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Genotoxicity of Npk Using Fluorescent Staining Methods On Peripheral Blood Mononuclear Cells (Pbmc)

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ABSTRACT

NPK (Nitrogen, Phosphorus, Potassium) is a common fertilizer that farmers have been using for several years. In 2020, a 15-15-15 NPK was found to induce micronuclei formation when exposed to Juvenile African Catfish Clarias gariepinus (Helen 2020). This study examines the genotoxicity of a 19-19-19 NPK on PBMCs using fluorescent staining methods through an MTT Assay, EtBr/AO Staining, Comet Assay, and Micronucleus Assay. The objective of the study is to determine the in vitro cytotoxicity of NPK. The study found that when PBMCs were exposed to the LC_{50} value of NPK for 24 hours, apoptosis and necrosis occurred, as well as several polynucleated cells. While there is a substantial amount of evidence for genotoxicity in an in vitro setting, more studies will be needed to conclude whether NPK causes in vivo cytotoxicity.

Introduction

The WHO defines the term mutation as the "permanent changes in the structure or amount of the genetic material of an organism that can lead to heritable changes in its function" (Boobis 2020). A mutagen is a chemical that causes heritable genetic changes by interacting with DNA. The process of causing a mutation is known as mutagenicity. Genotoxicity is a broader term that includes both mutagenicity and DNA damage. Genotoxins have the potential to cause DNA and chromosome damage, leading to germline mutations. Damaged DNA in somatic cells may result in a mutation that leads to a malignant transformation and cancer (Phillips 2009). However, it is still unclear exactly how most genotoxic substances induce genetic mutations.

Assessing the genotoxicity of NPK is vital to the entire human population as NPK is one of the most used fertilizers. In 2019, over 190 million tons of NPK had been consumed globally (Fernandez 2022). This large rate of consumption has the potential to adversely affect a large rate of our population if proven genotoxic. If NPK does cause genotoxicity, then the agricultural community can turn to safer fertilizers that provide equivalent yields as NPK for their desired crop.

Butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA), sorbic acid (SA), propyl gallate (PG), and sodium nitrate (SN), common chemicals used in food packaging, have shown to have genotoxic effects on chromosomes in a plant assay (Pandey 2014). There are currently no studies that show NPK genotoxicity on human cells. However, NPK was proven genotoxic on the Juvenile African Catfish Clarias gariepinus that were exposed to 2.26, 4.52, and 11.30 mg/L of a 15-15-15 NPK fertilizer (Helen 2020). Another study has shown that the NPK nanoparticle can increase the crop yield of wheat (Triticium aestivum L.). However, when the root-tip cells were examined, several types of chromosomal aberrations showed including multinuclei, micronuclei, abnormal metaphase, nucleus erosion, nucleus elongation, fragments, increased nuclear content, and others (Abdelsalam 2019).

When a genotoxic substance interacts with DNA structure and sequence, genetic material is damaged. The substance interacts at a specific location or base sequence of the DNA structure, causing breakage, fusion, deletion, lesions, mis-segregation, or non-disjunction leading to DNA damage and mutations (Mohamed 2017).

Researchers have developed several methods for identifying both in vitro and in vivo genotoxicity. Some common methods for detecting in vitro genotoxicity include the bacterial Ames test, DNA strand break measurements in cells (comet assay, alkaline unwinding and hydroxyapatite chromatography, alkaline elution), and cytogenetic assays (micronucleus and chromosomal aberration assays) (Dusinska 2012). As mentioned above, this study has used the MTT assay, EtBr/AO staining, comet assay, and micronucleus assay to determine if NPK induces genotoxicity in human PBMC cells.

Hypothesis

It was hypothesized that the LC_{50} concentration of NPK would cause genotoxicity in the PBMCs.
Specifically:

1. When the PBMCs are exposed to the LC_{50} concentration of NPK for 24 hours, apoptosis and cell death will occur. (shown through the EtBr/AO staining)
2. When the PBMCs are exposed to the LC_{50} concentration of NPK for 24 hours, tails will form during the comet assay signifying at least grade 1 DNA damage.
3. When the PBMCs are exposed to the LC_{50} concentration of NPK for 24 hours, several incidents of apoptosis, necrosis, and polynucleated cell formation will occur. (shown through micronucleus assay)

Materials and Methodology

Chemicals and Reagents

19-19-19 NPK was from Akshya (India), Fetal Bovine Serum (FBS), and antibiotic solution were from Gibco (USA), DMSO (Dimethyl sulfoxide), 96 well tissue culture plate and wash beaker were from Tarson (India). 1X PBS was from Himedia, (India), Penicillin/Streptomycin antibiotic solution, 3.8% sodium citrate, sterile IX PBS, RPMI 1640, RBC lysis buffer, LSM were purchased from Himedia, (India).

Cell Culture

PBMC was cultured in a liquid medium (RPMI 1640) supplemented 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin, and 100 µg/mL streptomycin, and maintained under an atmosphere of 5% CO_2 at 37°C.

PBMC Isolation

Human peripheral blood was collected from healthy volunteers in a 3.8% sodium citrate-treated tubes in accordance with established institutional guidelines. Briefly, mononuclear cells were isolated by layering peripheral blood onto Lymphocyte Separating Medium (LSM). The leukocyte enriched buffy coat containing the mononuclear cells was collected and the presence of erythrocytes was removed by lysing with 1% RBC lysis buffer.

MTT Assay

Materials Required

MTT (3-4,5 dimethylthiazol-2yl-2,5-diphenyl tetrazolium bromide) (5 mg/ml) were from Sigma (USA)

Procedure

Cell culture, and PBMC were cultured in liquid medium (RPMI 1640) supplemented 10% Fetal Bovine Serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37°C.

MTT Assay

NPK sample was tested for in vitro cytotoxicity, using PBMC cells by MTT assay. Briefly, the cultured PBMC cells were harvested by trypsinization, pooled in a 15 mL tube. Then, the cells were plated at a density of 1×10^5 cells/mL/cells/well (200 µL) into the 96-well tissue culture plate in RPMI 1640 medium containing 10% FBS and 1% antibiotic solution for 24 hours at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the NPK sample in a serum free RPMI 1640 medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 hours. After the incubation period, MTT (20 µL of 5 mg/mL) was added into each well and the cells incubated for another 2-4 hours until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1X PBS (200 µL). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added, and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and LC_{50} value was calculated using Graph Pad Prism 6.0 software (USA).

Cell viability % = Test OD/Control OD X 100

EtBr/AO Staining

Materials Required

Trypsin-EDTA was purchased from Gibco (USA), EtBr and Acridine orange was purchased from Sigma Aldrich (USA). Fluorescent Imaging System, BioRad, USA.

Procedure

Briefly, 1 x 10^5 cells/mL of PBMC cells were plated into a 96 well tissue culture plate and incubated for 24 hours in a RPMI 1640 growth medium. After incubation, the wells were washed and treated with 50 mM glucose and 7.017 mg/mL of NPK sample in a serum-free DMEM medium. The plate was incubated at 37°C at 5% CO_2 incubator for 24 hours. After incubation, 10 µL of 1 mg/mL acridine orange and ethidium bromide were added to the wells and mixed gently. Finally, the plate was centrifuged at 1500 rpm for 5 minutes and evaluated immediately within an hour and examined at least 100 cells by a fluorescent imaging system.

Comet Assay

Materials Required

Low melting agarose, and normal agarose were from Merk, 6 well tissue culture plates and wash beakers were from Tarson, (India).

Procedure

Briefly, PBMC cells were seeded in a 24-well plate at a density of 10,000 cells/well and incubated for 24 hours at 37°C in a humidified 5% CO_2 incubator. The wells were treated with 7.017 mg/mL of NPK sample in a serum-free RPMI 1640 medium for 24 hours in a CO_2 incubator. After incubation, the cells were collected in a

1.5 mL tube by centrifuging at 1500 rpm for 5 minutes and the supernatant was discarded, and comet assay was performed based on the protocol of Nandakumar et al with slight modifications. The microscopic slides were sequentially coated with 200 µL of 0.75 % normal melting agarose as the first layer and 100 µL of 0.5% low melting agarose as the second layer. The next step was to add 20 µL cell suspensions to 60 µL of 0.5% low melting agarose, which was distributed on the slides as the third layer. Then the slides were incubated in cell lysis buffer (2.5 M NaCl, 0.2 M NaOH, 100 mM Na_2EDTA, 10 mM Tris–HCl, 1% Triton X-100 and 10% dimethyl sulfoxide, pH =10.0) overnight at 4°C. After that, the slides were immersed in double-distilled water three times followed by a 20 min incubation of unwinding solution (3 M NaOH). Subsequently, the slides were placed in a horizontal gel electrophoresis tank containing electrophoresis solution (1 mM Na_2EDTA and 300 mM NaOH, pH =13). The electrophoresis was conducted at 50 V (1 V/cm, 300 mA) for 10 min. Then the slides were incubated in neutralization buffer (0.4 M Tris–HCl, pH =7.5) for 10 min followed by immersion in ultrapure water three times, and air-drying. The cells were stained with 50 µL of ethidium bromide (5 mg/L) and observed under a fluorescent imaging system (Bio-Rad-ZOE). All steps were carried out under dim light to minimize extra DNA damage.

Micronucleus Assay

Materials Required

KCl, Methanol, glacial acetic acid were purchased from Merk, (USA). Penicillin/streptomycin, Fetal bovine serum was purchased from Gibco, Thermofisher Scientific, (USA).

Procedure

Lymphocyte culture was set up using culture media 10 mL of RPMI 1640 culture medium supplemented with 10% fetal bovine serum, penicillin/streptomycin at a final concentration of 2 µg/mL. The wells were treated with 7.017 mg/mL of NPK samples in a serum-free RPMI medium for 24 hours in a CO_2 incubator. After 24 hours the cells were centrifuged (2000 rpm at 5 minutes) to remove the culture medium and lymphocytes were harvested. Then the cells were treated with a hypotonic solution of KCl 0.75 mL at 37°C for 20 mins. Cells are fixed with 3:1 methanol: glacial acetic acid. Slides were dried on hot plate at 56°C for 2 minutes. The slides were then stained using trypsin-Giemsa technique for chromosomal aberration analysis. The slides were observed under a microscope at 40X and the images were analyzed using an image spectrum software.

Statistical Analysis

The difference in estimated parameters between the groups was analyzed using one-way ANOVA with Bonferroni's test. Data expressed as mean ± SD. All parameters were analyzed at 95% confidence intervals and P value of <0.05 was considered to be statistically significant. Statistical analysis of the data was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego, California USA.

Results

Cytotoxicity Assessment by MTT Assay

The results of the MTT assay determined the LC_{50} concentration to be 7.017 mg/mL. Figures 1 and Table 1 show the cell viability percentage. As seen through Figures 2-8 as the concentration of NPK decreases, the viability increases.

Table 1. Cell Viability (%)

S. No	Tested sample concentration (mg/ml)	Cell viability (%) (in triplicates)			Mean Value (%)
1.	Control	100	100	100	100
2.	20 mg/ml	32.56	36.86	25.29	31.57
3.	18 mg/ml	33.72	36.03	35.20	34.98
4.	16 mg/ml	42.31	30.58	40.82	37.90
5.	14 mg/ml	42.64	37.68	43.14	41.15
6.	12 mg/ml	54.54	44.63	54.71	51.29
7.	10 mg/ml	49.92	70.08	37.35	52.45
8.	8 mg/ml	82.31	35.86	40.16	52.78
9.	6 mg/ml	46.94	52.23	76.20	58.45
10.	4 mg/ml	84.13	76.19	84.13	81.48
11.	2 mg/ml	56.36	153.39	56.36	88.07

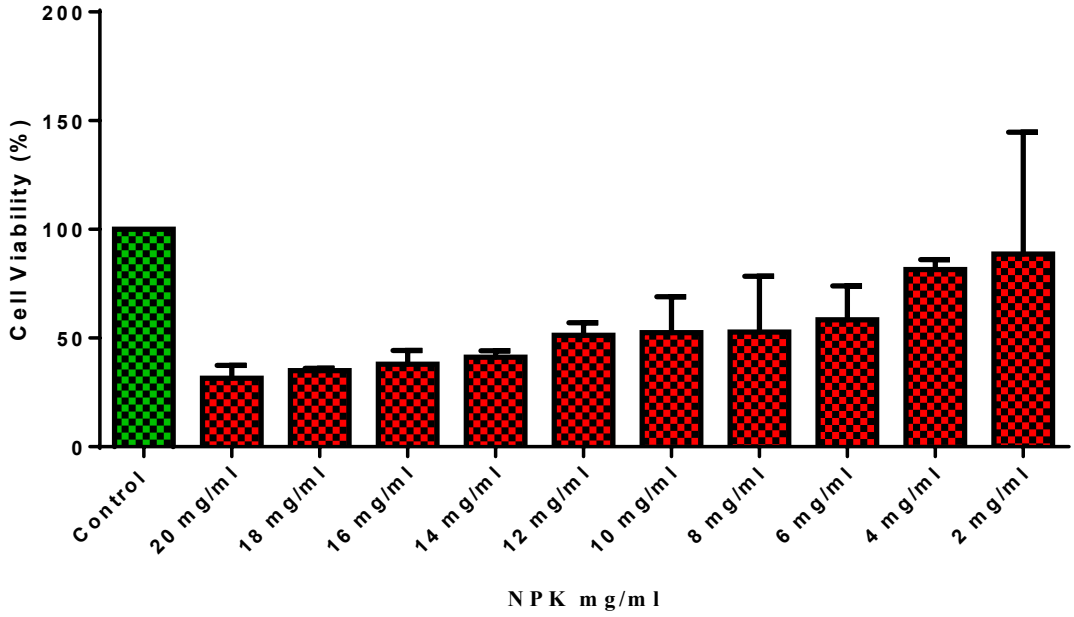

Figure 1. Cell Viability (%)

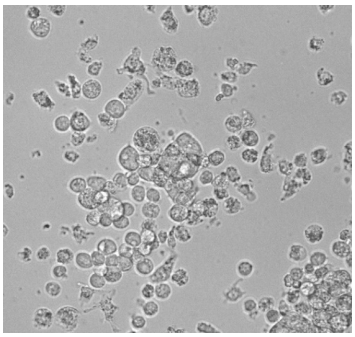

Figure 2. Control

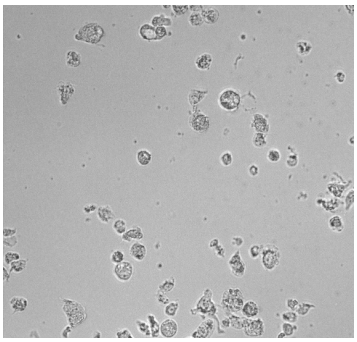

Figure 6. NPK 8 mg/mL

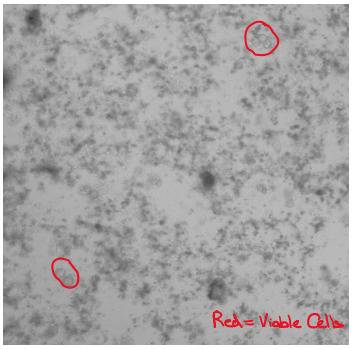

Figure 3. NPK 20 mg/mL

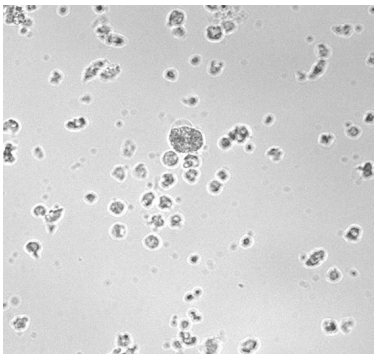

Figure 7. NPK 4 mg/mL

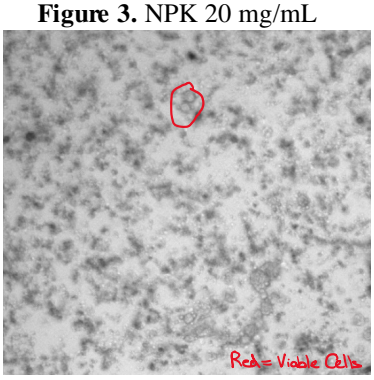

Figure 4. NPK 16 mg/mL

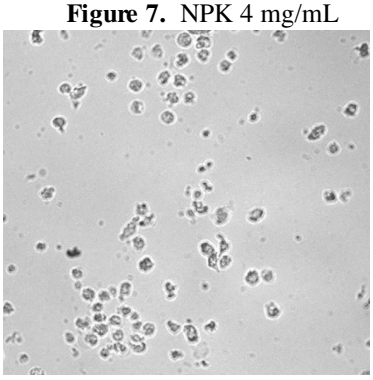

Figure 8. NPK 2 mg/mL

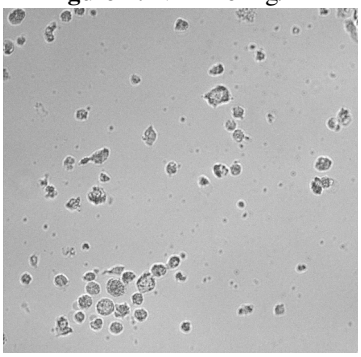

Figure 5. NPK 10 mg/mL

Figures 2-8. Images of control cells and NPK-treated cells

Live and Dead Analysis by EtBr/AO Staining

The results of the EtBr/AO staining showed on average 24 dead cells, 3.5 necrotic cells, 12 pro-apoptotic cells, 56.5 apoptotic cells, and 4 live cells. Figure 9 shows images of live control cells. Figure 10 shows cells treated with the LC_{50} concentration of NPK.

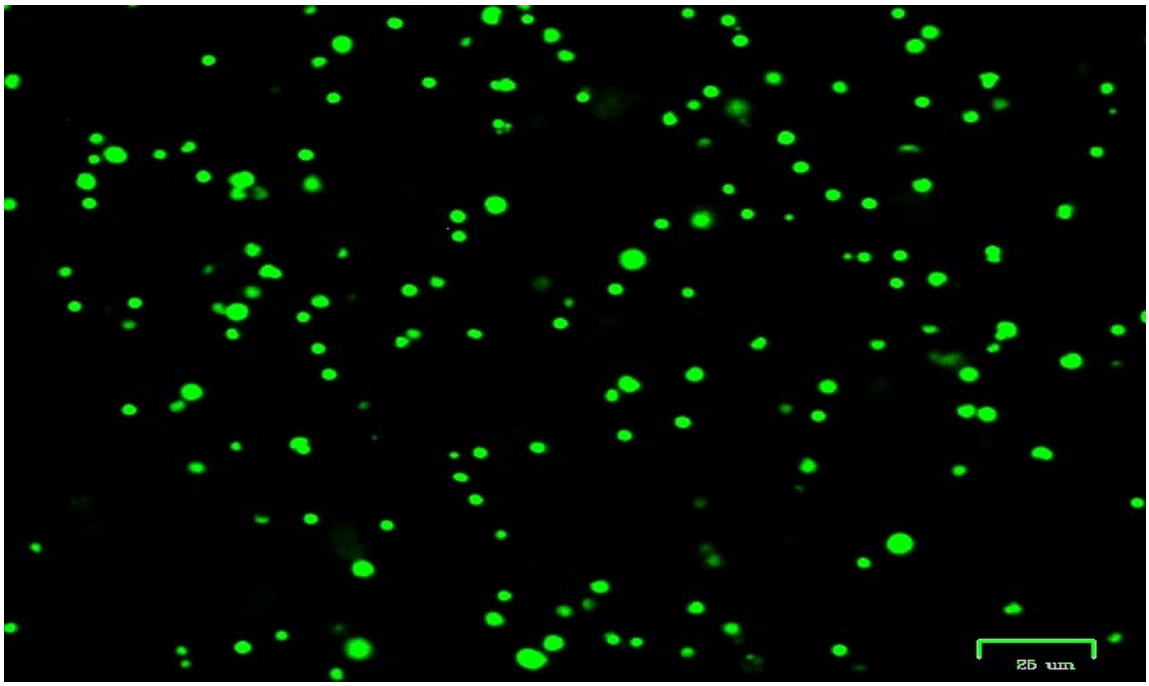

Figure 9. Control

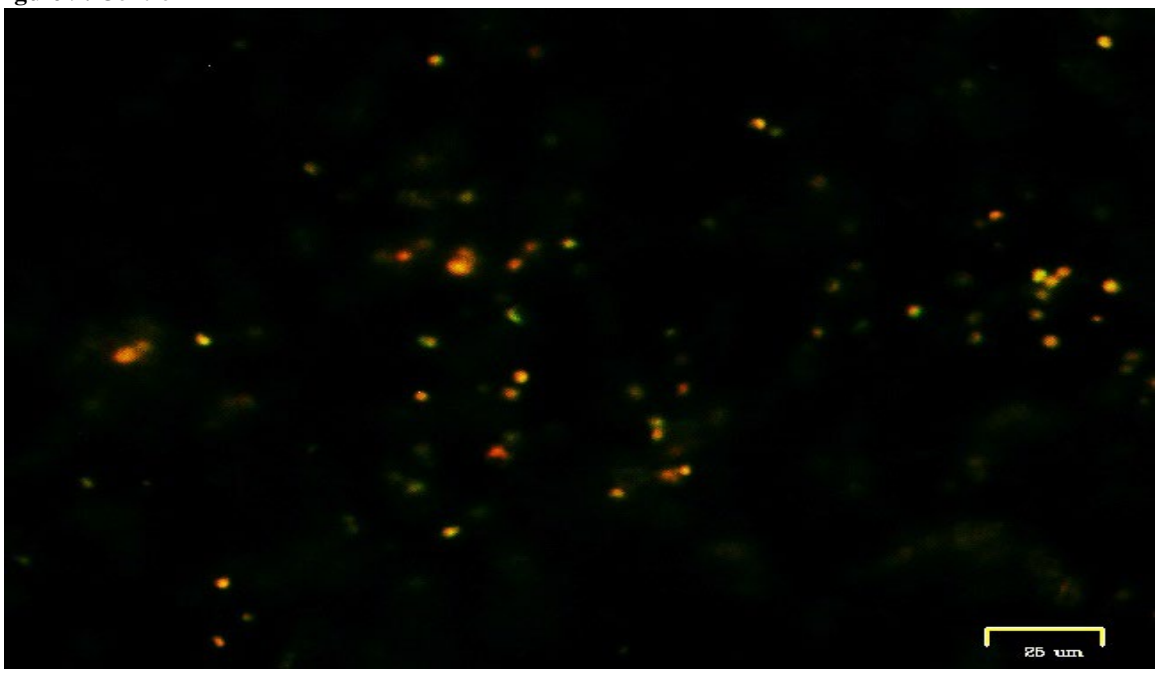

Figure 10. Treated with 7.017 mg/mL of NPK sample

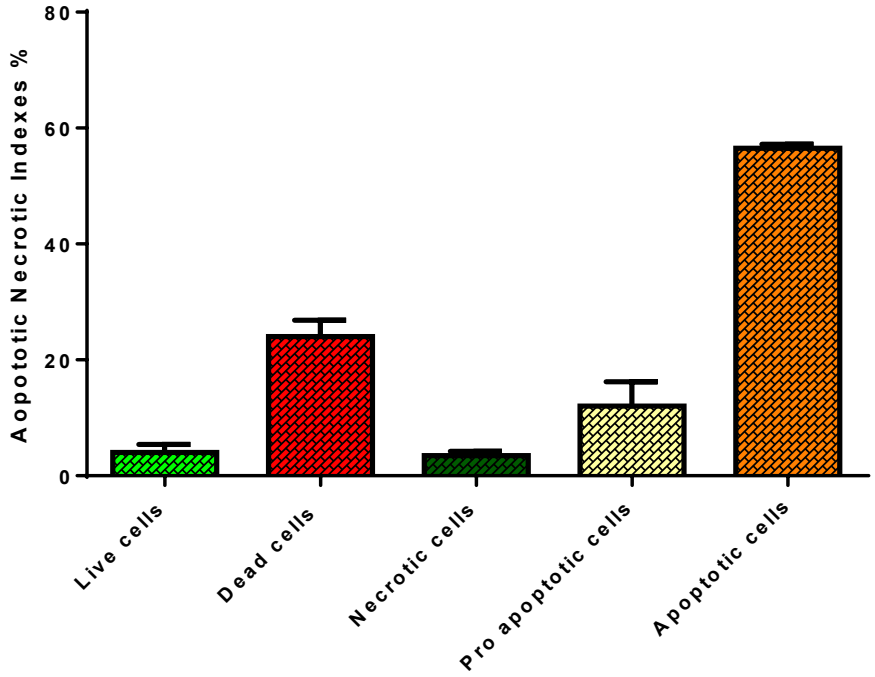

Figure 11. Apoptotic and Necrotic Index Percentages Chart

Table 2. Apoptotic and Necrotic Index of NPK

S. No	Dead cells	Necrotic cells	Pro-Apoptotic cells	Apoptotic cells	Live cells
1.	26	4	9	56	5
2.	22	3	15	57	3

Genotoxicity by Comet Assay

The comet assay concluded that 97.5% of the cells had a DNA damage event. Tail formation is visible in Figure 13.

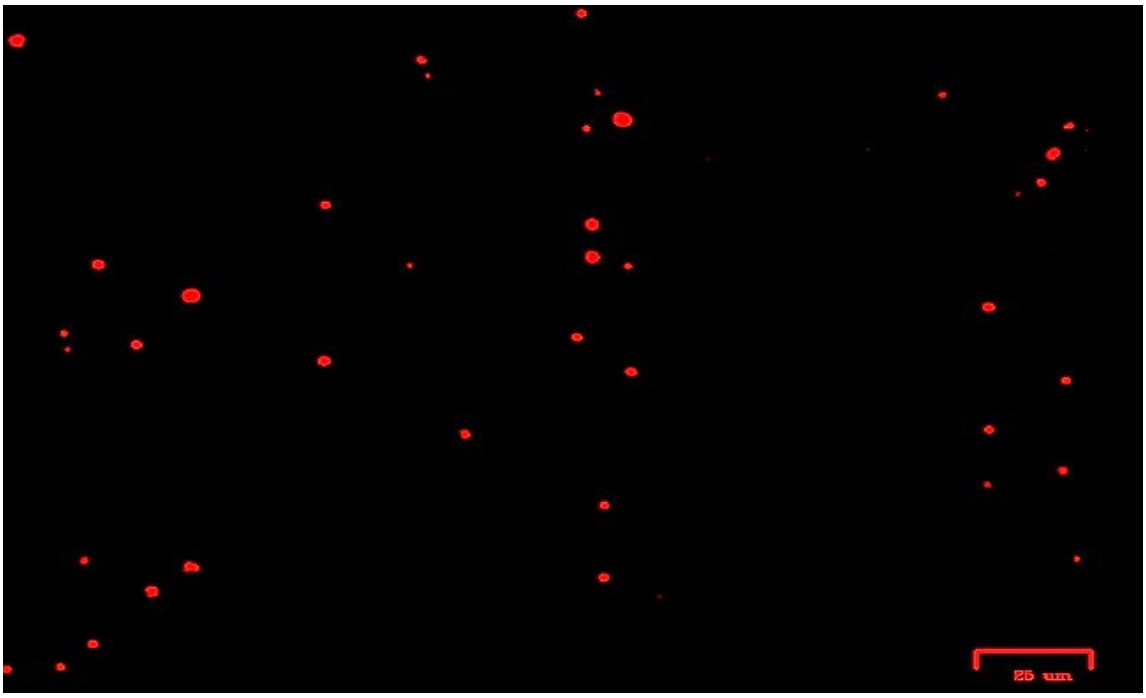

Figure 12. Control

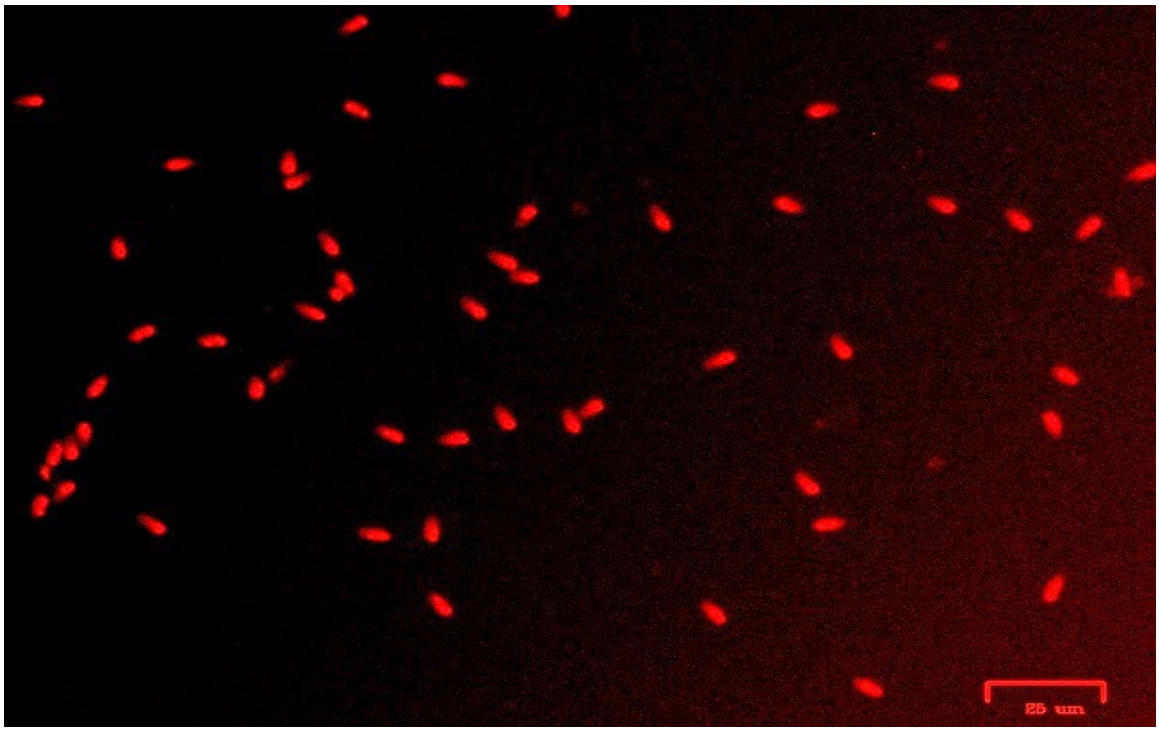

Figure 13. Treated with 7.017 mg/mL of NPK sample

Table 3. Comet Assay Results

S. No	Test sample	Number of comet events										DNA damage events (%)
		Class 0		Class 1		Class 2		Class 3		Class 4		
1.	Control	100	100	0	0	0	0	0	0	0	0	0
2.	Treated with NPK sample	2	3	0	0	74	76	20	14	4	7	97.5%

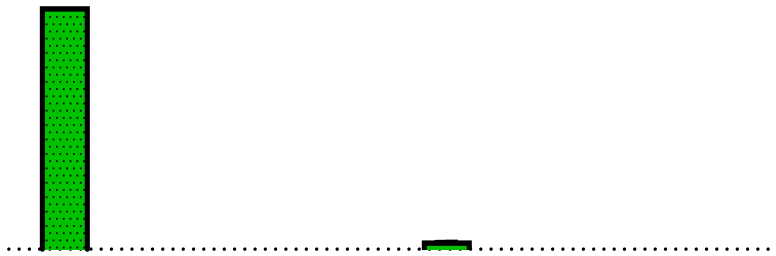

Figure 14. Chart of Comet Assay Results (1 = Control, 2 = Treated with NPK sample)

Micronucleus Assay Results

From the observed DNA damage events, apoptosis was the most common with a 41.935% occurrence. Figures 16-17 shows images of the observed DNA damage events present in Table 4.

Table 4. Micronucleus Assay Results

DNA damage events	Number of Events	% of Occurrence
Normal binucleated cells	1	3.225%
Binucleated cell with micronuclei	0	0%
Binucleated cell with nucleoplasmic bridge,	0	0%
Binucleated cell with bud	0	0%
Mononucleated cell with micronucleus	0	0%
Cell in necrosis	6	19.355%
Cell in apoptosis	13	41.935%
Polynucleated cells	11	35.484%

Figure 15. Chart of Micronucleus Assay Results

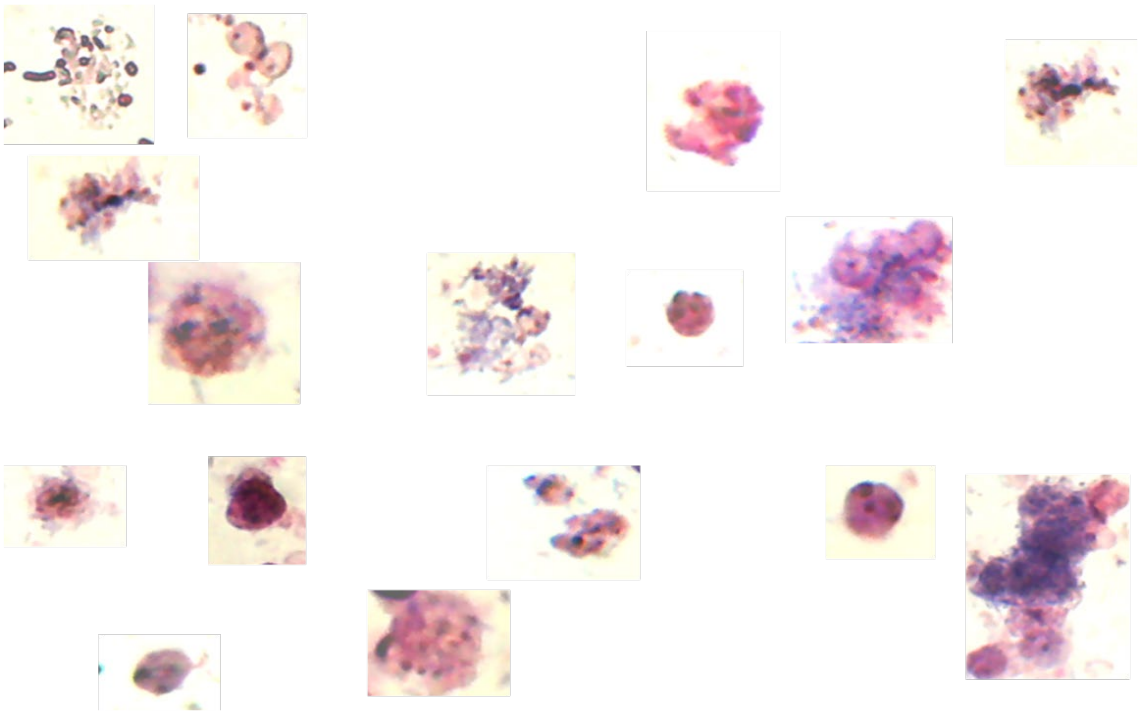

Figure 16. Images of cells found to have deviations

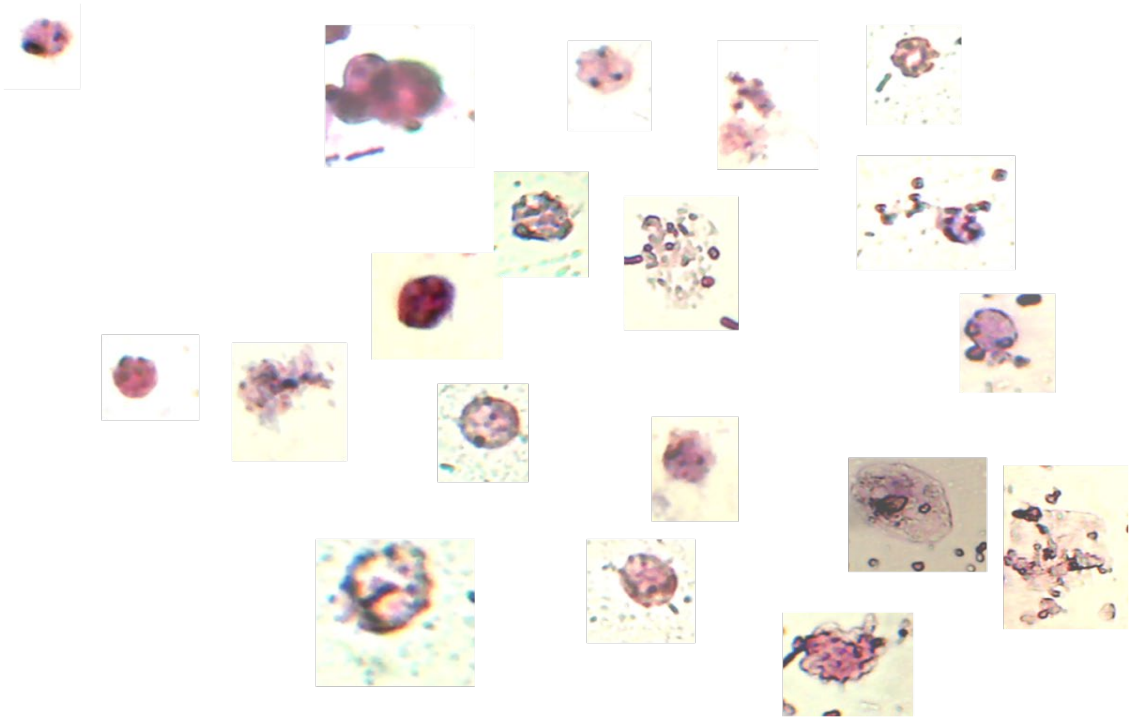

Figure 17. *Continued* Images of cells found to have deviations

Discussion

As mentioned before, there are no reported studies on the genotoxicity testing of NPK on human cells. There have been many agents studied for genotoxicity. In 2021, Sanz-Serrano and team studied genotoxicity of betaine on human cells. They found betaine not to be genotoxic.

Betaine is a food additive, primarily added in beverages, cereal products, confectionaries, or dairy products (Sanz-Serrano 2021). A key difference between the two studies is the cell culture used. The betaine study used a TK6 (human-derived lymphoblastoid cells), while the NPK study used PBMCs. Another difference includes the concentrations of betaine. Sanz-Serrano and team studied betaine, choline, and taurine at concentrations between 74-2000 µg/mL at 3 hours and 24 hours. The PBMCs in this study were treated with 7017 µg/mL NPK for 24 hours. Sanz-Serrano's study used 6 x 10^5 TK6 cells/mL while this study used 1 x 10^5 PBMC cells/mL. Similar processes were utilized for the Comet and Micronucleus Assays.

Sanz-Serrano and team added three different enzymes to their TK6 cells and treated the cells with the betaine for only 3 hours during the Comet assay. In this NPK study, 97.5% of the cells in the Comet assay had a DNA damage event. When comparing this statistic to the DNA damage in the 2000 µg/mL betaine study group, Sanz-Serrano and team concluded that "none of the three compounds showed statistically significant induction in DNA strand breaks or enzyme-sensitive sites."

In the micronucleus assay, when the sample was exposed to the 2000 µg/mL concentration of betaine (without S9), 0.78% of the cells had a DNA damage event (number found by averaging the 3 trials and converting to a percent: average was 7.8 micronuclei per 1000 cells). In this NPK study there was a high percentage of polynucleated cells as well as substantial amounts of apoptosis (41.935%) and necrosis (19.355%) in the cells. There was no micronuclei formation (as seen in Table 4 and Figures 16-18).

Conclusion

In an in vitro setting, the LC_{50} concentration of NPK clearly induces genotoxicity in human cells. However further studies need to be carried out to accurately conclude that NPK is genotoxic to humans. The next step would be to screen a large population of NPK-exposed farmers from several ethnicities and see if there are any genotoxic patterns through the comet and micronucleus assays. If the evidence once again suggests that NPK is genotoxic, then society can confidently make the switch to a safer fertilizer that benefits the crop, farmer, and consumer.

Acknowledgments

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

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