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Cytogenetic Effects of Ranitidine on Stem Cell L-Asparaginase Treated Mice

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Abstract- This study was established to investigate the cytogenetic effects of Ranitidine (RNT) on mice bone marrow stem cells with, before and after L-asparaginas (ASNase) enzyme chemotherapy. The negative control group was injected with 0.2 ml distilled water only for 24 hours, while drugs controls groups consists of RNT controls groups; was given (2 mg/kg or 4 mg/kg) RNT for 24 hours; and ASNase controls groups; was given 1000 U/kg ASNase for 24 hours. Furthermore, three combinations of RNT/ASNase were given at different exposure time. The results revealed appearance cytogenetic effects at ($p<0.05$) following treatments with RNT and ASNase. Both dosage of RNT caused wide genetic damage compared with other treatment groups. The combination between ASNase and RNT demonstrated appearance cytogenetic effects on mice bone marrow. This study concluded also that combination between RNT and ASNase have positive impact by reduce the aggressiveness of RNT on stem cells and enhancement the ASNase activity as a drug.

Keywords- *Mitotic index, chromosomal aberrations, micronuclei, Ranitidine, L-asparaginase*

I. INTRODUCTION

Peptic ulcer disease is main common health trouble, recorded as a important reason of death in more than 10,000 cases yearly (1). It is a chronic inflammatory state of upper gastrointestinal tract caused by auto digestion of epithelium by gastric acid (2). Histamine acting a major role in mediating gastrin-stimulated gastric acid secretion of stomach (3) by effect on histamine type2 receptors that found in gastric parietal cells (4). Restrain the action of histamine by histamine type2 receptors antagonists can decrease acid secretion that are contained within the stomach. This mechanism developed to treatment peptic ulcers (5). Furthermore, Histamine type2 receptors antagonists thought they are mediated in blocking the proliferative effect of tumors cells (6) because they are inhibition the trophic effects of histamine on gastrointestinal epithelial cells which involved in pathogenesis of carcinogenesis by enhanced rates of cell proliferation (7).

Ranitidine is an extremely prevalent histamine type2 receptors antagonist. It is exert in the most prominent effect on the acid secretion (8). Decrease volume of gastric juice is main clinical use of Ranitidine (9). It is may use in patients who undergo curative resection of colorectal cancer and cause prolong the survival of these patients (10). Reducing the frequency of ulcers and upper gastrointestinal symptoms the most important effectiveness of Ranitidine but it is not influential in preventing the global endoscopic deterioration caused by chemotherapy (11).

Many chemotherapy influence DNA, and the damage to DNA may product chromosomal aberrations causing chromosomal instability, and may lead to mutagenesis (12)(13). Enzymes can be used as chemotherapeutic agents and they are demonstrating a high degree of substrate specificity that can be very useful in restricting their cytotoxicity toward specific tissues (14).

L-asparaginase is the first enzyme have anti-leukemic activity (15), used in the acute lymphoblastic leukemia chemotherapy for almost 3 decades. So, it is an effective antineoplastic agent (16). Very efficient and low-cost sources of this enzyme are microorganism's source (17). The clinical action of this enzyme is attributed to the reduction of L-asparagine due to hydrolysis it to the aspartic acid and ammonia. Tumor cells unable to synthesize this amino acid therefore selectively killed by L-asparagine deficiency that result cell cycle arrest in G1 phase and apoptosis, all that leads to cell death (18).

Bone marrow is a main hematopoietic organ consists of hematopoietic cells in different stages of ripeness, involving erythrocytes, leukocytes and platelets (19). L-asparaginase differs from cytotoxic drugs in it causes little toxicity to bone marrow reduction (16) but it has many toxicity profile in the inhibition of protein synthesis or in immunological sensitization to a foreign protein (20). This study was aimed to evaluate the effects of Ranitidine on increasing the effect of L-asparaginase and vice versa.

II. MATERIALS AND METHODS

A. Ranitidine dose and concentration

Ranitidine (50 mg/2ml) was obtained from Al-Karamah Teaching Hospital as a vial. Two dosages were used in current study which was 2 mg/kg and 4 mg/kg as the same for human (21). These two dosages were prepared by diluted the drug with distilled water to gain a required concentration and dose , these were 0.05 mg/mouse equivalent to 2 mg/kg and 0.1 mg/mouse equivalent to 4 mg/kg.

B. L-asparaginase dose and concentration

L-asparaginase (10000 U) was being brought as a vial from Al-Karamah Teaching Hospital. For mouse injection (intraperitoneally), a dose of 1000 unit/kg was prepared by dissolving the drug in distilled water to gain the required concentration and dose, which equivalent to 25 unit/mouse (22).

C. Laboratory animals

The current study needed one hundred of Albino Swiss male mice. These mice were purchased from National Center for Drug Control and Research/ Ministry of Health/ Baghdad. Mice that used in the experiments have age from 8 to 12 weeks while the average rate of weights was (25±2) gm. Mice were ranked in 10 blocks each block consisting of 10 mice then placed in discrete plastic cages. Mice were kept in a room temperature of (23-25) ºC and fed with standard pellets and fresh water to avoid stressful conditions.

D. Administration of laboratory animals

The laboratory animals had been divided into two groups:

1) Control Groups

This group included four blocks each block consisted of 10 mice. These mice had been killed after 24 hours of treatment.

Block 1: (Negative Control): 0.25 ml of distilled water was injected intraperitoneally for each mouse.

Block 2: (RNT I Control): Ranitidine 2 mg/kg dosage: 0.2 ml Ranitidine was injected intraperitoneally for each mouse.

Block 3: (RNT II Control): Ranitidine 4 mg/kg dosage: 0.2 ml Ranitidine was injected intraperitoneally for each mouse.

Block 4: (L-asparaginase Control): 0.2 ml L-asparaginase was injected intraperitoneally for each mouse.

2) Treatment groups

Each group included 20 mice. These mice were injected at similar dose of L-asparaginase which about 1000 U/kg, but depending on the dose of Ranitidine divided into two sections each section included of 10 mice, first section (a) was treated with low dose 2 mg/kg and the second (b) was treated with high dose 4 mg/kg. These groups are illustrating as following:

Group I: The mice were injected with Ranitidine and L-asparaginase together at the same time and killed after 24 hours.

Group II: The mice were injected with L-asparaginase for 48 hours and Ranitidine for 24 hours then killed.

Group III: The mice were injected with Ranitidine for 48 hours and L-asparaginase for 24 hours then killed.

III. CYTOGENETIC EXPERIMENTS

A. Chromosome preparation from somatic cells of the mouse bone marrow

The experiment was conducted based on the way of Allen *et al*. (1977) (23). Mice were injected with colchicine for 2 hours before cervical dislocation sacrifice**.** Both femur bones were excised and gapped from the middle. By using warm phosphate buffer saline (PBS) rinsed all bone marrow in test tubes. Spin down test tubes by centrifuge at speed 2000 rpm for ten minute. Removal the supernatant flowed by added hypotonic solution (0.075 M of potassium chloride) to the test tubes. Suspension incubated for 20 min in water bath at 37º C with continuous shaking (to make cells fragile). Removal the supernatant after spin down the test tubes by centrifuge at speed 2000 rpm for ten minute was followed by treated pellet with freshly prepared fixative solution (Methanol: Glacial Acetic Acid, 3:1). The cells fixed by kept at 4º C in refrigerator for 20 minute then spin down at 2000 rpm for 10 minute by centrifuge. Re-fix the cells for three times that to get debris free white pellet. Few drops were dropped vertically on the clean slide then kept overnight to dry after that stained with (Giemsa's stain). Five slides were prepared for each animal. After preparation the slides, one hundred divided cells in metaphase stage of the mitotic division were scanned for each animal to detected types of chromosomal aberrations (break, fragment, gap, ring, polyploidy, acentric fragment).

IV. CYTOGENETIC ANALYSIS

A. Mitotic index (MI) assay

The five slides were prepared in the previous method were scanned again by light microscope with lens (40X) and counted 1000 of divided and non-divided cells on each slides. Then the percentage rate was calculated for only the divided cells (metaphase cells). It is illustrate in the following equation:

$$\text{Metaphase index (\%)} = \left(\frac{\text{Number of metaphase cells}}{\text{Total number of the cell(1000)}} \right) \text{x } 100$$

B. Chromosomal aberration (CA) assay

The prepared slides were examined under the oil immersion lens (100X) of light microscope for 100 divided cells per each animal, and the cells should be at the metaphase stage of the mitotic division where the chromosomal aberrations were clear and the percentage of these aberrations could be estimated.

C. Micronucleus (MN) assay

The trial was prepared depending on method of (Schmid, 1975) (24) as following: Mouse was killed via cervical dislocation. Both femur bones were excised and gapped from the middle. By using human plasma (heat inactivated) rinsed all bone marrow in test tubes. Spin down test tubes by centrifuge at speed 1000 rpm for five minute. Discard the

supernatant except little plasma was gently mixed with the pellet. One drop of suspension was utilized to make thin smear onto clean slide. Then slides left over night to dry in room temperature. Absolute methanol was used to fix the smear on the slides at five minute before the stain. The smear was stained with Giemsa stain for 15 minute after that rinsed with distill water and left to dry. Lastly, five slides were prepared for each one mouse. These slides were scanned under the oil immersion lens and recorded the numeral of MN in 1000 stem cells on each slide. The micronucleus index was gained by using the following equation:

$$\text{Micronucleus index} = \left(\frac{\text{Number of micronuclei}}{\text{Total count}} \right) \text{x } 100$$

V. STATISTICAL ANALYSIS

One way to analysis of variance was performed to assay whether group variance was significant or not. The data obtained were statistically analyzed using a 2 × 2 contingency table (X 2).

The comparison between groups was demonstrated by using SPSS version 16 software. The difference is considered significant when the probability of chi square value at $p<0.05$.

VI. RESULTS AND DISCUSSION

The results of mitotic index (MI) were sorted in table (1). The toxic effects of RNT (I and II) and ASNase caused significant differences in MI, this differences clearly showed by reducing its values (4.7%, 4.5% and 5.58%) respectively in mice bone marrow compared with negative control (6.32%). Also treatment groups (I, II and III) have significant different when compared with the negative control, RNT and ASNase. All these results were statistically significant ($p<0.05$).

TABLE I. PERCENTAGES OF MITOTIC INDEX IN BONE MARROW OF MICE FOR NEGATIVE CONTROL, RNT (I AND II), ASNASE AND TREATMENT GROUPS

Dose	No. of animal used	No. of cells examined	Mitotic Index	
			No. of dividing cells	%
Negative control 0.2ml of D.W	5	25000	1580	6.32
RNTI (2mg/kg)	5	25000	1175	[a*]4.7
RNTII (4mg/kg)	5	25000	1125	[a*]4.5
ASNase (1000mg/kg)	5	25000	1396	[a*]5.58
Treated group I (a) ASNase + RNTI (2mg/kg)	5	25000	936	[b*]3.74
Treated group I (b) ASNase + RNTI (4mg/kg)	5	25000	936	[c*]3.74
Treated group II (a) ASNase 48hrs. + RNTI (2mg/kg) 24hrs.	5	25000	1302	[b*]5.20
Treated group II (b) ASNase 48hrs. + RNTI (4mg/kg) 24hrs.	5	25000	1200	[c*]4.8
Treated group III (a) RNTII (2mg/kg) 48hrs. + ASNase 24hrs.	5	25000	899	[b*]3.59
Treated group III (b) RNTII (4mg/kg) 48hrs. + ASNase 24hrs.	5	25000	991	[c*]3.96

[a] Negative control vs. RNT (I and II) and ASNase , [b] Treated group vs. Negative control, RNTI and ASNase, [c] Treated group vs. Negative control, RNTII and ASNase, * Significant different at ($P < 0.05$).

TABLE II. PERCENTAGES OF DIFFERENT TYPES OF CHROMOSOMAL ABERRATIONS (CA) IN MICE BONE MARROW FOR NEGATIVE CONTROL, RNT (I AND II) ASNASE GROUPS AND TREATMENT GROUPS (I, II AND III)

Dose	No. of animals used	No. of cells examined	No. and types of chromosomal aberrations													
			Break		Fragment		Gap		Ring		Polyploidy		Acentric Fragment		Total	
			No.	%	No.	%	No.	%	No.	%	No.	No.	%	No.	%	No.
Negative control 0.2ml of D.W	5	500	24	4.8	69	13.8	21	4.2	1	0.2	0	0	73	14.6	188	37.6
RNTI (2mg/kg)	5	500	58	11.6	146	29.2	84	16.8	19	3.8	3	0.6	213	42.6	523	^{a*}104.6
RNTII (4mg/kg)	5	500	75	15	91	18.2	129	25.8	62	12.4	10	2	166	33.2	533	^{a*}106.6
ASNase (1000mg/kg)	5	500	25	5	39	7.8	45	9	41	8.2	4	0.8	71	14.2	225	^{a*}45
Treated group I (a) ASNase + RNTI (2mg/kg)	5	500	21	4.2	59	11.8	27	5.4	54	10.8	1	0.2	120	24	282	^{b*}56.4
Treated group I (b) ASNase + RNTI (4mg/kg)	5	500	19	3.8	40	8	96	19.2	34	6.8	7	1.4	89	17.8	284	^{c*}56.8
Treated group II (a) ASNase 48hrs. + RNTI (2mg/kg) 24hrs.	5	500	21	4.2	64	12.8	97	19.4	53	10.6	10	2	126	25.2	371	^{b*}74.2
Treated group II (b) ASNase 48hrs. + RNTI (4mg/kg) 24hrs.	5	500	10	2	82	16.4	102	20.4	49	9.8	7	1.4	158	31.6	408	^{c*}81.6
Treated group III (a) RNTII (2mg/kg) 48hrs. + ASNase 24hrs.	5	500	16	3.2	45	9	74	14.8	50	10	4	0.8	72	14.4	261	^{b*}52.2
Treated group III (b) RNTII (4mg/kg) 48hrs. + ASNase 24hrs.	5	500	26	5.2	52	10.4	107	21	56	11.2	4	0.8	55	11	300	^{c*}60

[a] Negative control vs. RNT (I and II) and ASNase , [b] Treated group vs. Negative control, RNTI and ASNase, [c] Treated group vs. Negative control, RNTII and ASNase, * Significant different at ($P < 0.05$).

The results of chromosomal aberrations (CAs) scored in table 2. Animals treated with Ranitidine (I and II) showed a high frequency of total chromosomal aberrations (104.6%, 106.6%) respectively in mice bone marrow cells, these findings were significant ($p<0.05$) when compared with negative controls (37.6%). As well as, the single dose of ASNase induced CAs after one day reached to (45%). There are significant differences at ($p<0.05$) when compared this value with the negative control. The combination between ASNase and RNT demonstrated the effects became less than RNT but higher than ASNase in treated group I (56.4%, 56.8%), treated group II (74.2%, 81.6) and treated group II (52.2%, 60%). These values accorded significant differences at ($p<0.05$) when compared with negative control.

TABLE III. PERCENTAGES OF MICRONUCLEI (MN) IN MICE BONE MARROW FOR NEGATIVE CONTROL, RNT (I AND II), ASNASE AND TREATMENT GROUPS

Dose	No. of animal used	No. of cells examined	Micronuclei	
			No. of MN	%
Negative control 0.2ml of D.W	5	25000	670	2.68
RNTI (2mg/kg)	5	25000	1190	a*4.76
RNTII (4mg/kg)	5	25000	1095	a*4.38
ASNase (1000mg/kg)	5	25000	808	a*3.23
Treated group I (a) ASNase + RNTI (2mg/kg)	5	25000	957	b*3.82
Treated group I (b) ASNase + RNTI (4mg/kg)	5	25000	957	c*3.82
Treated group II (a) ASNase 48hrs. + RNTI (2mg/kg) 24hrs.	5	25000	1012	b*4.04
Treated group II (b) ASNase 48hrs. + RNTI (4mg/kg) 24hrs.	5	25000	1040	c*4.16
Treated group III (a) RNTII (2mg/kg) 48hrs. + ASNase 24hrs.	5	25000	926	b*3.70
Treated group III (b) RNTII (4mg/kg) 48hrs. + ASNase 24hrs.	5	25000	940	c*3.76

[a] Negative control vs. RNT (I and II) and ASNase , [b] Treated group vs. Negative control, RNTI and ASNase, [c] Treated group vs. Negative control, RNTII and ASNase, *significant different at ($P < 0.05$).

Table 3 shows the results of micronuclei (MN). The frequency of MN in negative control showed a significant differences when compared with all positive groups and also treatment groups at ($p<0.05$).

After one day of single dose RNT show significant reduction in MI and high increase in CAs and MN that may result from abnormality in cell cycle (25) or from inhibition DNA synthesis. Reduction DNA synthesis, nuclei percentage and enhancement gastric mucosal damage had been associated with utilized RNT as a drug (26) (27). Methyl groups that consist in the structure of RNT considered important reason to appear cytogenetic effects in cells. Differences in the DNA repair systems for each change, chromatin remodeling (28) and methylation of histones protein that package chromatin (29) were happened undergo effect of methyl groups. Increase RNT dose synchronization with evaluated gap aberration and decline in overall rate of aberration that depended on DNA-breakage (break, fragment and acentric fragment) in bone marrow cells, that may related with hydrophilic cationic feature of RNT where net positive charge have with a large number of hydrogen bonding sites (30). This feature can reduce PH value and prevent gap break which can do at pH 13 (31). The structural nitrite in RNT (32) produces N-nitroso compounds which have genotoxic and carcinogenic effects (33) duo to induce DNA damage to form DNA fragmentation (34). Nitrite as a free radical (35) can induced clastogenic activity (36)(37). This activity created MN in bone marrow (38). So, RNT produce MN at different concentration (39). Figure (1) showed different chromosomal aberrations while figure (2) showed MN formation on mice bone marrow.

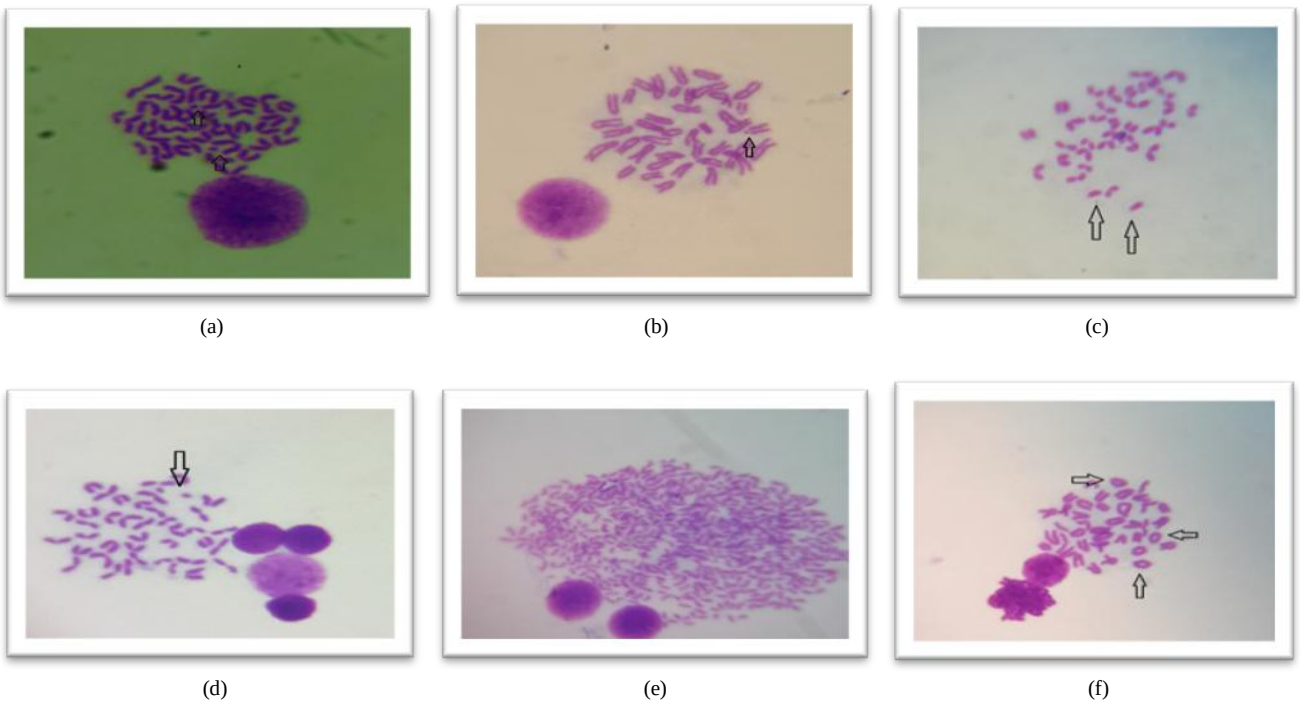

Figure 1. Chromosomal aberrations in mice bone marrow at (100X). (a) Break, (b) Gap, (c) Fragment, (d) Acentric Fragment, (e) polyploidy, (f) Ring

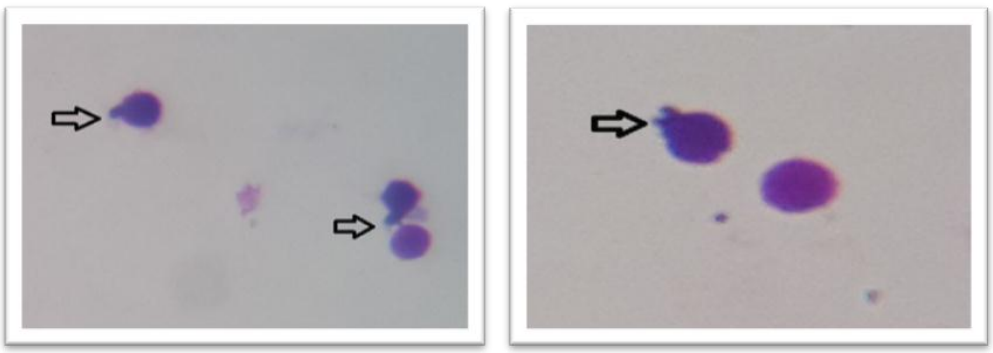

Figure 2. Formation Micronuclei in mouse stem cells at (100 X)

The animals were induced with single dose of ASNase at one day also occurred significant reduction in MI and increase in CAs and MN when compared with negative control. Long et al., (2001) demonstrated that ASNase have acute toxicities effect on peripheral leukocyte count and blood platelet count of normal mice (40). Catalyzes the hydrolysis of a L-aspargain (ASN) into L-aspartic acid and ammonia (15) by ASNase compensate in normal tissues by convert L-aspartic acid to ASN undergo action of asparagines synthetase and thus conventions L-glutamine (Gln) as the amine acid donor (41). ASNase have glutaminase side activity which mean deamidates Gln to L-glutamic acid (Glu) and ammonia (42). Gln and ASN are precursors of biosynthesis of purines and pyrimidines for DNA replication. Depletion in Gln and ASN by effect of ASNase could be significant inhibition of protein and DNA synthesis (43), then cells stopped in G1-phase of cell cycle that lead to decline in mitotic cells and MI value (16). That agreement with the result of current study. Furthermore, ammonia which released from hydrolysation of ASN and Gln, might contribute to the cytotoxicity of ASNase treatment (44). ASNase produce DNA fragmentation (45) and effect on protein synthesis by depletion asparagine which essential factor required for protein synthesis that can effect on packing protein synthesis (15). Also reduce the ability of cells to repair DNA damage one of the ASNase treatment disadvantage (46). All that possible created cytogenetic effect on mice bone marrow.

All treatment groups for MI, CAs and MN showed significant difference when compared with negative control, RNT and ASNase corresponding to it at ($p<0.05$). These results may duo to stimulate ASNase to action and at the same time inhibit the effectiveness of RNT. ASNase actions which stop cells in G1-phase of cell cycle (15) were enhanced by thiol compound (47). RNT contains a sulfur atom in their structure, this atom is effective and has scavenger properties (48). RNT also considered hydrophilic cationic drug (30), That lead to conceived that sulfur atom and hydrophilic traits may effect on activator site of the enzyme specially ASNase have the thiol group binding domain with high affinity towards free-SH group containing effectors and the most hydrophilic compounds would bind more effectively to the activator site of ASNase and convert it from one conformation to another to be more active (47). Add more inhibit the effectiveness of RNT back to ASNase antioxidant activity against nitrite and can remove their effect on cells (49). So, nitrite impact in RNT structure removing by ASNase.

VII. CONCLUSIONS

Data conclude that the Ranitidine has considerable genetic toxicity effects on mouse bone marrow stem cells. Whereas appear decrease in MI by reduce number of dividing cells, evaluate in the MN formation and increasing occurrence of CAs. Moreover, there is a reduction in MI and increase in CA and MN caused by L-asparaginase, which gives evidence for the genotoxic effect on mouse bone marrow stem cells. As well as, the combination between Ranitidine and L-asparaginase demonstrated wide detrimental effects on mouse chromosomes stem cells, that may come back to interact action of both drugs chemicals.

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