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Evaluation of Micronucleus, Nuclear Division Index and Sister Chromatid Exchanges in Human Lymphocyte for Local Samples of Al-Tuwaitha Region-Iraq

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Abstract: During the period February 2007 till May 2008. Micronucleus (MN) in binucleated lymphocytes, nuclear division index (NDI) and sister chromatid exchanges (SCEs) were performed among 183 persons living at Al- Tuwaitha region surrounding previously to the Iraqi Atomic Energy commission .This number included sixty samples from Eshtar village, aged 15 - 50 years, thirty one samples from Al-Wardia region, aged 17-61 years, fifty two samples from Al-Readh region, aged 18-62 years and forty samples from Al-Tameem region, aged 18 - 58 years. Control group, which included 100 samples aged 16 - 68 years from other regions of Baghdad was also studied. The results of the frequencies of MN and SCE were revealed a significant increase ($p<0.05$) in the males and females of human lymphocyte in these regions of studies as compared with the control group. While the results of the average of NDI were significant decrease ($p<0.05$) in the males and females of human lymphocyte in these regions of studies as compared with the control group. In addition, the results of MN , NDI and SCE for various ages and genders were compared in the studied groups. In conclusion, the results of our experiment suggest that the accumulation of genetic damage is detectable in peripheral lymphocytes of local samples of Al-Tuwaitha region. As well as, the increase frequencies of MN and SCE indicate the cumulative effect of low-level chronic exposure to radiological and chemical materials. The current results of MN and NDI frequency were within normal values according of the technical report of International Atomic Energy Agency (IAEA) No. 405 , 2001.

Key words: Cytokinesis-block micronucleus assay; Sister chromatid exchanges; Ionizing radiation; Human lymphocyte.

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تقييم فحوصات النوى الصغيرة، معامل الانقسام النووي والتبادل الكروماتيدي الشقيقي في الخلايا اللمفاوية لدم الإنسان لعينات محلية من منطقة التويثة–العراق

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الخلاصة: خلال المدة من شباط 2007 حتى أيار 2008، أجريت فحوصات النوى الصغيرة MN ،معامل الانقسام النووي NDI والتبادل الكروماتيدي الشقيقي في الخلايا اللمفاوية لدم الإنسان لـ 183نموذجا من أشخاص أصحاء طبيعيين يسكنون منطقة التويثة المحيطة بمنظمة الطاقة الذرية العراقية سابقا، وشملت هذه الدراسة 60 نموذجا من قرية عشتار بعمر 15 – 50 سنة ، 31 نموذجا من منطقة الوردية بعمر 17–61 سنة ، 52 نموذجا من منطقة الرياض بعمر 18– 62 سنة و40 نموذجا من منطقة التأميم بعمر 18– 58 سنة، إضافة إلى مجموعة السيطرة والتي شملت 100 نموذجا بعمر 16– 68 سنة من مناطق أخرى من أحياء بغداد. أظهرت النتائج وجود زيادة معنوية ($p<0.05$) في معدل تردد النوى الصغيرة MN في الخلايا اللمفاوية ثنائية النواة والتبادل الكروماتيدي الشقيقي لذكور وإناث المناطق المدروسة مقارنة مع مجموعة السيطرة. بينما لوحظ انخفاض معنوي ($p<0.05$) في معامل الانقسام النووي NDI في الذكور والإناث في هذه المناطق مقارنة مع مجموعة السيطرة. إضافة إلى ذلك، قورنت نتائج النوى الصغيرة ومعامل الانقسام النووي والتبادل الكروماتيدي الشقيقي لمختلف الفئات العمرية والجنس في للمجاميع المدروسة.نستدل من نتائج الدراسة وجود ضرر تراكمي في المادة الوراثية في الخلايا اللمفاوية لعينة من الساكنين في منطقة التويثة وقد تشير هذه الزيادة في المؤشرات المدروسة إلى التأثيرات المتراكمة من التعرض لجرع واطئة من المواد المشعة او الكيميائية. علما أن النتائج الحالية بالنسبة إلى تردد النوى الصغيرة ومعامل الانقسام النووي في الخلايا اللمفاوية ضمن المعدلات الطبيعية حسب التقرير الفني للوكالة الدولية للطاقة الذرية المرقم 405 لسنة 2001.

Introduction

Cytogenetic methods have become a valid tool to assess radiation damage and to support triage, medical treatment decisions, and prognosis of radiation casualties. Recently, several new biodosimetric methods overcoming some of these limitations have been proposed, including protein marker (analyses (1, 2, 3) and gene expression biomarker for application to ionizing radiation (4) .Micronuclei are formed from chromosomal fragments or lagging chromosomes at an anaphase (due to mitotic spindle damage) which are not included in the nuclei of the daughter cells. They are therefore seen as distinctly separate objects within the cytoplasm of the daughter cells (5,6). The in vitro cytokinesis-block micronucleus (CBMN) assay is a cytogenetic method based on the assessment of micronuclei in nucleated cells that have completed only one nuclear division (7,8). Sister chromatid exchange (SCEs) analysis is widely used to assess genetic damage, in spite of the fact that the mechanism involved in SCE origin and formation is not well understood. It is generally accepted that SCE represents the interchange of DNA replication products at homologous loci (9). The modulation of SCE frequencies has been used to detect potentially carcinogenic and mutagenic agents, and an increased

frequency of SCE can be an indicator of persistent DNA damage (10). Agents that induce SCE are also capable of inducing chromosomal aberration (11), but the reciprocal phenomenon may not be valid (12) .

The nuclear division index (NDI) is a marker of cell proliferation in cultures which is considered a measure of general cytotoxicity, the relative frequencies of the cells may be used to define cell cycles progression of the lymphocyte after mitogenic stimulation and how this has been affected by the exposure, the index is in itself not sufficiently robust for direct application as a biodosimeter. Nevertheless the assay is frequently employed as a useful research tool for in peripheral blood lymphocytes in order to detect the effect of radiological and chemical hazard in human blood of Al-Tuwaitha region.

Materials and Methods

Study population

In March 2007- June 2008 the researcher examined 183 healthy donor inhabited living the Al-Tuwaitha region which surround to the Iraqi Atomic Energy Commission (previously). These samples included 60 individuals selected randomly from population living in the Eshtar village (aged 15-50 years), 31 individuals selected randomly from population living in Al-Wardia region (aged 17-61 years), 52 individuals selected randomly from population living in Al- Raʿad region in Jesr-Diyala (aged 18-62 years), 40 individuals selected randomly from population living in Al-Tameem region (aged 18-58 years), compared with a sample consisted of 100 healthy normal individuals collected randomly from understanding the cell cycling kinetics of the cultures. It will indicate perturbations that may be caused by exposure to a mutagen such as radiation (13, 14). The lowest NDI value is 1.0, which occurs if all of the viable cells have failed to divide during the cytokinesis-block period and so, all will be mononucleated. If all viable cells complete one division there will be all binucleated, the NDI value can be greater than 2.0 if certain viable cells have completed more than one nuclear division during the cytokinesis-block phase and therefore contain more than two nuclei (8).

The aims of the present study was to determine MN frequency , NDI and, SCEs

population living in Baghdad a from way Al-Tuwaitha nuclear site (aged 16-68 years). Prior to the study, a questionnaire on life style, such as occupational history,smoking and drinking habits, health status, diet, etc., was applied to all individuals.

Cytogenetic procedures

All cytogenetic tests were performed by the standard cytogenetic procedure (15, 16) with minor modifications made in our laboratory. Heparinized whole blood samples were collected by venipuncture and cultured simultaneously for two different genotoxic analyses: sister-chromatid exchanges (SCEs), and micronucleus test (MN). Briefly, 0.5ml blood to 4.5 ml culture RPMI-1640 culture medium (sigma) supplemented with 20% fetal bovine serum (sigma) ,100 UI/ml penicillin (Sigma-Aldrich) and 0.1 mg/ml streptomycin (Sigma-Aldrich). Phytohemagglutinin (PHA) at a concentration of 10 µl/ml was used to stimulate lymphocyte proliferation. Blood

cultures were incubated at 37°C for 72 hours. Cytochalasin B (Sigma) was added 44 h after PHA stimulation at a concentration of 4.5 µg/ml to block cells at cytokinesis. At 72 h of incubation, the lymphocytes were harvested by centrifugation and fixed with methanol: acetic acid (3:1). The slides were prepared and stained with stained in 4% Giemsa solution for 20 min. In samples seeded for SCE test, the cells were cultured in the presence of 10 mg/ml bromodeoxyuridine (Sigma), to cultures and incubated at 37°C for 72 hours.. Lymphocyte cultures of SCE samples were stopped at second mitotic division with colchicine (final concentration: 16 mg/ml) which was added three hour before harvesting. Differential staining of sister chromatids was performed using fluorescence-plus-Giemsa (FPG) technique with Hoechst 33258 stain (Sigma) (9) .

Scoring criteria

Micronucleus test

Cytokinesis-block micronucleus assay was performed analyzing more than 500 binucleated cells was evaluated for the frequency of MN using 400 x magnification for surveying the slides while 1000 × magnification was used to confirm the presence or absence of MN in the cells. A total of 1000 living interphasic cells were used for assessment of mono-, bi-,and poly-nucleated cells and NDI was calculated according the formula: NDI = (M1 + 2 x M2 + 3 x M3 + 4 x M4) /N where M1-M4 represents number of cells with one to four nuclei founded, respectively, and N is the total number of scored cells (16).

Sister-chromatid exchanges test

The slides of SCE were prepared, as described previously (15), 50 metaphase chromosomal sets with well-differentiated sister chromatids per sample were analyzed. Counted chromatid exchanges between sisters chromatids were presented as mean number per cell and the standard error were calculated. Double (and multiple) exchanges of sister chromatids that occurred on same chromosomal arms were also counted and presented as average numbers per cell.

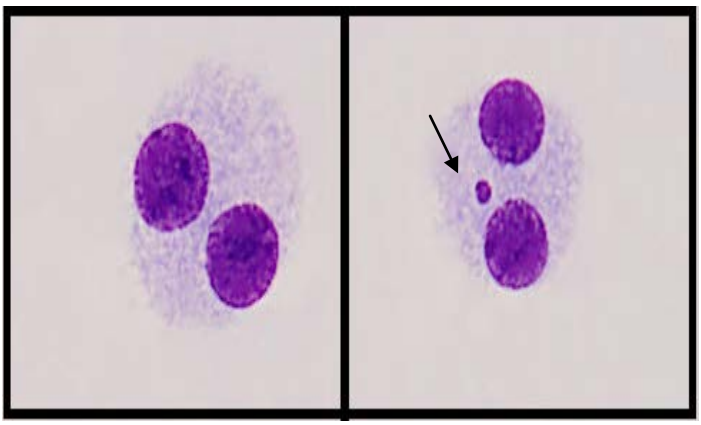
Data Analysis and Statistics

The data of this study were compiled into the computerized data file and frequency, distribution and statistical description (Mean, SE) were divided using SPSS statistical software. We used statistical analysis of variance (ANOVA) test and least significantly difference (LDS) test by probability of less than 0.05 ($p<0.05$) according to (17).

Results and Discussion

The frequencies of MN frequency ,SCE and NDI were performed on peripheral blood lymphocytes which were obtained from 183 individuals of resident living the Al-Tuwaitha region such as Eshtar village, Al-Wardia, Al-Raʿad and Al-Tameem region, then compared with 100 individuals control living in Baghdad a way from Al-Tuwaitha nuclear site. Micronuclei (MN) are formed from lagging chromosomal fragments or whole chromosomes at anaphase which are not included in the nuclei of daughter cells (Figure 1). They are therefore seen as distinctly separate small spherical objects that have the same morphology

and staining properties of nuclei, within the cytoplasm of the daughter cells (18) . SCEs frequencies were scored in metaphase chromosomes were identified by fluorescent plus giemsa staining (Figure 2).



-A- -B-

Figure (1): Binucleated lymphocyte cell without (A) and with 1 micronuclei (B) (1000X)

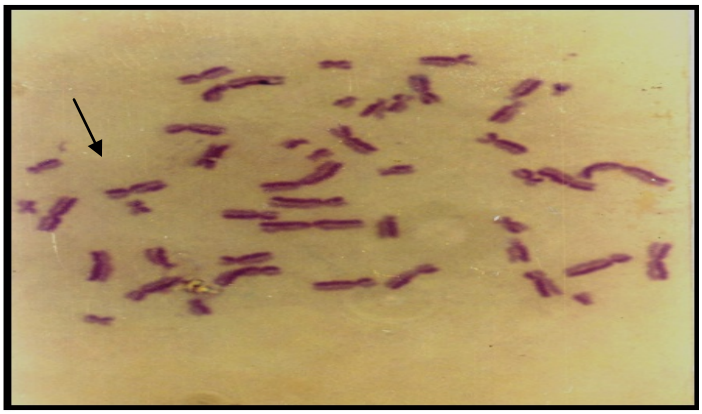
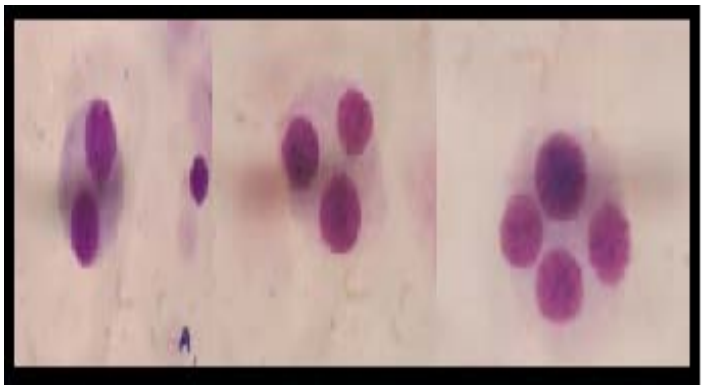


Figure (2): Microphotographs of sister chromatid exchanges in metaphase chromosomes by fluorescent plus Giemsa staining (1000X)

The NDI assay was performed according to the description by (14) , the NDI was calculated binucleated, trinucleated and quadrinucleated lymphocyte cell per 1000 lymphocytes (Figure 3).



-A- -B- -C-

Figure (3): Cytokinesis blocked human lymphocyte cell,(A) : Binucleated cell, (B): Trinucleated cell and (C): Qudrinucleated lymphocyte cell (1000X)

The mean values of SCE, MN and NDI of populations of resident living the Al-Tuwaitha region such as Eshtar village, Al-Wardia, Al-Raʿad and Al-Tameem region and control population are presented in Table 1-4. Both groups of populations of resident living the Al-Tuwaitha region had higher values of SCE and MN and lower NDI values in comparison with healthy subjects. The results of the frequency of MN measured in binucleated lymphocyte cell in residents living the these region surround to Al- Tuwaitha nuclear site and control group are summarized in Table 1..The average of MN per cell (Mean ± SE) for Eshtar village, Al-Wardia, Al-Raʿad and Al-Tameem region were 0.024 ± 0.0007 , 0.025 ± 0.0013 , 0.027 ± 0.0008, and 0.026 ± 0.0011 MN/cell , respectively when compared with the control 0.010 ± 0.0004 MN/cell. A significant increase ($P< 0.05$) in MN frequency was observed in this region as compared with the control. However, no significant variation ($P > 0.05$) was observed in MN frequency of Eshtar village ,Al-Wardia, Al-Raʿad and Al-Tameem region, (Table 1).

Micronuclei in mitotically active cells arise from structural chromosomal aberrations or disturbed function of mitotic spindle. That is why some authors consider that the follow up of MN frequencies in peripheral blood lymphocytes in the samples of human individuals could be a very effective test to estimate the effects of biological, physical and chemical agents (8) .According to our results, the MN background level is 8- 35 which is in agreement with IAEA manual reporting the background MN values to range 2 to 40 per 1000 BN cells (15) and other studies (13, 19, 20, 21). These results suggest that the frequency of MN can be used as a potential biomarker for assessing a specific environmental risk. Furthermore, studies of mutations at MN frequency have provided insights into several aspects of somatic mutations in vivo, including molecular mechanisms of mutagenesis, the relationship between DNA damage and mutation, as well as individual susceptibility factors such as DNA repair capacity (22,23) .

Table (1): Frequencies of MN, SCEs and NDI in the males and females of human lymphocyte resident living the Al- Tuwaitha region and control Compared to Control Individuals

Region	Age (year)	No. of samples	MN / Cells (Mean ± SE)	Nuclear Division Index (NDI) (Mean ± SE)	SCEs/cell
Eshtar Village	15-50	60	0.024±0.0007 b	1.150±0.0071 b	6.2 ± 0.1 a
Al-wardia region	17-61	31	0.025±0.0013 b	1.138±0.0132 b	6.28 ± 0.22 a
Al-Ra'ad region	18 -62	52	0.027±0.0008 b	1.146±0.0081 b	7.38 ± 0.20 b
Al-Tameem region	18-58	40	0.026±0.0011 b	1.164±0.0092 b	7.48 ± 0.45 b
Control (Baghdad)	16-68	100	0.010±0.0004 a	1.312±0.0114 a	5.9 ± 0.2 a

♦Similar latter in a column (for comparison between regions) mean there is no significant difference ($p < 0.05$), according to Duncan test.

The mean frequencies of SCEs per cell (Mean ± SE) for Eshtar village, Al-Wardia, Al-Raʿad and Al-Tameem region were 6.2 ± 0.1, 6.28 ± 0.22 , 7.38 ± 0.20 and 7.48 ± 0.45 SCEs / cell , respectively when compared with the control 5.9 ± 0.2 SCEs/cell.The values of SCEs per cell differed significantly but without statistical significance ($p < 0.05$) between populations of resident living the Al-Tuwaitha region such as Eshtar village, Al-Wardia, Al-Raʿad and Al-Tameem region and control population (Table 1). It is well established that SCE arises during replication of a damaged DNA and SCE assay is a sensitive method for identifying chemical and physical DNA-damaging agents (24) .

progression of the lymphocyte after mitogenic stimulation and how this has been affected by the exposure (13,14) .

Also, in the table 1 shown that the average of NDI (Mean ± SE) for Eshtar village , Al-Wardia, Al-Raʿad and Al-Tameem region were 1.150 ± 0.0071 , 1.138 ± 0.0132 , 1.146 ± 0.0081 and 1.164 ± 0.0092, respectively when compared with the control 1.312±0.0114 A significant decrease ($P > 0.05$) in NDI was observed in this region as compared with the control (Table 1). The nuclear division index as biomarker of cell proliferation in cultures which is considered a measure of general cytotoxicity, the relative frequencies of the cells may be used to define cell cycles.

The Frequencies of MN , SCEs and NDI in peripheral blood lymphocytes in the male of resident living the Al-

Tuwaitha region than the males of control group are shown in table 2. The average of MN frequency was significant higher (P< 0.05) in the males in this region as compared with the males of control group, but the difference between Eshtar village, Al-Wardia, Al-Raʿad and Al-Tameem region for MN and SCEs were no significant (P > 0.05). Also, the average of NDI was significant decrease (P> 0.05) in the male of resident living the Al- Tuwaitha region when compression to average of NDI in the males of control group, but the NDI average difference between Eshtar village, Al-Wardia, Al-Raʿad and Al-Tameem region were no significant (P > 0.05). The increase frequencies of MN, SCEs and NDI indicate the cumulative effect of low-level chronic exposure to radiological and chemical materials. The current results of frequency MN , SCEs and NDI within of normal values according of the technical report of International Atomic Energy Agency (IAEA) No. 405 , 2001. The risk of cancer formation associated with increased micronucleus frequencies in human population is under intensive investigation, and it is of significant importance to evaluate related biomedical changes possibly incurred in these exposed people. The presence of an interaction between smoking habit and occupational exposure to genotoxic agents should be always tested.

Table (2): Mean ± SE of MN /Cell and NDI in the males of human lymphocyte resident living the Al- Tuwaitha region and control

Region	Age (year)	No. of samples	MN / Cells (Mean ± SE)	Nuclear Division Index (NDI) (Mean ± SE)	SCEs/cell
Eshtar Village	15-50	40	0.025±0.0009 b	1.142±0.0080 b	6.0 ± 0.09 a
Al-wardia region	18-60	18	0.024±0.0013 b	1.137±0.0155 b	6.11 ± 0.20 a
Al-Ra'ad region	18 -62	30	0.027±0.0012 b	1.144±0.0106 b	7.00 ± 0.10 b
Al-Tameem region	18-55	24	0.024±0.0013 b	1.164±0.0129 b	7.18 ± 0.30 b
Control (Baghdad)	16 -62	47	0.009±0.0005 a	1.312±0.0157 a	5.8 ± 0.1 a

♦Similar latter in a column (for comparison between regions) mean there is no significant difference (p < 0.05), according to Duncan test.

(Table 3) shown that the SCE and MN were significant increase (P< 0.05) in the females of human lymphocyte resident living the Al- Tuwaitha region as compared with the females of control group. Also, the MN showed different significant (P < 0.05) in female of human lymphocyte resident living in the Al-Wardia, Al-Raʿad and Al-Tameem region, as compared with the female of human lymphocyte residents living in Eshtar village .while the SCE showed different significant (P < 0.05) in female of human lymphocyte resident living in the Al-Raʿad and Al-Tameem region, as compared with the female of human lymphocyte residents living in Eshtar village and Al-Wardia (Table 3). In the present study, the MN frequency increase in the population of the Al-Tuwaitha region, due to the presence of low doses radioactive materials in this region because of its proximity to the nuclear site in that region. Micronuclei frequency increases as the age naturally; there are well known ageing events which take place in the genetic material, too, As we could notice in our study, in males the process is going differently than in females, where the MN frequency is even more intense, the events taking place in direct connection with the exposure duration to the harmful environment. In the study of detection of radiological and chemical of public exposure to uranium-235 at Al-Tuwaitha nuclear research site (24) . Moreover, the two most important factors influencing MN background frequency, besides dietary factors (25) and exposure to a wide range of environmental clastogens and aneugens, are age and gender (26, 27) .

Table (3): Mean ± SE of MN /Cell and NDI in the females of human lymphocyte resident living the Al- Tuwaitha region and control

Region	Age (year)	No. of samples	MN / Cells (Mean ± SE)	Nuclear Division Index (NDI) (Mean ± SE)	SCEs/cell
Eshtar Village	17-45	20	0.022±0.0011 c	1.165±0.0137 b	6.15 ± 0.10 a
Al-wardia region	17-61	13	0.025 ± 0.0013 b	1.140±0.0237 b	6.18 ± 0.2 a
Al-Ra'ad region	19-61	22	0.027±0.0012 b	1.149±0.0128 b	7.11 ± 0.12 b
Al-Tameem region	25-58	16	0.027±0.0021 b	1.164±0.0130 b	7.24 ± 0.20 b
Control (Baghdad)	17 -68	53	0.010± 0.0006 a	1.311±0.0156 a	5.9 ± 0.1 a

♦Similar latter in a column (for comparison between regions) mean there is no significant difference (p < 0.05), according to Duncan test.

Also, the average of NDI was significant decrease (P > 0.05) in the female of human lymphocyte resident living the Al- Tuwaitha region when compression to average of NDI in the males of human lymphocyte control group .(Table 4), but the NDI average difference between Eshtar village, Al-Wardia, Al-Raʿad and Al-Tameem region were no significant (P> 0.05). Decrease in the NDI shows increased cytotoxicity of chemotherapy, the tested of NDI based on the fact that this marker estimates general toxicity (14,28) . The proportion of binucleated cells may be used as a biomarker of the lymphocytes mitogen response, immune functions and cytostatic effects of various studied agents (29). (Table 5) shown that the comparison between gender and MN , SCE and NDI requencies (Mean ± SE) in the human lymphocyte resident living the Al-Tuwaitha region and control group. The separation on genders shows that MN mean was significant increase (P< 0.05) in the males of residents living in Eshtar village as compared with females in this group and controls. Also, the MN showed no significant difference (P> 0.05) between males and females in the Al-Wardia, Al-Raʿad and Al-Tameem region and control., while, the separation on genders shows that MN and SCEs mean is no significant (p < 0.05) in females than in males of control group.

Agents that directly induce DNA strand breaks (like physical agents) are weak inducers of SCE, in contrast to S-dependent agents, which efficiently induce SCE and CA (30). However, it has been reported that human populations exposed to ionizing radiation. also present increased frequencies of SCE (31,32). In spite of many reports in the literature about SCE analysis, neither the biological effect of SCE nor the mechanisms that lead to their formation are clearly understood. The present results observed for the radiation workers indicate an increase in the SCE frequencies in parallel to higher CA frequencies, which can be attributed to the low level of radiation exposure.

These MN values are in agreement with the large scale study of Fenech (16) of variables influencing baseline micronucleus frequencies: 0.31 MN / 1000 / year. For a female control population a more prominent increase of 0.58 MN /1000 /year was found (33, 34). Regarding NDI , statistical analysis showed that there was no significant difference (P > 0.05) between males and females in each Al- Tuwaitha region and control groups (Table 5). The difference of MN and NDI frequencies may be due to a different sensitivity to environmental mutagen for males and females. This result of the current study has been compatible with other studies examining the effect of gender found no evidence of any difference (14, 34.35) , even by age group.

Table (4): Comparison between gender and MN /Cell and NDI (Mean ± SE) in males and females of human lymphocyte resident living the Al- Tuwaitha region and control

Region	Age (year)	No. of samples	Gender	MN / Cells (Mean ± SE)	Nuclear Division Index (NDI) (Mean ± SE)	SCEs/cell
Eshtar Village	17-45	20	♀	0.022 ± 0.0011*	1.165±0.0137	6.15 ± 0.10
	15-50	40	♂	0.0009 ± 0.026	1.142±0.0080	6.0 ± 0.09
Al-wardia region	61-17	13	♀	0.0016±0.027	1.140±0.0237	6.18 ± 0.2
	18-60	18	♂	0.0013±0.025	1.137±0.0155	6.11 ± 0.20
Al-Ra'ad region	19-61	22	♀	0.0011±0.027	1.149±0.0128	7.11 ± 0.12
	18-62	30	♂	0.001±0.027	1.144±0.0106	7.00 ± 0.10
Al-Tameem region	25-58	16	♀	0.0023±0.029	1.164±0.0130	7.24 ± 0.20
	55-18	24	♂	0.0018±0.029	1.164±0.0129	7.18 ± 0.30
Control (Baghdad)	17-68	53	♀	0.0005±0.013	1.311±0.0156	5.9 ± 0.1
	16 -62	47	♂	0.0004±0.011	1.312±0.0157	5.8 ± 0.1

*Significant differences between genders in same region and control(males and females) using F-test.

Conclusion

In conclusion, the results of our experiment suggest that the accumulation of genetic damage is detectable in peripheral lymphocytes of local samples of Al-Tuwaitha region. As well as , The results indicated is a possibility that there of using the changes in the mean of MN and SCEs as biomarkers for the assessment of DNA damage in the human peripheral blood lymphocytes of population living in the Al- Tuwaitha region, We also found that NDI was the important parameter which reflected proliferation capacity as a measure of genome damages in population. This results of MN and NDI frequencies within of normal values according of the technical report of International Atomic Energy Agency (IAEA) No. 405 , 2001.

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