EVALUATION OF GENOTOXIC POTENTIAL OF ASPARTAME

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Summary

The present study was planned to investigate the genotoxic potential of aspartame. Bone marrow and peripheral blood micronucleus, bone marrow chromosomal aberration and sperm morphology test were conducted. In micronucleus and chromosomal aberration tests the drug was administered in four different doses (250, 455, 500 and 1000 mg/kg) in a single dose (p.o) to four different groups of animals. (455 mg/kg, selected on the basis of conversion of human adequate daily intake (ADI) dose which is 50 mg/kg to animal dose). Cyclophosphamide (CP), single dose was injected (i.p) at 100 mg/kg to one group which was used as a standard and distilled water treated group was maintained as control. Bone marrow slides were prepared at 24, 48 and 72 h respectively in all groups. In sperm morphology test the drug was administered in four different doses (250, 455, 500 and 1000 mg/kg) in a single dose (p.o) to four different groups of animals everyday for one week. Cyclophosphamide (CP), single dose was injected (i.p) at 100 mg/kg to one group which was used as a standard and distilled water treated group was maintained as control. Percentage of micronucleated polychromatic erythrocytes, percentage of total aberrations induced and percentage of abnormal sperms were determined and the results were analyzed by one-way ANOVA followed by Dunnet's test. Aspartame at doses 455, 500 and 1000 mg/kg showed significant (P<0.01) increase in the number of micronucleated polychromatic erythrocytes, total aberrations and abnormal sperms in 24, 48 and 72 h treatments.These results clearly indicate aspartame to be clastogenic.

Keywords: Aspartame, chromosome aberrations, Micronucleus Tests.

Running title: Aspartame Genotoxicity.

Introduction

Aspartame (or ASP) is the name for an artificial, non-saccharide sweetener, *aspartyl-phenylalanine-1-methyl ester*; i.e., a methyl ester of the dipeptide of the amino acids aspartic acid and phenylalanine. This sweetener is marketed under a number of trademark names, including Tropicana Slim, Equal, NutraSweet, and Canderel, and is an ingredient of approximately 6,000 consumer foods and beverages sold worldwide. In the European Union, it is also known under the E number (additive code) E951. Aspartame is also one of the sugar substitutes used by people with diabetes.

Aspartame has been the subject of vigorous public controversy regarding its safety and the circumstances around its approval. Many studies have recommended further investigation into the possible connection between aspartame and diseases such as brain tumors, brain lesions, and lymphoma.[1] These findings, combined with alleged conflicts of interest in the approval process, have engendered vocal activism regarding the possible risks of aspartame. In 1995, FDA Epidemiology Branch Chief Thomas Wilcox reported that aspartame complaints represented 75% of all reports of adverse reactions to substances in the food supply from 1981 to 1995. A total of 92 different symptoms and health conditions were reported by physicians and consumers. Questions have been asked about brain cancer, lymphoma, and genotoxic effects such as DNA-protein crosslink's, chromosome aberrations etc. There is debate in the scientific and medical community as to whether these symptoms are caused by short-term or long-term exposure to aspartame. It is not only the results of the research that have been questioned, but the design of the research that led to specific outcomes. For example, in human research of aspartame, it is usually provided in slow-dissolving capsules. But the concentration of aspartate in the blood from ingesting aspartame using slow-dissolving capsules is much lower than that from ingesting liquid aspartame (such as in carbonated beverages).[2]

Although the United States Food and Drug Administration (US FDA) and European Food Standards Authority (EFSA) have given a clean bill of health to aspartame, the Cesari Maltoni Cancer Research Center, European Ramazzini Foundation of Oncology and Environmental Sciences in Italy have confirmed the carcinogenetic effect of aspartame. A review of literature claims that 100% of industry funded (either whole or in part) studies concluded that aspartame is safe, 92% of independently funded studies have found that aspartame has the potential for adverse effects.[3] So further studies need to been conducted on aspartame to evaluate its genotoxic effect (e.g.: chromosome aberration, micronucleus and sperm morphology tests). Hence the present work is taken to evaluate the genotoxic effects of aspartame.

Materials and methods:

Drugs and chemicals

Aspartame, Bovine albumin serum, May Grunewald's stain, Geimsa stain and colchicine were procured from Himedia, Mumbai. Cyclophosphamide was procured from Alkem Laboratories, Mumbai and Eosin Y stain from Loba Chemie, Mumbai. All other chemical reagents used were of analytical grade.

Aspartame was given by oral route and cyclophosphamide was injected (i.p) at a dose of 100 mg/kg. Swiss albino mice of either sex, weighing 25-30 gm, procured from KSHEMA, Mangalore were used in the present experiments. Animals were maintained on standard rodent feed with drinking water *ad libitum*, and were acclimatized for 1 week to laboratory conditions. They were housed, handled and sacrificed at the end of the experiments in accordance with the guidelines of CPCSEA and the study was approved by the institutional animal ethical committee (Ref No: KSHEMA/AEC/075/2008). In this study, peripheral blood and bone marrow were collected 24, 48 and 72 h after treatment for micronucleus and chromosomal aberration tests. For sperm morphology test the cauda epididymis was dissected after 1 week of treatment with aspartame.

Total 6 groups (n=6) were maintained including one treated with cyclophosphamide (positive control) and another with distilled water (negative control).

Bone marrow and peripheral blood micronucleus test

The drug was administered in four different doses (250, 455, 500 and 1000 mg/kg) in a single dose (p.o) to four different groups of animals. (455 mg/kg, selected on the basis of conversion of human adequate daily intake (ADI) dose which is 50 mg/kg to animal dose). Cyclophosphamide (CP), single dose was injected (i.p) at 100 mg/kg to one group which was used as a standard and distilled water treated group was maintained as control. Bone marrow slides were prepared at 24, 48 and 72 h respectively in all groups. Bone marrow and peripheral blood preparations were made by using the modified method of Schmid (1973).[4] Here instead of fetal calf serum, 5% bovine albumin prepared in buffered saline (pH 7.2) was used as suspending medium (Seetharam *et.al*, 1983).[4] The animals were sacrificed by cervical dislocation. The tibia and femur bones were removed and the bone marrow cells were flushed with a syringe containing 5% bovine albumin solution. The suspension was mixed properly and centrifuged at 1000 rpm for 8 min. The supernatant was discarded and a thick cell suspension was made in bovine albumin solution. A drop of cell suspension was placed on absolutely clean slide and smeared with the help of a spreader slide. The slides were air dried. Soon after drying, the slides were fixed in absolute methanol for 10 min. For peripheral blood micronucleus test, blood smears were prepared on clean glass slides. Staining was done by using May Grunewald's and Geimsa stain at time between 3-24 h after fixation. The slides were kept in May Grunewald's stain, freshly diluted with equal volume of phosphate buffer (pH-6.8) for 15 min. The slides were transferred to Geimsa, freshly diluted with phosphate buffer (1:6) and kept for 10 min. The slides were rinsed for several times in buffer. Finally the slides were kept in buffered water for 5 min. The slides were then air dried and scanned for the presence of micronuclei (MN) in polychromatic (PCE) and normochromatic (NCE) erythrocytes under the microscope.

About 2000 PCE and corresponding NCE from each animal treated and controls were scanned for the presence of MN. The PCE/NCE ratio was also determined in each group.[4]

Bone marrow chromosomal aberration test

The drug was administered in four different doses (250, 455, 500 and 1000 mg/kg) in a single dose (p.o) to four different groups of animals. Cyclophosphamide (CP), single dose was injected (i.p) at 100 mg/kg to one group which was used as a standard and distilled water treated group was maintained as control. Bone marrow slides were prepared at 24, 48 and 72 h respectively in all groups. Experimental animals were injected with 0.2 to 0.3 ml of 0.025% mitotic arrestant colchicine (i.p). The animals were kept as such for 90 min for the colchicine to react with the cells. After this period, they were sacrificed by cervical dislocation. The marrow cells from the femur and tibia bones were flushed with 0.56% KCl by using a syringe. The marrow suspension was thoroughly mixed and transferred to a centrifuge tube and incubated at room temperature for 20 min. At the end of this period the suspension was centrifuged at 1000 rpm for 8 min. The supernatant was discarded and the pellet was dispersed in the fixative (1:3 acetic acid methanol mixture) and was kept for one hour. After this period the marrow suspension was again centrifuged. This cycle of incubation and centrifugation was repeated 3-4 times and finally a thick suspension was made in the fixative. Using a dropper, about 2-3 drops of the final cell suspension was dropped on the absolutely clean, prechilled slides. The slides were flame dried and stained with buffered Geimsa of pH 6.8. For staining, 20-25 drops of stock Geimsa was taken in a coupling jar and about 30-35 ml of buffer solution of pH 6.8 was added. The prepared bone marrow slides were immersed in this diluted stain for about 20 min. After staining the excess stain was washed with the buffer solution. The slides were air dried and observed under the microscope. One hundred metaphases from each animal were analysed for the presence of structural and numerical aberrations (breaks, fragments, rings, stickiness etc.). The percentage of total chromosomal aberrations were calculated.[5]

Sperm morphology test

The four doses mentioned earlier were repeated here. Aspartame was administered daily for a period of one week. After one week the animals were sacrificed by cervical dislocation. The reproductive tract was exposed and both the cauda epididymis were removed. It was placed in a watch glass containing 1ml of phosphate buffered saline (PBS). Both the cauda epididymis were minced in the PBS using a wire mesh and forceps. The suspension obtained was filtered through two layers of muslin cloth, to remove the tissue fragments. The filtered suspension was stained with 1% aqueous eosin Y (10:1). After 30 min a drop of the suspension was taken on a clean slide and a uniform smear was made. The slides were air dried. The slides were observed under the microscope to score different types of abnormal sperms. 2000 sperms per animal were examined for each treatment and control groups.[4]

Statistical analysis

Results were expressed as mean ± S.E.M. In bone marrow micronucleus test, the chromosomal damage was measured as the percentage of Micronuclei present in polychromatic erythrocytes (MNPCE) per 2000 PCE (%MNPCE). In bone marrow chromosomal aberration test, the chromosomal damage was measured as percentage of total aberration induced. In sperm morphology test, the changes in morphology were expressed as percentage of abnormal sperms present. For statistical analysis one way ANOVA followed by Dunnet's test was done. $P<0.05$ was considered to be significant.

Results

A 250 mg/kg dose of aspartame did not induce any gaps in the chromosomes and so did the controls. But higher doses produced statistically significant amount of gaps. A 250 mg/kg dose of aspartame did not produce significant amount of aberrations as compared to controls while higher doses of aspartame induced significant amount of chromosomal aberrations. This indicates a dose dependent increase in induction of chromosomal damage.

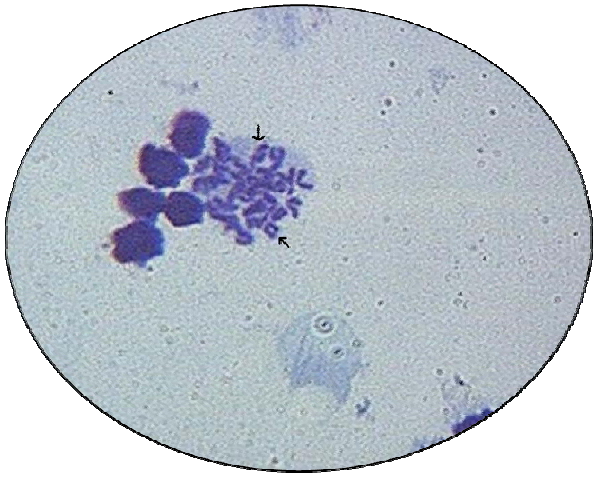

Figure 1: Photomicrograph of mice bone marrow chromosomes after 24 h of treatment with a single dose (1000 mg/kg) of aspartame in 100X microscope, showing breaks (arrow mark)

A 250 mg/kg dose showed 0.9% of total aberration after 24 hours, 0.95% after 48 h and 5.6% after 72 h [Table 1].

Table 1: Incidence of percentage of total aberrations in various treated and control groups.

Treatment groups (mg/kg) (n=6)	**%Total Aberration in 24 h**	**%Total Aberration in 48 h**	**%Total Aberration in 72 h**
Control	0.4 ± 0.02	2.3 ± 0.1	1.8 ± 0.21
Cyclophosphamide (100 mg/kg) i.p	60.65 ± 0.18[a]	73.1 ± 1.43[a]	39.9 ± 0.91[a]
Aspartame (250 mg/kg) p.o	0.9 ± 0.03[NS]	0.95 ± 0.04[NS]	5.6 ± 0.55[a]
Aspartame (455 mg/kg) p.o	7.3 ± 0.02[a]	4.05 ± 0.05[b]	12.25 ± 0.20[a]
Aspartame (500 mg/kg) p.o	5.4 ± 0.07[a]	5.65 ± 0.23[a]	13.6 ± 0.33[a]
Aspartame (1000 mg/kg) p.o	14.4 ± 0.07[a]	18.7 ± 0.65[a]	28.8 ± 0.82[a]

ANOVA followed by Dunnet's test. **a =** P<0.01 compared to control; **b** = P<0.05 compared to control; **NS** = statistically not significant (P>0.05)

A 500 mg/kg dose [Figure 2] produced 5.4% total aberration after 24 h, 5.65% after 48 h and a significantly higher 13.6% after 72 h [Table 1].

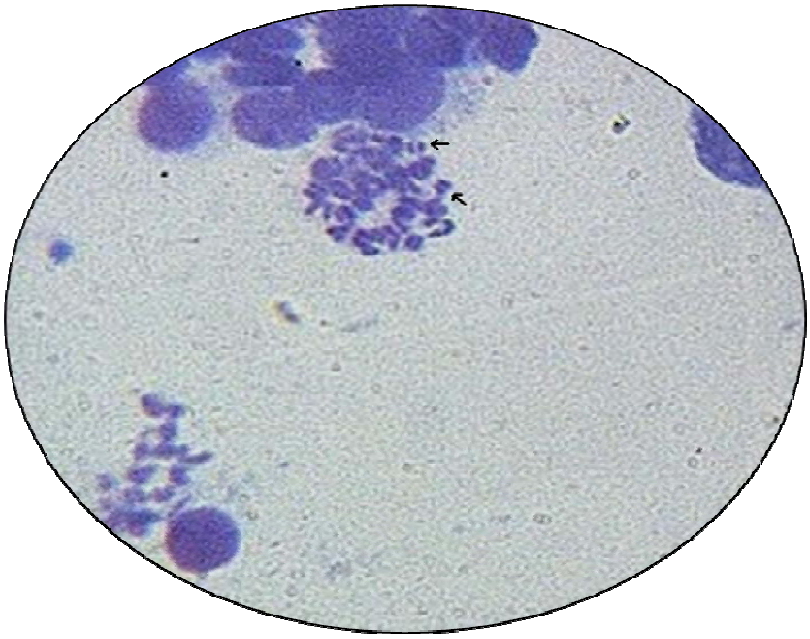

Figure 2: Photomicrograph of mice bone marrow chromosomes after 24 h of treatment with a single dose (500 mg/kg) of aspartame in 100X microscope, showing breaks (arrow mark)

A 1000mg/kg [Figure 1] dose also showed a time dependant increase in the aberrations. A dose of 455 mg/kg which is equivalent to the adequate daily intake (ADI) of 50mg/kg in humans showed 7.3% aberrations after 24 h, 4.05% after 48 h and 12.25% after 72 h [Table 1].

The results of bone marrow micronucleus test showed insignificant percentage of micronuclei in PCE at a dose of 250 and 455 mg/kg after 24 h. But higher doses indicated a significant increase in the percentage of micronuclei in PCE as well as changes in the PCE/NCE ratio. After 48 and 72 h all doses (except 250mg/kg in 48 h) showed statistical significance as compared to controls [Table 2].

Table 2: Incidence of percentage of micronuclei in polychromatic erythrocytes (%MNPCEs) in bone marrow of various treated and control groups

Treatment groups (mg/kg) (n=6)	**%MNPCE ± S.E.M in 24 h**	**%MNPCE ± S.E.M in 48 h**	**%MNPCE ± S.E.M in 72 h**
Control	0.17 ± 0.017	0.18 ± 0.02	0.20 ± 0.01
Cyclophosphamide (100 mg/kg) i.p	2.8 ± 0.016[a]	1.64 ± 0.02[a]	0.80 ± 0.01[a]
Aspartame (250 mg/kg) p.o	0.19 ± 0.01	0.21 ± 0.02	0.36 ± 0.01[a]
Aspartame (455 mg/kg) p.o	0.54 ± 0.06[a]	0.51 ± 0.18[a]	0.53 ± 0.16[a]
Aspartame (500 mg/kg) p.o	0.21 ± 0.01	0.53 ± 0.01[a]	0.67 ± 0.02[a]
Aspartame (1000 mg/kg) p.o	0.23 ± 0.12[a]	0.74 ± 0.19[a]	0.76 ± 0.12[a]

ANOVA followed by Dunnet's test. **a =** P<0.01 compared to control; **b** = P<0.05 compared to control; **NS** = statistically not significant (P>0.05)

The results of peripheral blood micronucleus test also showed insignificant percentage of micronuclei in PCE at a dose of 250 and 455 mg/kg after 24 h. But higher doses indicated a significant increase in the percentage of micronuclei in PCE as well as changes in the PCE/NCE ratio. After 48 and 72 h all doses (except 250mg/kg in 48 h) showed statistical significance as compared to controls [Table 3].

Table 3: Incidence of percentage of micronuclei in polychromatic erythrocytes (%MNPCEs) in peripheral blood of various treated and control groups

Treatment groups (mg/kg) (n=6)	**%MNPCE ± S.E.M in 24 h**	**%MNPCE ± S.E.M in 48 h**	**%MNPCE ± S.E.M in 72 h**
Control	0.16± 0.018	0.17 ± 0.02	0.19 ± 0.01
Cyclophosphamide (100 mg/kg) i.p	1.9 ± 0.015[a]	1.62 ± 0.02[a]	0.78± 0.01[a]
Aspartame (250 mg/kg) p.o	0.20±0.01[NS]	0.20±0.02[NS]	0.34± 0.01[a]
Aspartame (455 mg/kg) p.o	0.32±0.01[NS]	0.49 ± 0.17[a]	0.51± 0.15[a]
Aspartame (500 mg/kg) p.o	0.65± 0.05[a]	0.51 ± 0.01[a]	0.65± 0.02[a]
Aspartame (1000 mg/kg) p.o	0.34 ± 0.02[a]	0.72 ± 0.17[a]	0.74 ± 0.10[a]

ANOVA followed by Dunnet's test. **a =** P<0.01 compared to control; **b** = P<0.05 compared to control; **NS** = statistically not significant (P>0.05)

Table 4: Percentage of abnormal sperms induced by various treated groups and controls

Treatment groups (mg/kg) (n=6)	**Time in weeks**	**%Abnormal sperms ± SEM**
Control	1	1.28 ± 0.08
Cyclophosphamide (100 mg/kg) i.p	1	4.3 ± 0.83[a]
Aspartame (250 mg/kg) p.o	1	1.98 ± 0.22[a]
Aspartame (455 mg/kg) p.o	1	1.49 ± 0.45[a]
Aspartame (500 mg/kg) p.o	1	2.05 ± 0.29[a]
Aspartame (1000 mg/kg) p.o	1	2.52 ± 0.13[a]

ANOVA followed by Dunnet's test. **a =** P<0.01 compared to control; **b** = P<0.05 compared to control; **NS** = statistically not significant (P>0.05)

The results of sperm morphology test also indicated a dose dependant increase in number of sperm abnormalities. Out of 2000 sperms counted per animal, on an average 1.98% abnormal sperms were noted in 250 mg/kg treated animals, 2.05% in 500 mg/kg, 2.52% in 1000 mg/kg, 1.49% in 455 mg/kg and 4.30% abnormal sperms in cyclophosphamide treated animals [Table 4].

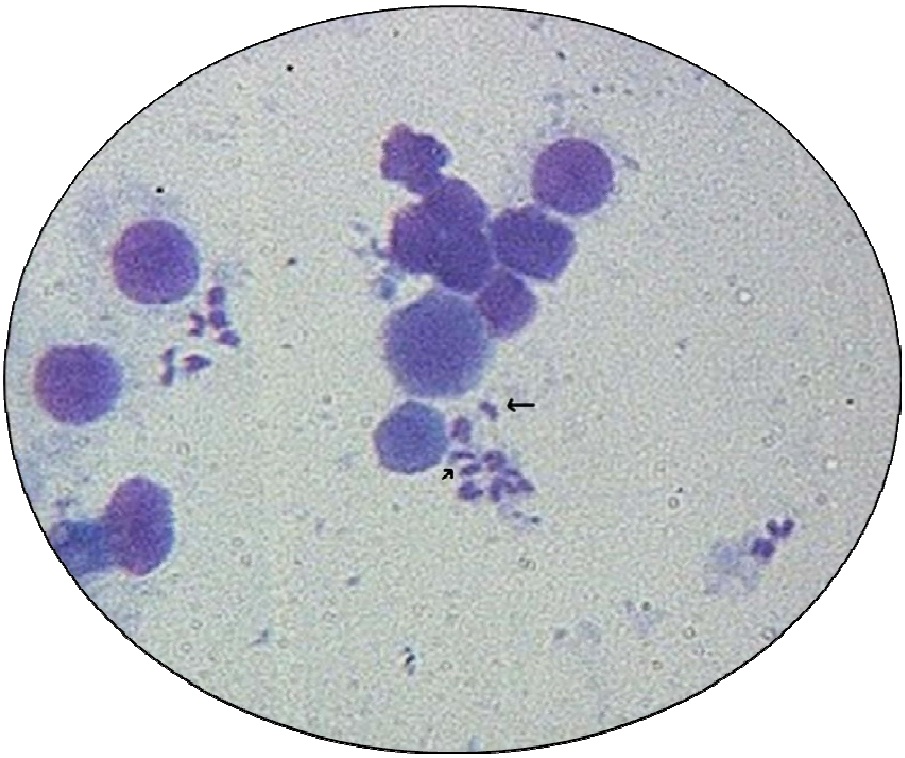

Figure 3: Photomicrograph of mice bone marrow chromosomes after 24 h of treatment with a single dose (100 mg/kg) of cyclophosphamide in 100X microscope, showing breaks (arrow mark)

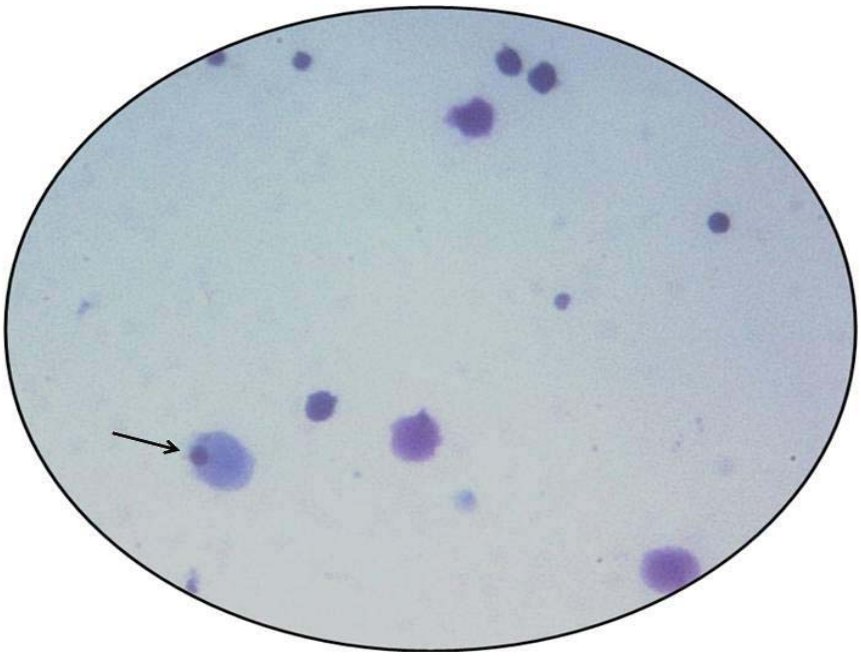

Figure 4: Photomicrograph of mice bone marrow micronucleus after 24 h of treatment with a single dose (1000 mg/kg) of aspartame in 100X microscope, showing micronuclei (arrow mark).

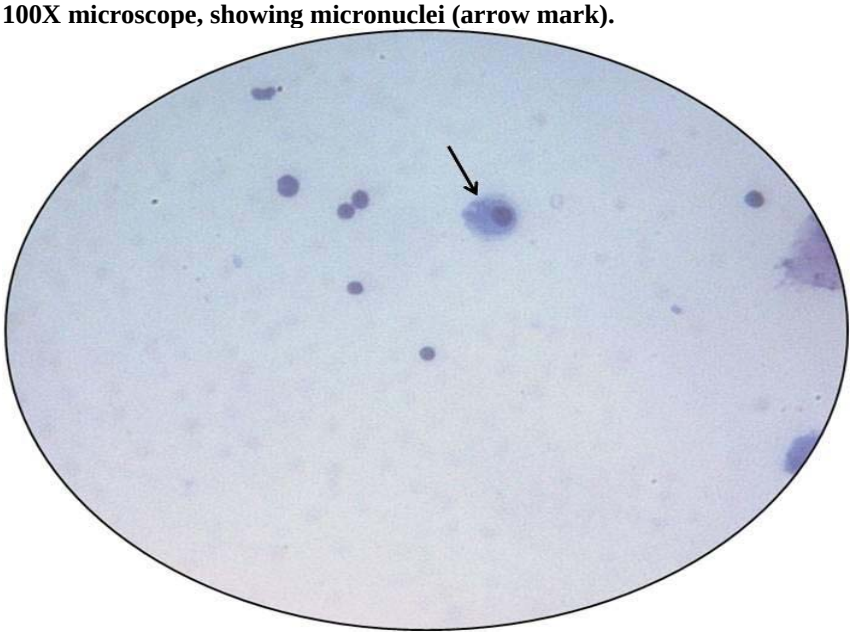

Figure 5: Photomicrograph of mice bone marrow micronucleus after 48 h of treatment with a single dose (500 mg/kg) of aspartame in 100X microscope, showing micronuclei (arrow mark).

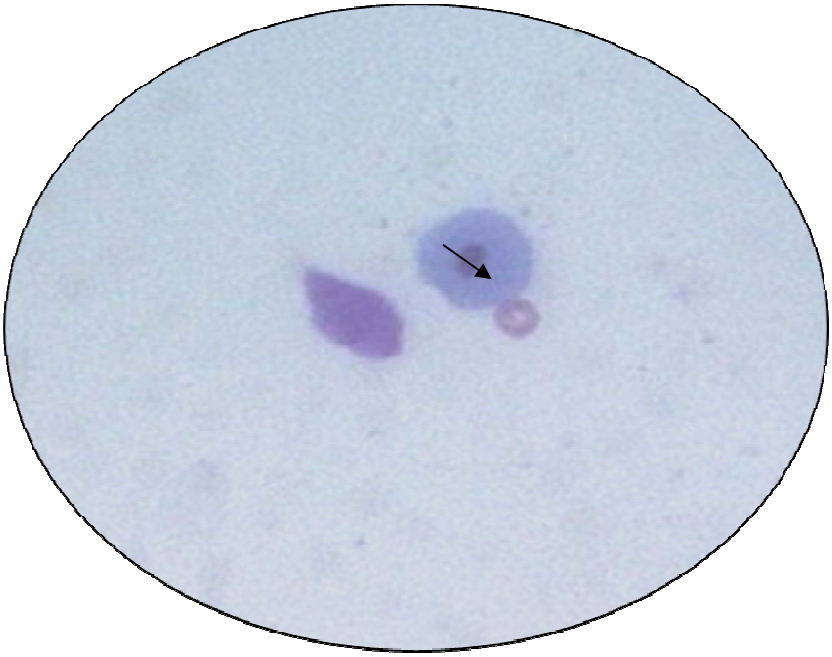

Figure 6: Photomicrograph of mice peripheral blood micronucleus after 24 h of treatment with a single dose of (455 mg/kg) of aspartame, showing micronuclei (arrow mark).

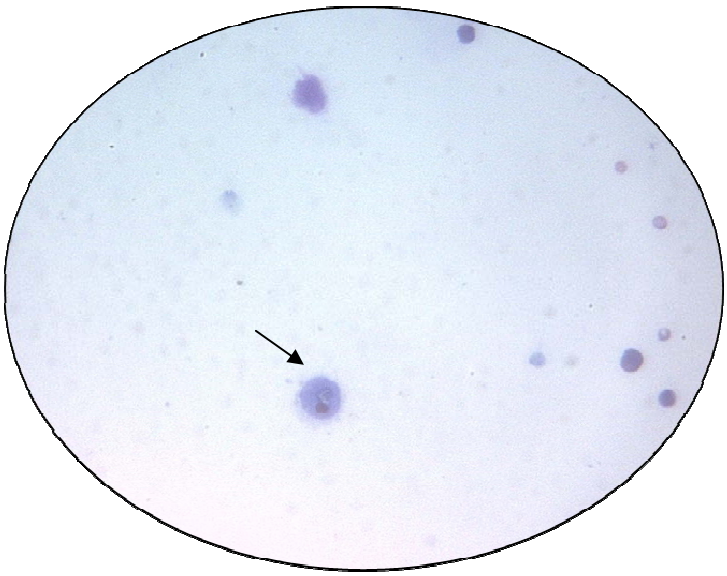

Figure 7: Photomicrograph of mice peripheral blood micronucleus after 48 h of treatment with a single dose of (500 mg/kg) of aspartame, showing micronuclei (arrow mark).

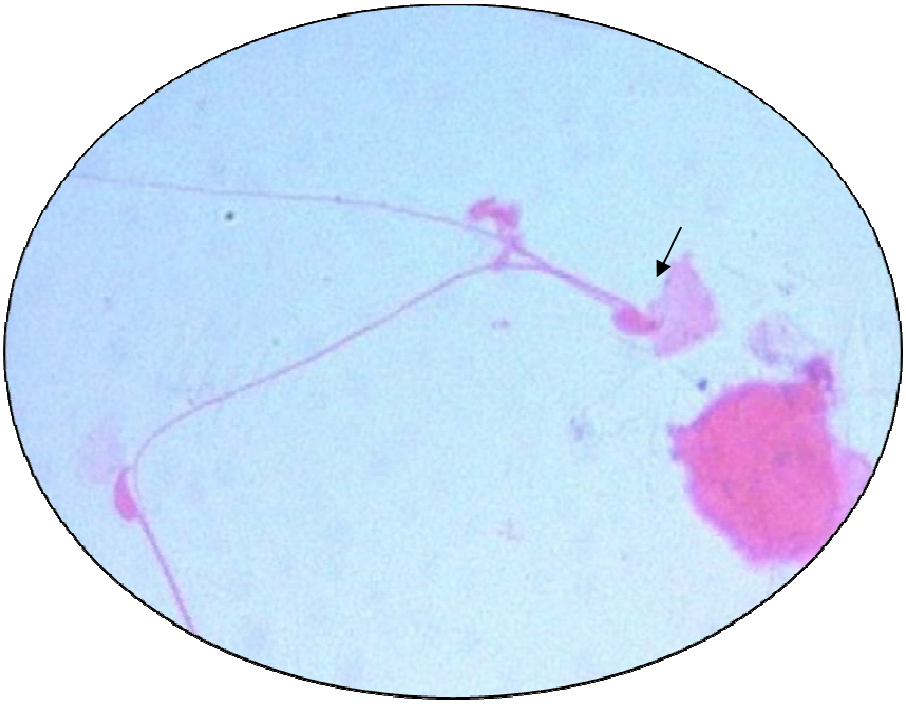

Figure 8: Photomicrograph of eosin yellow stained mice sperms obtained from the cauda epididymis seen in a 100X microscope, showing a double tailed sperm (arrow mark).

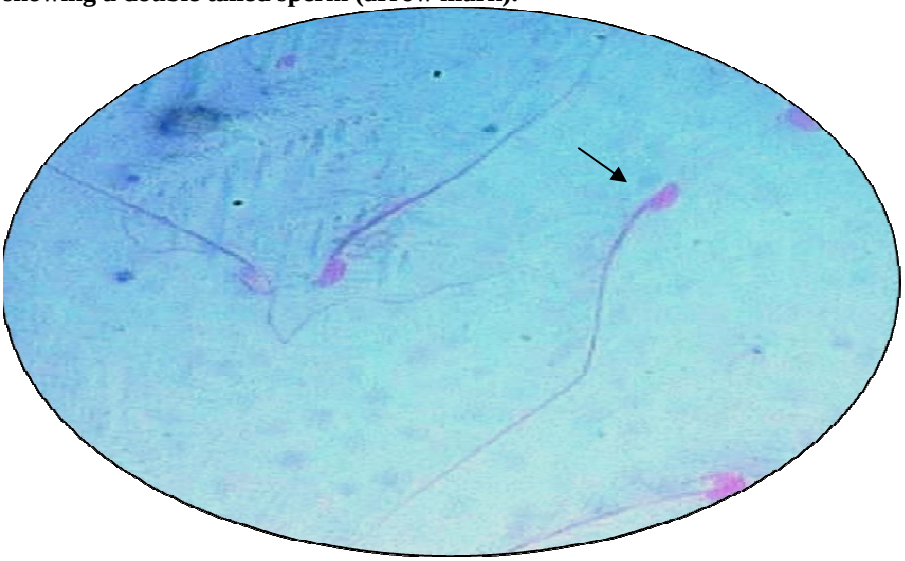

Figure 9: Photomicrograph of eosin yellow stained mice sperms obtained from the cauda epididymis seen in a 100X microscope showing hook less sperms (arrow mark).

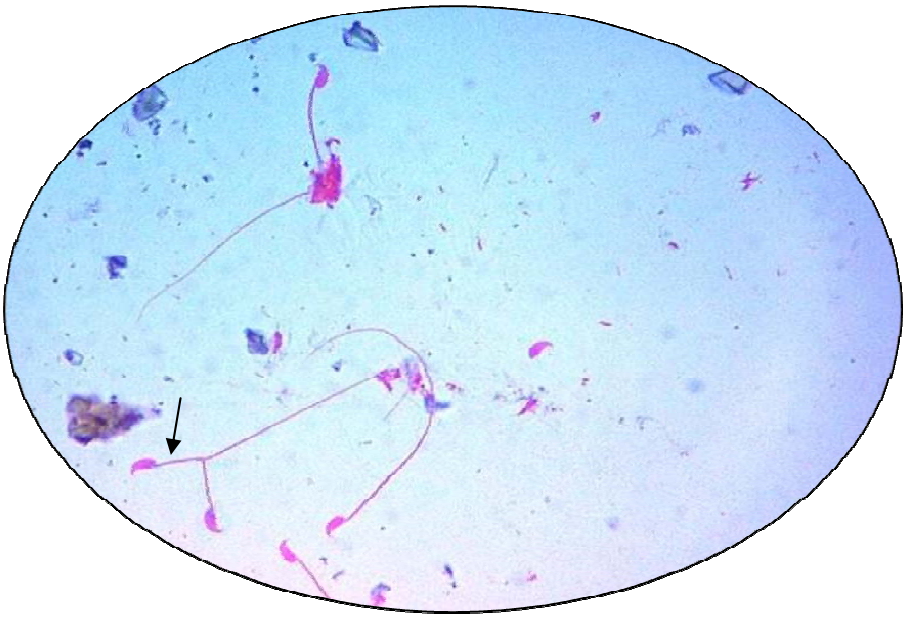

Figure 10: Photomicrograph of eosin yellow stained mice sperms obtained from the cauda epididymis seen in a 100X microscope showing a double headed sperm (arrow mark).

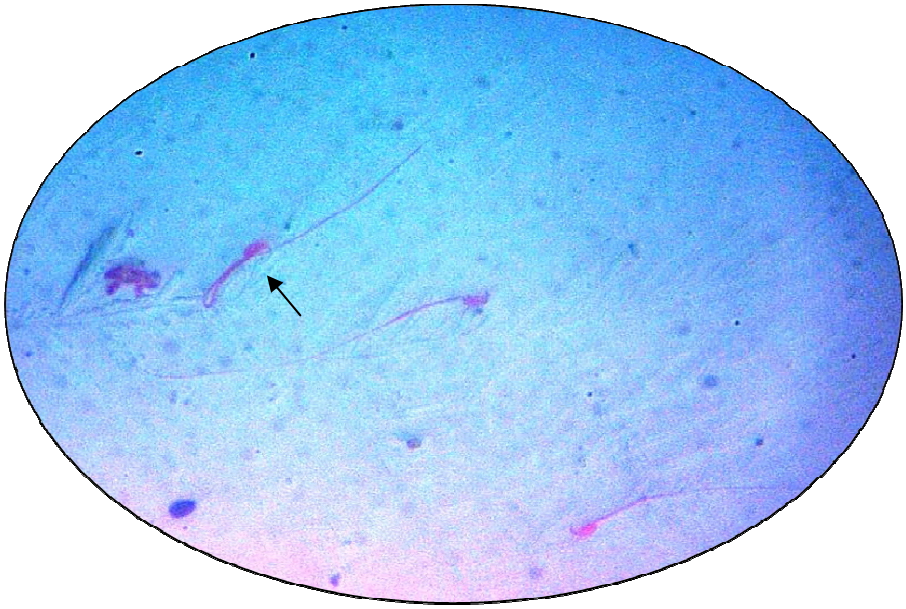

Figure 11: Photomicrograph of eosin yellow stained mice sperms obtained from the cauda epididymis seen in a 100X microscope showing a folded sperm (arrow mark).

Discussion

The induction of chromosomal aberrations has been considered as a reliable indication of the mutagenic activity of any agent or chemical. This of course emanates from the fact that nearly all genetic information in eukaryotes is encoded in a linear sequence, in the microscopically visible mitotic and meiotic chromosomes and any agent that is capable of disrupting this sequence will cause genetic changes by rearranging the ordered array of information. Implicit in this reasoning is that any agent that induces structural chromosome aberration will consequently pose genetic risk of some magnitude.

Various types of the cells can be used for scoring the chromosomal aberrations. Spermatogonial cells, spermatocytes, oocytes, early embryos, bone marrow cells etc. The frequency of aberrations like chromatid and ischromatid breaks; gaps, rings, exchange, multiple aberrations and fragmentation are usually considered to assess the genotoxic effects produced by different agents. There are limited studies on these aspects in the Indian context.[6] Previous studies on genotoxicity of aspartame have been employed on human lymphocytes.[7] In this study, aspartame significantly induced chromosomal aberrations and micronucleus formation and showed effect by decreasing the mitotic index. Ahmed and Thomas (1992) reported that phenylalanine and methanol, which are metabolic products of aspartame, have a genotoxic risk for humans.[7] There are also several contradictory studies about genotoxicity and carcinogenicity of aspartame mostly on human lymphocytes and chromosomes.[7] However, it must be taken into account that aspartame induced chromosomal aberration, micronuclei formation and sperm abnormalities in a dose dependant manner. It is not possible to conclude that aspartame is safe according to these results. Therefore, it is necessary to be careful when using it in food and beverages as a sweetener.

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