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Relative Genotoxic Assessment of Structural Configuration of Nitro Explosives and Their Amines Using Comet Assay in Earthworms

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ABSTRACT: Nitro explosives are toxic in nature and hence the methods of their disposal need to be improved, such that they do not impose any hazards to the environment. Earthworms *Dicogaster curgensis*, were used as model organisms in this study. Earthworms were exposed to the nitro explosives (tri nitro toluene and tri nitro phenol) and to their corresponding amines, to compare the genotoxicity of the nitro and amino derivatives. It was observed that amino groups attached to carbon atoms in the aromatic rings, aided in reduction of genotoxicity as compared to the parent nitro explosives. We confirmed that the use of filter paper contact method, followed by comet assay is a sensitive method to evaluate the DNA damage induced by tri-nitro toluene and tri-nitro phenol in earthworm coelomocytes. In this paper, we also discuss the possibility that increased toxicity of nitro aromatic explosives might be attributed to the presence of a nitro group, specifically at 2' position in the aromatic ring.

KEY WORDS: Nitro explosives, Aromatic ring, Genotoxicity, Earthworm, Comet Assay.

Abbreviations:

- Tri Nitro Toluene (TNT)
- 4 amino 2,6 di nitro toluene (4-ADNT)
- 2 amino 4,6 di nitro toluene (2-ADNT)
- 2,4, di amino 6 nitro toluene (2,4-DANT)
- Tri nitro phenol (TNP)
- 2 amino 4,6 di-nitro phenol, (2-ADNP)

I. INTRODUCTION

In spite of extensive research carried out to study the ecotoxicity of trinitro explosives, their disposal still remains a major concern. A recent study by Fuchs *et al* (2011) [5] suggests that the use of filter paper contact method and comet assay is a sensitive method to evaluate the DNA damage induced by tri nitro toluene in earthworm coelomocytes. Using this as the basis of our work, we compared the genotoxicity of trinitro toluene (TNT), trinitro phenol (TNP) and their amines; it seems that the increase in genotoxicity of nitro aromatic explosives might be attributed to the presence of a nitro group specifically at 2' position in the aromatic ring.

The work regarding toxicity of TNT, TNP and their reduction compounds has been reported. Robidoux and co-workers (2002) [18] used lysosomal neutral red retention time (NRRT) assay for lysosomal membrane stability and the total immune activity (TIA) assay to assess the toxic effects of TNT *in vivo* on *Eisenia andrei*, in studies using filter paper

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contact method. Lachance *et al* (2004) [9] tested the toxicity and bioaccumulation of TNT, 2-ADNT, 4-ADNT, 2,4-DANT and 2,6-DANT in *Eisenia andrei*, using amended sandy loam forest soil and observed the order of toxicity as 4-ADNT > TNT > 2-ADNT. Gong et al (2007) [6] exposed *Eisenia fetida* to a gradient of TNT-spiked soils and studied the molecular toxicological mechanism of TNT by performing gene expression by 4032 cDNA microarray. They also found reduced production of earthworm juveniles at higher doses of TNT. The toxicity of TNP has been recorded earlier, too [8]. A comparative study however, of TNT, TNP and their metabolites, using DNA damage as a biomarker has never been reported. We performed this study and hypothesized about the relation between positional specificity of nitro groups and toxicity of the explosives.

Earthworms were preferred for this study owing to their short life span, availability, and ease in breeding. Single cell gel electrophoresis, also known as the comet assay [20], was used in this study as it is a rapid, sensitive and relatively simple method to detect DNA damage at the level of individual cells. With this technique, the toxicity of heavy metals & other pollutants to earthworms has been studied by S.A. Rankine and co-workers (2004) [16] and in our laboratory, by R.S. Manerikar et al (2007, 2008) [12,13].

Explosives like TNT are disposed by techniques like open detonation, open burning and incineration, which is hazardous to the environment. Since it is known that nitro groups lead to increase in toxicity of nitro explosives Bailey and Spanggord, 1983 [1], we tried to further understand the correlation between the positional specificity of the nitro groups and the genotoxicity of nitro explosives. This study will help to evaluate and improve the current disposal methods of nitro explosives by converting the aromatic nitro groups into the amino derivatives, which are substantially less hazardous to the ecosystem.

II. MATERIALS AND METHODS

1.1. Chemicals: TNT was provided by the High Energy Materials Research Laboratory, Pune, India. TNP and other chemicals were of analytical grade and bought from Sigma Aldrich (St. Louis, Mo, USA).

1.2. Conversion of nitro explosives into corresponding amino compounds: The reduction reactions for TNT were carried out as described by I. L. Finar (1969) [4]. The isolated amines were characterized by neutral reduction test and primary aromatic amine diazotization test (Finar, 1969) [4]. The isolated amino compounds were further confirmed by comparing with the available data on melting point, color, aromatic primary amine diazotization test, neutral reduction test, IR, NMR, and mass spectral reports, Graham et al., 2004, [7]; and Yinon, 1990, [22].TNP was reduced with aqueous sodium sulphide method, which yielded 2-ADNP. It was further isolated with methanol as solvent and characterized as described earlier (Finar, 1969) [4].

1.3. Animals: *Dichogaster curgensis* were maintained under laboratory conditions of 12/12 h dark–light cycle, 40–50% humidity, and temperature at 22 ± 2° C on dried, homogenized cattle manure. Adult, healthy worms (average weight, ~ 250-300 mg) with well developed clitellum were selected.

1.4. Preparation of experimental sets and exposure of worms: Moist filter paper contact test was performed according to OECD guidelines (1984) [14, 15, 20]. A filter paper (45 cm^2) was inserted in each glass vial containing 1 ml of test compounds in methanol, to obtain a concentration of 0.02 µM/cm^2. After evaporation of solvent, papers were moistened with 1 ml distilled water, and a single earthworm was put in each vial. The vials were covered with finely woven cotton cloth and sealed with rubber band to prevent worms from escaping during exposure. Similarly, control earthworms were exposed in vial without any test compound. All vials were incubated for 72 hrs at 22 ± 2° C in dark conditions. A set of three worms were exposed to each compound, and the experiments were performed in triplicates.

1.5. Coelomocyte harvesting: A simple, non-invasive technique described by Eyambe et al (1991) [3], was used to harvest coelomocytes. The cell viability was checked using tryphan blue exclusion method (Strober, 2001) [21] and final cell density was adjusted to 1×10^6 cells/ml with PBS for use in comet assay.

1.6. Alkaline comet assay: The comet assay was carried out as per the protocol described by Manerikar et al (2008) [12,13].

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1.7. Statistical analysis: Data were expressed as the mean ± S.E.M. Significant differences between the results of the different treatment groups were determined using one-way ANOVA and Tukey's multiple comparison test. Values of $p < 0.05$ were considered significant. All statistical analyses were carried out using Graph Pad Prism 6.0 software (USA).

III. RESULTS AND DISCUSSION

One of the parameters used to evaluate genotoxicity of the test compounds was % DNA in tail (Figure 1A). The average % DNA in tail value for TNT was higher (33.05 ± 0.65) as compared to its amine derivatives, which were 30.62 ± 0.58, 13.17 ± 0.4, and 14.39 ± 0.38 for 4-ADNT, 2-ADNT and 2,4-ADNT respectively, while control was at 7 ± 0.25 %. A similar trend can be seen in case of Olive Tail Moment too (Figure 1B). These results suggest that the parent tri nitro compounds are more toxic as compared to their amine derivatives. This result is in accordance with the studies done on fathead minnows, by Bailey *et al* (1983) [1].

It was expected that substituting two nitro groups with amino groups should further reduce the toxicity. However, 2,4-DANT shows marginally more % DNA in tail and olive tail movement values as compared to 2-ADNT, suggesting that 2,4-DANT is more toxic than 2-ADNT. This could be due to the difference in redox potential of the two compounds. The redox potential of 2,4-DANT is -0.502 V, which is more as compared to that of 2-ADNT, i.e. -0.417 V , R. G. Riefler and B. F. Smets, 2000 [17].

Comparing the % DNA in tail and Olive tail moment parameters of TNT, 4-ADNT and 2-ADNT, the order of toxicity obtained is TNT > 4-ADNT > 2-ADNT. The number of nitro groups is more in TNT as compared to its derivatives (Figure 2), thereby accounting for its higher toxicity as compared to 4-ADNT and 2-ADNT. However, in spite of the differences in the structural configuration of TNT and 4-ADNT (Figure 2), there does not seem to be a substantial difference in their toxicities. Moreover, in case of 4-ADNT and 2-ADNT, there is a significant difference in their toxicities (2-ADNT's values are less than half of 4-ADNT's values), even though both the compounds contain one amino group and two nitro groups. The only difference between them is in the positioning of the nitro and amino groups in the aromatic ring structure (Figure 2). In 4-ADNT, the nitro groups are at 2nd and 6th positions; while in 2-ADNT the nitro groups are at 4th and 6th positions. Hence, from this data, and by studying the earlier literature, we hypothesize that presence of nitro group at the second position in the aromatic ring substantially increases (almost doubles) the toxicity of the nitro explosives. TNT and 4-ADNT, both have a nitro group at the second position, and both exhibit relatively higher toxicities.

TNP and 2-ADNP induced significant ($p < 0.05$) DNA damage in earthworm coelomocytes; their % of DNA in tail values (16 and 14 % respectively) are almost twice as compared to that of control (7 %). 2-ADNP shows significantly less values as compared to TNP; this is suggestive of decreased toxicity of 2-ADNP. Similar observations were recorded for their olive tail moment values too.

IV. CONCLUSION

In case of the aromatic ring compounds TNT and TNP, presence of three nitro groups resulted in increased toxicity, as seen in the % DNA in tail and olive tail movement values in the present study. The additional amino group present in 4 ADNT, 2, 4 DANT and 2 ADNP leads to relative reduction in toxicity, as compared to the nitro group present at their parent explosive compounds TNT, 2ADNT and TNP, respectively. Finally, it can be concluded that change from nitro group to amino group with suitable method could reduce toxicity of nitro explosives, thus helping in their ecologically safe disposal.

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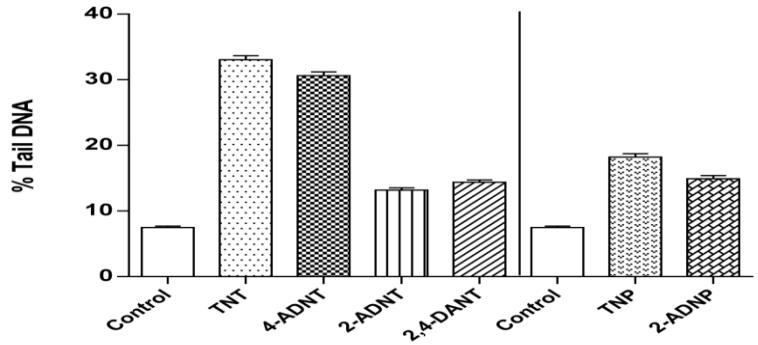
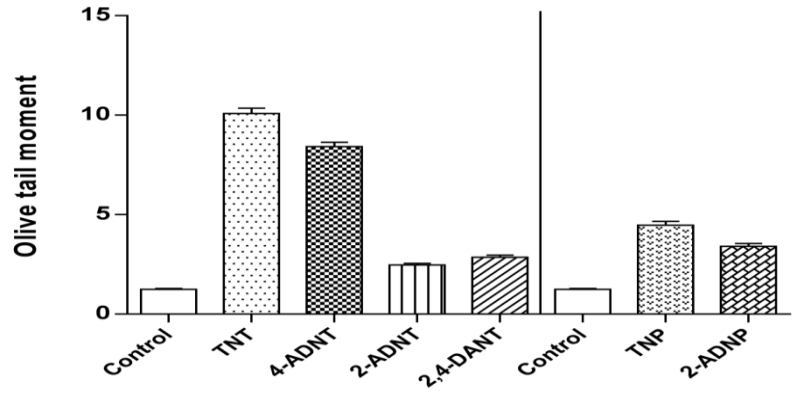
Observations on Comet assay parameters for compounds under study:

Figure 1(A): Extent of DNA damage observed as % DNA in tail.



Extent of DNA damage in *Dichogaster curgensis* coelomocytes, measured as % DNA in tail, after exposure to 0.02 µM concentrations of placebo and the test compounds as per the sets. Means were compared with ANOVA and Tukey's multiple comparison test (Graph Pad Prism 6.0 software). Values of $p < 0.05$ were considered significant. Y bars represent mean standard error (± S.E.).

Figure 1(B): Extent of DNA damage observed as olive tail moment.



Extent of DNA damage in *Dichogaster curgensis* coelomocytes, measured as olive tail moment, after exposure to 0.02 µM concentrations of placebo and the test compounds as per the sets. Means were compared with ANOVA and Tukey's multiple comparison test (Graph Pad Prism 6.0 software). Values of $p < 0.05$ were considered significant. Y bars represent mean standard error (± S.E.).

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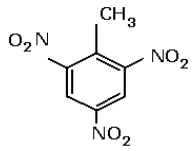
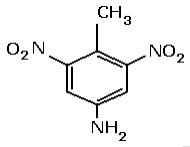
Figure 2: Structures for compounds under study:

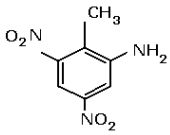
Tri-Nitro Toluene [TNT]



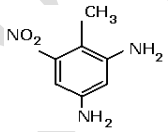
4 Amino 2,6 Di-Nitro Toluene [4-ADNT]



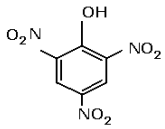
2 Amino 4,6 Di-Nitro Toluene [2-ADNT]



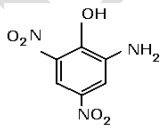
2,4 Di-Amino 6 Nitro Toluene [2,4-DANT]



Tri-Nitro Phenol [TNP]



2 Amino 4,6 Di-Nitro Phenol [2-ADNP]



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