HIGH SCHOOL EDITION
Journal of Student Research
Volume 12 Issue 1 (2023)

Effects of Diisononyl Phthalate Exposure on Colorectal and Leukemia Cancer Cell

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ABSTRACT

Phthalates (PAEs), also known as phthalates, are collectively known as phthalimide esters and are mainly used as plasticizers (plasticizers), added to plastics to enhance elasticity, transparency, durability and longevity. Diisononyl phthalate (DiNP), a type of PAEs, is commonly used in a large variety of plastic items, and is listed as a substance "known to the State of California to cause cancer" under Proposition 65 legislation.[1] PVC plastics have become ubiquitous in our modern society. DiNP leaches from PVC-based consumables, eventually leading to deposition. DiNP leached from consumables can eventually be deposited in certain tissues through inadvertent application. Studies on cancer cells leukemia cancer cells and colorectal cancer cells have demonstrated health risks in human populations exposed to DiNP, including endocrine disorders, reproductive damage, and tumorigenesis. In this research, we studied the effect DiNP causes on the leukemia cancer cell and colorectal cancer cell. The results show that DiNP has a proliferative effect on immune cells and an inhibitory effect on colorectal cancer cells. We do not know what specific pathway or mechanism DiNP could activate; However, it is highly likely that DiNP causes damage because it can trigger the proliferation of immune cells, or lesions, by modulating some of the organelles or receptors in immune cells. This effect is beneficial in non-immune cancers, but harmful in cancers that's immune cell related, in this case, leukemia.

Introduction

Phthalate Family and Properties

Phthalate, or phthalate ester, are esters of phthalic acid. They are produced industrially by the acid catalyzed reaction of phthalic anhydride with excess alcohol. The synthesis of phthalate could be illustrated as below:

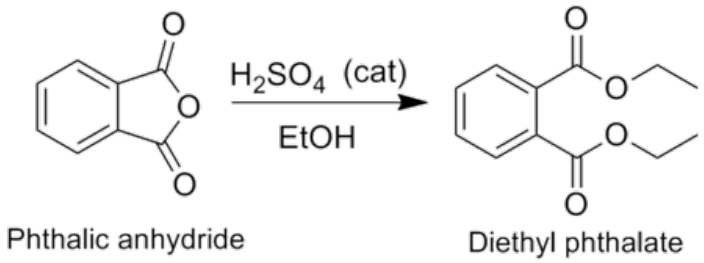

Figure 1.

[1] "State of California, Chemicals known to the state to cause cancer or reproductive toxicity, January 3, 2014" (PDF). Archived from the original (PDF) on 2014-01-10.

The synthesis of diethyl phthalate is illustrative of this: Phthalates are categorized as "high" or "low," depending on molecular weight. Lower-molecular-weight phthalates are derived from C3-C6 alcohol, and high phthalates include those with 7-13 carbon atoms in their chemical backbone, which gives them increased permanency and durability. There are over 18 types of phthalate, the most common ones includes Di(2-ethylhexyl) phthalate (DEHP), Mono(2-ethylhexyl) phthalate (MEHP), diisononyl phthalate (DiNP) and diisodecyl phthalate (DiDP). In this research, DiNP was chosen as the primary reactant. [2]

Phthalates are one of the most commonly used plasticizers in the world. When added to plastics, primarily polyvinyl chloride(PVC), or vinyl, phthalates allow the long polyvinyl molecules to slide against one another, therefore making them flexible and pliant. [3]

Phthalates Usage

Phthalates are commonly used in a variety of consumer goods, including plastic toys, baby products(lotion, shampoo, powders and leathers), building materials(vinyl, flooring, wall paper, paint, glue and adhesives), scented producers(candles, detergent and air freshers), art supplies(paint, clay, wax and ink), enteric coating of pharmaceuticals, automobiles, medical equipment(tubing, blood bags, plastics in the NICU), cosmetics and other personal care products. They are also found in many types of food, including dairy products, meats, fish, oils & fats, baked goods, infant formula, processed foods, and fast foods– from contamination from the processing or packaging. [4]

Phthalates' Impact on Health

Phthalates, as a widespread environmental pollutant and endocrine disruptor, its threat to human health is highly concerning. Phthalates are toxic to most human body functions, including the reproductive system, early infant development, the human digestive system, the lymphatic system, the immune system, the antioxidant system, and the nervous system. Phthalates have ultimately been shown to cause a variety of diseases, including leukemia, mental illness, lung cancer, breast cancer, liver cancer, and colorectal cancer. Diisononyl phthalate (DiNP) is a mixture of chemical compounds consisting of various isononyl esters of phthalic acid. It is listed as a substance "known to the State of California to cause cancer" under proposition 65 legislation.

Colorectal cancer(CRC), or colon cancer, is the third most commonly diagnosed cancer and the second leading cause of cancer deaths in the United States. Colorectal cancer starts with polyps in the colon or rectum. Excluding skin cancers, colorectal cancer is the third most commonly diagnosed cancer in the United States.It's a disease in which cells in the colon or rectum grow out of control. The American Cancer Society (ACS) provides an estimate for the number of colorectal cancer cases in the United States for 2022 of 151,030 new cases of colorectal cancer, and 52,580 deaths of colorectal cancer during 2022. The lifetime risk of developing colorectal cancer is about 1 in 23(4.3%) for men and 1 in 25(4.0%) for women. [5]

[2] Wang, Y., & Qian, H. (2021). Phthalates and Their Impacts on Human Health. Healthcare (Basel, Switzerland), 9(5), 603. https://doi.org/10.3390/healthcare9050603

[3] BrickAdmin. (2021, July 15). What are phthalates?: Uses, benefits, and Safety Facts. ChemicalSafetyFacts.org. Retrieved September 15, 2022, from https://www.chemicalsafetyfacts.org/phthalates/

[4] BrickAdmin. (2021, July 15). *What are phthalates?: Uses, benefits, and Safety Facts*. ChemicalSafetyFacts.org. Retrieved September 15, 2022, from https://www.chemicalsafetyfacts.org/phthalates/

[5] Colorectal cancer statistics: How common is colorectal cancer? American Cancer Society. (n.d.). Retrieved September 15, 2022, from https://www.cancer.org/cancer/colon-rectal-cancer/about/key-statistics.html

Phthalate in Environment

Phthalates are easily released into the environment. In general, they do not persist due to rapid biodegradation, photodegradation, and anaerobic degradation. Outdoor air concentrations are higher in urban and suburban areas than in rural and remote areas. They also pose no acute toxicity.

Because of their volatility, DEP and DMP are present in higher concentrations in air in comparison with the heavier and less volatile DEHP. Higher air temperatures result in higher concentrations of phthalates in the air. PVC flooring leads to higher concentrations of BBP and DEHP, which are more prevalent in dust. A 2012 Swedish study of children found that phthalates from PVC flooring were taken up into their bodies, showing that children can ingest phthalates not only from food but also by breathing and through the skin. [6]

Colorectal Cancer and Phthalate

Colorectal cancer, also known as colon cancer or rectal cancer, refers to cancer that occurs anywhere in the colon. Colorectal cancer is associated with a high-fat, low-fiber diet, chronic inflammation of the colon, colorectal adenomas, genetic factors and other factors such as: schistosomiasis, pelvic radiation, environmental factors (e.g. molybdenum deficiency in the soil), and smoking. According to current studies, it is likely that non-genetic rectal cancer is strongly related to the dysregulation of the intestinal microbiota in the human body. Dysbiosis situations can induce colonic carcinogenesis through a chronic inflammation mechanism. When these inflammatory responses become chronic, cell mutation and proliferation can result, often creating an environment that is conducive to the development of cancer. Scientists also find out that phthalate mixture induced increases in HT29 proliferation of 10.94% at 33.33 ppt and 60.87% at 3.33 ppt, whereas this proliferation relation at lower concentrations was not found for DLD1 cells. Previous studies also demonstrate preliminary information regarding the low dose induction of proliferation of the cancer cells by phthalate mixtures. Because non-monotonic dose responses are still being debated, further studies are required to re-evaluate the reference doses defined by governments for phthalate. [7] [8]

Leukemia Cancer and Phthalate

Leukemia is a group of malignant clonal diseases of hematopoietic stem cells. Clonal leukemia cells accumulate in the bone marrow and other hematopoietic tissues due to mechanisms such as uncontrolled proliferation, impaired differentiation, and impaired apoptosis, and infiltrate other non-hematopoietic tissues and organs while suppressing normal hematopoietic function. Clinical manifestations include varying degrees of anemia, bleeding, infectious fever, and enlargement of the liver, spleen, lymph nodes and skeletal pain. There are many causes of leukemia, including viral, chemical, radiological, or genetic factors. Some chemicals have a leukemogenic effect. The incidence of leukemia is higher in people exposed to benzene and its derivatives than in

[6] BrickAdmin. (2021, July 15). What are phthalates?: Uses, benefits, and Safety Facts. ChemicalSafetyFacts.org. Retrieved September 15, 2022, from https://www.chemicalsafetyfacts.org/phthalates/

[7] Yurdakok Dikmen B, Alpay M, Kismali G, Filazi A, Kuzukiran O, Sireli UT. In Vitro Effects of Phthalate Mixtures on Colorectal Adenocarcinoma Cell Lines. J Environ Pathol Toxicol Oncol. 2015;34(2):115-23. doi: 10.1615/jenvironpatholtoxicoloncol.2015013256. PMID: 26081030.

[8] Chen HP, Pan MH, Chou YY, Sung C, Lee KH, Leung CM, Hsu PC. Effects of di(2-ethylhexyl)phthalate exposure on 1,2-dimethyhydrazine-induced colon tumor promotion in rats. Food Chem Toxicol. 2017 May;103:157-167. doi: 10.1016/j.fct.2017.03.014. Epub 2017 Mar 8. PMID: 28284729.

the general population. Leukemia has also been reported to be induced by nitrosamines, pautozone and its derivatives, and chloramphenicol. Certain antitumor cytotoxic drugs, such as nitrogen mustard, cyclophosphamide, methyl benzyl hydrazine, VP16 and VM26, have leukemogenic effects.[9] [10] [11]

Limitation and Gaps of Current Studies

Current studies have confirmed the effect of phthalate, a drug that has been shown to have an effect on colorectal cancer and leukemia. However, insufficient in vivo data have led to the fact that the pathway through which phthalate induces cells is still unknown. Available studies suggest that phthalate intake can cause a range of cancers including heart tumors, lung cancer, stomach cancer, brain cancer, and leukemia. And the effects continue into the behavior of future generations and infants in the fetus. But scientists have not been able to explain the exact cause of this link. [12]

Aims of Research

The main objective of the experiment was to investigate the effect of phthalate on colorectal cancer and leukemia, trying to find out which cellular pathways are induced by phthalate and whether different concentrations of the drug cause different levels of effects.

Materials and Methods

Preparation Before Treatment

Solution Preparation

The chemical solution of phthalate obtained from Aldrich (USA) is phthalate that has been dissolved in ethanol. Through the serial dilution method, the same amount of phthalate solution was put into 9, 99, 999 and 9,999 times its dose of MEM cell media solution, respectively, resulting in a 10%, 1%, 0.1%, and 0.01% solution of phthalate and cell media. This solution will later become the major source fluid in the following experiment. Meanwhile, whenever this source fluid is treated in a 96 well cell plate, it is diluted by 20 times due to the cell medium, and 200 times if added in a 6 well plate.

[9] Duan XL, Ma CC, Hua J, Xiao TW, Luan J. Benzyl butyl phthalate (BBP) triggers the malignancy of acute myeloid leukemia cells via upregulation of PDK4. Toxicol In Vitro. 2020 Feb;62:104693. doi: 10.1016/j.tiv.2019.104693. Epub 2019 Oct 17. PMID: 31629899.
[10] Manz, P., Cadeddu, R. P., Wilk, M., Fritz, B., Haas, R., & Wenzel, F. (2014). Impact of Di(2-ethylhexyl)phthalate on migration rate of human promyelocytic leukemia cells (HL-60). Clinical hemorheology and microcirculation, 58(1), 241–246. https://doi.org/10.3233/CH-141903
[11] Wang, L. S., Liu, H. J., Zhang, J. H., & Wu, C. T. (2002). Purging effect of dibutyl phthalate on leukemia cells involves fas independent activation of caspase-3/CPP32 protease. Cancer letters, 186(2), 177–182. https://doi.org/10.1016/s0304-3835(02)00324-5
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Cell Culture and Treatments

Colo320DM (ATCC CRL-1593.2™) and U937 (ATCC CCL-220™) cell lines were obtained from the American Type Culture Collection(ATCC). Colo320DM colorectal cancer cells were grown in a MEM medium solution (SIgma-Aldrich, USA) at 37°C in an incubator at 5% CO2 and at about 60% humidity. DiNP mixture in acetonitrile stock solution was prepared from the standards. Before experiments,the old medium was removed from the flask, and 4 ml of trypsin was added. Then, the flask was put in the incubator for 4 minutes. After taking the flask out of the incubator, clap the flask to detach the cell from the bottom of the flask, add 5 ml of fresh MEM to neutralize the trypsin, and transport the combined liquid into a tube. After that, centrifuge the tube with 3000 rpm for 4 minutes with a tube of water on its opposite side which would create a cell pellet at the bottom of the tube. This cell line prepared an in vitro basis which can be later used for assays.

Molecular Docking

Molecular Docking was performed by using the PyRx v.08 software to test the binding affinity between DiNP and receptors(estrogen alpha receptor, estrogen beta receptor, Fas receptor, DR3 and DR 6 receptor, PDE protein, P53 protein, g-protein coupled receptor[GPCRs], Protein Tyrosine Phosphatase 1B [PTP1B]). And the five receptors that had the highest binding affinity with DiNP were chosen. Then, molecular docking between those receptors and their known ligands was conducted. Finally, the binding affinity between DiNP and those 5 receptors and the binding affinity between those seven receptors and their known ligands is compared to assess how well these receptors bound to DiNP.

All macromolecules were acquired from the Protein Database Bank (https://www.rcsb.org/) and downloaded as .pdb files. DiNP and all other ligands were retrieved from the PubChem Compounds Database (https://pubchem.ncbi.nlm.nih.gov/) as .sdf files which were later converted to .pdb files through the SMILES Translator (https://cactus.nci.nih.gov/translate/).

Cytotoxicity Assays

MTT Assay

The MTT cell Proliferation ASsay was conducted to measure DiNP's effect on cancer cell's survival rate, for it is able to measure the cell proliferation rate and the cell's viability under treatment that is based on the ability of NADPH-dependent cellular oxidoreductase enzymes to reduce the tetrazolium dye(MTT) to its insoluble formazan(purple) . For the MTT assay, 10 milliliter of MTT solution was added to each well that contained DiNP, and the plate was put in the incubator at 37°C for 1.5h. MTT will react with succinate dehydrogenase in the cells and form formazen. The amount of formazan is proportional to the amount of cells survived. Cell viability was measured using a microplate reader at 595 nm.

LDH Assay

This assay measures the cytosolic enzyme lactate dehydrogenase(LDH), which is released upon necrosis. A coupled enzymatic reaction that results in the conversion of a tetrazolium salt (INT) into a red color formazen will occur with the release of LDH. Cultured cells were incubated with DiNP (DiNP 10%, DiNP 1%, DiNP 0.1% and DiNP 0.01% respectively) for 24 h to induce toxicity. Afterwards 35 uL of media are transferred from the treated plate into two fresh 96 well plates labeled as "released LDH Plate" and "Total LDH Plate". Then add 10 uL of LDH lysis buffer to each well in the original plate and put the original plate in the incubator at 37 celsius for 45 minutes. Simultaneously, also add 30 uL of LDH substrate to the "released LDH Plate " and put it in a box at room temperature to keep it away from light for 30 minutes.

After 45 minutes of incubation, the original plate was taken out from the incubator. Transfer 30 uL from each well from the original plate to each well in the "Total LDH Plate". Then, add 30 uL of LDH substrate to each well in the "Total LDH Plate" and put it in the box to keep it away from light for 30 minutes.

Now the "Released LDH Plate"that has been sitting in the box for 30 minutes was taken out from the box, and was added with 30 uL of the LDH stop solution to prevent more reaction between LDH and LDH substrate. Afterwards, the "Released LDH Plate" was put in a microplate reader. Absorbance of red color formazan at 490 nm and 650 nm is measured to identify the cytotoxic effect of DiNP.

After leaving the "Total LDH Plate" in the box for 30 minutes, take it out and add 30 uL of stop solution to each well in the plate. Then, use the microplate reader to read the "Total LDH Plate" also at 490 nanometers and 655 nanometers and subtract the numbers acquired from the reading at 655 nanometers from the numbers acquired from the reading at 490 nanometers. Finally, calculate the percent toxicity of DiNP using this formula:

(Sample released LDH - control released LDH)/(sample total LDH - control released LDH)*100

Cell Attachment Assay

Cell attachment assay measures cell binding either to immobilized ligands or to cell monolayers in flat-well microtiter plates under static conditions. For the cell attachment assay, a 24 well tissue culture plate (Costar, Cambridge, MA) was prepared. The well was coated with 2µl of substrate fibronectin (Sigma-Aldrich, St. Louis, MO) to create a protein layer for the binding of inflammatory cells. Cells were counted with a hemocytometer and brought up to a density of 5x10^5 /mL of media. Afterwards, 0.5 mL of suspended cells were put in each well and treated with concentration of DiNP 10%, DiNP 1%, DiNP 0.1%, DiNP 0.01% respectively, and 1µM TPA and 1µM LSP as the positive controls, and a negative control well with no treatment.

Treated cells were then incubated at 37°C for 24 hours. Following this, unattached cells and their media were removed by aspiration. Attached cells were fixed and stained with Diff Quick(Baxter Scientific), which were later measured using the java-based image processing program Image J. After the images were uploaded to Image J, they were first turned into 8 bit black and white pictures. Followed by adjusting the threshold, the images were subtracted from their background. In the last part, the images are denoised and then set to count 10 units of colored part as a cell by the ICTN tool. The ICTN tool calculates the number of cells in the whole image and after the calculation is done, the results are recorded and analyzed using google sheets.

Caspase-3 Colorimetric Assay

The Caspase-3 Colorimetric Assay measures Caspase-3's activities to assess whether DiNP is able to induce apoptosis. Caspase are enzymes that control apoptosis and cell inflammation (Fan et al., 2005): when activated, caspase will execute apoptosis, so if DiNP is able to induce apoptosis, caspase will be activated. Moreover, a chromophore p-nitroaniline (pNA) solution will be added, and it will cause a deeper color change if there is more caspase and a lighter color change if there is less caspase (Caspase Colorimetric Apoptosis Assay, 2019).

Following the cell culture procedures, transfer the cells to a six-well plate, and treat the cells with their respective treatments (add 5 uL of DiNP 0.1 uM, DiNP 1 uM, DiNP 10 uM, and DiNP 100 uM to the wells in four different rows). Then, incubate the cells in the incubator for 24 hours. After 24 hours, the cells are collected in 1.5 ml tubes with 50ul of lysis buffer in each tube. Following the protocol of the assay kit provider (Caspase Colorimetric Apoptosis Assay, 2019), 50ul of assay buffer, 45 ul of lysis buffer, and 5ul of cell lysate are added to each well of a 96-well plate. Then, add 5 microliter of pNA substrate solution, mix the contents in each well, and take readings at times 0, 15 minutes, 45 minutes, and 1 hour with a microplate reader (iMark, USA) at 415 nanometers. Finally, calculate the percent change of the caspase activity over the hour by this formula: (sample average absorbency - control average absorbency)/control average absorbency * 100

Statistic Analysis

Multiple T-Tests were conducted to compare the means between the control and samples with different treatments, and p-values were calculated with a significance level if the p-value is 0.05 or lower. Additionally, standard deviations calculated by the Standard Deviation Calculator are shown by the error bars on graphs.

Results

Molecular Docking

Macromolecules	Average Binding Affinity to Phthalate (KD)
Human Serum Albumin	-7.15
Human Topomerase II alpha receptor	-6.6625
Glucocorticoid receptor	-6.375
Death Receptor 5	-5.0444
Estrogen Alpha receptor	-4.5

MTT Assay

MTT Colon

DiHP induces a decrease in the rate of survival in colorectal cancer cells(Colo320), which means it causes cell death of the colorectal cancer cell.

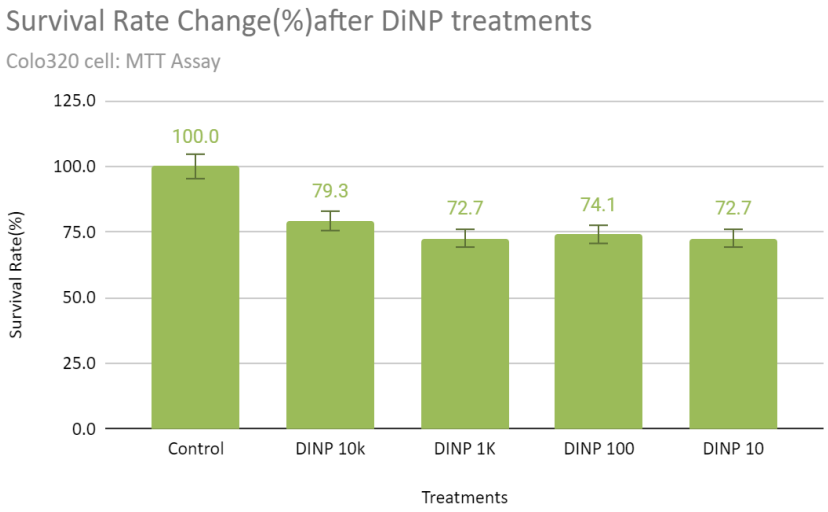

Figure 2.

MTT Leukemia

DiHP induces an increase in the rate of survival in leukemia cancer cells(U937), which means it causes proliferation of leukemia cancer cells.

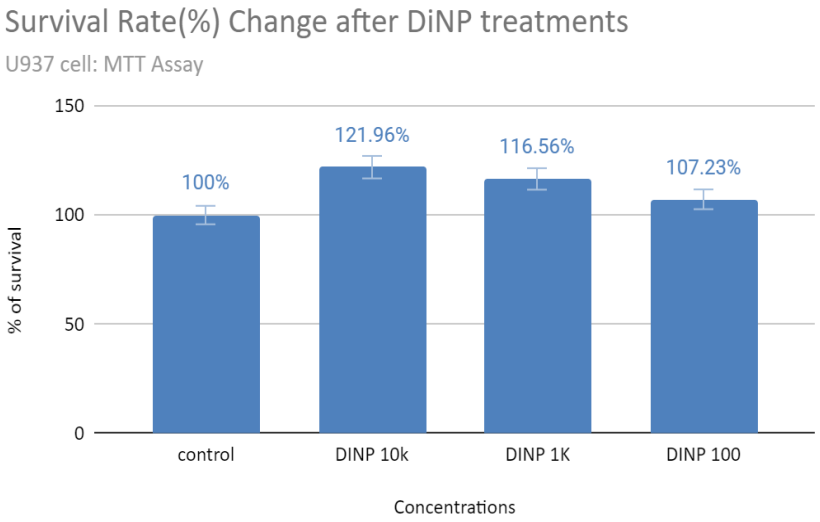

Figure 3.

LDH Assay

LDH Colon

The p-value calculated by t-test for DiNP 10k, DiNP 1k, DiNP100, and DiNP10 are 0.17, 0.38, 0.67, 0.68, respectively, which were all higher than 0.05. Therefore the results were non-significant.

DIHP 10k	DIHP 1K	DIHP 100	DIHP 10
0.1693879739	0.3844209239	0.6661742	0.6834282818

LDH Leukemia

The p-values for DiNP 1k and DiNP 100 calculated by t-test were 0.305 and 0.316, (higher than 0.05) which means the result of DiNP 1k and DiNP 100 is not significant. Hence, although their results show a decreasing trend of cytotoxicity on the graph, these data tend not to be passed over in silence.

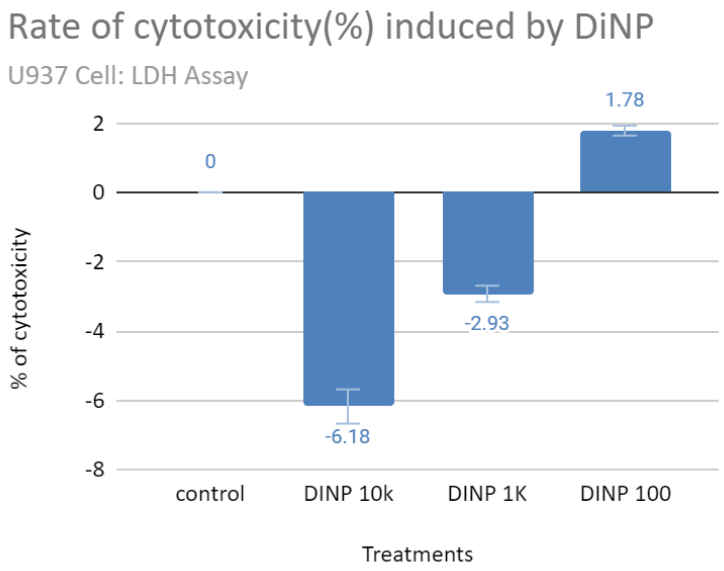

Figure 4.

Cell Attachment Assay

Lipopolysaccharide(LPS) and tissue-type plasminogen activator(TPA) are two types of bacteria that induce cell adhesion. In this assay, the two types of bacteria are used as positive control, and the negative control received no treatments.

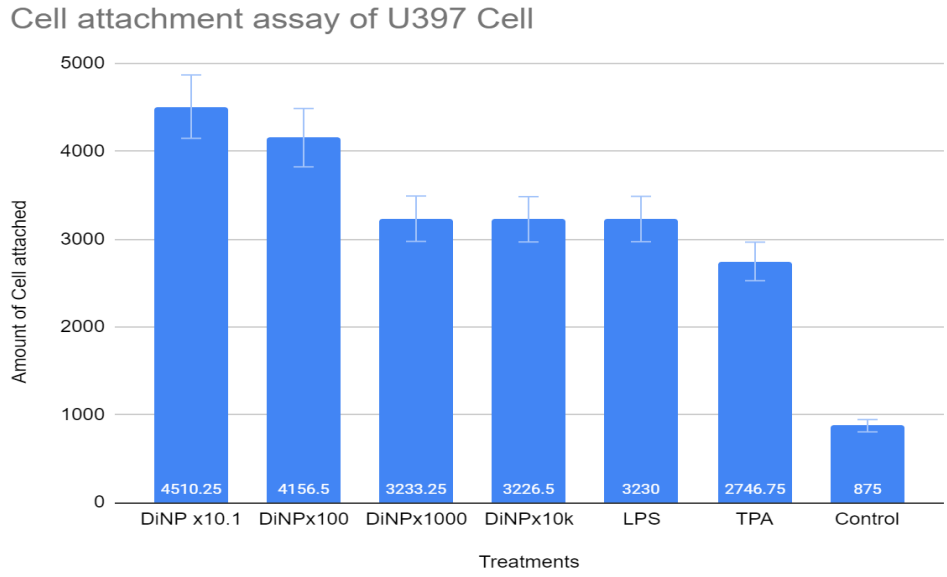

Figure 5.

Caspase-3 Colorimetric Assay

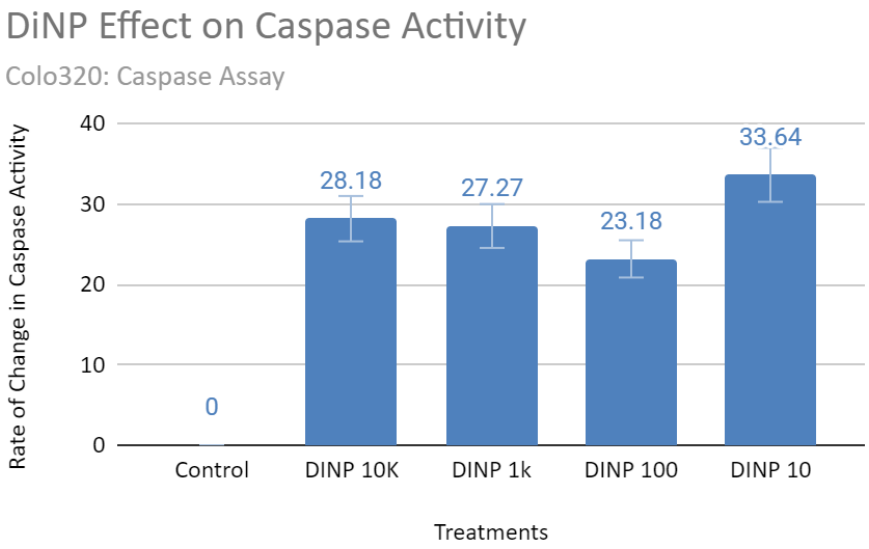

Figure 6.

Discussion

Phthalate Effect on Colorectal Cancer Cell

LDH and MTT Colon

Dose-response plots for the MTT assay are derived from optical density measurements. As shown in Figure 1, compared to the control group, cell viabilities in all concentrations of DiNP solution were found to be significantly different (p value < 0.05). In addition, DiNP 100 and DiNP 1k, which had a p-value lower than 0.005, are considered to be highly significant with a decrease percent of cell viability in colorectal cancer cells for 74.1% and 72.7% respectively. According to the results, it can be concluded that DiNP caused a decrease in colorectal cancer cell viability(average decrease of -25.7% in cell viability) , which means it most likely led to the death of colorectal cancer cells. When the responses at these concentrations were fitted to a suitable regression line, a half maximal inhibitory concentration (IC50) value of 0.0015 was calculated for Colo320 cells. The formula for IC 50 is listed as below: $Y = 0.2954 + (0.4067 - 0.2954)/(1 + [x/0.0015]^{0.6741})$.

The p-value of LDH toxicity assay was not significant. The analysis of this result will be carried out in three ways, firstly 1: a visual comparative analysis of the results for mtt and ldh: it is clear from the experimental results that phthalate at concentrations of 10%, 1%, 0.1%, and 0.01% did not cause very significant tumor cell necrosis, but caused significant apoptosis of rectal tumor cells. This means that the drug induces cell death by apoptosis as opposed to directly affecting the cells themselves. If this drug is used to treat rectal cancer tumors, it is not advisable to trigger inflammatory reactions in the surrounding tissues to avoid greater harm to the patient.

Analysis for ldh: Although the results of ldh toxicity test are not significant, the average data of ldh show that the toxicity of the drug presents a does non-monotonic situation --- that is, the lower the drug concentration the greater the toxicity, and the higher the drug concentration the less the toxicity. This may be the reason why tumor cells become resistant to higher concentrations of drugs more quickly. And tumor cells that have developed drug resistance are going to develop more rapidly than those that have not.

The analysis of the data combining ldh, mtt and comparing leukemia tumor cells.

The results of mtt compared with ldh showed that the lower the drug concentration, the higher the cell activity; the lower the drug concentration, the higher the cell activity.

In relation to the dose non-monotonic situation just seen in the colon ldh toxicity assay and in another tumor cell, the data suggest that there may be a threshold for cellular tolerance of phthalate drugs. When the drug concentration is increased and exceeds a certain threshold, apoptosis is directly induced in the tumor cells. When the dose is less than a certain level, it triggers cellular necrosis.

Caspase Assay Colo-3

Endocrine-disrupting chemicals (EDCs) have challenged traditional concepts in toxicology, because EDCs can have effects at low doses with a non-monotonic dose-response relationship that is not predicted by effects at higher doses.22 Considering the potential cumulative effects of multi-exposure of xenobiotics at lower doses, new strategies for risk assessment should be evaluated.12 Among EDCs, phthalates are widely distributed; which leads to appreciable exposures in the general population through several sources as a mixture. In the current study, the effects of the low-dose phthalate mixture (DBP, BBP, DEHP, DiNP, DNOP, and DiDP) exposure on colorectal adenocarcinoma cell line were investigated in vitro. Even though the current study has the limitation of providing in vitro data that might not carry over to in vivo conditions, the findings through MTT assay and wound healing migration assay reveal that cell viability/proliferation were increased by phthalate treatment at concentrations less than 33.33 ppt. The induction of the proliferation rate at lower doses was statistically significant ($p < .05$) for HT29; however, similar results were not found in DLD1 cells. The differences between DLD1 and HT29 for the low-dose

Phthalate Effect on Leukemia Cancer Cell

MTT Leukemia and LDH Leukemia

LDH is released into the surrounding culture medium upon cell damage or lysis during apoptosis and necrosis; therefore, as an indicator for cell membrane integrity, an LDH assay was performed. The results reveal that LDH leakage was found to be lower in treated cells when compared to control groups in both cell lines ($p < .05$) (Fig. 2). However, these decreases were found to be dose-independent, where no differences were observed between concentrations.

Cell Attachment Assay Leukemia

Because crystal violet stains DNA, it is useful for obtaining quantitative information about the relative density of cells adhering to multi-well cluster dishes for cytotoxicity and viability assays. The amount of FIG. 1: MTT test results of DLD1 and HT29 cells treated with phthalate mixtures ($p < .05$). Volume 34, Number 2, 2015 In Vitro Effects of Phthalate Mixtures on Colorectal Adenocarcinoma Cell Lines 119 dye taken up by the mono-layer and the intensity of the color produced after solubilization of the dye are proportional to cell number.21 No significant differences were observed for DLD1 and HT29 cells within the increased doses of phthalate mixture (Fig. 4). B. Wound Healing In the wound healing migration assay, we observed that at lower concentrations (especially at 3.33 ppt), HT29 cells migrated faster than in control medium ($p < .05$). Wound closure increased 38.10% ± 1.14% compared to control groups. The results were found to be consistent with the MTT assay results. For DLD1 cells, wound healing decreased from 1.15- to 2.96- fold, indicating the cytotoxicity of the compound; the decrease was highest at 33.33 ppt (Figs. 5 and 6)

Conclusion

Data collected from cancer cell studies are sufficient to raise concerns about the potential carcinogenicity of DINP. However, the epidemiological evidence in humans does not appear to be sufficient to support the attribution of carcinogenicity to DINP exposure. In addition, it remains unclear
It is not clear which cellular processes, including activation of nuclear receptors such as death receptor, fas1, ERα and AhR, are involved.

The cause of DINP carcinogenesis may be related to its attack on immune cells and the properties of the p53 pathway. The targeting of immune cells resulted in the proliferation of cancer cells in the immune system, but caused a decrease in colorectal cancer cells. In addition, redox alterations and epigenetic disturbances may also be the main pathways leading to DINP carcinogenesis. One should be concerned about the use of DEHP in food and plastic products.

Acknowledgments

I would like to thank my advisor for the valuable insight provided to me on this topic.

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