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Geno- cytotoxicity study of antiretroviral drug used in HIV- therapy

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Abstract: The present study deals with the evaluation of the Geno-cytotoxic effect of the abacavir at different doses 100 mg/kg, 300 mg/kg and 600 mg/kg (b.w) by using peripheral blood micronucleus assay(tail cut method), sperm abnormality assay and chromosomal aberration assay. In the Peripheral blood micronucleus assay there was increase in the Micronuclei in the peripheral blood of group II & group III in drug treated rats ($p<0.01$), significantly as compared to control group but not more than the standard. In sperm abnormality assay, administration of abacavir for 28 consecutive days, result marked decrease in sperm count, testis weight but not significant effect was observed in morphological study of sperm. *In vivo* chromosomal aberration assay, all drug treated group showed significant value ($p<0.01$) as compared to control and in chromosomal aberrations the percentage of abbreviation significantly ($p<0.01$) increased at 300 and 600mg/kg & at 100mg/kg showed ($p < 0.05$) significant value. Thus it can be concluded that abacavir induces abnormality in genetic makeup and fertility function because of its genocytotoxic nature

Keywords: Geno-cytotoxicity, Antiretroviral, HIV, sperm abnormality assay, Abacavir

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Nucleoside reverse transcriptase inhibitors (NRTIs) are known for causing hematologic disorders, myopathy, cardiotoxic effects, peripheral neuropathies, and hepatotoxic effects (Lewis et al. 1992). These heterogeneous adverse effects of NRTIs are related to defective mitochondrial DNA replication secondary to the NRTI-induced deleterious inhibition of the mitochondrial DNA polymerase gamma (Lewis et al. 1995). NRTIs also have been clearly associated with peripheral lipodystrophy, which is characterized by lipoatrophy of the face, legs, and arms. To date, reports of liver involvement associated with NRTI-based therapy are rare compared with the wide use of NRTIs (Huyen et al. 2003). These effects can also lead to the toxicity of the genome can which in turn cause change in cellular functions, cancer, and cell death (Sancar et al. 1994). Genotoxicity effect may lead to cellular malignant transformation (Albertini et al. 2000). A cytotoxic compound can undergo various cell fates ie necrosis of the cells, decrease in cell viability and apoptosis (Zimmer et al. 2005). Abacavir (Abc) is a nucleoside analog reverse transcriptase inhibitor that is used in combination with other anti-retroviral agents to treat human immunodeficiency virus (HIV) infected patients and acquired immunodeficiency syndrome (AIDS) patients (Li et al. 2013). Abc is phosphorylated in a unique stepwise manner to produce the active moiety, carbocyclic guanosine triphosphate The mechanism of anti-HIV activity for Abc has been shown to be substrate inhibition of HIV reverse transcriptase by carbocyclic guanosine triphosphate, resulting in chain termination and interruption of the viral replication cycle (Kumar et al. 1999). Based on the above facts it was decided to evaluate the geno-cytoxic effects of Abc as it is widely used in treatment of HIV, so as to establish its toxicological profile.

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MATERIALS AND METHODS

Animal

Wistar rats (150-250 gm) of either sex were bought from Animal House NIPER, Mohali. They were kept in the

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Animal House of Institute of Pharmaceutical sciences, Kurukshetra University, Kurukshetra and Housed at standard conditions of temperature (22±1°) and 12/12 h light & dark cycle. They were fed with standard pellet diet (Ashirwad industries, Roper, Punjab) and had free access to water. Fasting animals were used during the experiment. Permission for conduct of these experiments was obtained from IAEC.

Acute toxicity study
Toxicity study of all dose of Abc was performed as per OECD guideline No. 423

Pharmacological investigation
Wistar rats were divided into 5 groups each groups have 5 animals. Group I (negative control) received distilled water, Group II (standard group) received cyclophosphamide (Cyc) and Group III-V received different dose concentration of Abc 100mg/kg, 300mg/kg and 600mg/kg for 28 days.

Peripheral blood micronucleus assay
Peripheral blood micronucleus assay (tail cut method) as suggested by Queiroz et al., (2013) was used. After 28 days of treatment blood was collected from tail tip and blood smear was prepared on pre- cleaned slide and slide was allowed to dry in room temperature and fixed in absolute methanol for 5 minute. After fixation slide was stained with acridine orange (14 mg/40ml) for 5 min followed by three washing with phosphate buffered pH 6.8. It can be stain with Giemsa 20 %. After drying, slide viewed under 100X (oil immersion) fluorescent microscope in filter tube.

Chromosomal aberration assay
After 28 days of treatment, two hours before sacrifice animals were injected 0.2 ml of colchicine (4 mg/kg b.w.). Bone marrow cells were collected from both the femora by flushing in 0.075M KCl, pre warmed at 37 °C, and incubated at 37 °C for 20 min. The materials were centrifuged at 1000 rpm for 10 min and fixed in cold fixative (acetic acid–methanol 1:3). Centrifugation and fixation were repeated three times at an interval of 15 min. Finally, the cells were resuspended in 0.5 ml of fixative and dropped on clean chilled slides, flame dried and stained in 10% buffered giemsa stain. The slides were coded. Mitotic index (MI) was calculated from 1000 cells/animal and expressed in percentage. 1200 metaphases per dose were observed for the presence of chromosomal aberrations. Different types of aberrations were scored separately (Tice et al. 1987).
Scoring of aberrations

Hundred well spread metaphase were scored per animal (500 metaphase per treatment group) at random. All aberrations like chromatid breaks, chromosomal breaks, Polyploidy were considered equal regardless of the number of breakages involved. Chromosomal aberrations cells (CA/cell) were calculated including and excluding gaps (Tice et al. 1987).

Formula of percentage mitotic index calculation

$$\text{Percentage of mitotic index} = \frac{\text{No. of dividing cells}}{\text{Total no of cells}} \text{X } 100$$

Sperm abnormality assay
8 weeks old male Wistar Rats were used for the experiments. One post treatment sampling time i.e., 28 days was used for the study. Animals were sacrificed after 28days of treatment by cervical dislocation and the testes were dissected out and weighed. Both the cauda epididymis were removed and placed in a watch glass containing 1 ml phosphate buffered saline (pH=7.2). The cauda epididymides were minced thoroughly and the suspension obtained was filtered through a fine mesh cloth to remove tissue debris and stained with 1% giemsa for about 20 minutes. A drop of the sperm suspension was smeared on a clean slide. Two thousand sperms per animal were scored from each group for the presence of sperm shape abnormalities following the criteria (Wyrobek et al. 1975). Types of abnormal sperms observed were banana, hook less, folded and Sperm count was done with the help of the haemocytometer. Weight of testis was also calculated (Vega et al. 1998), (Kempinas et al. 1988).

Statistical analysis
Data are presented as Mean ± SEM (n=5) and analyzed by ANOVA followed by Dunnett's t-test using graph pad and obtained in the study were compared with the vehicle control group. The p value <0.05 were considered statistically significant.

RESULTS

Acute Toxicity Study
The drug was found to be safe at the dose of 6000mg/kg according to guideline 423.

Peripheral blood micronucleus assay
Abc induced MN (micronuclei) in dose dependent manner in peripheral blood of rats. The normal cells without MN and MN induced by Abc in rat peripheral blood reticulocytes stained with acridine orange are shown by photomicrograph in Fig 1(a) and (b) respectively. The values of dose dependent induction of MN by Abc in peripheral blood of rat are given in Table 1.

Sperm abnormality assay
In sperm abnormality assay morphology of the sperm, sperm counts were studied. In sperm morphology assay, three morphological changes were observed amorphous head, hook-less sperm, folded type. There was increase in morphological abnormalities in Abc treated animals in dose dependent manner. The normal and abnormal structures of sperm are shown in Figure 2(a) and (b) respectively. In sperm count assay, the number of sperms per epididymis was decreased in Abc treated animals in dose dependent manner. The micrographic pictures of sperm count in neburer's chamber of various groups are shown in Fig (c) and (d). The weight of testis was less in Abc treated groups as compared to control group. The values of sperm abnormality assay are given in Table 2.

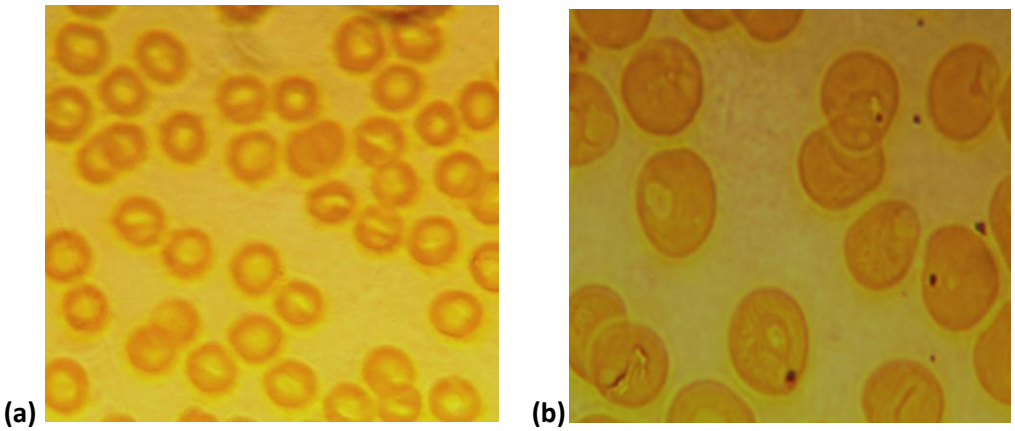

Figure 1(a) Control group show no MN in peripheral blood, (b) Showing the formation of micronuclei in peripheral.

Table 1 Dose dependent induction of MN by Abacavir in peripheral blood of rat

Treated Group	Duration (Day)	Dose (mg/kg)	Peripheral blood MN
I	28	DW	25.8 ± 6.0
II	28	50	156 ± 19.6**
III	28	100	61 ± 2.5**
IV	28	300	89 ± 3.6**
V	28	600	137 ± 17.6**

All the values are shown as mean ± SEM (n=5).* p<0.05, ** p<0.01, ***p<0.001 indicate the level of statically significance. DW-distilled water

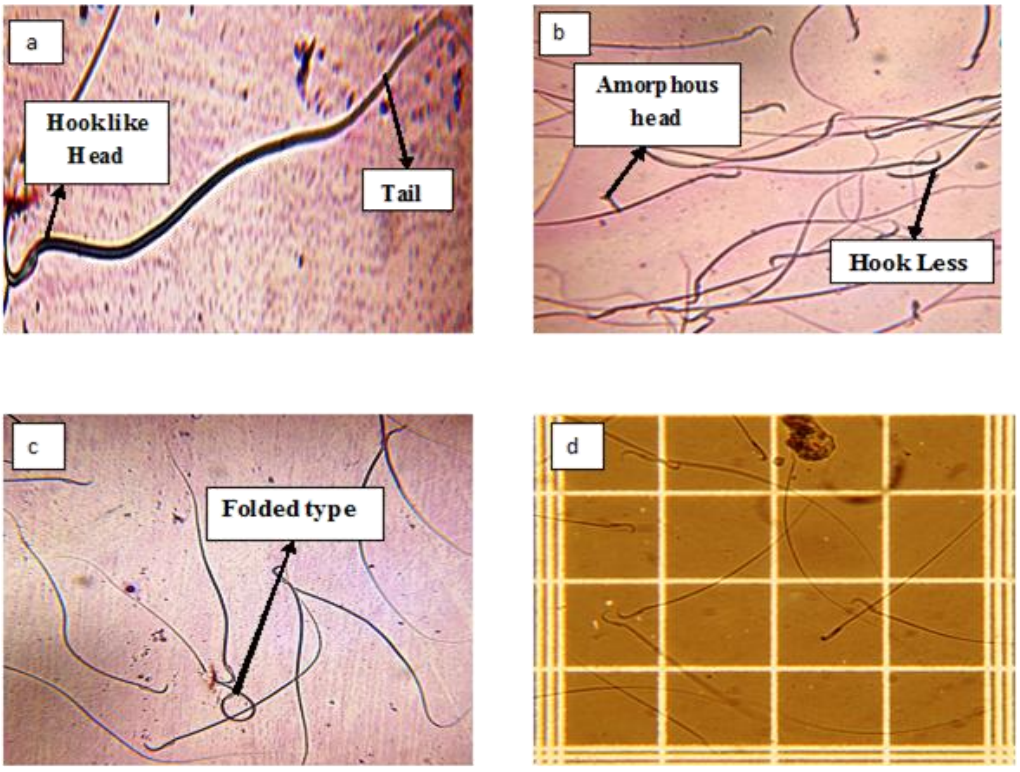

Figure 2 (a) Showing the normal structure of sperm, **(b)** Showing the hook-less and amorphous head type abnormality, **(c)** Showing the folded type abnormality, **(d)** Showing the Sperm count in Neubaur's Chamber

Table 2 Effect of Abacavir on morphological abnormality, weight of testis and sperm count

Treated Group	Dose (mg/kg)	Amorphous Head	Hook-Less	Folded	Total	Percentage of Abnormal sperm	Weight of the testis after treatment	Sperm count/ epididymis
I	DW	21	33	27	81	0.75 ± 0.02	1.1 ± 0.05	41 ± 2.0
II	50	130	150	90	370	3.1 ± 0.24**	0.2 ± 0.07**	31 ± 2.2*
III	100	31	42	37	110	1.0 ± 0.01^{ns}	0.5 ± 0.2*	31 ± 3.0*
IV	300	38	46	40	124	1.2 ± 0.009*	0.4 ± 0.1*	31 ± 2.1*
V	600	51	63	33	147	1.4 ± 0.012**	0.5 ± 0.2*	31 ± 2.5*

All the values are shown as mean ± SEM (n=5).* p<0.05,** p<0.01,***p<0. 001 indicate the level of statically significance

Chromosomal aberration assay

Hundred well spread metaphase were scored per animal (500 metaphases per treatment) at random. All aberrations like chromatid breaks, chromosomal breaks, polyploidy were considered equal regardless of number of breakages involved. The different types of abnormalities in chromosome are index of Abc treated animals was less as compared to the control animals. The % age mitotic index in the bone marrow cells of wistar rats of all the groups are given in Table 3.

Table 3 Effect of Abacavir on mitotic index

Groups	Dose (mg/kg)	Number of cells analysed	Number of dividing cells	Percentage of mitotic index
I	DW	5000	420	8.4 ± 0.53
II	50	5000	65	1.3 ± 0.50**
III	100	5000	329	6.56 ± 0.10**
IV	300	5000	215	4.3 ± 0.63**
V	600	5000	79	1.58 ± 0.14**

All the values are shown as mean ± SEM (n=5).* p<0.05,** p<0.01,***p<0.001 001 indicate the level of statically significance

The chromosomal aberrations in the Abc treated groups were more as compared to control group. Absolute frequency of cells with number of chromosomal aberrations in rats is given in Table 4.

DISCUSSION

The present study deals with the evaluation of the Geno-cytotoxic effect of the Abc at different concentrations 100 mg/kg, 300 mg/kg and 600 mg/kg by using peripheral blood micronucleus assay (tail cut method), sperm abnormality assay and chromosomal aberration assay.

Micronuclei provide an indirect measurement of induction of structural chromosomal aberrations (Tilmant et al. 2013), (Wolf et al. 1997). These are small chromatin containing bodies arising from small chromatin-containing bodies arising from the chromosome fragments or from the chromosome that failed to incorporate into daughter nuclei following the mitosis. In the Peripheral blood micronucleus assay (tail cut method), the ratio of Peripheral blood MN showed, there was increase in the Micronuclei in the peripheral blood of group II & group III in drug treated rats (p<0.01), significantly as compared to control group but not more than the standard. The ratio of Peripheral blood MN, significantly (p<0.01) increased in dose dependent manner except at 100 mg/kg dose of Abc which is non-significant. Further thus the significant increase in the incidence of MN following the Abc administration could be partially attributed to its spindle-poison effect, as evidenced from the occurrence of the multiply tiny dot like MN in several erythrocytes (Bird et al. 1982).

The sperm abnormality assay was performed for evaluating the spermatoxic effect in sperm abnormality assay; three different parameters included are studied. Morphology of the sperm, sperm count, and testis weight (Grigg et al. 2007). Administration of Abc for 28 consecutive days, result marked decrease in sperm count, testis weight but not significant effect was observed in morphological study of sperm. It has been reported that decrease in sperm count, testis weight, are valid for induction of male infertility in animals. The result suggests that Abc has toxicity to male reproductive organs.

In rats *in vivo* chromosomal aberration assay is the one of the most frequently used test for the quantification of genetic damage induced by a chemical in somatic cells. Mitotic assay show, all

Table 4 Absolute frequency with number of chromosomal aberration cells of rat at 28-days after Abacavir treatment.

Group	Dose(mg/kg)	Total AM	Break		Polyploidy	Percentage of the chromosomal aberration
			CtB	ChD		
I	DW	12	6	4	2	2.52 ± 0.6380
II	50	120	7	37	6	29.6 ± 10.502**
III	100	64	43	14	7	12.7 ± 1.93
IV	300	119	57	47	15	21.3 ± 2.49**
V	500	111	61	39	11	18.94 ± 4.0**

All the values are shown as mean ± SEM (n=5).* p<0.05,** p<0.01,***p<0.001 001 indicate the level of statically significance. CtB-chromatid break, ChD-chromatid deletion, AM-aberrant metaphase

significant value ($p<0.01$) as compared to control and in chromosomal aberrations the % age of abbreviation significantly ($p<0.01$) increased at 300 and 600mg/kg & at 100mg/kg showed ($p < 0.05$) significant value. Similar effects have also been showed by other retroviral drugs (Guérard et al. 2013).

Conclusion

More recent drugs should have examined for their genotoxicity as required by regulatory agencies worldwide, but the results obtained often are not published in open scientific literature and therefore are not available to scientific community. From a drug development point of view, the distinction between DNA interaction and non DNA interaction is extremely important having obvious implication for risk assessment based on threshold consideration. So, genotoxicity assays have become an integral component of regulatory requirements. Genotoxicity evaluation is necessary as there is high correlation between genotoxicity and carcinogenic potential of any compound. There was decrease in number of micronuclei in Abc treated groups as compared to the control group. In Sperm abnormality assay there was decrease in sperm count and weight of testis. In Chromosomal aberration assay there was increase in the frequency of the chromosomal aberrations as compared to control group. Abc induced a depression in mitotic activity and increased the frequency of chromosomes aberration, so that genotoxic effect are observed in both chromosomal aberration and micro-nucleus assay. Thus it can be concluded Abc induces abnormality in genetic makeup and fertility function because of its genocytotoxic nature.

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