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GENOTOXICITY OF BLOXIVERZ (NEOSTIGMINE METHYLSULFATE) ON BONE MARROW CELLS OF MOUSE

Dr. D. Rathna Prabha* and Dr. M. Ganga Raju[a]

*Department of Zoology, Tara Govt. College, Sanga Reddy, Medak (Dist.), Telangana State.
[a]Department of Pharmacology, Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, Telangana State.

***Corresponding Author: Dr. D. Rathna Prabha**

Department of Zoology, Tara Govt. College, Sanga Reddy, Medak (Dist.), Telangana State.

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ABSTRACT

Neostigmine is used in post operational care to relieve the patient from anaesthetic effects. Today it is the most common agent used for the reversal of effects of other agents used for neuromuscular blocks, like Suxamethonium chloride, Atracurium etc. In our study, *in vivo* experiments were conducted on bone marrow of Swiss albino mice to assess the genotoxic effects of the drug on somatic chromosomes and mitotic index. Human therapeutic doses were converted to mice body weight basis ranging from minimum to maximum (2, 8 &20µg.) Experiments were conducted in single and cumulative dose series for 24, 48 and 72hr period. Slides were prepared from bone marrow of control and treated mice by following standard air drying technique and stained with 2% Giemsa. Mitodepression induced by Neostigmine is more in cumulative when compared to single dose series. In both series this effect is more in 24 hr period without any statistical significance. There is a gradual decrease after 48 and 72 hr period. The drug did not induce chromosome aberrations for all periods and doses to a significant extent. The observations indicate that Neostigmine is not genotoxic to bone marrow cells of mouse.

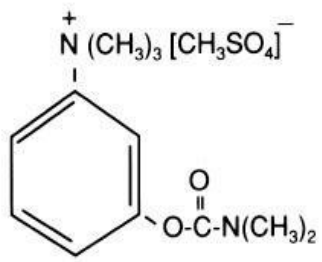
KEYWORDS: Neostigmine, Genotoxicity, Chromosome aberrations, Cytogenetic protocols.

1. INTRODUCTION

In the modern scientific world**,** with an increasing knowledge in the field of science and technology, various chemical substances have been introduced to serve human requirements for a comfortable life. These include drugs, cosmetics, food additives, insecticide, pesticides and other environmental substances. Nearly 1000 new ones are added annually to this ever increasing list.[1]

Rapid advances in pharmacology and chemotherapy have helped in introducing new compounds in human medicines, possessing improved efficacy. Such compounds are generally tested for their mutagenic potentials before releasing them into the market. Drugs and chemicals are used in common life to cure many human diseases. An accumulation of these or their biodegraded products in tissues may issue serious genetic damages to the precious genetic apparatus. Further such hazards may get transmitted to future generations also. Among the available battery of tests used to assess the drugs for causing genetic disorders, *in vivo* cytogenetic protocols are regarded as reliable assays.[2-4]

Neostigmine is used to relieve patients from anaesthetic effects after surgery. Acetylcholine is a neurotransmitter. Its function is impaired by anaesthetic drugs by blocking it by Acetyl cholinesterase activity. This can be reversed by anti-anaesthetic drugs like Neostigmine. It blocks Acetyl cholinesterase and relieves Acetylcholine which resumes its neurotransmitter function. The usual dose of Neostigmine ranges from 0.5 to 2 mg. The maximum dose limit is 5mg.for human being. It is readily absorbed and eliminated from the body. Approximately 80% of the drug was eliminated in urine and 50% as unchanged form and 30% as its metabolites within 24 hrs. It is metabolized by microsomal enzymes of in the liver. It is reported that it causes, allergic reactions, muscle cramps, rashes, urticaria, respiratory depression, respiratory arrest, bronchospasm and weakness.[5] Neostigmine is chemically designated as m-hydroxyphenyl trimethyl ammonium methyl sulfate dimethyl carbamate, having the following structural formula:



$C_{13}H_{22}N_2O_6S$ 334.39

It is listed as an essential drug by W.H.O and approved by FDA in 2013. So far very few experimental studies have been conducted for genetic potentials of Neostigmine. These include experiments with *in vitro* bacterial reverse mutation assay (Ames test), *in vitro* chromosome aberration assay and *in vivo* micronucleus assay. The results revealed that Neostigmine was not mutagenic. Long term animal studies have not been performed to evaluate the carcinogenic potential of Neostigmine. Studies on the effect of Neostigmine on fertility have not been performed.[5] These studies show that the available information on genotoxicity of Neostigmine is fragmentary. Hence we have studied the genotoxic effects of Neostigmine by using standard mammalian system, like Mouse.

Monitoring therapeutic compounds using mammalian somatic cells as test system has considerable significance in assessing risks to human health.[6,7] In such analysis chromosome aberrations serve as biological dosimeters in evaluating mutational damage.[8] Actively proliferating and mitotically dividing bone marrow cells provide maximum information on damage induced by the drug. *In vivo* studies are the most recommended and highly reliable methods of genotoxic evaluation.[9-11] *In vivo* somatic studies are particularly important because damage caused in somatic cells may lead to malignant growth. The present study results have great significance from the genotoxic point of view and the results obtained can be extrapolated to human beings.

2. MATERIALS AND METHODS

Drugs and chemicals

Neostigmine was purchased from market which is manufactured by VHB Medi. sciences Ltd, Mumbai. All other reagents and chemicals were of analytical grade.

Animals

Swiss albino mice of 8-10 week old were used for the activity. They were kept in polypropylene cages at 25±2º C, with relative humidity 45-55% under 12h light and dark cycles. All the animals were acclimatized to the laboratory conditions for a week before use. They were fed with standard animal feed and water *ad libitum*. The institutional animal ethical committee of Gokaraju Rangaraju College of Pharmacy, Hyderabad, with Reg. No. 1175/PO/ERe/S/08/CPCSEA approved the study.

In vivo experiments were conducted on mice, since they possess anatomical and physiological conditions similar to those of human beings.[12] Hence the results obtained can be applied to human beings. Doses were formulated according to the literature[13] and converted to mice body weight basis ranging from minimum to maximum (2, 8 & 20 µg). Swiss albino mice strains were purchased from standard suppliers (Albino Research & Training Institute, Hyderabad). 8-10 week old pathogen free Swiss albino mice were injected with 3 doses (2, 8 & 20 µg) of Neostigmine (purchased from market which is manufactured by VHB Medi. sciences Limited and Marketed by NEON LABORATORIES LIMITED Mumbai) in 0, 5 ml quantities. Experiments were conducted in single and cumulative dose series.

In single dose experiments the drug was administered only once. Where as in cumulative dose experiments, the doses were repeated for three times with 24hr. interval between injections, intended to see periodical effects of the drug. Control animals received the same volume of sterile double distilled water. Control and treated animals of both series were maintained under identical experimental conditions. Animals were observed for general behavior, like food and water intake.

There was no difference between control and treated animals in both series of experiments. Whereas the animals received highest concentration initially were inactive for about 1hr after that they became normal.

The experimental and control animals were injected with 0.5ml of 0.05% Colchicine on either side of the lower abdomen to ensure equal distribution, two and half hr before sacrificing them in order to arrest the cells at metaphase. Control and experimental animals were sacrificed at 24, 48 and 72hr. periods, following the last injection of the drug. Slides were prepared from bone marrow by following standard air drying technique and stained with 2% Giemsa. 5000 cells were scored for computing mitotic index to see the effect of the drug on cell division. Pillai and Sinha's[14] 2x2 contingency was employed for evaluating the significance of the data obtained on mitotic index values.

100 well spread non-overlapping metaphases were screened and analyzed for each dose and period under oil immersion objective for chromosome aberrations. Structural and other type of chromosome abnormalities were tabulated by following the scheme of Adler[15] by taking into consideration the total aberrations with and without gaps for interpretation of the action of the drug on chromosomes. Woolf's modified chi-square test of Li[16] was used for evaluating the significance of the data obtained on chromosome aberrations.

3. RESULTS AND DISCUSSION

Mitotic Index and effect on cell division

Table 1 & 2 reveals the quantitative data on mitotic index induced by the drug in bone marrow cells along with their X2 values for single and cumulative experiments respectively. Results reveal that Neostigmine reduced the rate of cell division as evidenced by low Mitotic Index values compared to control was showing its mitodepressive effect. Mitodepression is more in cumulative compared to single dose series. There is a gradual decrease in mitodepression after 24 hr period in both series of experiments. There is dose and period response in both series of experiments (Tables 1 & 2, Graphs 1 & 2). However the data is statistically insignificant.

Table 1: Quantitative data on mitotic index and mitodepression induced by Neostigmine in bone marrow cells of Mouse.*(single dose series)

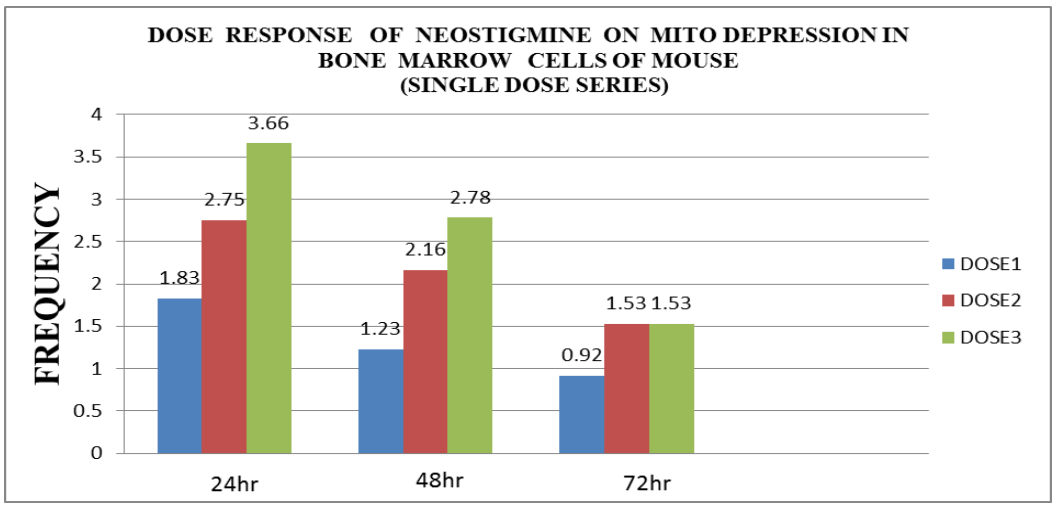
Period (hr)	Dose (µg)	No. of cells n division	Mitotic index	Mito depression	X2 values
24	Control	327	6.54	-	-
	2	321	6.42	1.83	0.06
	8	318	6.36	2.75	0.13
	20	315	6.30	3.66	0.23
48	Control	323	6.46	-	-
	2	319	6.38	1.23	0.07
	8	316	6.32	2.16	0.08
	20	314	6.28	2.78	0.14
72	Control	325	6.50	-	-
	2	322	6.44	0.92	0.01
	8	320	6.40	1.53	0.04
	20	320	6.40	1.53	0.04

*computed from 5000 cells for each dose and period.
X2 value significant at 5% level (Expected value: 3.84).

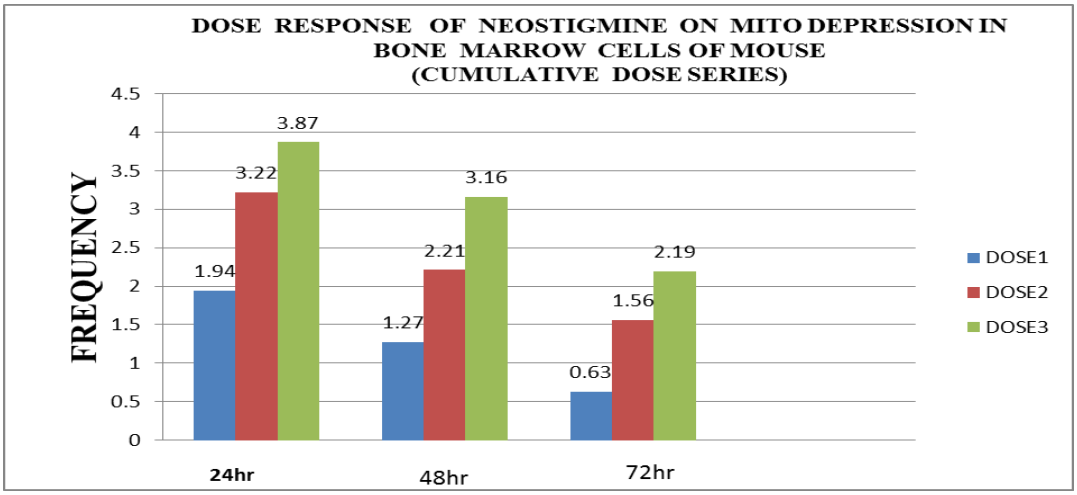
Table 2: Quantitative data on mitotic index and mitodepression induced Neostigmine in bone marrow cells of mouse*(cumulative dose series)

PERIOD (hr)	Dose (µg)	No. of cells n division	Mitotic index	Mito depression	X2 values
24	Control	310	6.20	-	-
	2	304	6.08	1.94	0.06
	8	300	6.00	3.22	0.17
	20	298	5.96	3.87	0.25
48	Control	316	6.32	-	-
	2	312	6.24	1.27	0.03
	8	309	6.18	2.21	0.08
	20	306	6.12	3.16	0.17
72	Control	320	6.40	-	-
	2	318	6.36	0.63	0.007
	8	315	6.30	1.56	0.04
	20	313	6.26	2.19	0.08

*computed from 5,000 cells for each dose and period.
X2 value significant at 5% level (Expected value: 3.84).



GRAPH 1



GRAPH 2

Thus emphasizing its insignificant effect on cell division process. This could be due to rapid elimination of the drug in short period from the system.[13] It also suggests that the drug and/or its metabolites are not affecting the energy building process of cell division.

Chromosome Mutations

Whenever cells get exposed to drugs, they may enter the interior of the cells and cause genetic damages also. These damages at chromosome level are referred as chromosome mutations. These chromosome mutations are important dosimeters to assess genetic damage.[8] These include structural, Numerical and other abnormalities. Among the structural chromosome anomalies, compared to control (photo 1), gap (Arrow G in photo2) represents discontinuation of the chromatid. Break (Arrow in photo3) is seen as broken chromatid piece away from the main chromosome axis. Terminal deletions are identified by the unequal lengths of chromatids. Numerical abnormalities include polyploidy (Photo 7) cells, wherein the total number of chromosome complements changes. Counts of such cells denote the action of a drug and its metabolites on the synthesis of proteins required for the construction of spindle apparatus.

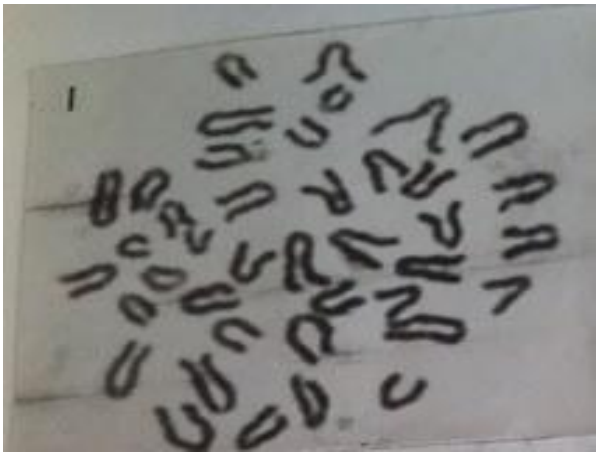


Photo. 1:- A metaphase spread from bone marrow tissue of mouse showing 40 normal chromosomes.

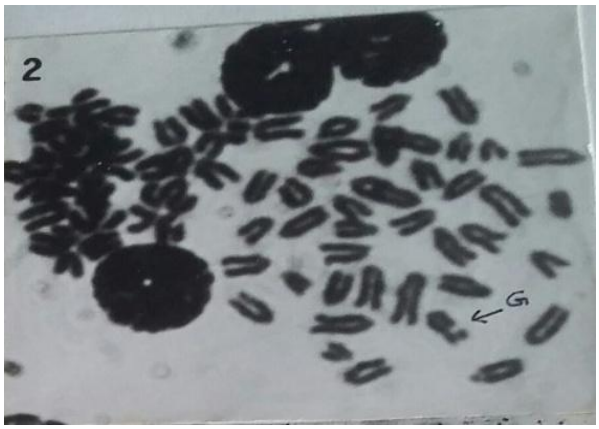


Photo. 2:- A metaphase spread from bone marrow tissue of mouse, in which arrow 'g' indicates a chromatid gap.

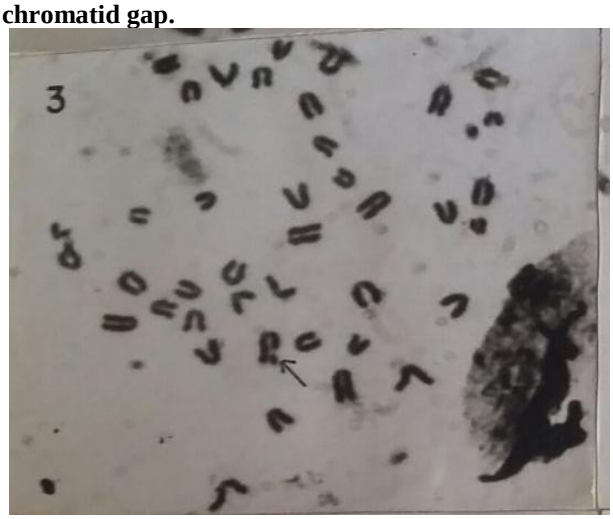


Photo. 3:- A metaphase spread from Bone marrow tissue of mouse, In which a chromatid break is indicated by the arrow.

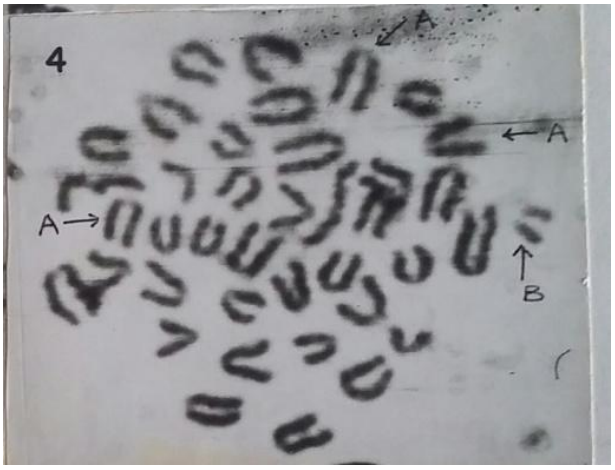


Photo. 4:- A metaphase spread from bone marrow tissue of mouse, In which a and b arrows indicate early chromatid Separations.

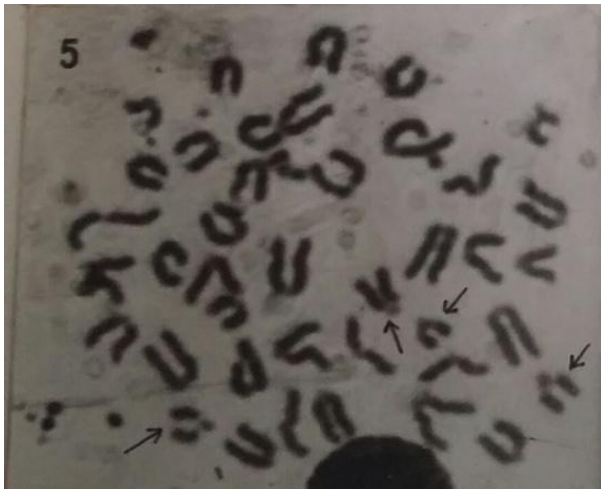


Photo. 5:- A metaphase spread from bone marrow tissue of mouse, in which arrows indicate "rabbit ear" chromosomes.

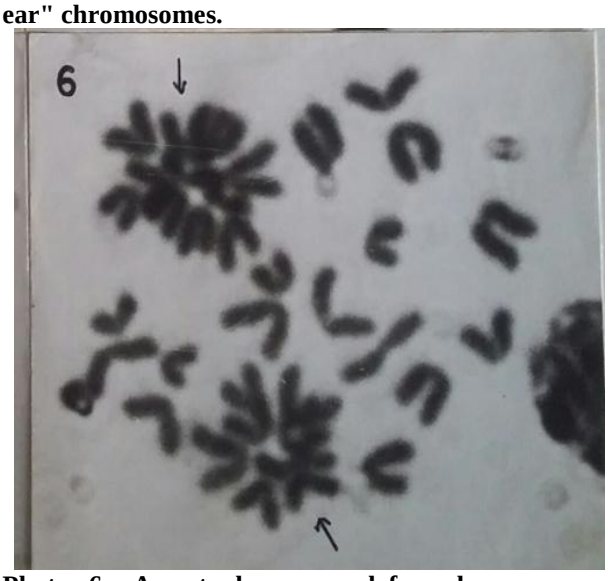


Photo. 6:- A metaphase spread from bone marrow tissue of mouse, in which arrows indicate achrocentric assosiations.

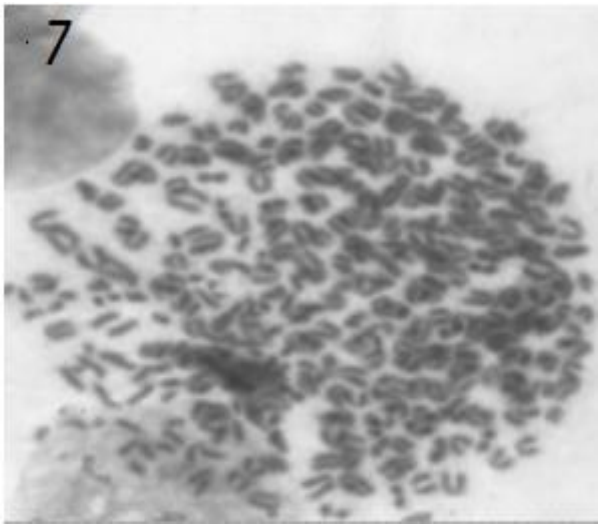


Photo. 7:- A Polyploid spread from bone marrow tissue of mouse.

The other chromosome abnormalities like chromatid separations (A&B Arrows in photo 4), rabbit ear chromosomes (Arrows in photo 5) and acrocentric associations (Arrows in photo 6) represent the action of a drug and/or its metabolites on heterochromatin regions which lie adjacent to the centromere. In early chromatid separations two chromatids of a chromosome separate from one another quite early compared to the rest of the chromosome compliment (arrow in photo 4). In rabbit ear chromosomes the two chromatids separate from the centromere & the region appears like a dot in the center (arrow in photo 5). In acrocentric association two or more chromosomes occur in groups, with centromeres of all in a group directed towards each other and more towards the center (arrow in photo 6).

Table 3: Distribution of chromosome mutations induced by Neostigmine in bone marrow cells of Mouse * (Single dose Series)

Period [Hr]	Dose (µg)	Structural abnormalities				Total		numerical Abnormalities	Other Abnormalities		
		Gaps	Breaks	Fragments	Terminal Deletions	With Gaps	Without Gaps	Polyploid Cells	Early Chromatid Separations	Rabbit ear Chromosomes	Acrocentric associations
24	Control	0	0	0	1	1	1	0	2	14	2
	2	0	0	0	0	0	0	0	3	14	2
	8	0	0	0	0	0	0	0	5	15	3
	20	1	1	1	1	3	2	1	7	17	3
48	Control	0	0	0	0	0	0	0	1	12	2
	2	0	0	0	0	0	0	0	3	14	3
	8	0	1	0	0	1	1	0	3	16	2
	20	0	0	1	0	1	1	0	4	16	3
72	Control	0	0	0	0	0	0	0	2	11	2
	2	0	0	0	0	0	0	0	2	12	2
	8	0	0	0	1	1	1	0	2	14	2
	20	0	0	0	0	0	0	0	2	14	2
*Computed from 100 Metaphases for each Dose & Period.											

Table 4: Distribution of chromosome mutations induced by Neostigmine in bone marrow cells of Mouse * (Cumulative Dose Series)

Period [Hr]	Dose (µg)	Structural abnormalities				Total		numerical Abnormalities	Other Abnormalities		
		Gaps	Breaks	Fragments	Terminal Deletions	With Gaps	Without Gaps	Polyploid Cells	Early Chromatid Separations	Rabbit ear Chromosomes	Acrocentric chromosomes
24	Control	0	0	1	0	1	1	0	2	11	2
	2	0	0	0	0	0	0	0	3	15	2
	8	0	0	1	1	2	2	0	3	16	2
	20	1	1	0	1	3	2	1	5	16	3
48	Control	0	0	0	0	0	0	0	3	9	1
	2	0	0	0	0	0	0	0	3	12	2
	8	0	0	1	0	1	1	1	3	14	2
	20	0	0	1	1	2	2	0	4	15	3
72	Control	0	0	0	0	0	0	0	1	8	2
	2	0	0	0	0	0	0	0	2	9	2
	8	0	0	0	0	0	0	0	2	9	2
	20	0	0	0	0	0	0	0	2	11	3
*Computed from 100 Metaphases for each Dose & Period.											

Table 3 and 4 represents the quantitative data on chromosome abnormalities induced in bone marrow cells of mouse after single and cumulative dose experiments respectively. Among structural chromosome mutations gaps, breaks, fragments and terminal deletions were observed rarely in both series of experiments. The statistically insignificant values indicate that Neostigmine and/or its metabolites are not genotoxic. The innocuous nature may be due to rapid elimination the drug from the system after 24 hr.[13]

The insignificant data on polyploidy cells indicate that it is not inhibiting the synthesis of proteins required for the construction of spindle apparatus. The statistically insignificant values on other abnormalities emphasize that the drug and/or its metabolites do not bring any damaging effect on the chromosome structure involving heterochromatin.

CONCLUSION

The present study revealed that Neostigmine is ineffective on cell division and not genotoxic in somatic tissue. These observations supports that the use of Neostigmine is safe from genotoxic point of view. The present studies are of great interest in the context of the observations of Cohen **et al** (1969), that "negative chromosome findings with drugs that enjoy wide spread use should be published".

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