

The following is an addendum to Chapter 9

9 PRECLINICAL REPRODUCTIVE AND GENETIC TOXICOLOGY CENTER

The National Center for Preclinical Reproductive and Genetic Toxicology has been established at the institute with the help of funding received from the Department of Science and Technology and the Indian Council of Medical Research. All assays/test systems required to undertake reproductive and genetic toxicity of a drug/chemical/compound have been standardized and majority of them are in place. The necessary infrastructure has been

established (Annual Report 2006-07). The objectives set during the reporting period were to i) evaluate genetic and reproductive toxicity of Bisphenol an endocrine disrupter, ii) provide services to outside agencies/ pharmaceutical industry for toxicological evaluation of their compounds, and iii) conduct workshops on Genetic Toxicity.

The progress made during the reporting period are highlighted below.

9.2 Genetic Toxicity studies of an endocrine disrupter, Bisphenol A (*Funded by Dept. of Science & Technology, Govt. of India and Indian Council of Medical Research*)

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Duration	:	2006-2009

Over the past few decades there have been increasing incidences of testicular cancer, cryptorchidism, hypospadias, and a decline in sperm count. All these disorders are thought may be due to increasing exposure to endocrine disruptors, which are known to mimic the action of natural hormones and have potential to disrupt endocrine functions in animals and human. Bisphenol A (BPA) is one of the weak estrogenic endocrine disrupter, an important chemical used in the manufacture of polycarbonate plastics, epoxy resins, dental sealant, epoxy-lined food and drink containers etc. Wide spread use of BPA in consumer products has lead to a great public concern since adverse effects of BPA on human and animal reproduction are suspected due to its estrogenic activity. The presence of Bisphenol A in serum, urine, amniotic fluid and umbilical cord blood of human has recently generated keen interest in carrying out in-depth research to evaluate low dose effects of BPA on reproductive

function and understand mechanism of its action. An association of BPA with recurrent miscarriages, PCO, obesity, diabetes has been reported, which has generated keen interest among the scientists in understanding its effect not only on reproduction but also on genetic material. As far as genotoxic effects of BPA are concerned very little information is available and majority of the data is based on in-vitro experiments. According to existing guidelines (OECD and ICH) *in vitro* data needs to be substantiated by in-vivo experimental model. Hence the study was undertaken to determine genotoxic and mutagenic potential of BPA at environmentally relevant exposure dose level suggested to be safe for humans by National Toxicology Program (USA)

Adult Holtzman male and female rats were gavaged with various doses of BPA over a period of 6 days and blood as well as bone marrow was sampled at 24 h after the last treatment. Bone marrow cells were processed for micronucleus analysis and

blood lymphocytes for evaluation of DNA damage by comet assay. A vehicle control group received sesame oil whereas positive control group was administered with Cyclophosphamide (40 mg/kg). Another group of male and female rats were treated separately with same doses of Bisphenol A over the period of six days and one and half

hours prior to sacrifice were injected intraperitoneal with colchicine and bone marrow was sampled and processed for chromosome analysis.

The data obtained from this study clearly showed that Bisphenol A significantly increased the formation of micronuclei (Fig.152 and Table 22) and also increased

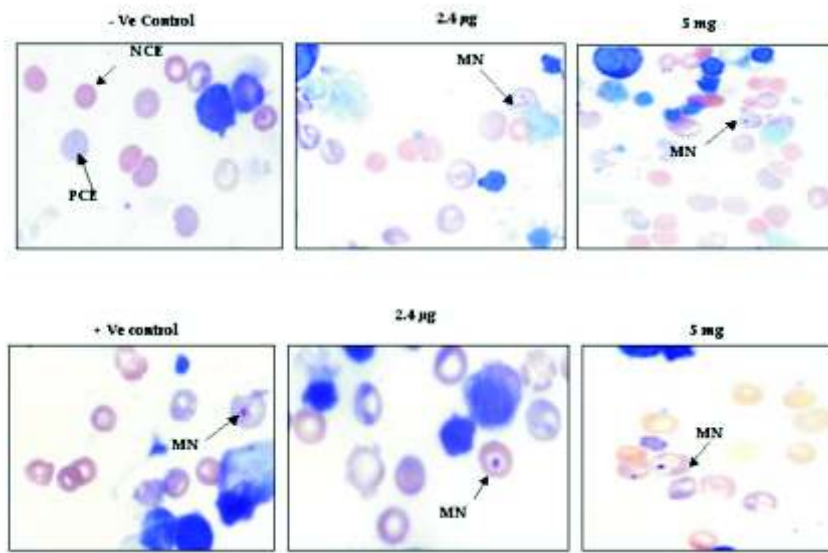


Fig 152: Presence of micronucleus (MN) in bone marrow cells of rat

Table 22: Induction of Micronuclei in Bone Marrow Erythrocytes following BPA Exposure.

Dose/(kgbw)	Male (n=5) (Mean \pm SD)		Female (n=5) (Mean \pm SD)	
	%MNPCE	%PCE	%MNPCE	%PCE
Sesame oil ^a	0.10 \pm 0.07	46.7 \pm 1.1	0.10 \pm 0.07	46.1 \pm 0.5
2.4 μ g	0.37 \pm 0.14	44.9 \pm 1.9	0.31 \pm 0.12	45.3 \pm 1.1
10 μ g	0.61 \pm 0.06 ^{**}	44.4 \pm 2.2	0.50 \pm 0.06 ^{**}	44.9 \pm 1.9
5 mg	0.95 \pm 0.26 ^{***}	44.3 \pm 1.9	0.96 \pm 0.22 ^{***}	43.6 \pm 1.2
50 mg	1.87 \pm 0.30 ^{***}	43.8 \pm 2.3	1.75 \pm 0.24 ^{***}	43.1 \pm 1.6
(40 mg) CP ^b	2.96 \pm 0.30 ^{***}	40.6 \pm 2.1	2.90 \pm 0.36 ^{***}	40.3 \pm 1.8

MNPCE, micronucleated polychromatic erythrocyte; PCE, polychromatic erythrocyte; (Mean \pm SD, * p < 0.05,

** P < 0.01, *** p < 0.001). one-way ANOVA and Tukey test was used for statistical analysis.

^a Vehicle control

^b Positive control, cyclophosphamide

chromosomal aberrations (numerical as well as structural) as compared to the control group. Fig.153-155 and Table 23 and 24

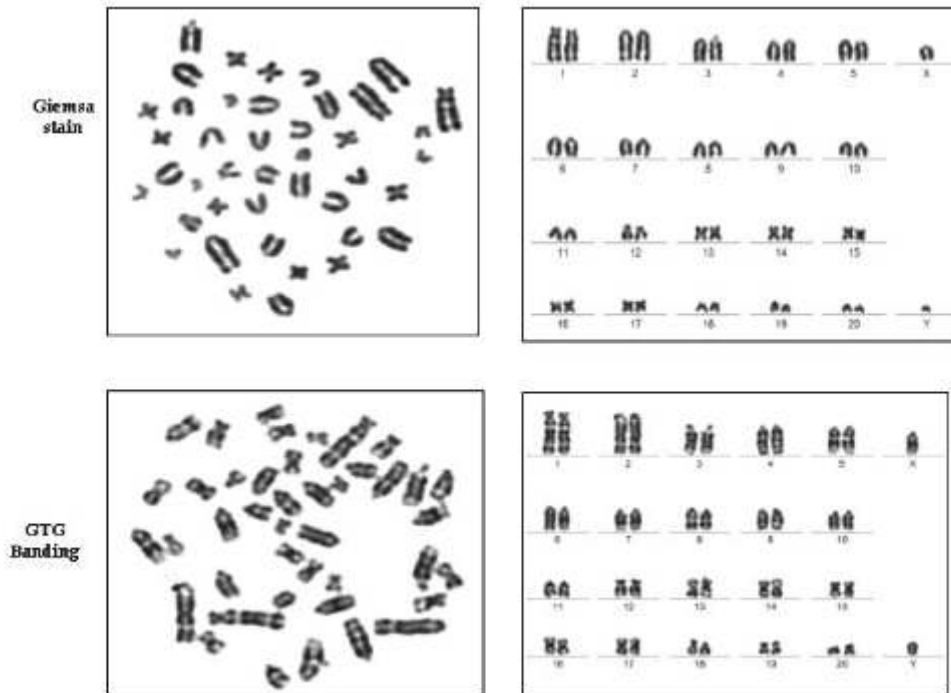


Fig. 153: Metaphase spread of bone marrow cells in control rat.

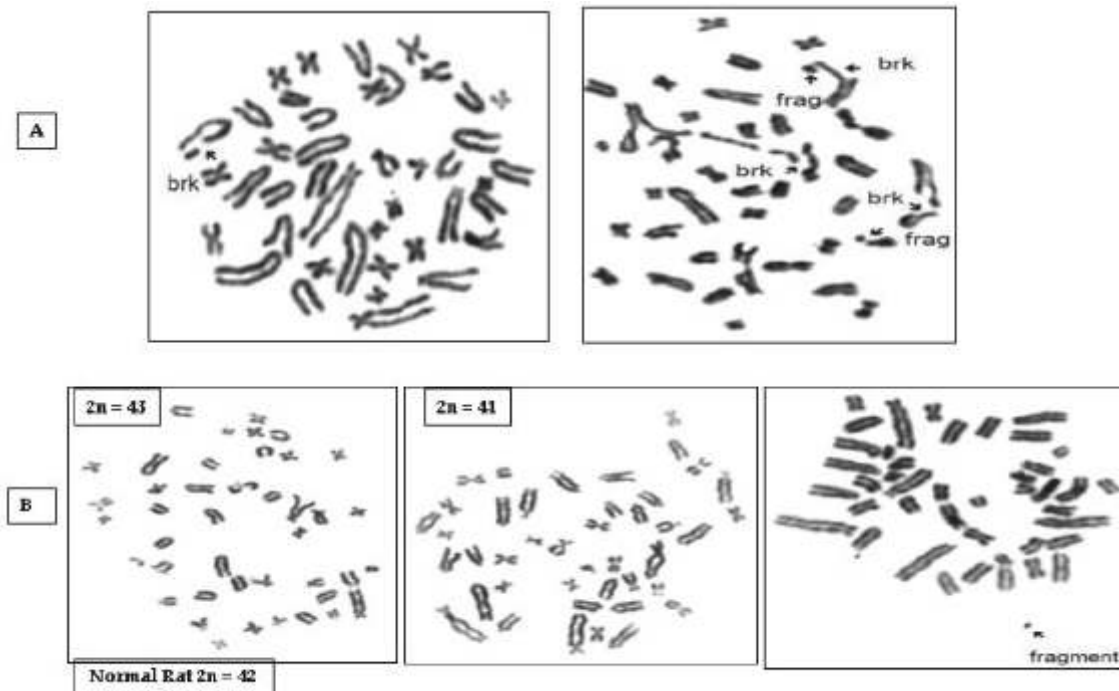


Fig.154: Chromosomal aberration in bone marrow cells of cyclophosphamide treated rats (A) Bisphenol A induced aneuploidy in rat bone marrow cells(B).

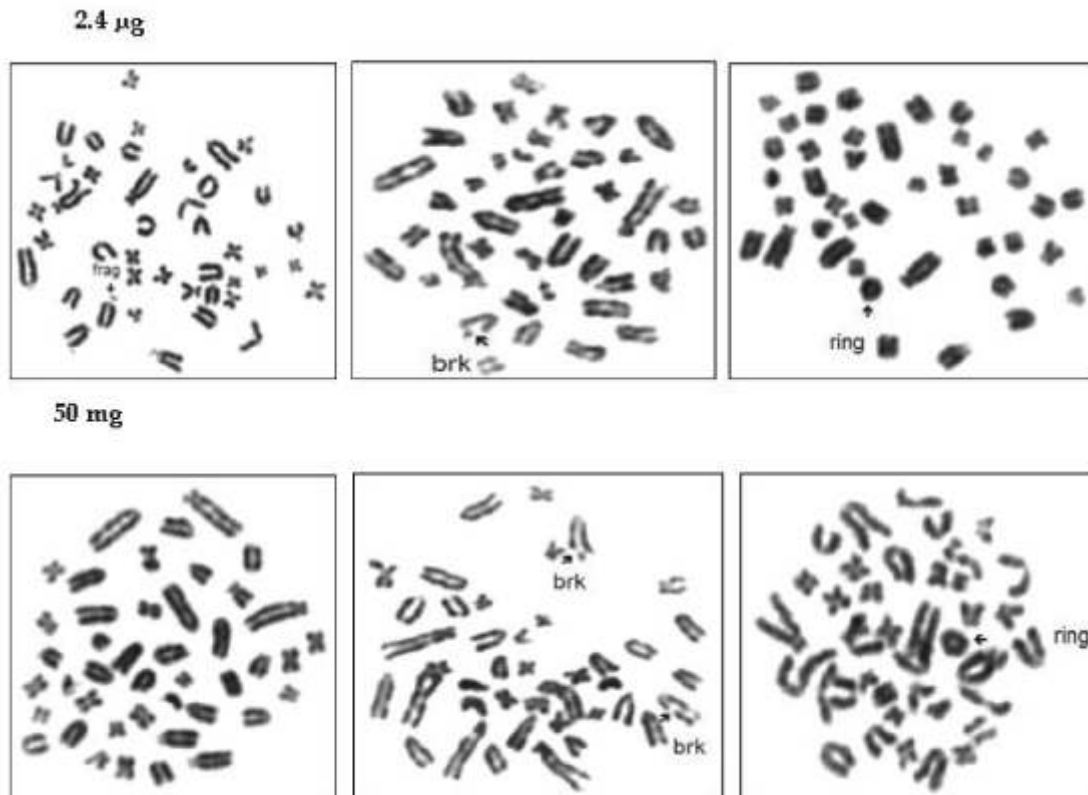


Fig.155: Chromosomal aberration in bone marrow cells of BPA treated male rats.

Table 23: Frequency of chromosomal aberration in male rat bone marrow cells following BPA

Dose/(kgbw)	%NA (n=5) (Mean ± SD)	B	G	F	R	Total SCA	% SCA (n=5) (Mean ± SD)
Sesame oil ^a	1.8 ± 0.83	7	9	6	2	24	4.6 ± 1.14
2.4 µg	4.2 ± 0.83	15	11	10	4	38	6.8 ± 1.30
10 µg	6.4 ± 1.14*	16	16	15	6	53	8.4 ± 1.51*
5 mg	8.4 ± 1.14***	27	25	21	5	78	14.4 ± 1.67***
50 mg	11.0 ± 1.56***	34	27	23	4	88	17.6 ± 1.14***
(40 mg) CP ^b	16.2 ± 1.92***	38	36	31	10	115	23 ± 1.00***

B, chromatids and chromosomes breaks; G, chromatids and chromosomes gaps; F, chromatids and chromosomes fragments; R, rings. All values are expressed as (Mean ± SD, *p < 0.05, ** P < 0.01, ***p < 0.001), one-way ANOVA and Tukey test was used for statistical analysis.

NA= Numerical aberrations; SCA: Structural chromosomal aberrations;

^a Vehicle control, ^b Positive control, cyclophosphamide

Table 24: Frequency of chromosomal aberration in female rat bone marrow cells following BPA exposure.

Table 3: Frequency of chromosomal aberration in female rat bone marrow cells following BPA Exposure

Dose /(μ g/bw)	% NA (n=5) (Mean \pm SD)	B	C	F	R	Total SCA	% SCA (n=5) (Mean \pm SD)
Sesame oil ^a	1.6 \pm 1.14	3	6	4	1	19	3.8 \pm 1.64
2.4 μ g	3.4 \pm 1.14	13	11	8	1	33	5.6 \pm 1.14 ^b
10 μ g	4.3 \pm 1.43 ^{***}	16	15	12	4	47	8.6 \pm 1.14 ^{***}
5 mg	6.6 \pm 1.51 ^{****}	24	20	17	2	63	11.6 \pm 1.51 ^{****}
50 mg	9.6 \pm 1.67 ^{****}	31	26	18	4	79	15.4 \pm 1.67 ^{****}
(40 mg) CP ^b	13.8 \pm 1.92 ^{****}	39	34	24	8	105	21 \pm 2.30 ^{****}

B, chromatids and chromosomes breaks; C, chromatids and chromosomes gaps; F, chromatids and chromosomes fragments; R, rings. All values are expressed as (Mean \pm SD, ^bp < 0.05, ^{***}p < 0.01, ^{****}p < 0.001). one-way ANOVA and Tukey test was used for statistical analysis.

NA= Numerical aberrations; SCA: Structural chromosomal aberrations;

^a Vehicle control, ^b Positive control, cyclophosphamide

DNA damage was significantly increased in blood lymphocytes of BPA treated rat as compared to control by comet assay. In the alkaline comet assay, an increase in DNA strand breaks leads to greater DNA migration out of the nucleus and into the tail of the comet. The majority of lymphocytes obtained from control animals exhibited very little comet tail where as highly significant dose dependent increase in tail

length and DNA damage intensity were observed among Bisphenol A-exposed animals (Fig 156 and Table 25 and 26). The mean percentages of the induced DNA damage (tail DNA percentage, TP) and the olive tail moment (OTM, the product of tail length and percentage of DNA in the tail) were highly significant at all the dose of BPA in comparison to control.

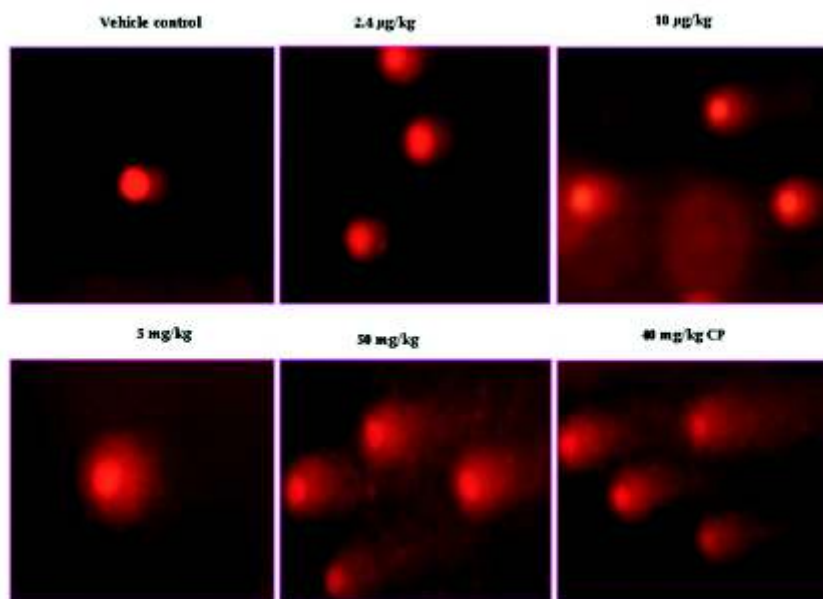


Fig.1 Comet assay in lymphocytes cells obtained from control and BPA treated rats

Fig. 156: Comet assay in lymphocytes cells obtained from control and BPA treated rats.

Table 25: Assessment of DNA damage in lymphocytes by comet assay in-vivo exposure of BPA in male rats.

Dose/(kgbw)	TMO (Mean \pm SD)	TM (Mean \pm SD)	TL (Mean \pm SD)	TD (Mean \pm SD)
Sesame oil ^a	1.028 \pm 0.25	1.71 \pm 0.18	5.96 \pm 1.13	14.21 \pm 0.89
2.4 μ g	1.80 \pm 0.58	2.55 \pm 0.64	9.48 \pm 0.88	19.97 \pm 3.26
10 μ g	2.76 \pm 0.08***	5.28 \pm 1.41***	12.83 \pm 1.09***	32.28 \pm 1.24***
5 mg	5.36 \pm 1.06***	10.44 \pm 1.97***	23.69 \pm 2.02***	41.04 \pm 2.83***
50 mg	6.81 \pm 0.95***	16.01 \pm 1.72***	33.65 \pm 4.83***	47.39 \pm 2.00***
(40 mg) CP ^b	8.56 \pm 0.52***	19.91 \pm 2.75***	37.43 \pm 5.19***	52.56 \pm 0.62***

TMO = Tail Moment Olive, TM = Tail Moment, TL = Tail Length, TD = $\frac{1}{2}$ Tail DNA

All values are expressed as (Mean \pm SD, *p < 0.05, ** P < 0.01, ***p < 0.001). one-way ANOVA and Tukey test was used for statistical analysis.

^a Vehicle control

^b Positive control, cyclophosphamide

Table 26: Assessment of DNA damage in lymphocytes by comet assay after in-vivo exposure of BPA in female rats.

Dose / (kgbw)	TMO (Mean \pm SD)	TM (Mean \pm SD)	TL (Mean \pm SD)	TD (Mean \pm SD)
Sesame oil ^a	0.92 \pm 0.46	1.62 \pm 0.32	6.35 \pm 1.96	12.95 \pm 2.79
2.4 μ g	2.11 \pm 0.70	3.86 \pm 1.50	12.58 \pm 0.85	23.80 \pm 5.49
10 μ g	3.14 \pm 0.45***	6.30 \pm 0.84***	16.28 \pm 3.58***	31.53 \pm 5.72***
5 mg	4.81 \pm 0.44***	11.12 \pm 1.31***	21.23 \pm 5.25***	39.75 \pm 5.43***
50 mg	6.51 \pm 0.90***	14.38 \pm 2.57***	27.43 \pm 4.63***	45.00 \pm 2.46***
(40 mg) CP ^b	8.05 \pm 0.52***	18.23 \pm 2.65***	36.40 \pm 5.32***	51.38 \pm 2.66***

TMO = Tail Moment Olive, TM = Tail Moment, TL = Tail Length, TD = $\frac{1}{2}$ Tail DNA

All values are expressed as (Mean \pm SD, *p < 0.05, ** P < 0.01, ***p < 0.001). one-way ANOVA and Tukey test was used for statistical analysis.

^a Vehicle control

^b Positive control, cyclophosphamide

These results demonstrate that micronucleus induction, chromosomal aberration in bone marrow erythrocytes as well as DNA damage in lymphocyte of rat treated with different oral doses of BPA showed a significant increase of micronuclei, chromosomal aberrations and DNA damage confirming that BPA has genotoxic activity.

The study will have high impact on modifying the current standard doses that are used in traditional toxicological studies for risk assessment for BPA as well as for other endocrine disrupters. It will also reduce the uncertainty regarding the prevalence and potential environmental toxicological impact of low dose effects of BPA on genotoxicity.

9.2 Evaluation of genotoxic potential of Niscar gel, a microbicide *(Funded by Dept. of Science & Technology, Govt. of India and Indian Council of Medical Research)*

Niscar gel is a combined gel formulation of Nisin (a natural food preservative exhibiting antibacterial and spermicidal activities) and Carrageenan (a known anti HIV agent). Its efficacy towards antifertility and antimicrobial potency was studied extensively at the Institute and was found to be an effective microbicide having contraceptive properties. Currently Niscar gel is the focus of clinical trials. In view of this genetic toxicity of Niscar gel was undertaken.

Female rats were gavaged three different doses of Niscar gel and controls were administered with vehicle (1 percent polycarbophil) and a group of rats treated with cyclophosphamide served as a positive control. Animals were gavaged with the respective test compound for 3 days; these animals were sacrificed 24 hrs after last dosing. Bone marrow samples were collected and processed further for detecting chromosomal aberrations and micronucleus formation. Blood samples were collected in heparinized vials and processed further for comet assay.

Niscar gel did not show any effect on metaphase chromosomes. Further, no significant difference was observed between control and

treated animals with respect to frequency of micronucleus formation as well as ratio of polychromatic erythrocytes to Normochromatic erythrocytes. Comet assay did not show any DNA damage in lymphocytes. These results demonstrated that Niscar gel is a non-genotoxic compound.

9.3 Evaluation of genotoxic potential of biodegradable polymeric nano particles *(Funded by Dept. of Science & Technology, Govt. of India and Indian Council of Medical Research)*

During the past two decades significant advances have been made in the development of biodegradable polymeric materials for biomedical applications. Degradable polymeric biomaterials are preferred candidates for controlled/sustained release drug delivery vehicles, developing therapeutic devices such as temporary prostheses, three-dimensional porous structures as scaffolds for tissue engineering. A novel biodegradable polymer, polyethylene sebacate, is synthesized by ICT, Mumbai. A collaborative project entitled "Custom-designing Targeted, Efficient and Safe Nano-particulate Veterinary Drug Delivery System" was formulated and submitted to DBT for funding. Concept proposal was accepted in principal by DBT and on 14th September 2007 project was defended. Toxicological study was undertaken to evaluate genotoxic potential of the same.

Groups of rats and mice each were treated with three different PES doses 100mg, - 3000mg/kg BW. PES was suspended in polyethylene glycol 400 (PEG 400) and was gavaged to rat as single dose and in split dose. PEG400 was used as the solvent control. Animals were sacrificed by cervical dislocation 24 hours after final treatment. The samples were processed further for detecting chromosomal aberrations and micronucleus formation and DNA damage. The PES nanoparticles did not show any genotoxicity in Micronucleus assay, Comet assay and Chromosomal aberration test respectively.