



STUDY ON GENOTOXIC EFFECTS OF METRONIDAZOLE AND TINIDAZOLE IN ALBINO MICE

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ABSTRACT

Objective: The present study was planned to explore and compare the Genotoxic potential of metronidazole and tinidazole in Swiss albino mice.

Methodology: Genotoxicity testing was carried out using single cell gel electrophoresis (comet assay) and micronuclei assay. In acute study only single dose was given and in sub acute study, doses were repeated for 7 days. Blood sample was collected by retro orbital puncture and bone marrow was collected from femur after sacrificing the animal. Blood sample was used for comet assay and bone marrow sample for micronuclei assay.

Results: Increase in comet tail length and percentage of micro nucleated polychromatic erythrocytes (% MnPCE) was observed in groups treated with single and multiple doses of Cyclophosphamide. The same effect was seen to some extent metronidazole whereas tinidazole did not show any significant changes.

Conclusion: The results indicate that metronidazole showed mild Genotoxic effect while tinidazole was devoid of genotoxicity.

KEY WORDS: Metronidazole, tinidazole, Cyclophosphamide, comet assay, micronucleus assay, genotoxicity.



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INTRODUCTION

Genetic toxicology is defined as the study of adverse effects on the process of heredity [5]. As a discipline of toxicological research; it has been given the task of detecting mutagenic chemicals using an array of tests. Genotoxic profile of a drug can be established by *in vitro* and *in vivo* tests designed to detect genetic damage directly or indirectly by various mechanisms. Preclinical toxicity testing of a new molecule is an essential step in the drug discovery and development. In addition to general toxicity testing, now it is mandatory for a new molecule to be screened for its potential genotoxicity from regulatory point of view [1]. The biology of genetic toxicity has intrinsic appeal for physicians and scientists associated with drug development. It gives vital clues about the inherent capacity of drugs to cause DNA damage and also defines the threshold dose capable of causing DNA damage. Various DNA damage biomarkers like micronucleus test, chromosomal aberration test, single cell gel electrophoresis test, unscheduled DNA synthesis assay, point mutation assay, tunnel assay etc., have been summarized by Fenech[4]. Metronidazole and tinidazole are the commonly used antimicrobials in clinical practice. Metronidazole is a prototype Nitroimidazoles with broad spectrum activity against protozoa and many anaerobic bacteria. Information regarding metronidazole is well documented. Of late the gene interaction studies are gaining more importance. Metronidazole is known to act through its reductive activation of the nitro groups by susceptible microorganisms [10]. It exerts cytotoxicity by damaging DNA and other critical biomolecules through reduction of nitro group which is electrophilic and thereby found to inhibit cell mediated immunity to induce mutagenesis. This can predict its carcinogenicity and possibly its genotoxicity. In high doses metronidazole is reported to be carcinogenic in rodents [6] and mutagenic in bacteria [15]. It has been reported not only metronidazole and also its hydroxy metabolite is potentially genotoxic and carcinogenic [13].

Presence of nitro group has been suggested to be responsible for its mutagenic and genotoxic activity [9]. On the other hand metronidazole has been reported to be non-genotoxic in mice in some reports [6]. Similarly, tinidazole has been suggested to possess genotoxic and cytotoxic potential [8]. However, there appears to be controversy regarding its genotoxicity. There is paucity of information regarding genotoxicity of tinidazole. Therefore, the present study was planned to confirm the genotoxicity potential of metronidazole and tinidazole, in their maximal therapeutic equivalent doses in albino mice using micronuclei assay and single cell gel electrophoresis (comet assay).

MATERIALS & METHODS

Drugs & Chemicals

Metronidazole and Tinidazole were obtained as gift samples from Centaur Pharmaceuticals Ltd, Promed lab. Ltd. & Wockhardts Pharm. Ltd. respectively. Cyclophosphamide (Endoxan @) was purchased from local market. Low melting Agarose (LMA), Normal Melting Agarose (NMA) and fetal bovine serum were obtained from HIMEDIA. Histopaque was obtained from Sigma (St. Louis, MO, USA). All other chemical reagents used were of analytical grade. The clinically used maximum doses of these drugs were computed to mice equivalents with the help of the table devised by Paget and Barnes and were found to be 78mg/kg for Metronidazole, 52mg/kg for tinidazole and 40mg/kg for Cyclophosphamide. All the drugs were administered orally except Cyclophosphamide which was administered (i.p.) in the volume of 10ml/kg.

Experimental Animals

Swiss albino mice of either sex, weighing 20-25gm procured from central animal house of the institute were used in the present experiments. Animals were maintained on standard rodent feed (Amrut feeds) with drinking water *ad libitum* and maintained on a 12-hour light/dark

cycle (8.00AM to 8.00PM). In addition, mice were acclimatized for 1 week to laboratory environment. They were housed, handled and sacrificed at the end of experiment in accordance with the guidelines of CPCSEA and the study was approved by Institutional animal ethical committee. Animals are divided into different groups for acute and subacute studies for individual drugs. Each group consisted of 5 animals. Animals in the control group received equal volume of normal saline.

Study design

In acute study, blood and bone marrow samples were collected 24 hrs after the single dose treatment, whereas in sub-acute study doses were repeated for every 24 hrs for 7 times and the samples were collected 24 hrs after the last dose.

***In vivo* micronucleus assay**

24hrs after the last dose of drug treatment the animals were sacrificed by overdose of ether anesthesia and the bone marrow was collected for micronucleus assay as described by Schimid W [2]. Both the femora were removed *in toto*, by cutting through pelvis and tibia and the bones were then freed from muscle fibers by the use of gauze and fingers. Femur is separated from other bones and muscles; the epiphysis is cut open by gentle traction. The bone marrow is aspirated with a help of needle and syringe of appropriate size. Then the femur was submerged completely in fetal bovine serum (5ml) contained in a centrifuge tube and subsequently, the marrow was aspirated by several gentle aspirations and flushing. This process was also repeated from the distal end of the femur and the tube was shaken, so that bone marrow cells get into the serum as a fine suspension and not settle down in the form of gross particles. Then the tube was centrifuged at 1000 revolution per minute for 5 minutes to isolate the bone marrow cell as a pellet. This pellet was suspended in 1-2 drops of foetal bovine serum, and drops of this suspension were put on glass slides so as to prepare bone marrow smears. These slides were air dried and stained with undiluted May Gruenwald (MG)

stain for 3 min followed by diluted MG stain (1:1; with distilled water) for 2 minutes. Later, slides were stained with diluted geimsa stain (1:6; with distilled water) for 10 min. The slides were then rinsed in distilled water and backside was cleared by methanol and then they were air dried and mounted permanently with cover slips. These slides were observed under microscope (oil immersion) for the presence of micronuclei in 2000 polychromatic erythrocytes (MnPCE) per animal. Bone marrow toxicity was assessed by the incidence of polychromatic erythrocytes (PCE) per 200 total erythrocytes.

Comet assay (Single Cell Gel Electrophoresis)

24 hrs after the last dose drug treatment, about 0.5ml of blood was collected from retro orbital sinus, and was mixed with double amount of phosphate buffer solution (PBS) and was processed for SCGE (comet assay) as described earlier by Rojas E. etal 1999 [3]. The blood sample was then gently layered over the histopaque in a centrifugation tube which was centrifuged at 1000 revolution per minute for 25-30minute to obtain a white band containing lymphocytes. This band is now aspirated out into a micro-centrifuge tube with the help of pasture pipette and is mixed with 0.5% LMA (to form as a second layer on the slides). Frosted glass slides are taken to form a 3 layered agar bed- bottom (first) layer is composed of 0.67% NMA; middle (second) layer consists of 0.5% LMA in which lymphocytes were suspended earlier. Finally the middle layer was covered by 0.5% LMA to form top (third) layer. These slides were then kept in lysis buffer (2.5M NaCl, 100mM EDTA, 10mM Tris HCl, 10% DMSO, 1% Triton X100, pH =10) for 2-3hr / overnight. After that slides were incubated for 20 min in electrophoresis buffer (100mM EDTA, 300mM NaOH, pH>13) prior to electrophoresis at 25 V and 300mA carried out for 1hr. After electrophoresis, slides were washed 2-3 times with neutralization buffer (0.4M Tris HCl pH>7.5) and were air dried. These slides were then immersed in absolute ethanol for 3 min to precipitate the DNA. Air dried slides were then immersed in fixing solution (75gm trichloroacetic

acid, 25 gm zinc sulphate, 25 gm glycerol in 500ml distilled water) for 10 minutes and then were washed with double distilled water for 6-8 times before they were air dried for about 1 hour. Subsequently, silver staining was carried out by the procedure of Nadin et al [18], where 68ml of staining solution B (100mg ammonium nitrate, 100mg silver nitrate, 500mg tungstosilic acid, 250 µl of formaldehyde in 500 ml double distilled water) was mixed with 32 ml of staining solution A (25 gm sodium carbonate in 500 ml double distilled water) and poured into a couplin jar. The slides were dipped vertically in these staining filled couplin jars until grayish color developed on the slides. Staining was stopped by dipping the slides in stopping solution (1% glacial acetic acid) for 5 minutes. Whole procedure was carried out in dim light to minimize artificial DNA damage. All the slides were coded before evaluation and then were observed under microscope (45X). 100 comets were scored per animal, (50 in each of two replicate slides). By trypan blue exclusion method, samples with less than 90% viable lymphocytes were rejected. Comet head diameter and total comet length were measured using ocular micrometer which was calibrated with the help of stage micrometer and tail length

was calculated by formula: Comet tail length (µm) = total comet length – head diameter.

Statistical analysis

All the results were expressed as Mean ± S.E.M. To compare different groups with saline group, one-way analysis of variance (ANOVA) was done, followed by Dunnet's post hoc test. $P < 0.05$ was considered to be significant.

RESULTS

In in-vivo micronucleus assay, the incidence % MnPCE per 2000 polychromatic erythrocytes (PCE) was calculated in all the drug treated groups both in acute as well as subacute studies. In acute study mean % MnPCE was, 0.515 ± 0.007 , 0.160 ± 0.012 , for metronidazole, and tinidazole respectively in Normal saline, treated groups respectively. However, in Cyclophosphamide treated group the mean % MnPCE was significantly ($P < 0.01$) increased with 3.53 ± 0.039 in contrast to other drug treated groups. Similar results were also obtained in the subacute study groups (Table 1 & 2).

Table 1
Incidence of % MnPCE (in 2000PCE) in acute & sub-acute studies.

S.no	Treatment group mg/kg (n =5)	Acute study		Sub-Acute study	
		% MnPCE Mean ± S.E.M	%PCE Mean ± S.E.M	%MnPCE Mean ± S.E.M	%PCE Mean ± S.E.M
1.	Control (saline) 8ml/kg	0.100 ± 0.105	58.60 ± 1.364	0.110 ± 0.012	55.50 ± 1.000
2.	CP 40mg/kg	3.53 ± 0.039	56.20 ± 1.158	3.555 ± 0.383	53.40 ± 1.269
3.	MTZ 78mg/kg	0.515 ± 0.007	56.20 ± 1.158	0.525 ± 0.008	54.60 ± 1.275
4.	TDZ 52mg/kg	0.160 ± 0.012	56.40 ± 1.264	0.170 ± 0.011	55.50 ± 1.345

One-way ANOVA, $F_{5, 24} = 536.1$ (acute study); 201.1 (sub-acute study); $P < 0.0001$

* $P < 0.0001$ (student's 't' test)

Table 2
Dunnett's Multiple Comparison Test

S.no	Group comparisons	P value for Acute study	P value for Sub-acute study
1.	Control vs. CP	P < 0.01	P < 0.01
2.	Control vs. MTZ	P<0.01	P<0.01
3.	Control vs. TDZ	P>0.05	P>0.05

In comet assay, mean comet tail length was calculated in all the drug treated groups both in acute as well as subacute studies. In acute study, the saline treated (negative control) group showed a mean value of 3.056 ± 0.184 which did not differ significantly from that of the groups treated with tinidazole. Metronidazole

showed a mean value of 7.605 ± 0.128 . As expected, Cyclophosphamide treated group showed a significant ($P < 0.01$) increase in comet tail length with the mean of 23.030 ± 0.446 in contrast to other drug treated groups. Similar results were also observed in subacute study groups (Table 3 & 4).

Table 3
Comet tail length (μm) in acute & sub acute study

S.no	Group [n =5]	Treatment	Tail length (μm)		Cell viability %
			Acute	Sub-acute	
1.	Negative control	Normal saline	3.056 ± 0.184	3.247 ± 0.104	>96
2.	Positive control	Cyclophosphamide	23.030 ± 0.446	28.24 ± 0.436	>95
3.	Group 1	Metronidazole(MTZ)	7.605 ± 0.128	7.937 ± 0.402	>97
4.	Group 2	Tinidazole (TDZ)	3.555 ± 0.095	3.791 ± 0.203	>95

One-way ANOVA, $F_{5, 24} = 481.7$ (acute study); 458.8 (Sub-acute study); $P < 0.0001$
* $P < 0.0001$

Table 4
Dunnett's Multiple Comparison Test

S.no	Group comparisons	P value for Acute study	P value for Sub-acute study
1.	Control vs. CP	P<0.01	P<0.01
2.	Control vs. MTZ	P<0.01	P<0.01
3.	Control vs. TDZ	P>0.05	P>0.05

Comparing acute and subacute study groups, it was found that there was a significant increase in comet tail length & % MnPCE in only Cyclophosphamide treated group ($P < 0.01$) among all the treatment groups. (Fig. 1 & 2)

Figure1
Percentage of micronucleated polychromatic erythrocytes in various groups

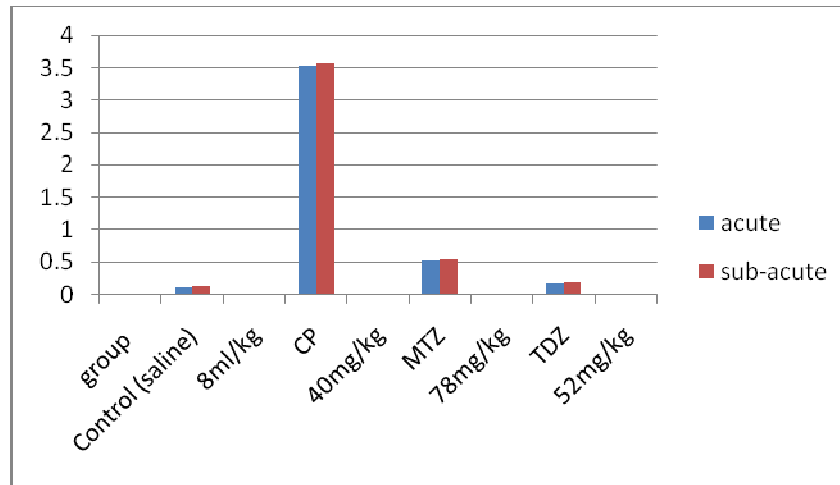
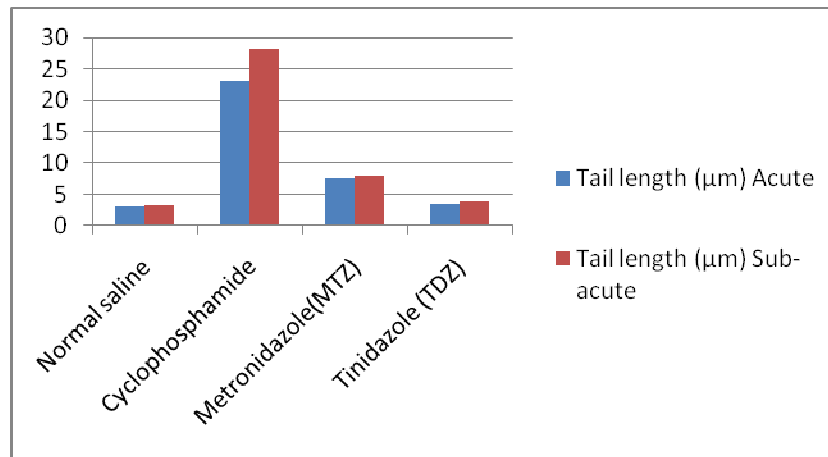


Figure 2
comet tail length in in various groups



DISCUSSION

In summary, metronidazole in clinical equivalent doses have significant genotoxicity while the corresponding doses of tinidazole did not have genotoxic potential in mice as observed in the present study. Metronidazole is known to act through its reductive activation of the nitro groups [10] by susceptible microorganisms in high doses metronidazole has been reported to be carcinogenic in rodents and mutagenic in bacteria [15, 16]. It has been reported not only metronidazole but also its hydroxy metabolite is potentially genotoxic and carcinogenic [13]. Presence of nitro group is has been suggested

to be responsible for its mutagenic and genotoxic activity [9]. The reports regarding the genotoxicity of Nitroimidazoles is controversial because some reports mention it as genotoxic while some other mention it as non-genotoxic. In our study it is confirmed to be genotoxic. Metronidazole is a prototype Nitroimidazoles. Similarly, tinidazole has been suggested to posses' genotoxic and cytotoxic potential [8]. However there appears to be controversy regarding its genotoxicity [17]. Therefore, the present study was planned to confirm and compare the genotoxicity potential of

metronidazole and tinidazole in their therapeutic equivalent doses in albino mice. Mechanisms like oxidative DNA damage caused by metronidazole in hepatocytes and antioxidants have been shown to protect the hepatocytes against such damage [14]. It is well known that DNA damage and apoptosis are physiological phenomenon and damaged DNA either gets repaired or leads to apoptosis. Logically, when damage exceeds the capabilities of repair process or when normal repair mechanisms fail, genotoxicity manifests in the form of either formation of MnPCE or comets. It has been suggested that physiological levels of steroids are necessary for induction of micronuclei by metronidazole, since pretreatment with mifepristone (RU 486) or adrenalectomy prevented formation of micronuclei by metronidazole. These reports lead to debate whether steroids potentiate

genotoxicity of metronidazole or vice versa. It appears that an elevated corticosterone level is not mandatory for induction of micronuclei formation indicating a permissive role of corticosterone to accomplish metronidazole genotoxicity. It has been reported that curcumin and garlic are known to reverse the genotoxic effects of cisplatin[20]. In the present study clinical equivalent doses of nitroimidazoles were used. In clinical practice metronidazole and tinidazole are widely used in protozoal infections. The findings of the present study if extrapolated to humans clearly indicate that metronidazole could be genotoxic in clinical doses. The genotoxic (mutagenic) potential of metronidazole observed in present study is of great clinical concern. However, such toxicity of the concerned nitroimidazoles needs to be confirmed clinically.

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