Cytogenetic effects of magnevist and methotrexate on mice bone marrow

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التأثيرات الوراثية الخلوية للماكنفست والميثوتركسايت على نخاع عظم الفئران

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المستخلص

صممت هذه الدراسة للتعرف على التأثيرات الوراثية الخلوية للماكنفست على نخاع العظم للفئران المختبرية قبل وبعد العلاج بالميثوتريكسايت بجرعة 2.5 ملغ/ كغ. حقنت مجموعة المراقبة السلبية مع 0.2 مل من الماء المقطر. حقنت الماكنفست وحده إلى الفئران داخل الصفاق بجرعة 0.6 ملغ/ كغ لمدة 24 ساعة و 48 ساعة. وبالمثل، حقنت الميثوتركسايت فقط عن طريق الوريد بجرعة 2.5 ملغ/ كغ لمدة 24 ساعة و 48 ساعة، والتي اعتبرت الضوابط الإيجابية. وعلاوة على ذلك، اعطيت مجموعات مختلفة من الميثوتركسايت/ الماكنفست من حيث نقاط زمنية مختلفة من العلاج. وأظهرت نتائج الاستقراء عالية (P<0.05) من الانحرافات الكروموسومية وتكوين النويات الصغيرة وانخفاض في مؤشر الإنقسامية من العلاجات التالية: الماكنفست لوحده، الميثوتركسايت لوحده، الميثوتركسايت / الماكنفست سوياً، مقارنة مع مجموعة التحكم المقابلة. ومن المثير للاهتمام، أظهرت الماكنفست مع المعالجة بالميثوتركسايت أعلى حث في جميع نقاط النهاية البيولوجية من المجموعات الأخرى. واستنتجنا إلى أن تأثير المزيج العالي قد ينتمي إلى زيادة في التأثير السلبي للميثوتركسايت التي تتعززت بالماكنفست والعكس بالعكس.

الكلمات المفتاحية:معامل الانقسام, التشوهات الكروموسومية, النويات الصغيره, ماكنفست, ميثوتركسايت.

Abstract

This study was established to investigate the cytogenetic effects of magnevist on mice bone marrow before and after methotrexate (MTX) 2.5 mg/kg. The negative control group was treated with 0.2 ml distilled water. Magnevist was given alone to the mice intraperitoneally with 0.6 mg/kg for 24 hrs and 48 hrs. Similarly, MTX was only intravenously injected with 2.5 mg/kg for 24 hrs and 48 hrs, which were considered as positive controls. Moreover, different combinations of magnevist/ MTX were given in terms of different time points of treatment. Results showed high induction (p<0.05) of chromosomal aberrations (CAs) and micronuclei (MN) and reduction in mitotic index (MI) of following treatments: magnevist, MTX and magnevist/MTX combination, compared to corresponding control groups. Interestingly, the combination magnevist and MTX treatment demonstrated higher induction in all biological end points than other groups. We concluded that the high combination effects may belong to increase in the aggressiveness of MTX that enhanced by magnevist and vice versa.

Key words: Mitotic index, chromosomal aberrations, micronuclei, magnevist, methotrexate.

Introduction

The magnetic resonance imaging (MRI) contrast agents is playing an important roles to diagnose diseases, therefor demand for new MRI contrast agents, with high sensitivity and important functionalities, are necessary. Inorganic nanoparticles possess unique characteristics like, large surface area, easy surface functionalization, excellent contrasting effect, and other size-dependent properties, so they usually used as MRI contrast agents (1). Magnetic resonance imaging (MRI) has good properties that allow us to visualize the internal structure of the various organs in human body and their functions. The distinction between the soft tissues of human body can be done using MRI, which gives a better view than computed tomography (CT). Also the ionizing radiation is not used in MRI which means there is no any side effect which can arise from this radiation as in CT. The magnetic moment can be developed using magnevist, which is a paramagnetic agent (2). Magnevist (gadopentetate dimeglumine) is one of the brand names for a gadolinium-based magnetic resonance imaging (MRI) contrast agent. Magnevist classify as extracellular gadolinium-based contrast agents (Gd-CAs) most widely used contrast agents for MR imaging (3). Although there are a limitation for using chemotherapeutic drug application according to its high damage to noncancerous tissues but the evidences for therapeutic efficiency of high dose chemotherapeutic injection are increasing importantly every day (4). Methotrexate (MTX) is identified as chemotherapeutic, formerly known as amethopterin, the trade name is Trexall or Rheumatrex. MTX empirical formula is $C_{20}H_{22}N_8O_5$ and the molecular weight is 454.44 g/mol (5). It is used with low dose to treat some autoimmune diseases like psoriasis and rheumatoid arthritis and with high dose to treat different types of cancer (e.g. solid organ tumors and leukemia) (6). MTX strongly hampers the function of dihydrofolate reductase (DHFR) that helps the conversion of dihydrofolate to the active tetrahydrofolate. During the DNA synthesis, the folic acid is necessary. Therefor when using MTX, that would lead to inhibit the DNA, RNA and proteins synthesis. During the S-phase of the cell cycle, some cells (like malignant and myeloid cells, gastrointestinal and oral mucosa) are dividing very quickly, which result in replication of DNA and lead to hamper the growth and proliferation of these cells. Recent studies demonstrated that the higher cytotoxic effect of MTX appears during this phase (7). Many study also demonstrated that MTX cause high induction to chromosome aberrations (CAs), micronuclei (MN) also cause high reduction to mitotic index (MI) (8, 9). As it is known, that the bone marrow is considered as a major hematopoietic organ, which is composed of hematopoietic cells in various stages of ripeness, including erythrocytes, leukocytes and platelets (10). This study was aimed to evaluate the effects of magnevist on increasing the effect of methotrexate and vice versa.

Materials and methods
Magnevist dose and concentration

Magnevist (Payer, Germany), single dose of the magnevist was used (0.6 mg/kg). This dose adoption from leaflet belongs to Bayer HealthCare Pharmaceuticals Company. It was purchased from Al-Karamah Teaching Hospital as vial. For mouse injection (intraperitoneally), a dose of 0.6 mg/kg was prepared by diluted the drug in distilled water to prepare the required dose and concentration (11).

Methotrexate dose and concentration

Methotrexate (Ebewe, Austria) (50 mg), it was administered by intravenously, and it's obtained from Al-Karamah Teaching Hospital as vial. For mouse injection, a dose of 2.5 mg/kg was tested by diluted the drug in distilled water to prepare the required dose and concentration. Such concentration has been found to be genotoxic of bone marrow of mouse (12).

Laboratory animals

Eighty Albino Swiss male mice were gained from National Center for Drug Control and Research / Ministry of Health / Baghdad. Their ages ranged between (8-12) weeks and weighting (25 ±2) gm. They were divided into 8 groups; each group was put in separated plastic cages under optimal conditions in the animal house of College of Science-University of Wasit.

Administration of laboratory animals

All animals' studied groups were divided according to types, materials and injected time as follow:

Control group.

Group I: Negative control, (10 mice) treated with 0.2 ml of (D.W.). Mice bone marrow samples were taken for cytogenetic analysis (MI, CA, and MN).

Magnevist study groups

Group I: Positive control 1, the animals were treated with 0.2 ml magnevist 0.6 mg/kg and sacrificed after 24 hrs. (10 mice).

Group II: Positive control 2, the animals were treated with 0.2 ml of magnevist 0.6 mg/kg and sacrificed after 48 hrs. (10 mice).

Methotrexate study Groups

Group I: Positive control 1, the animals (10 mice) were treated with 0.2 ml of MTX 2.5mg/kg and sacrificed after 24 hrs.

Group II: positive control 2, the animals (10 mice) were treated with 0.2 ml of MTX 2.5mg/kg and sacrificed after 48 hrs.

The interaction studies

Group I: Treatment group 1, the animals (10 mice) were treated with Magnevist (0.6mg/kg) and Methotrexate (2.5 mg/kg) at the same time and sacrificed after 24 hrs.

Group II: Treatment group 2, the animals (10 mice) were treated with Magnevist (0.6 mg/kg) for 48 hrs. and Methotrexate (2.5 mg/kg) for 24 hrs. and then sacrificed.

Group III: Treatment group 3, the animals (10 mice) were treated with Methotrexate (2.5 mg/kg) for 48 hrs. and Magnevist (0.6 mg/kg) for 24 hrs. and sacrificed.

The mice bone marrow samples were taken for cytogenetic analysis (MI, CA, and MN).

Cytogenetic experiments

Chromosome preparation from somatic cells of the mouse bone marrow

The experiment was done according to Allen *et al* (13). Colchicine was injected 2 hr. before sacrifice. Mice were sacrificed by cervical dislocation. It was dissected and both of femur bones were excised. Bone marrow was aspirated by flushing with phosphate buffer saline (PBS) in the centrifuge tube. The suspension was flushed in the tube properly to get good cell suspension and centrifuged for 10 min at 2000 rpm. Supernatant was discarded and the pellet was treated with pre-warmed (37°C) KCl (0.56%) and shaken well. Suspension incubated in a water bath at 37°C for 20 min. Pellet was treated with freshly prepared fixative solution (Methanol: Glacial Acetic Acid, 3:1) and shacked well then centrifuged for 10 min at 2000 rpm. Fixative was repeated 3 times to get debris free white pellet. Few drops from the tube were dropped vertically on the slide. Slides were kept overnight to dry then stained with (Giemsa's stain) and observed under microscope in 40 x and then in 100 x magnifications. A total of 100 well spread metaphase plates were scored for chromosomal aberrations) gap, chromatid break, polyploidy, acentric fragment, ring and fragmentation (were counted and data of scoring was expressed as percentage chromosomal aberrations.

Cytogenetic analysis

1-Mitotic index (MI) assay

The slides were examined under high power (40x) of light microscope, and (1000) of divided and non-divided cells were counted and the percentage rate was calculated for only the divided ones (metaphase cells) according to the following equation:-

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

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.

3-Micronucleus MN assay

This experiment was done according to method of Schmid (14) as follow:-

The femur bone cleaned from tissues and muscles, then gapped from the middle with a forceps in a vertical position over the edge of a test tube by a sterile syringe, (1 ml) of human plasma (heat inactivated) was injected so as to wash and drop the bone marrow in the test tube. Then the test tubes were centrifuged at speed of 1000 rpm (5 min). The supernatant was removed, and one drop from the pellet was taken to make a smear on a clean slide. The slides were kept at room temperature for (24 hr.). The slides were fixed with absolute methanol for (5 min.), then stained with Giemsa stain for (15 min), then washed with D.W and left to dry. Two slides for each animal were prepared for micronucleus test. The slides were examined under the oil immersion lens, and at least 1000 polychromatic erythrocytes (PCE) were examined for the presence of micronucleus. The micronucleus index was obtained using the following equation:

$$\text{Micronucleus index} = \left(\frac{\text{Number of micronucle i}}{\text{Total count of PCE}} \right) \times 100$$

Statistical analysis

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (Two-sample T-test) using the computer program Minitab release (14.12) discovery Copyright 2004. The difference was considered significant when the probability value was less than $p < 0.05$.

Results and discussion

The results of metaphase test are presented in table (1). There are significant differences when we compare between negative control and positive control (I, II, III and IV) and these differences were due to the toxic effect of MTX alone and magnevist alone too by reducing the mitotic index (MI). And also there is a significant different when we compare the treatment groups (I, II and III) with negative control (0.2 ml of D. W.). All these results were statistically significant ($p<0.05$).

Table (1): Percentages of mitotic index in bone marrow of mice for negative control, positive control groups and treatment groups (Mean ± SE)

[a] Positive control groups vs. Negative control, [b] Treatment groups vs. Negative control,

Groups	Mitotic index % $M\pm$SE
Negative control (0.2 ml of D.W.)	6.600 ± 0.30
Positive control I (Magnevist for 24 hr.)	[a*]5.440 ± 0.43
Positive control II (Magnevist for 48 hr.)	[a*]5.540 ± 0.26
Positive control III (MTX for 24 hr.)	[a*]3.620 ± 0.12
Positive control IV (MTX for 48 hr.)	[a*]3.620 ± 0.15
Treatment group I (MTX + Magnevist for 24 hr.)	[b*]3.720 ± 0.037
Treatment group II (Magnevist 48 hr. - MTX 24 hr.)	[b*]3.700 ± 0.12
Treatment group III (MTX 48 hr. - Magnevist 24 hr.)	[b*]3.600 ± 0.11

*Significant at ($p<0.05$).

Table (2): Percentages of different types of chromosomal aberrations (CA) in bone marrow of mice for negative control, positive control groups and treatment groups (Mean ± SE)

Experimental Groups	Chromosomal aberrations %						Total %
	Acentric Fragment %	Ring %	Polyploidy %	Break %	Fragment %	Gap %	
Negative control (0.2 ml of D.W.)	5.40 ± 1.75	0.000 ± 0.000	0.000 ± 0.000	0.200 ± 0.200	5.000 ± 0.707	1.000 ± 0.447	11.60 ± 1.05
Positive control I (Magnevist for 24 hr.)	15.80 ± 2.24	1.600 ± 0.510	2.200 ± 0.970	3.200 ± 0.917	20.40 ± 3.36	10.20 ± 3.01	[a*]53.40 ± 3.23
Positive control II (Magnevist for 48 hr.)	16.00 ± 3.86	0.400 ± 0.400	1.000 ± 0.548	0.600 ± 0.400	39.40 ± 2.09	24.40 ± 6.10	[a*]81.80 ± 6.56
Positive control III (MTX for 24 hr.)	15.20 ± 3.50	2.600 ± 0.510	1.200 ± 0.374	0.000 ± 0.000	14.80 ± 3.12	9.00 ± 3.36	[a*]42.80 ± 2.79
Positive control IV (MTX for 48 hr.)	13.80 ± 1.46	1.000 ± 0.447	1.000 ± 0.548	1.000 ± 0.632	18.80 ± 3.26	27.40 ± 4.69	[a*]63.00 ± 4.60
Treatment group I (MTX + Magnevist for 24 hr.)	11.40 ± 1.47	0.800 ± 0.200	1.800 ± 0.583	9.80 ± 4.41	19.00 ± 2.37	36.60 ± 2.16	[b*]79.40 ± 5.42
Treatment group II (Magnevist 48hr. - MTX 24 hr.)	18.60 ± 0.400	0.200 ± 0.200	1.600 ± 0.510	0.600 ± 0.400	37.00 ± 4.92	27.00 ± 7.01	[b*]85.00 ± 6.44
Treatment group III (MTX 48 hr. - Magnevist 24 hr.)	12.40 ± 2.38	0.000 ± 0.000	1.000 ± 0.316	5.60 ± 2.25	37.40 ± 4.12	33.40 ± 6.29	[b*]89.80 ± 6.72

[a] Positive control groups vs. Negative control, [b] Treatment groups vs. Negative control, *Significant at ($p < 0.05$).

Table (3): Percentages of micronuclei (MN) in bone marrow of mice for negative control, positive control groups and treatment groups (Mean ± SE)

Groups	Micronucleus % $M \pm SE$
Negative control (0.2 ml of D.W.)	2.620 ± 0.12
Positive control I (Magnevist for 24 hr.)	[a*]6.620 ± 0.17
Positive control II (Magnevist for 48 hr.)	[a*]7.440 ± 0.21
Positive control III (MTX for 24 hr.)	[a*]6.380 ± 0.20
Positive control IV (MTX for 48 hr.)	[a*]7.240 ± 0.19
Treatment group I (MTX + Magnevist for 24 hr.)	[b*]9.140 ± 0.34
Treatment group II (Magnevist 48hr. - MTX 24 hr.)	[b*]9.420 ± 0.18
Treatment group III (MTX 48 hr. - Magnevist 24 hr.)	[b*]9.380 ± 0.17

[a] Positive control groups vs. Negative control, [b] Treatment groups vs. Negative control, *Significant at ($p < 0.05$)

The results of chromosomal aberrations present in table 2. Animals treated with magnevist positive control (I and II) with dose (0.6 mg/kg) showed a high frequency of total chromosomal aberrations (53.4%, 81.8%) respectively in mice bone marrow cells, these findings were significant ($p < 0.05$) when compared with negative controls (11.6%). As well as, the animals that treated with MTX positive control (III and IV) demonstrated significant differences (42.8%, 63%) respectively in mice bone marrow cells, when compare with negative control. When the same dose of magnevist was given together with MTX, chromosomal aberrations were increased, these results showed significant value ($p < 0.05$), there was an induction for all treatment groups when compared with negative control.

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 ($p < 0.05$). The positive control (I and II) showed a significant reduction in MI and a high increase in CAs and MN. The reason for this results was due to the toxic effect of magnevist that cause DNA damage by produce hydroxyl radicals (•OH), this suggested by Yamazaki *et al* (15) and Kim (16). The cytotoxic and mutagenic impact for •OH can react with the deoxyribose DNA backbone and bases. Thus, it is probably cause a lot of lesions. The react which happen between •OH and DNA bases by add pi bonds to the electron-rich. The pi bonds are situated between C5-C6 of pyrimidines and N7-C8 in purines (17). The first event that appeared after the reaction between •OH with the deoxyribose sugar backbone represented by the removal of hydrogen from a deoxyribose carbon [18]. Thus creates a 1'-deoxyribosyl radical. After that, the radical may react with molecular oxygen and resulting in a peroxyl radical, which can be reduced and dehydrated to form a 2'-deoxyribonolactone and free base. A deoxyribonolactone is mutagenic and resistant to repair enzymes. Thus, a basic site is created (19). Fiechter and his colleagues showed in their study on cardiac magnetic resonance imaging (CMR) on human lymphocyte DNA integrity, here the aim of this study was to analyze DNA double-strand breaks (DSBs) in human blood lymphocytes before and after CMR examination with using gadolinium contrast agents and he found that the DNA damage was back to gadolinium contrast agents (20). The figure below showed different chromosome aberrations from mice bone marrow for positive control (I and II).

(A)   (B)

(C)   (D)
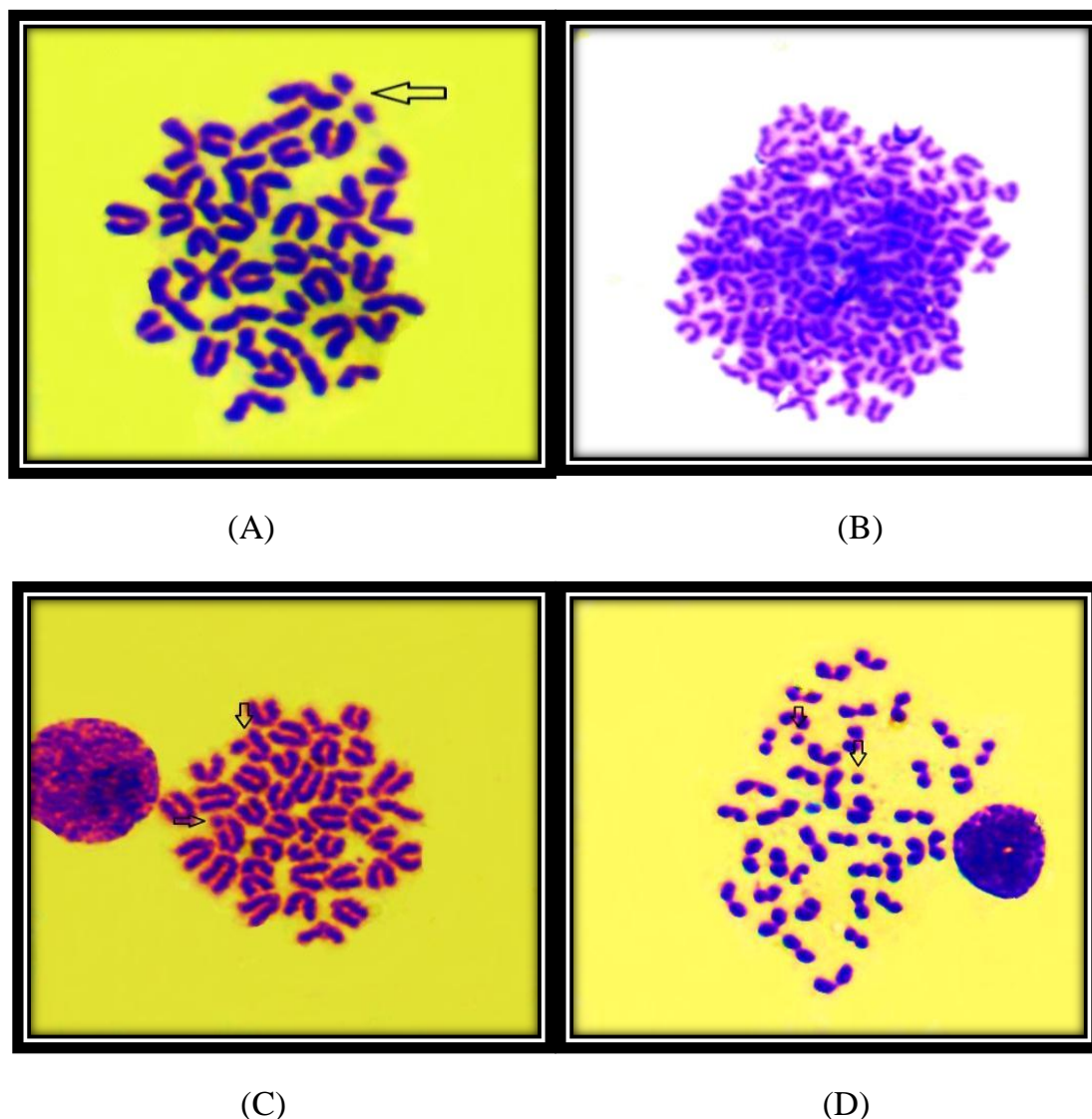
Figure (1): Chromosomal aberrations in mice bone marrow at (100x) injected only with magnevist. A: acentric fragment B: polyploidy C: break D: fragment.

In the positive groups (III and IV), there is a clear increase in the value of CAs and MN and a significant reduction in MI when compared with negative control. The reduction of MI for mice that treated with single dose (2.5 mg/kg) is consistent with the results of Jafer and Shubber (9) that showed decreasing in MI were observed after treatment with MTX because of resistance lymphocytes to the drug (MTX), arise as a result of a mutation in the gene site for dihydrofolate reductase (DHFR) (21). On the other hand, Jalal (8) reported that MTX cause decrease in MI because of the toxic effects of the MTX due to its ability to overlap with the genetic material DNA from through the lack of an enzyme (DHFR) key in the process of growth and cell division (22). The results of CAs were in agreement with Al-Shimary who indicated that the MTX has the ability to induce the chromosomal abnormalities (23). Also these results are in agreement with the results of Hassan *et al* who

reported that MTX is one of chemotherapeutic drugs that also cause increase in chromosomal aberrations (24). As well as, these results were supported by Jalal (8) when showed an increase in CAs due to toxic effects of the drug through the lack of an enzyme (DHFR) key as it leads to deplete rules nitrogen deficient oxygen triple phosphate (dNTP) involved in building DNA, resulting in an objection reform process damage the automatic molecule DNA in addition to it leads to damage to this molecule, which leads to chromosomal changes (25). The study of Hemeida and Mohafez showed a strong evidences support a role for reactive oxygen species (ROS) in the pathogenesis of MTX damages (26). The significant increase in MN to the positive groups (III and IV) were due to the intracellular accumulation of the drug resulting in a continuous inhibition of deoxyribonucleotide triphosphate (dNTPs) synthesis, subsequently causing genetic lesions due to the inhibition of DNA repair. However, because of an insufficient of dNTPs remain, the genetic lesions induced by MTX are expressed by micronuclei (27, 28, and 29). Our results almost similar to the results of Novakovic *et al* who studied the effect of methotrexate in vivo on frequency of micronuclei in peripheral blood lymphocytes (30). Figure 2 demonstrate the formation of micronucleus of mice bone marrow by magnevist.

All treatment groups for CAs and MN showed significant difference when compared with negative control and positive controls corresponding to it at (p<0.05). Significant increase in chromosomal aberrations back dramatically to nested action of MTX and Gd-DTPA, which Gd-DTPA was produced hydroxyl radicals and elevated cellular oxidative stress, MTX effect on the antioxidant enzymes by inhibiting its action, this led to increasing and inducing free radicals. Additionally, the effect of MTX on the cell cycle (especially S phase). The significant differences between treatment groups with negative control and positive control groups corresponding to them, may to the nested effect of MTX and Gd-DTPA, which Gd-DTPA induce micronuclei and MTX cause an increase in MN because it's effect on the synthesis of DNA and cause chromosome damage (CAs), thus induce MN formation.
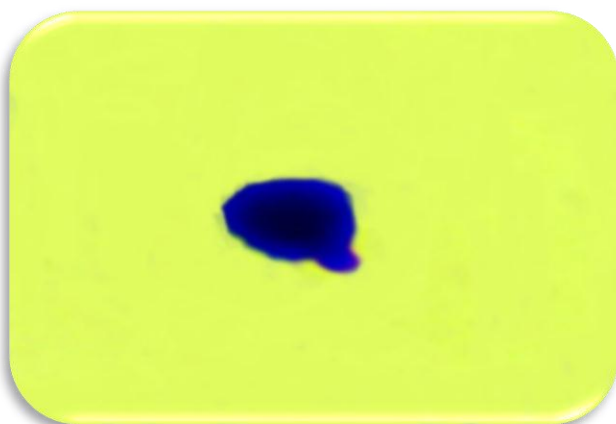
Figure (2): Magnevist formation MN at (100x).

Conclusions

Data conclude that the magnevist is a genotoxic contrast agent, which shows significant genotoxic effects on mice bone marrow stem cells. Moreover there is a significant reduction in MI and significant increasing in CA and MN caused by methotrexate, which gives evidence for the genotoxic effect of methotrexate in mouse bone marrow stem cells. As well as, both magnevist and methotrexate as a combined treatment demonstrated a very aggressive effects on mice stem cells, which may belongs to the interact action of both chemicals.

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