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Progestin (norethisterone)-induced genetic damage in mouse bone marrow

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Summary

Primolut-N tablets containing norethisterone were assessed for their in vivo genotoxic effect on the bone marrow cells of Swiss albino mice. The chromosomal aberration assay and the micronucleus test were employed for the study. Statistically significant increases in chromosomal aberrations were induced by doses $\geqslant 3.0$ mg/kg/day. The maximum frequency of aberrations was induced at 24 h, thereafter decreasing with increasing time. But the drug Primolut-N did not induce a significant increase in the number of micronuclei in bone marrow erythrocytes at any of the doses and time intervals studied.

Interest in the genotoxic effects of sex hormonal drugs has increased during the last few decades. Oral contraceptives (OCs), drugs with varying concentrations of progestins and oestrogens, have been reported to be mutagenic in various plant and animal test systems (Hakeem and Amer, 1965; Murthy and Prema, 1983; Kabarity and Mazrooei, 1984; Shyama et al., 1991). The genotoxic effect of oestrogens is almost established (IARC, 1979; Ivett and Tice, 1981; Rudiger et al., 1984).

Williams et al. (1968) reported that progesterone causes alterations in meiotic chromosomes, chiefly sticky degeneration and improper spreading with clumping of chromosomes. Epidemiologic studies by Pike et al. (1983) suggested an increased incidence of breast cancer in women who used OCs which contain high potency progestins. However, Rohrborn and Hansmann (1974) found only a slight, but not significant increase of non-disjunction in the oocytes of mice after 1 mg norethisterone acetate treatment. McDenough (1985) points out the importance of studies on progestins, because of their widespread use in in vitro ferilisation programmes. Von Eickstedt and Lang (1987) have discussed the toxic effect of progesterone in pregnant humans. The mechanism of action of contraceptive progestin at the molecular level is discussed in detail by Perezpalacios et al. (1992). The present work was undertaken to add more information about the genotoxic effects of progesterones by studying the mutagenic potential of Primolut-N on the bone

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marrow cells of Swiss albino mice, using chromosomal aberration analysis and the micronucleus (MN) test.

Materials and methods

Test chemicals

Primolut-N (Schering AG, Germany; batch No. 913) was purchased from German Remedies Ltd. (Bombay, India). Cyclophosphamide (Endoxan, Asta-Werke AG, Germany; batch No. 707032) was purchased from Kandelwal Laboratories (Bombay, India). Both drugs were dissolved in sterile distilled water just before use. The concentrations were adjusted so that the oral administration of 0.2 ml/10 g body weight of the animal provided the required dose of the drug. All other chemicals used were of reagent grade.

Primolut-N tablets were dissolved to form drug suspensions of various doses, namely, 0.3, 1.5, 3.0, 6.0, 12.0, 18.0, 24.0 and 30.0 mg/kg body weight. They were administered to Swiss albino mice for 15 consecutive days using an oral catheter. The lowest dose, 0.3 mg/kg, represents the human therapeutic dose. Dose–response analysis was carried out for the above doses 24 h after the final feeding. Time–response studies were done using mice fed 3.0 mg/kg/day, 6, 12, 24, 48, 96 h and 1, 2 and 3 weeks after the final feeding.

Animals fed a single dose of 50 mg/kg of cyclosphosphamide (CP) served as positive controls. Time–response studies of CP were carried out at 6, 24 and 48 h after feeding. Mice fed sterile distilled water served as negative solvent controls.

Experimental animals

Adult Swiss albino mice (*Mus musculus*), produced from the Indian Institute of Science (Bangalore, India), were acclimatised for 2 weeks, reared and bred in the departmental animal house. From their progeny, 8–10-week-old healthy females (average body weight 25 g) were selected for the present study. The animals were reared in polypropylene cages (290 × 220 × 140 mm) bedded with paddy husk and were maintained at a temperature of 28°C (±2°C) and 50% (±6) humidity. The mice were fed standard mouse pellets (Lipton India Ltd.) and water ad libitum.

Since Primolut-N is used by women only, female mice were employed for the study. Two sets of three female mice were used for each of the experimental groups studied, the first set being

TABLE 1

PERCENTAGE [a] FREQUENCY OF CHROMOSOMAL ABERRATIONS INDUCED BY VARIOUS DOSES OF PRIMOLUT-N AT 24 h OF TREATMENT

Treatment	Dose (mg/kg b.w.)	MI [b]	Classical aberrations				Non-classical	Total ± SEM
			BS	TS	MA	Total ± SEM	S & P ± SEM	
Control	–	3.74	3.47	–	–	3.47 ± 8.86	1.49 ± 0.25	4.96 ± 1.53
Primolut-N	0.3	3.66	4.58	0.7	–	5.28 ± 1.32	3.87 ± 0.39	9.15 ± 1.89
Primolut-N	1.5	3.20	1.94	–	–	1.94 ± 0.53	9.42 ± 1.10 **	11.36 ± 1.98
Primolut-N	3.0	3.60	2.60	–	–	2.60 ± 0.73	11.73 ± 1.20 ***	14.33 ± 2.03 **
Primolut-N	6.0	3.57	3.55	1.29	–	4.84 ± 1.24	7.10 ± 0.81 *	11.94 ± 1.96 *
Primolut-N	12.0	3.20	5.32	1.97	–	7.29 ± 1.83	7.00 ± 0.75 *	14.29 ± 2.02 **
Primolut-N	18.0	3.30	4.21	1.87	–	6.08 ± 1.62	9.82 ± 0.93 ***	15.9 ± 2.13 **
Primolut-N	24.0	3.20	3.85	0.85	–	4.78 ± 1.03	11.11 ± 1.13 ***	15.81 ± 2.16 **
Primolut-N	30.0	2.97	1.63	–	–	1.63 ± 0.63	13.88 ± 1.39 ***	15.51 ± 2.46 **
CP	50.0	2.42	4.67	5.61 ***	1.87	12.15 ± 2.11 **	9.35 ± 7.82 **	21.5 ± 2.42 ***

[a] From 100 metaphases/animal.

[b] From 2000 cells/animal.

Three animals were used for each experimental group. MI, mitotic index; BS, breaks; TS, translocation; S & P, stickiness and pulverisations; MA, multiple aberrations.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

used for chromosome analysis and the second for the MN test.

Chromosome analysis

Mice were injected intraperitoneally with 0.2 ml of 0.025% colchicine, 1.5 h before they were killed by cervical dislocation. Bone marrow chromosomal preparations were made according to the method of Tjio and Whang (1962), using 0.56% potassium chloride as hypotonic solution. Flame-dried slides were coded and stained with buffered 10% Giemsa (pH 6.8). 100 metaphase spreads per animal were analysed. Breaks were identified as unstained regions present along the chromatids wider than the thickness of the chromatids that bear them. Transfer of the terminal portion of one chromosome to another leading to unequal length of arms was scored as a translocation. Improper spreading with stickiness and clumping of chromosomes was identified as stickiness. The extreme of stickiness represented by sticky degeneration of the chromosome was noted as pulverisation. Stickiness and pulverisations were scored together. The mitotic index (MI) was calculated by analysing 2000 cells per animal.

Micronucleus test

Experimental animals were killed at different time intervals. Bone marrow preparations were made according to the method of Schmid (1973), with a slight modification, i.e., the fetal calf serum was replaced by 5% bovine albumin solution in

TABLE 2

PERCENTAGE [a] FREQUENCY OF CHROMOSOMAL ABERRATIONS INDUCED BY 3.0 mg/kg OF PRIMOLUT-N AT DIFFERENT TIME INTERVALS AFTER TREATMENT

Treatment	Time	MI [b]	Classical aberrations				Non-classical aberrations	Total ± SEM
			BS	TS	MA	Total ± SEM	S & P ± SEM	
Control	6 h	3.73	1.43	0.72	–	2.15 ± 0.56	2.51 ± 0.31	4.66 ± 0.43
Primolut-N	6 h	3.57	1.64	0.23	–	1.87 ± 0.37	0.95 ± 0.11	2.82 ± 0.23
CP	6 h	2.90	3.80	2.53	1.90	8.23 ± 1.34 *	1.27 ± 0.14	9.50 ± 0.95
Control	12 h	3.60	1.99	0.33	–	2.32 ± 0.49	2.32 ± 0.24	4.64 ± 0.48
Primolut-N	12 h	3.50	4.03	1.57	–	5.60 ± 0.63	2.24 ± 0.26	7.84 ± 0.81
Control	24 h	3.65	3.47	–	–	3.47 ± 0.43	1.49 ± 0.13	4.96 ± 0.51
Primolut-N	24 h	3.60	2.60	–	–	2.60 ± 0.38	11.73 ± 1.13 ***	14.33 ± 1.30 **
CP	24 h	2.42	4.67	1.87	15.61 ***	12.15 ± 1.21 **	9.35 ± 0.91 ***	21.5 ± 2.11 ***
Control	48 h	4.20	3.96	–	–	3.96 ± 0.40	0.50 ± 0.11	4.46 ± 0.45
Primolut-N	48 h	4.10	3.91	2.28 *	–	6.19 ± 0.06	3.26 ± 0.34	9.45 ± 0.96
CP	48 h	2.68	3.70	1.23	3.70 ***	8.63 ± 1.23	7.41 ± 0.74 ***	16.84 ± 1.60 ***
Control	96 h	3.68	1.54	0.31	–	1.85 ± 0.16	1.85 ± 0.21	3.70 ± 0.38
Primolut-N	96 h	3.62	3.91	0.65	–	4.56 ± 0.34	2.93 ± 0.31	7.49 ± 0.75
Control	1 week	4.20	3.96	–	–	3.96 ± 0.42	0.50 ± 0.08	4.46 ± 0.43
Primolut-N	1 week	3.60	2.33	–	–	2.33 ± 0.34	1.33 ± 0.13	3.66 ± 0.37
Control	2 weeks	3.86	3.45	–	–	3.45 ± 0.43	1.48 ± 0.15	4.93 ± 0.51
Primolut-N	2 weeks	3.20	0.99	–	–	0.99 ± 0.10	3.62 ± 0.36	4.61 ± 0.43
Control	3 weeks	3.75	0.99	0.33	–	1.32 ± 0.23	2.30 ± 0.23	3.62 ± 0.37
Primolut-N	3 weeks	3.58	1.99	–	–	1.99 ± 0.21	1.00 ± 0.12	2.99 ± 0.31

[a] From 100 metaphases/animal.
[b] From 2000 cells/animal.
Three animals were used for each experimental group.
For abbreviations see Table 1.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

phosphate-buffered saline (PBS) (Seetharama Rao et al., 1983). Smears were stained with May-Grünwald Giemsa and analysed for the presence of MN in both polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs). 2000 PCEs and the corresponding number of NCEs per animal were analysed for the presence of MN. The ratio of PCEs to NCEs (P/N) was calculated for all treated and control groups.

Statistical analysis

Statistical analysis of the data on chromosomal aberrations was performed using the chi-square test (Li, 1966). For the micronucleus test results, the Mann-Whitney *U*-test (Siegel, 1956) was employed.

Results

Chromosome analysis

Dose–response assay. Table 1 summarises the results of the analysis of chromosomal aberrations induced by various doses of Primolut-N, 24 h after a 15-day treatment. Aberrations such as breaks, translocation, stickiness and pulverisations were observed. Statistically significant increases in chromosomal aberrations were noted at doses $\geqslant 3.0$ mg/kg. A dose-dependent increase in total aberrations was also noted. Among the various aberrations noted, stickiness and pulverisations were very frequent and were found in significant quantities at all doses studied except the lowest (0.3 mg/kg). This showed a sudden increase from 0.3 to 1.5 mg/kg and after 3.0 mg/kg this showed a decrease up to 12.0 mg/kg. No significant numbers of translocations were induced by any doses of Primolut-N. Breaks were not found in significant quantities at any of the doses studied. CP (50 mg/kg) also induced significant frequencies of aberrations. Metaphases with fragmentations and multiple aberrations were observed only in CP-treated mice. The MI values are also presented in Table 1. No significant changes occurred in MI both in the Primolut-N- and in the CP-treated animals.

Time–response assay. The results of the analysis of chromosomal aberrations induced by Primolut-N (3.0 mg/kg) at different time intervals are summarized in Table 2. Significant increases in aberration frequencies were noted 24 h after treatment; later on the frequencies decreased. Of the various aberrations, stickiness and pulverisa-

TABLE 3

PERCENTAGE [a] FREQUENCY OF MICRONUCLEI INDUCED BY VARIOUS DOSES OF PRIMOLUT-N AT 24 h OF TREATMENT

Dose (mg/kg b.w.)	% PCE	% NCE	% MN in PCE ± SEM	% MN in NCE ± SEM	P/N ratio ± SEM
Control	48.21	51.79	0.24 ± 0.033	0.12 ± 0.012	0.94 ± 0.069
Primolut-N					
0.3	47.36	52.64	0.29 ± 0.062	0.14 ± 0.032	0.90 ± 0.032
1.5	54.27	45.73	0.45 ± 0.032	0.27 ± 0.056	1.19 ± 0.031
3.0	54.65	45.35	0.38 ± 0.053	0.15 ± 0.032	1.21 ± 0.046
6.0	48.08	51.92	0.31 ± 0.034	0.13 ± 0.024	0.93 ± 0.060
12.0	47.16	52.84	0.30 ± 0.035	0.10 ± 0.023	0.89 ± 0.035
18.0	46.92	53.08	0.35 ± 0.046	0.16 ± 0.032	0.88 ± 0.072
24.0	46.75	53.55	0.31 ± 0.008	0.19 ± 0.026	0.88 ± 0.038
30.0	46.09	53.91	0.32 ± 0.093	0.22 ± 0.058	0.86 ± 0.032
CP					
50.0	38.75	61.25	3.63 ± 0.115 *	1.42 ± 0.093 *	0.63 ± 0.012

[a] From 2000 cells/animal.
Three animals were used for each experimental group.
* $P = 0.05$.

tions were found in significant quantities at 24 h. Translocations were increased at 48 h. CP induced significant aberrations at all time intervals studied, the highest frequency being at 24 h. No significant difference in the MI values were noted either for the Primolut-N- or for the CP-treated mice.

Micronucleus test

Dose–response assay. Table 3 indicates the frequency of MN induced by various doses of Primolut-N at 24 h after 15 days of treatment. Primolut-N did not induce a statistically significant increase in the number of MN compared to the negative controls. No significant difference in the P/N ratio was observed. CP induced significant frequencies of MN.

Time–response assay. The frequencies of MN induced by Primolut-N (3.0 mg/kg) at different time intervals are represented in Table 4. No significant increase in micronucleated erythrocytes was noted at any of the time intervals studied. The P/N ratio did not show any significant change. CP induced a significant frequency of MN.

Discussion

The above data indicate that Primolut-N is capable of inducing chromosomal aberrations in bone marrow cells of mice and the effect is both dose- and time-dependent. Stickiness and pulverisations were the major types of aberrations. Still, this drug failed to induce significant frequencies of MN at any of the doses and time intervals studied.

TABLE 4

PERCENTAGE [a] FREQUENCY OF MICRONUCLEI INDUCED BY 3.0 mg/kg OF PRIMOLUT-N AT DIFFERENT TIME INTERVALS AFTER TREATMENT

Time	Treatment	% PCE	% NCE	% MN in PCE ± SEM	% MN in PCE ± SEM	P/N ratio ± SEM
6 h	Control	51.00	48.92	0.32 ± 0.034	0.19 ± 0.042	1.07 ± 0.009
	Primolut-N	49.27	50.73	0.23 ± 0.062	0.19 ± 0.093	0.97 ± 0.054
	CP	44.31	55.69	0.93 ± 0.086	0.48 ± 0.043 *	1.80 ± 0.024
12 h	Control	49.14	50.86	0.31 ± 0.073	0.18 ± 0.036	1.03 ± 0.008
	Primolut-N	48.56	51.44	0.27 ± 0.088	0.14 ± 0.033	0.95 ± 0.042
24 h	Control	50.80	49.20	0.34 ± 0.033	0.19 ± 0.012	1.01 ± 0.051
	Primolut-N	48.08	51.92	0.38 ± 0.053	0.15 ± 0.032	1.21 ± 0.031
	CP	38.75	61.25	3.63 ± 0.015 *	1.43 ± 0.093 *	0.63 ± 0.012
48 h	Control	47.36	52.64	0.32 ± 0.036	0.22 ± 0.039	1.04 ± 0.043
	Primolut-N	45.83	54.17	0.18 ± 0.052	0.11 ± 0.022	0.85 ± 0.052
	CP	36.34	63.66	3.17 ± 0.126 *	1.28 ± 0.113 *	0.57 ± 0.011
96 h	Control	52.34	47.66	0.36 ± 0.008	0.21 ± 0.008	1.02 ± 0.043
	Primolut-N	43.97	56.03	0.12 ± 0.032	0.07 ± 0.032	0.79 ± 0.048
1 weeks	Control	52.40	47.60	0.36 ± 0.072	0.19 ± 0.042	1.10 ± 0.033
	Primolut-N	46.24	53.76	0.30 ± 0.008	0.16 ± 0.042	0.87 ± 0.062
2 weeks	Control	51.35	48.65	0.34 ± 0.003	0.21 ± 0.003	1.06 ± 0.042
	Primolut-N	46.71	53.29	0.32 ± 0.039	0.12 ± 0.042	0.90 ± 0.068
3 weeks	Control	51.48	48.52	0.37 ± 0.052	0.22 ± 0.082	1.06 ± 0.034
	Primolut-N	50.57	49.43	0.25 ± 0.000	0.17 ± 0.008	1.03 ± 0.009

[a] From 2000 cells/animal.
Three animals were used for each experimental group.
* $P = 0.05$.

The mutagenicity of the drug is mainly dependent on the amount of the drug and its metabolites reaching the target organs and the yield is also dependent on the efficiency of the DNA repair of the affected cells in the test organisms.

Progesterone was reported to induce a significant increase in chromosomal aberrations by Williams et al. (1968). Our present observation is consistent with those results. Stickiness and pulverisations were the most frequent type of aberration noted in our present study. This agrees with the sticky degeneration and improper spreading and clumping of chromosomes induced by progesterone (Williams et al., 1968), as well as the increased amount of non-disjunction induced by norethisterone acetate in the oocytes of mice (Rohrborn and Hansmann, 1974).

Anovlar 21, an OC which contains the progestin norethisterone acetate, induces considerable stickiness in the chromosomes of plants and animals (Hakeem and Amer, 1965; Kabarity and Mazrooei, 1984; Shyama et al., 1991). Our present observation of the induction of stickiness and pulverisation by Primolut-N agrees with the above results where an OC with the progestin norethisterone acetate is used.

The present time–response assay indicates that stickiness and pulverisation show a sudden increase from 12 h to 24 h, followed by a rapid decrease with time. This fully agrees with the statement of Savage (1975) that stickiness is a transient phenomenon which will disappear after some time.

Various biochemical and other related interpretations are given by many investigators to explain the induction of stickiness (Darlington, 1942; De Robertis et al., 1948; Hsu et al., 1965, as quoted by McGill et al., 1974; Giri et al., 1981). From these different views, it is inferred that chemically induced stickiness is a very complicated phenomenon, involving physical, mechanical and physiological properties of the chromosomes.

Chromatid breaks were seen more frequently than chromosome breaks in the metaphase plates analysed. This indicates that the damage affects the DNA strand in its late S phase or after the DNA has replicated or duplicated (Bird et al., 1982).

The time–response analysis revealed significant increases in chromosomal aberrations at 24 h of treatment with Primolut-N and decreased later on. This suggests that the drug and/or its metabolites were active during this period. According to Shyama et al. (1991) the decline in the frequency of aberrations observed at the later time intervals may be due to some or all of the following reasons: (i) repair of damaged genetic material; (ii) elimination of the drug and its metabolites from the body; (iii) elimination of the cells/chromosomes with damaged genetic material; and (iv) inactivation of the drug and/or its metabolites. A decline in SCEs in women within 3 months after discontinuation of the OCs was noted by Murthy and Prema (1983). They are of the opinion that the DNA damage leading to SCE is a temporary phenomenon.

Primolut-N did not produce significant frequencies of MN at any of the doses or time intervals studied in the present investigation. But it showed a significant mutagenic effect at high doses as far as chromosomal damage is concerned. This positive mutagenic effect in chromosomal analysis and the negative results in the MN test observed in the present investigation in Primolut-N-treated mouse bone marrow requires clarification. Usually, chromosomal breaks and lagging of chromosomes result in MN. Primolut-N induced a very low frequency of chromosomal breaks in mice. This may be the major reason for the non-induction of MN by this drug. Recently Savage (1988) has even suggested the possibility of a decreased number of MN compared to the acentric fragments observed in the same individual. Secondly, stickiness and sticky degeneration, i.e., pulverisation of chromosomes, induced by Primolut-N may be due to the degeneration of their nucleoproteins; hence the non-induction of MN by this drug. Kliesch et al. (1981) reported that these two tests are not equally sensitive. However, negative results in the MN test will not always prove lack of mutagenicity, as observed by Matter and Grauwiler (1974) with MNNG. Although MNNG is a potent mutagen in lower organisms, mammalian cells in vitro and the host-mediated assay, it does not induce MN in mouse bone marrow. In the present context it is worth noting that Jellema and Schardien (1975)

stated that 'Despite that observed relationship between chromosome lagging or fragmentation at metaphase and formation of MN, comparison of two methods for assaying cytogenetic effect may not be valid or necessarily useful, since there are several confounding factors in such comparison'. The cells examined in the MN test are exclusively of the erythroid series while those examined by cytogenetic analysis are composed of all marrow cells undergoing mitotic division. The present observations suggest that for a complete evaluation of mutagenic effects one has to use a battery of test systems, since no single test can be expected to detect all mutagens. Hence, from the present data, it may be concluded that Primolut-N induces chromosomal aberrations, but not MN, in bone marrow cells of mice.

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