining. When incubated with 0.01% glutaraldehyde,

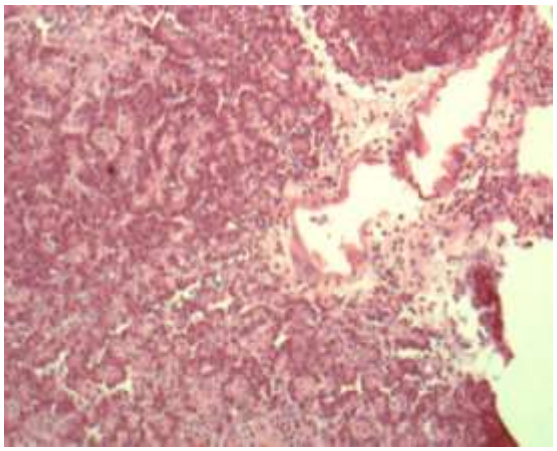
p53 displayed forms that migrated as the monomer and at least two species migrating in the vicinity the 92-kDa marker. The mobility of these forms relative to the marker proteins suggested that they were p53 dimmers.

Culturing Of Bladder Cells:

Cells are procured from the bladder cancer tumor aspirates when the surgury is performed from the ethically recourses and then are transferred througd HPSS transportation media to the cultivation facility and then using standard reference protocols(media composition) cancer cells are cultivated and passaged. the cellular material is subjected to cellular lysis to basic form to pool up the cell lysis for further protein extraction procedures similar to the total proton extraction.







Tissue sections are first oxidized by periodic acid. The oxidative process results in the formation of aldehyde groupings through carbon-to-carbon bond cleavage. Free hydroxyl groups should be present for oxidation to take place. Oxidation is completed when it reaches the aldehyde stage. The

aldehyde groups are detected by the Schiff reagent. A colorless, unstable dialdehyde compound is formed and then transformed to the colored final product by restoration of the quinoid chromophoric grouping.

Naphthol acid phosphate is hydrolyzed by acid phosphatases present in the tissue, and napthol derivatives are produced. The naphthol derivatives couple with an unstable diazonium salt, hexazonium pararosanilin, to produce a red azo dye to mark the sites of enzyme activity.

Discussion

Chlordane is a central nervous system stimulant. The liver and kidney are the other organs affected by chlordane. A sudden onset of convulsions preceded by vomiting. Seizures caused by cyclodiene pesticides may appear as long as 48 hr after exposure and then may appear periodically over several days following the initial episode. Tonic-clonic convulsions usually are accompanied by confusion, in coordination, excitability and in some instances coma, hypotension and respiratory failure. Do not give fats, oils or milk since these will enhance absorption from the intestinal tract. Accidental poisoning can occur in children, formulating workers, suicide attempts, individuals who live in chlordane treated residences. Individuals with a history of convulsive disorders would be expected to be at increased risk from exposure.

Routes of exposure include ingestion, inhalation, dermal and eye contact. out of the various damages chlordane caused to the human body the most important thing which attracted international research communities attention is bladder cancer which is caused by chlordane exposure. most of the subject who are exposed to chlordane are suspected to exhibit such types of cancers more often. it is very little bit known about the role of p53 in suppressing the carcinogenicity caused by chlordane in certain cells which compromise in performing normal functions in human bladder. in order to study this particular cascade events which are been switched on by p53.

This project concentrated on the extraction of proteins directly from the bladder cancer blood samples and bladder cancer cell lysis which are

cultivated which are taken from bladder cancer subjects exposed to chlordane. in order to cross confirm wether the tumour protein p53 which is present in the bladder cancer subjects do have it active domain to perform its anticarcinigenic functions in its body or not. most of the studies on p53 concentrates on gene sequencing studies but this particular study is done only to find whether the active specificity of p53 against the specific antibody is restored in the cells or not to perform active apoptosis or not. of the various subjects studied only one subject have shown active p53 specificity, reaming all have not shown any kind of p53 specificity to antibody.

CONCLUSION

By the studies performed in this project one can confirm that there is a major requirement of research to be done in the molecular aspects in the level of nucleic acid expressions to further role of p53 in chlordane exposed subjects. the data procured from this project can give an assumption that bladder cancer subjects who procure the same due to the exposure of chlordane have the conditions worse due to p53 malfunctions. the main objective of the project is to study the specificity of the p53 protein .so p53 has not shown any specificity against the antibodies which are used to study the same. in this prospective one can easily conclude that the bladder cancer subjects (chlordane exposed) do not have an active p53 to perform ant carcinogenic role(suppressor gene inactive) for subjects against cancer cells.

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