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Research Article

Genotoxicity Studies of Diclofenac Sodium in the Bone Marrow and Germ cells of Laboratory Mice

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Abstract

The genotoxic potential of Diclofenac Sodium (DC) in terms of induction of chromosomal aberration (CA), micronucleated polychromatic erythrocytes (MNPCE) in bone marrow and sperm abnormality in germ cell of mice has been investigated in Swiss albino mice (*Mus musculus*). Cyclophosphamide (CP) 40 mg/kg was used as clastogen in positive control while multiple doses of DC (1.5, 2.5 and 3.5 mg/kg) were given orally in test groups. Bone marrow and germ cells were sampled at 4, 13, 26 and 40 weeks after treatment. Significant structural chromosomal aberrations and sperm abnormalities were induced with all the selected doses at after 26 and 40 weeks exposure. Also a significant number of MNPCEs were produced with higher dose (3.5 mg/kg) after the a period of 13, 26 and 40 weeks as the chromosomal fragments produced ended up as micronuclei. The PCE/NCE ratio and the mitotic index decreased indicating that DC prevents cell division in mouse bone marrow. Thus, it can be concluded that prolonged use of Diclofenac sodium at high doses is genotoxic in both somatic cells as well as the germinal cells of mice.

Keywords: Diclofenac sodium; Genotoxicity; Chromosomal aberrations; Micronucleus; Sperm abnormality

Introduction

In order to provide a broad coverage of the mutagenic and presumably carcinogenic potential of a chemical, information is required on genotoxic effects at different levels, e.g., the gene, the chromosome and the cellular apparatus necessary for chromosome segregation. A number of testing procedures, both *in vitro* and *in vivo* have been designed to assess the effects of chemicals on the genetic material, consequently to assess the risk to living organisms including humans.

It is an established fact that many substances with an anti-inflammatory action influence DNA metabolism [1,2] and thus can give rise to damage in the genetic material. Diclofenac sodium (DC) is an aryl acetic Non-Steroidal Anti-Inflammatory Drug (NSAID), sold in ample amounts annually in several countries [3]. It is frequently prescribed for symptomatic treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, primary nocturnal enuresis for long-term and in chronic pain associated with cancer [4,5]. Since DC is recommended for both short term and the long term treatments, according to Furberg [6] long term treatment requires documentation of long term safety and efficacy, including indices of genotoxicity. Unlike other adverse reactions like hepatic toxicity [7-9] which appear soon after marketing, the development of a genetic damage or tumor may appear after more than 10 and even 20 years of exposure; the results of epidemiological studies are therefore available late and are obtained at expense of patients. Thus, genotoxicity testing has become a crucial component of safety evaluation for drugs and chemicals. Compared to two year animal carcinogenicity trials, the genotoxicity testing battery provides sensitive, relatively simple, fast and economical tool for detection of genetic damage [10]. Because of the widespread human exposure to DC, it was thought proper to obtain more insight into the genotoxic potential of DC, by using the mouse bone-marrow chromosomal aberration, micronucleus test and sperm abnormality assay at different doses and different time intervals.

Materials and Methods

Animals

Laboratory bred Swiss albino mice (8–12 weeks old) were procured from the institutional animal house and were acclimatized for 7 days under standard husbandry conditions (i.e., room temperature of 25 ± 5°C, relative humidity of 45–55%, and a 12-hour light-dark photoperiod), with *ad libitum* access to food (commercial mouse pellets) and water throughout the experimentation period. Approval from the local institutional animal ethical committee was taken before starting of the experiments. All protocols and experiments were conducted in strict compliance of ethical principles and guidelines provided by the committee for the purpose of control and supervision of experiments on animals.

Drug and chemicals

Diclofenac sodium (CAS Registry No. 15307-86-5) was received as a gift sample from ACME Pharmaceuticals Ltd., Mehsana, Gujarat, India. Cyclophosphamide (CPA; Endoxan-N) was purchased from Cadila Health Care Ltd. (Goa, India), and colchicine, Giemsa stain, May Grunwald stain and Bovine Serum Albumin (BSA) were purchased from Hi Media Laboratories Pvt Ltd. (Mumbai, India). All other chemicals used for the study were of reagent grade and purchased from commercial sources.

Dose

The recommended oral dose of DC for adult is 100- 200 mg/day, commonly prescribed to treat symptoms like osteoarthritis, ankylosing

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spondylitis (100 to 150 mg/day) and rheumatoid arthritis (150 to 200 mg/day but not more than 225 mg) [11]. Taking into consideration 50 kg as an average weight of human body [12] and maximum human prophylactic dose 200 mg/day, the limit of the drug per day is 4.0 mg/kg body weight. Keeping this in view and according to Preston [13] three doses 1.5, 2.5, and 3.5 mg/kg/day which are equivalent to 75 mg, 125 mg and 175 mg per day of human dose of DC respectively were selected. Cyclophosphamide (CP) (40 mg/kg body weight/day) was used in positive control group. Solutions of DC was prepared in distilled water just before use and administered orally.

Experimental protocol

The experimental protocol is same for CA assay, MN assay and sperm abnormality assay. For each assay, the animals were divided into 5 groups; each group consisting of 4 subgroups of 5 animals each; treated daily for 4, 13, 26 and 40 weeks. Separate negative and positive control groups were used for each sampling period.

The group distribution is as follows,

Group I: Negative Control (0.2 ml, distilled water).

Group II: Positive Control (40 mg/kg b.w. /day, CP).

Group III: Animal treated with DC-I (1.5 mg/kg/day)

Group IV: Animal treated with DC-II (2.5 mg/kg/day).

Group V: Animal treated with DC-III (3.5 mg/kg/day)

Solution of DC was prepared in distilled water just before use and administered orally. Positive control groups received CP intraperitoneally 24 hours before tissue sampling.

***In vivo* chromosome aberration assay:** The *in vivo* mammalian chromosome aberration test was conducted according to OECD guidelines for the testing of chemicals [15]. Animals were given 0.4 ml of 0.05% colchicine intraperitoneally 90 minutes before sacrifice. The animals were sacrificed at 4, 13, 26 and 40 week time points (for different groups) after the last dose, by cervical dislocation. Bone marrow preparations for metaphase cells were obtained by the standard technique [16]. The slides were stained in 5% buffered Giemsa, air-dried and mounted in DPX. The slides were coded and scored blind. Mitotic Index (MI) was obtained by counting the number of mitotic cells in 1000 cells per animal, and expressed as percentage [17]. Five hundred well spread metaphases per dose were scored for presence of chromosomal aberrations (CAs). Data of chromosomal aberrations/cell (CA/cell) were evaluated including gaps and excluding gaps [18]. Chromosomal aberrations were classified into categories like chromatid and isochromatid gaps, chromatid and isochromatid breaks, ring, dicentric ring, deletion, exchange, fragmentation stickiness, and acentric fragments were considered equal regardless of the number of breakages involved.

Mouse bone marrow micronucleus assay: The mouse bone marrow micronucleus assay was conducted according to OECD guidelines for the testing of chemicals [19] and the standard technique [20]. Animals were sacrificed at 4, 13, 26 and 40 weeks after dosing, by cervical dislocation. Both femur bones were removed and bone marrow collected in tubes containing 0.2 ml of 5 % bovine serum albumin and centrifuged at 1000 r.p.m. for 5 min. The smears were prepared and allowed to air dry, prior to fixation and staining with May-Gruenwald/ Giemsa solutions. Observations were made by means of light microscopy at 1000× magnification to assess the presence of micronuclei within Polychromatic Erythrocytes (PCE). Slides were coded and scored blind, and 1000 PCEs per animal were examined for the presence of micronuclei. The ratio polychromatic erythrocytes/ normochromatic erythrocytes (PCE/NCE) was calculated by counting a total of 1000 erythrocytes per animal. The values were expressed as the PCE/NCE ratio of the total erythrocyte counts to determine a reduction of erythroblast proliferation [21].

Sperm abnormality assay: Mice from each group were sacrificed by cervical dislocation and their cauda epididymis was removed. Sperm suspensions was obtained by mincing the cauda in 2 ml of phosphate-buffered physiological saline, pipetting the resulting suspension, and filtering it through muslin cloth to remove tissue fragments. A fraction of each suspension was then mixed (10:1) with 1% aqueous eosin Y (H_2O), and 30 minutes later, smears were made, allowed to dry in air and mounted under a coverslip with Permount mounting medium. One thousand sperms per animal were assessed [22], for morphological abnormalities, which included hookless, amorphous, folded, banana shape and two tail abnormality.

Statistical analysis: For statistical evaluation of the experimental data one-way ANOVA followed by Dunnett's multiple comparison tests was performed for the chromosomal aberrations/cell, the mitotic index micro nucleated cells and sperm abnormality. The difference between the control and experimental groups was analyzed by using Prism software (PRISM, 1997) as "a posteriori" test were used in all the experiments. The significance of differences was examined at the *p*-value 0.05 as significant.

Results

A careful examination of the animals for observable symptoms of clinical toxicity twice a day throughout the experimentation revealed that animals tolerated the highest dose without any toxic symptoms. No observable sign of toxicity was seen and the observed clinical condition of animals was found normal (including body weight) throughout the study.

Table 1 presents Mitotic Index (MI) data recorded in the bone marrow cells after administration of 0.2 ml distilled water (vehicle control) and 1.5, 2.5 and 3.5 mg/kg b.w of DC at 4, 13, 26 and 40 weeks sampling regimens. A general trend of mitotic depression as indicated by reduction in MI value as compared to control, was detected significantly even at the lowest dose (1.5 mg/kg b.w.) of DC at 13, 26 and 40 weeks of sampling time.

Dose related increase in abnormal metaphases and CAs/cell (both including and excluding gaps) were recorded at all the sampling times (Table 2). A statistically significant ($p < 0.01$) increase was observed for 3.5 mg/kg b.w. dose of DC even at minimal (4 week) exposure but the low dose (1.5 mg/kg) did not produce any sign of abnormality even ~~on~~ after 13 weeks of exposure. The metaphase analysis of the bone marrow cells revealed the presence of various types of aberrations such as gaps, chromatid and isochromatid breaks, ring, di-centric ring, deletion, exchange, fragmentation, stickiness and acentrics in varying frequencies in DC treated animals. Chromosome breaks were more frequent than other types of aberrations.

Table 1: The mitotic index in the bone marrow cells of Swiss albino mice treated with Diclofenac sodium.

Groups	Dose (mg/kg/day)	No. of metaphase analyzed	No. of dividing cells	% Mitotic Index
4 WEEKS				
NC	--	5000	409	8.180±0.540
PC	40	5000	61	1.220±0.259**
4DC-I	1.5	5000	381	7.620±0.370
4DC-II	2.5	5000	319	6.380±0.709**
4DC-III	3.5	5000	273	5.460±1.180**
13 WEEKS				
NC	--	5000	417	8.340±0.351
PC	40	5000	53	1.060±0.288**
13DC-I	1.5	5000	304	6.080±0.517**
13DC-II	2.5	5000	264	5.280±0.687**
13DC-III	3.5	5000	213	4.260±0.991**
26 WEEKS				
NC	--	5000	398	7.960±0.666
PC	40	5000	50	1.000±0.274**
26 DC-I	1.5	5000	297	5.940±1.477**
26 DC-II	2.5	5000	205	4.100±0.644**
26 DC-III	3.5	5000	179	3.580±0.807**
40 WEEKS				
NC	--	5000	411	8.220±0.277
PC	40	5000	52	1.040±0.230**
40 DC-I	1.5	5000	238	4.760±0.868**
40 DC-II	2.5	5000	198	3.960±0.673**
40 DC-III	3.5	5000	177	3.540±0.802**
RECOVERY STUDY				
S NC		5000	413	8.260±0.358
S-40 DC-III		5000	209	4.180±0.653**†

Data are expressed as mean±SD (n = 5). Mitotic index (%) = number of dividing cells per total number of cells observed × 100.
Abbreviations: NC: Negative control; PC: Positive control; DC: Diclofenac sodium; S: Satellite sampling.
Significance: * $p < 0.05$; ** $p < 0.01$ significant when compared with the NC. † $p < 0.05$ significant when compared with the 40 DC-III.

Data on micro nucleated erythrocytes in bone marrow cells of mice are presented in Table 3. The results show that at the 4 week sampling, percent MNPCE in the bone marrow of mice was not affected by treatment with any of the selected doses of DC. However, the 13, 26 and 40 week exposure with 3.5 mg/kg, significantly increased MNPCE in mice. The response can be directly correlated to bone marrow toxicity, as increasing bone-marrow suppression (reduced the PCE/NCE ratio) is observed at these exposure periods.

The results of the sperm morphology (Table 4) show a statistically significant ($p < 0.01$) increase in percentage of abnormal sperms on exposure to 3.5 mg/kg b.w. of DC for 13 or more weeks. Exposure at low and middle dose levels (1.5 and 2.5 mg/kg b.w.) of DC did not produce any abnormality up to 13 weeks. However, exposure with middle and high dose levels (2.5 and 3.5 mg/kg b.w.) of DC for 26 weeks or more led to induction of significant frequencies of abnormal sperms.

Discussion

NSAIDs are pharmaceuticals used for pathological conditions that often require long-term administration. NSAIDs are used for the relief of mild-to-moderate pain, and for chronic inflammatory disorders. Among the various adverse reactions that these drugs may cause, the occurrence of genotoxic and/or carcinogenic effects cannot be excluded [23]. According to the OECD guidelines, the drugs that are used extensively and over a long duration of time need to be tested extensively for mutagenicity, carcinogenicity, teratogenicity and other types of complication on the host system [24]. In our study, we have investigated the potential of DC to induce CA and MNPCE in bone marrow and frequencies abnormal sperm in germ cell of mice. Cyclophosphamide, the positive control chemical in the present study, is a covalent DNA binding agent [25]. The important factor for the therapeutic and the toxic effects of CP is the requirement of the metabolic activation by the hepatic microsomal cytochrome P_{450} mixed function oxidase system [26]. Phosphoramide mustard and acrolein are the two active metabolites of CP. CP's antineoplastic effects are associated with the phosphoramide mustard, while the acrolein is linked with its toxic side effects [27]. Acrolein interferes with the tissue antioxidant defense system [28], produces highly reactive oxygen free radicals [29] and suppresses SOD, GPx and CAT activities [30] and is mutagenic to mammalian cells [31]. The induction of significantly ($p < 0.001$) high percentages of aberrant metaphases, CAs (excluding gaps), MN per thousand PCEs in mouse bone marrow, and abnormal sperm by CP (40 mg/kg b.w. of mice) in the present study, are in complete agreement with its earlier reported clastogenicity.

There is limited information on the genotoxic effect of DC. The relevant data has not been published in peer-reviewed journals, in some cases the tests were conducted under the oversight of authoritative bodies, such as the U.S. National Toxicology Program; in the other cases the genotoxicity and carcinogenicity data are those

Table 2: The chromosomal aberration assay in the bone marrow cells of Swiss albino mice treated with Diclofenac sodium.

Groups	Dose mg/kg /day	No. of metaphase analyzed	Total AM	Gap[a]	Break		Ring	DR	D	Ex	Frag	St	AF	CA/cell	
					CtB	ChB								Including cells with gap	Excluding cells with gap
4 WEEKS															
NC	--	500	17	6	4	2	3	1	2	-	-	-	2	0.040 ± 0.019	0.028 ± 0.015
PC	40	500	362	102	89	28	96	58	51	30	24	11	36	0.986 ± 0.081**	0.812 ±0.137**
4DC-I	1.5	500	40	9	9	4	5	4	6	2	-	-	5	0.088 ± 0.013	0.070 ±0.007
4DC-II	2.5	500	51	6	12	9	5	5	9	3	-	-	9	0.110 ± 0.023*	0.098 ±0.022
4DC-III	3.5	500	80	13	15	8	19	13	9	5	1	-	7	0.180 ±0.023**	0.154±0.023*
13 WEEKS															
NC	--	500	23	8	7	2	3	2	2	-	-	-	3	0.056 ± 0.018	0.040 ± 0.019
PC	40	500	481	96	104	8	98	57	60	29	22	10	28	1.014 ± 0.061**	0.808 ± 0.147**
13DC-I	1.5	500	57	10	12	9	12	7	10	3	-	1	7	0.142 ± 0.033*	0.122 ± 0.026
13DC-II	2.5	500	88	8	21	10	11	7	21	12	1	1	10	0.204 ± 0.059**	0.178 ± 0.057*
13DC-III	3.5	500	146	20	44	9	18	9	39	13	-	1	19	0.344 ± 0.043**	0.302 ± 0.036**
26 WEEKS															
NC	--	500	31	13	11	3	2	1	3	1	-	-	2	0.072 ± 0.015	0.046±0.009
PC	40	500	472	109	91	27	92	43	52	22	18	12	37	1.018 ± 0.077**	0.794 ± 0.081**
26 DC-I	1.5	500	109	9	26	11	14	12	26	7	2	3	9	0.240 ± 0.052**	0.222 ± 0.046*
26 DC-II	2.5	500	170	19	61	23	22	18	48	10	-	-	25	0.454 ± 0.045**	0.416 ± 0.042**
26 DC-III	3.5	500	270	37	84	12	34	31	57	44	3	7	29	0.696 ± 0.119**	0.622 ± 0.124**
40 WEEKS															
NC	--	500	28	9	10	3	3	1	2	1	-	-	2	0.062± 0.008	0.044± 0.009
PC	40	500	481	106	94	18	83	62	61	25	21	12	30	1.004± 0.081**	0.802 ± 0.134**
40DC-I	1.5	500	156	19	34	21	21	8	41	10	4	2	19	0.358± 0.057**	0.320± 0.050**
40DC-II	2.5	500	244	25	44	15	58	35	44	20	9	11	20	0.562± 0.062**	0.512± 0.058**
40DC-III	3.5	500	295	36	72	21	61	41	54	23	13	11	23	0.700 ± 0.064**	0.628± 0.058**
RECOVERY STUDY															
S NC	-	500	30	7	9	6	4	2	2	-	-	-	3	0.066 ±0.011	0.052 ±0.008
S-40DC-III	-	500	211	23	46	22	49	31	42	13	10	8	18	0.504 ± 0.038**	0.458 ± 0.043**

[a]Includes both chromatid and isochromatid gap.

Data are expressed as mean±SD (n = 5). Abbreviations: NC: Negative control; PC: Positive control; AM Number of aberrant metaphases; CtB: Chromatid break; ChB: Chromosome break; DR: Dicentric ring; D: Deletion; Ex: Exchange; frag: fragmentation; St: Stickiness; AF: Acentric fragments; S: Satellite sampling. Significance: * $p < 0.05$; ** $p < 0.01$ significant when compared with the NC.

reported by the Physician's Desk Reference [32] or in the final package insert approved by the Center for Drug Evaluation and Research of the Food and Drug Administration. Unfortunately, this additional unpublished information is often incomplete; in particular, the results of genotoxicity assays are usually reported without any information of the doses that have been tested. Kullich and Klein [33] reported that various NSAIDs, including DC, in cytogenetic investigations did not reveal any genetic effects during a treatment period of two weeks. Using *in vitro* bacterial reversion test in the different dose range of DC, with several test strains of *Salmonella typhimurium* TA98, TA100, TA1535, TA1538 and *Bacillus subtilis*, it was found that that the mutagenicity of DC still remains questionable [34].

Accordingly, the genotoxicity of DC like gene mutation, (mouse lymphoma cell assay) [35], DNA repair test [36], ames bacterial reverse mutation [35], chromosomal aberrations, chinese hamster bone-marrow cells *in vivo* and chromosomal aberrations, male mice germinal cells *in vivo*, dominant lethal test in mice [35], SCE human lymphocytes *in vivo* [33] gives negative results. The results of present investigations for short duration studies at lower dose range are in agreement with the above findings. However, in contrast to the above cited reports, the findings of the present study indicate that the long term use of higher dose of DC acts as a clastogen *in vivo*.

A cytogenetic marker, such as Chromosomal Aberrations (CAs), is one of the most validated and widely used end-point for the quantification of the biological effects of DNA damaging agents. The test has been recommended for routine analysis, and data obtained are considered highly relevant in the human context [37]. In present study, DC at high doses induced significant increase in chromosomal aberrations per cell that increased with extended time intervals. The induction of chromosomal aberration is a complex cellular process and its mechanism (s) is not completely understood [38–40], however it is believed that structural chromosomal aberrations may result from: (i) direct DNA breakage, (ii) replication on a damaged DNA template, and (iii) inhibition of DNA synthesis, and other mechanisms such as topoisomerase II inhibition [41]. Further, significantly higher frequency of chromosomal aberrations observed at 26 weeks and 40 weeks of the treatment might be due to involvement of secondary metabolites.

It has been suggested that an *in vivo* micronucleus test should be carried out to evaluate the genotoxicity hazard of any substance if it is positive in either a reverse mutation assay or a chromosomal aberration assay or both assays [42]. Micronuclei appear in cells due to chromosomal damage during the last mitosis and they are the reliable indicators of genotoxicity of exogenous agents [43]. DC at high dose

Table 3: The micronucleus assay in the bone marrow cells of Swiss albino mice treated with Diclofenac sodium.

Groups	Dose (mg/kg b.w)	Individual animal Scores/1000PCE	% MNPCE (mean ±S.D.)	PCE/NCE (mean ±S.D.)
4 WEEKS				
NC	--	3, 5, 5, 1, 2	3.20±1.789	1.011±0.091
PC	40	20, 34, 29, 26, 28	27.40±5.079**	0.668±0.052**
4 DC-I	1.5	2, 2, 1, 3, 4	2.40±1.140	1.004±0.042
4 DC-II	2.5	3, 2, 4, 2, 3	2.80±0.837	0.932±0.040
4 DC-III	3.5	6, 4, 4, 5, 6	5.00±1.000	0.836±0.035**
13 WEEKS				
NC	--	2, 3, 6, 1, 3	3.00±1.871	1.196±0.263
PC	40	23, 38, 19, 24, 25	25.80±7.190**	0.527±0.126**
13 DC-I	1.5	3, 2, 4, 1, 6	3.20± 0.837	0.922±0.019*
13 DC-II	2.5	10, 7, 8, 8, 7	8.00± 1.225	0.852±0.042**
13 DC-III	3.5	9, 14, 11, 12, 13	11.80± 1.304**	0.708±0.015**
26 WEEKS				
NC	--	8, 5, 6, 4, 1	6.60±2.408	1.278±0.176
PC	40	34, 25, 41, 28, 39	33.40±6.878**	0.469±0.058**
26 DC-I	1.5	4, 3, 6, 5, 7	5.00±1.000	0.846±0.041**
26 DC-II	2.5	11, 12, 12, 15, 9	11.80±1.304	0.778±0.053**
26 DC-III	3.5	15, 20, 17, 19, 22	18.60±1.517**	0.714±0.068**
40 WEEKS				
NC	--	8, 1, 3, 9, 3	4.80±3.493	1.293±0.123
PC	40	43, 31, 29, 34, 33	34.00±5.385**	0.342±0.111**
40 DC-I	1.5	9, 6, 8, 10, 8	8.20±1.095	0.804±0.045**
40 DC-II	2.5	10, 12, 15, 16, 18	14.20±1.643**	0.696±0.017**
40 DC-III	3.5	27, 20, 23, 25, 20	23.00±3.082**	0.614±0.032**
RECOVERY STUDY				
S-NC	-	2, 8, 2, 4, 7	4.60±2.793	1.294±0.130
S-40 DC-III	-	12, 15, 13, 16, 20	15.40±1.517**	0.754±0.028**

Data are expressed as mean±SD (n = 5).

Abbreviations: NC: Negative control; PC: Positive control; MNPCE: Micronucleated polychromatic erythrocytes; PCE: Polychromatic erythrocytes; NCE: Normochromatic erythrocytes; DC: Diclofenac sodium; S: Satellite sampling. Significance: * $p < 0.05$; ** $p < 0.01$ significant when compared with the NC.

level increased the micronuclei frequencies in all sampling times and similar effects were seen with both lower doses of DC as dosing durations increased. It means DC produces chromosomal fragments that end up as micronuclei since it is known that micronuclei arise from the lagging fragments and whole chromosomes during cell division [20,41].

When evaluating the genotoxic effects of any agent in an organism, it is highly relevant to study the genotoxic effects on germinal cells as well, because this will provide information on transmissible genetic damage from one generation to another [45]. The change in sperm parameters probably arises from interference by the test substance with the genetically controlled differentiation of sperm cells. These abnormalities might result from naturally occurring errors in the differentiation process or the consequence of an abnormal chromosome complement /chromosomal aberrations [22,46,47]. Data of sperm abnormality test show that DC induced abnormalities in sperms in dose and time dependent manner, which pointed towards the positive correlation between the cytogenetic damage and sperm abnormality as previously reported in mice [48,49].

The determination of proliferation rates and mitotic indices in bone marrow cells proved to be a very useful and sensitive indicator of the cytostatic and cytotoxic action of various environmental hazards or therapeutic agents [50]. Similarly the micronuclei test used in this study also detects cytotoxic effects by the PCE/NCE relationship. The PCE/NCE ratio is regarded as an indicator for toxicity affecting the cellular integrity of the bone marrow too [51]. When healthy proliferation of bone marrow cells is affected by a toxic agent, the PCE/NCE ratio may decrease [52]. DC is found to decrease the MI and PCE/NCE ratio indicating its cytotoxic potential [20,44]. Our findings are in agreement with the reports which suggest that DC can cause cellular toxicity, p53-related genotoxicity, and apoptotic effects in medaka tissue and in cultured rat gastric mucosal cells [53,54].

In present study, very large number of gaps, breaks and acentric fragments in bone marrow cells were scored which may be considered to induce micronuclei formation, particularly the chromosomal breaks and acentric chromosomal fragments. It was confirmed that DC at high dose exerts its genotoxic effect after exposure for 26 and 40 weeks. The previous studies on DC, have reported it to be non genotoxic [33-36] probably because very low drug concentrations and different genotoxic endpoints were considered in the test systems. Different repair capacities of the various cell types used may also be responsible for the discrepancies.

The association between specific cytogenetic alterations and tumorigenesis is strong [55]. Indeed, it is this relationship that is used as one justification for including cytogenetic endpoints in toxicological evaluations of industrial chemicals, and development of new pharmaceutical and therapeutic compounds [56]. In long-term carcinogenesis assay, rats doses up to 2 mg/kg/day and mouse

Table 4: Sperm abnormality assay results in mice treated with Diclofenac sodium.

Group	Dose mg/kg b.w	Abnormal sperms	Amorphous	Banana	Hook less	Double Folded	Two tailed	% of abnormal sperm
4 WEEKS								
NC	--	94	52	9	30	3	-	1.880±0.497
PC	40	1031	412	202	331	57	29	20.620± 1.381**
4 DC-I	1.5	107	61	8	34	4	-	2.140±0.416
4 DC-II	2.5	108	57	8	41	2	-	2.160±0.336
4 DC-III	3.5	129	58	24	41	5	1	2.580±0.396
13 WEEKS								
NC	--	103	72	6	21	4	-	2.060±0.439
PC	40	986	418	198	268	79	23	19.720±2.420**
13 DC-I	1.5	110	59	8	42	1	-	2.200±0.436
13 DC-II	2.5	171	87	10	71	3	-	3.420±0.672
13 DC-III	3.5	305	135	21	146	2	1	6.100±0.696**
26 WEEKS								
NC	--	117	63	9	42	3	-	2.340±0.577
PC	40	1200	503	113	463	80	41	24.000±2.171**
26 DC-I	1.5	146	79	10	55	2	-	2.920±1.083
26 DC-II	2.5	273	149	30	90	2	2	5.460±0.573**
26 DC-III	3.5	485	273	52	151	6	3	9.700±0.752**
40 WEEKS								
NC	--	130	53	21	46	9	1	2.600±0.758
PC	40	1213	519	101	460	99	34	24.260±4.458**
40 DC-I	1.5	288	137	53	104	3	1	5.760±1.146*
40 DC-II	2.5	451	249	61	134	5	2	9.020±0.563**
40 DC-III	3.5	685	317	111	203	44	10	13.700±0.660**
RECOVERY STUDY								
S- NC		128	52	18	49	7	2	2.560 ±0.607
S-40 DC-III		487	224	91	136	31	5	9.740±0.598**

Data are expressed as mean±SD (n = 5).
Abbreviations: NC: Negative control; PC: Positive control; DC: Diclofenac sodium.
Significance: * $p < 0.05$; ** $p < 0.01$ significant when compared with the NC.
Five animals per group (representing a about of 5000 sperm cells) were analyzed for the presence of sperm abnormalities.

carcinogenicity study, oral DC at doses up to 0.3 mg/kg/day in males and 1 mg/kg/day in females was not tumorigenic [23]. In contrast some authors have reported that exposure of DC in three different tissues of male medaka fish can lead to carcinogenic and/ or apoptotic potential [53].

However, carcinogenicity study of DC in mice is negative while present results shown positive, this inconsistent result can be explained by certain limitations like, for extended exposures, stable aberrations (especially reciprocal translocations) can be induced in progenitor cells and transmitted through cell division to be recovered in peripheral lymphocytes [56]. Thus, they will accumulate over an extended exposure and this may responsible for carcinogenicity. In order to utilize fully the genotoxicity data for carcinogeicity risk assessment for a specific chemical it is necessary to establish the mechanism of induction of the tumors, and the role of chromosome alterations in initiation and progression. Although the induced reciprocal translocations can be considered a reliable surrogate for carcinogenicity however it was not investigated in this study, but in present study, we found more numbers of unstable aberrations, particularly chromatid-type damage (gaps, breaks and acentric fragments in bone marrow cells) resulting from DC exposures and cells with micronuclei containing chromosome fragments are also expected to be unstable. Present study is limited to investigation of neoplastic conversion (DNA alteration) while the subsequent step neoplastic development (DNA expression) is beyond the scope.

Including detoxication, is an important characteristic of any substance being tested, and the pattern of metabolic activation may be different between *in vivo* and *in vitro* experiments [57]. Accordingly, the metabolisms of DC produce reactive intermediates which are capable to bind covalently and modifying the proteins [58,59]. The absorbed DC is rapidly metabolized by mammalian enzymes cytochrome P-450 [60,61] to a number of major and minor reactive metabolites [62,63]. The major oxidative metabolic pathways for DC are the hydroxylation at position 4, and 5, and to a much lesser extent the formation of 3-hydroxy- and 4, 5-dihydroxydiclofenac. The 4- and 5-hydroxy derivatives are the major reactive metabolites, both present as glucuronide and sulfate conjugates. These active metabolite could be expected to cause oxidative injury to the mitochondria which may act as an early signal triggering mitochondrial dysfunction that lead to a impair Mitochondrial Permeability Transition (MPT) resulting in generation of Reactive Oxygen Species (ROS) and induced DNA damage [64,65]. This MPT has also been shown to be important in DC-induced cytotoxicity, resulting in generation of ROS, mitochondrial swelling, inability of mitochondria to produce ATP and oxidation of NADP and protein thiols [62,63,66].

Based on the evidence generated during the study it can be fairly concluded that DC at high doses with extended time intervals acts as

a clastogen *in vivo* and produces chromosomal fragments that end up as micronuclei and germ cell toxicity.

Conflict of Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of the manuscript entitled, "The genotoxic and cytotoxic effects of Diclofenac sodium in the mouse bone marrow".

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