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Research Article

Genotoxic evaluation of ornidazole and secnidazole in albino mice: an experimental study

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ABSTRACT

Background: The present study was planned to explore the genotoxic potential of some commonly used antimicrobials like ornidazole and secnidazole in swiss albino mice.
Methods: Therapeutic equivalent doses of ornidazole and secnidazole were given by intra peritoneal route. Single dose in individual groups of mice (n=5 in each) was administered for acute study. Doses were repeated every 24 hrs for 7 times in additional groups of mice (n=5 in each) for sub-acute study. Cyclophosphamide served as positive control while normal saline as negative control. After 24 hrs of single dose (acute study) and last dose of drug administration in sub-acute study, about 0.5 ml of blood was collected by retro orbital sinus for comet assay as described earlier (Rojas E et al, 1999) and later the mice were sacrificed to aspirate the femoral bone marrow for micronucleus test as described earlier by described by Schmid W (1975). In comet assay, the total comet length and head diameter was measured under microscope using ocular & stage micrometer to calculate comet tail length. In micronucleus assay, the stained bone marrow tissue smears were scored for the frequency of micronucleated polychromatic erythrocytes (MnPCE) and also the ratio between polychromatic erythrocytes (PCE) to normochromic erythrocytes (NCE) was obtained.
Results: It was analyzed by one-way ANOVA followed by Dunnet's multiple comparison tests. Significant ($P< 0.01$) increase in comet tail length and percentage of micronucleated polychromatic erythrocytes (% MnPCE) was observed in groups treated with single and multiple doses of Cyclophosphamide whereas ornidazole and secnidazole treated groups did not show any significant changes.
Conclusions: The results indicate that Ornidazole and secnidazole are devoid of genotoxicity.

Keywords: Ornidazole, Secnidazole, Cyclophosphamide, Comet assay, Micronucleus assay, Genotoxicity

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]

Nitroimidazoles like ornidazole and secnidazole are the commonly used antimicrobials widely used in clinical practice. Ornidazole is a prototype nitroimidazole 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. Ornidazole is known to act through its reductive activation of the nitro groups by susceptible microorganisms.[9] It exerts cytotoxicity by damaging DNA and other critical biomolecules through reduction of nitro group which is electrophilic,[10] 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 is has been suggested to be responsible for its mutagenic and genotoxic activity.[9] On the other hand metronidazole has been reported to be non-genotoxicity in mice in some reports.[19]

Similarly, tinidazole has been suggested to posse's genotoxic and cytotoxic potential.[8] However, there appears to be controversy regarding its genotoxicity. Reports regarding genotoxicity of ornidazole are very scanty few reports mentioning it as nongenotoxic[19] there is paucity of information regarding genotoxicity of secnidazole and ornidazole. Therefore, the present study is planned to confirm the genotoxicity potential of metronidazole and tinidazole, and to explore the genotoxic effects of ornidazole and secnidazole in their maximal therapeutic equivalent doses in albino mice using micronuclei assay and single cell gel electrophoresis (comet assay).

In view of controversial reports the above mentioned nitroimidazole antimicrobial agents were explored for their genotoxic potential in albino mice using single cell gel electrophoresis (comet assay) and micronucleus assay.

METHODS

Drugs & Chemicals

Ornidazole and Secnidazole were obtained as gift samples from Centaur Pharmaceuticals Ltd, Promed lab. Ltd. & Wockhardt 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 39mg/kg for ornidazole, 52mg/kg for secnidazole 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. The animals were divided in groups (n=5 in each) to receive one of the treatments, both in acute as well as sub-acute studies. Control animals received equal volume of normal saline.

Study design

In acute study, blood and bone marrow samples were collected 24 hr after the single dose treatment, whereas in sub-acute study doses were repeated for every 24 hr for 7 times and the samples was 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 Schimd W.[2] Both the femora were removed *in toto*, by cutting through pelvis and tibia and the bones were then freed from muscle fibres by the use of gauze and fingers. By gentle traction, the distal epiphysis was torn off, together with the rest of the tibia and the surrounding muscle. The proximal end of femur was carefully shortened with scissors until a small opening to the marrow canal becomes visible. The bone marrow was aspirated with the syringe (needle) of appropriate size by inserting it for few mm into the proximal part of the marrow canal. 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 shaked, 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 - SCGE)

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. et al 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 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 sub-acute studies. In acute study mean % MnPCE was, 0.515 ± 0.007, 0.160 ±0.012, 0.165± 0.010, 0.165± 0.010, 0.100± 0.105 for metronidazole, tinidazole, ornidazole and secnidazole respectively in Normal saline, treated groups respectively. However, in cyclophosphamide treated group the mean % MnPCE was significantly ($P<0.01$) increased with 3.53± .039 in contrast to other drug treated groups. Similar results were also obtained in the sub-acute study groups (Table 1 and Table 2).

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

Sr. 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± .039	56.20 ± 1.158	3.555± 0.383	53.40 ± 1.269
5.	ORZ 39mg/kg	0.165± 0.010	57.24±1.164	0.175± 0.008	55.76±1.152
6.	SDZ 52mg/kg	0.165± 0.010	56.45±1.245	0.170± 0.008	54.34±1.236

One-way ANOVA, $F_{5, 24} = 536.1$ (acute study); 201.1 (sub-acute study); $P<0.0001$ *$P<0.0001$(Student't' test)

Table 2: Dunnett's multiple comparison test.

Sr. 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. ORZ	P>0.05	P>0.05
3.	Control vs. SDZ	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 sub-acute 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, ornidazole and secnidazole. 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 sub-acute study groups (Table 3 and Table 4).

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

Sr. 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 3	Ornidazole(ORZ)	3.473 ±0.114	0.362 ±0.242	>95
4.	Group 4	Secnidazole(SDZ)	3.329± 0.073	3.359 ±0.073	>98

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.

Sr. 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. ORZ	P>0.05	P>0.05
3.	Control vs. SDZ	P>0.05	P>0.05

Comparing acute and sub-acute 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.

DISCUSSION

In summary, ornidazole and secnidazole do not have genotoxic potential in mice as observed in the present study. Ornidazole 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] Presence of nitro group is has been suggested to be responsible for its mutagenic and genotoxic activity.[7,10] On the other hand metronidazole has been reported to be non-genotoxicity in mice[6] and human[17] studies. Ornidazole is a prototype nitroimidazole. Similarly, tinidazole has been suggested to posses' genotoxic and cytotoxic potential.[8] However there appears to be controversy regarding its genotoxicity.[17] There is paucity of information regarding genotoxicity of secnidazole. Therefore, the present study was planned to explore the genotoxicity potential of nitroimidazoles like metronidazole, secnidazole, secnidazole and ornidazole 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. Dexamethasone[12] and estrogen[11] have been reported to produce micronuclei formation in *in vitro* studies even in absence of morphine. 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 permissive role of corticosterone to accomplish metronidazole genotoxicity.

In the present study clinical equivalent doses of nitroimidazoles were used. In clinical practice, ornidazole and secnidazole 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. However, such a toxicity of the concerned nitroimidazoles needs to be confirmed clinically.

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Ethical approval: The study was approved by the institutional animal ethics committee

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