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Original research article

Dependence of micronuclei assay on the depth of absorbed dose

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ARTICLE INFO

Article history:
Received 29 August 2016
Received in revised form
7 March 2017
Accepted 22 August 2017
Available online 17 September 2017

Keywords:
Biological dosimetry
Micronuclei
Blood
Gamma radiation
Correction factor

ABSTRACT

Aim: The purpose of the present study is to investigate the dependence of micronuclei response on the depth of absorbed dose.

Background: One of the most common cytogenetic methods used for radiation dosimetry is micronuclei (MN). Being less complex and faster than other methods are two remarkable advantages of the MN method which make it suitable for monitoring of population. In biological dosimetry based on the micronuclei method, the investigation into the dependence of response on the depth in which dose is absorbed is significant, though has received less attention so far.

Materials and methods: Blood samples were poured in separate vials to be irradiated at different depths using a linear accelerator system.

Results: According to the results, MN, as a function of the absorbed dose, had the best fitness with the linear–quadratic model at all depths. Furthermore, the results showed the dependence of MN response on the depth of absorbed dose. For doses up to 2 Gy, the maximum difference from the reference depth of 1.5 cm was related to the depth of 10 cm; however, by increasing the absorbed dose, the response associated with the depth of 20 cm showed the maximum deviation from the reference depth.

Conclusions: Consequently, it is necessary to apply a correction factor to the biological dosimetry. The correction factor is dependent on the depth and the absorbed dose.

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http://dx.doi.org/10.1016/j.rpor.2017.08.008

1. Background

There is considerable evidence that ionizing radiation affects cells directly or indirectly.[1] In general, these effects increase the frequency of apoptosis, micronucleation, DNA strand breaks and mutations, altered levels or activity of regulatory proteins and enzymes, reduced clonogenic efficiency, and oncogenic transformation.[2] Cytogenetic biodosimetry is a methodology based on the measurement of radiation induced biological effects visible at the cytogenetic level in order to correlate them with the dose of radiation.[3] Since the mid-1960s, biological dosimetry using chromosome damage biomarkers has been a valuable dose assessment method, especially when there are difficulties in interpreting data given by physical dosimetry or in cases of radiation overexposure with or without physical dosimetry data.[4] Nowadays, chromosomal aberrations in Peripheral Blood Lymphocytes (PBLs) is being studied for the dosimetry of ionizing radiations.[5] Metaphase analysis,[6–8] G-banding technique,[9] Premature Chromosome Condensation (PCC),[10] FISH (fluorescence in situ hybridization)[11] and micronuclei are amongst the conventional cytogenetic methods used for the dosimetry of radiation. Cytokinesis-block micronucleus (CBMN) assay was developed by Fenech and Morely in 1985.[12] MN method is less complex and faster than other methods which makes it suitable for monitoring large populations.[13,14]

Micronucleus incorporates in cytoplasm in the form of a small nucleus along with the main nucleus. This small nucleus originates from centromere-free chromatin elements (acentric fragment) or lagging chromosomes, which are not transferred to the daughter nucleus during mitosis, around which a nuclear coating is formed in telophase.[12] In this method, the cytokinesis of the cells that have accomplished a mitosis through Cytochalasin B stops; therefore, they are easily identified based on their bi-nucleate appearance.[15] Micronuclei are usually counted in peripheral blood lymphocytes which have accomplished a mitosis due to the stimulation of phytohemagglutinin (PHA).[16] With regard to the fact that irradiation is a strong clastogenic factor and, hence, a strong induction of micronuclei, it has been proven that CBMN is a reliable, valid and standard method to determine occupational, medical or accidental exposure to ray in the field of radiation biology. For instance, a study on chromosomal damage to people who were occupationally exposed to low-levels of ionizing radiations showed that the frequency of MN in employees was higher than in the control group.[17] In another study on the applicability of the micronuclei method to estimate the radiation dose in patients undergoing radiotherapy, the results indicated that MN assay, up to 2 Gy, was in a favorable agreement with the results of in vitro. Nevertheless, the frequency of MN in patients undergoing radiotherapy was lower than the in vitro conditions in higher doses.[18]

The direct and indirect effect of irradiations on the Song et al.[19] studied the extent of damage caused by radiation to lymphocytes in in vivo conditions after irradiation with alpha particles. They found that micronuclei assay can be used as a biological dosimeter for Internal Alpha Immunotherapy. In a survey on 10 workers who had been irradiated with a ^{60}Co source, the estimated dose by MN method was in an agreement with the measured dose by the dicentric method.[20] The accuracy of the MN method is also to the extent that illustrates the irradiation effect on patients undergoing radiotherapy at different stages of treatment.[21] They also found that the frequency of MN in the patients with cancer before radiotherapy is higher than healthy control groups. During the middle stages of radiotherapy, the frequency of MN was more than twice as much as the frequency of the pre-treatment stage while at the post-treatment stage, MN increased so that it was completely different from the pre-treatment and in-treatment stages.[21] Taghavi-Dehaghani et al. studied 26 patients with breast cancer to assess their sensitivity to gamma rays. The results showed that the frequency of MN, induced by radiation, was significantly higher in patients with early reactions than in patients with late reactions after being radiated with the dose of 4 Gy.[22]

2. Aim

The investigation into the dependence of biological dosimetry response on the depth of the absorbed dose is of utmost importance, though has received little attention so far. In most cases of MN application including estimation of the absorbed dose in nuclear accidents and chromosomal damages to patients undergoing radiotherapy,[23–26] the receiving point of the absorbed dose is located at different depths of the body. Nevertheless, the calibration curve is obtained at a specific depth (maximum depth); therefore, it is necessary to investigate the dependence of MN response on the depth of the absorbed dose. The purpose of the present study is to investigate the dependence of micronuclei response on the depth of the absorbed dose.

3. Materials and methods

3.1. *Sampling*

For the purpose of blood sampling, 25cc of fresh blood was obtained by venipuncture from a healthy non-smoker 29-year-old female. However, it has been proved that smoking had no significant effect on the micronucleus yields.[27] In order to neutralize the effect of individual differences in the results, all samples were collected from one individual.[28–31] The blood samples were poured in separate heparin vials, 1 cc each, under laminar hood in sterilized conditions. One of the vials was used as a non-irradiated/control and the other 20 vials were divided into four pentamerous groups. In order to investigate the effect of depth on the same dose, 4 separate vials were considered.

3.2. *Irradiation*

The pentamerous groups of the blood-containing vials, in four similar containers, were placed at the depths of 1.5 cm, 5 cm, 10 cm and 20 cm in water. The dimensions of the containers were chosen big enough to consider the effect of radiation scattering (length: 35 cm, width: 23 cm, height: 24 cm).

The source surface distance (SSD) was 100 cm for each four groups. Also, the Field size was 10 cm × 10 cm for each four

groups. The irradiation was conducted using a linear accelerator system (Campact Elektra AU051) with energy of 6 MeV and a dose rate of 350 MU/min. Delivered absolute dose values at the vial positions were verified using a calibrated Farmer-type chamber (0.6 cm^3, PTW, Germany) based on the IAEA TRS 398 protocol. The procedure of irradiation was similar for each four containers. That is, at first, the container of five vials was irradiated with a specific dose. Then, one vial was removed from the container and the remained four vials were irradiated with the same dose once more. The same procedure was conducted for the other three vials so that the final vial was irradiated five times more than the first removed vial.

The vials received different doses in the order of being removed from the container; that is, the vials respectively received the doses of 0.97 Gy, 1.94 Gy, 2.91 Gy, 3.88 Gy and 4.85 Gy at the depth of 1.5 cm, the doses of 0.92 Gy, 1.84 Gy, 2.76 Gy, 3.68 Gy and 4.6 Gy at the depth of 5 cm, the doses of 0.84 Gy, 1.68 Gy, 2.52 Gy, 3.36 Gy and 4.2 Gy at the depth of 10 cm, and the doses of 0.72 Gy, 1.44 Gy, 2.16 Gy, 2.88 Gy and 3.6 Gy at the depth of 20 cm.

As mentioned earlier, SSD was considered to be constant, which caused a difference in the distance between the source and the vials according to the different depths of water. In another trial, four vials were irradiated at the depths of 1.5 cm, 5 cm, 10 cm, and 20 cm by maintaining the distance between the source and the vials constant and choosing the same dose of 2 Gy.

3.3. *Micronuclei assay*

After irradiation, 4.5 mL of the RPMI-1640 culture medium (Gibco) containing 20% fetal calf serums, 20 μL/mL phytohemagglutinin (Gibco), 50 U/mL penicillin, 50 mg/mL streptomycin and 2 mM glutamine (Sigma) were added to each 0.5 mL blood in the culture vials. All cultures were duplicated, i.e. two culture vials containing cellar culture medium were considered for each sample. The cultures were incubated in a CO_2-containing incubator (Gallenkamp, ENG) at 37 °C ± 1 °C in an atmosphere humidified by 5% CO_2 and 95% air for 72 h.

In order to stop the cells at the stage of cytokinesis, Cytochalasin B (Sigma, USA) with an ultimate concentration of 6 μg/mL was added to the medium after 44 h since the start of cultivation and the stimulation of lymphocytes. The cells were harvested and centrifuged at 1200 rpm for 10 min by a centrifuge (Sigma, USA) after 72 h since the start of cultivation. Afterwards, the solution of 0.075 molar KCL (Merck, Germany) was added to the cells, and was once again centrifuged at 1000 rpm for 7 min. After a short-term hypotonic shock, cells were treated 3 times with a fixative (a mixture of acetic acid and methanol in the volume ratio of 1:6) and were transferred on a clean glass slide. All the slides were stained with Giemsa solution 10% (Merck, Germany) and coded by an individual.

To determine the frequency of micronuclei in binuclear cells, the slides were examined by an optical microscope (Nikon, YS 100) at 40× magnification. The micronuclei which were healthy, had a diameter between 1/16 and 1/3 of the main nucleus, and did not overlap with or connect to the main nucleus were enumerated.[16] For each sample, 4000 binuclear cells (2000 binuclear cells from each duplicated culture) were examined to record the frequency of micronuclei. To evaluate the standard deviation, each experiment was repeated at least three times.

3.4. *Statistical investigation*

In this research the one-way analysis of variance (ANOVA) technique was used to compare means of MN for all four depths in 2 Gy dose. The ANOVA test can be used for the case of a quantitative outcome with a categorical explanatory variable that has two or more levels of treatment.[32] In an ANOVA omnibus test, a significant result indicates that at least two groups differ from each other but it does not identify the groups that differ.[33] So an ANOVA is generally followed by evaluating all the pairs of means in order to decide which ones show a significant difference. This approach called for pairwise comparisons. The most common method of pairwise comparisons is the Tukey test.[33,34]

4. Results

The vials were irradiated at different depths and doses in a water container. The frequency of MN significantly increased at all depths by dose rise (at 95% confidence level). As illustrated in Fig. 1(a), the obtained dose–response curves, obtained at all depths, showed the best fitness with the linear–quadratic models. The variations of the micronuclei response as a function of absorbed dose through a quadratic equation are comparable with the studies of Prosser et al.[35] and Silva et al.[36] Likewise, they obtained a quadratic equation for the dose–response curve in the micronuclei method.

As displayed in Fig. 1(a), for doses up to 2 Gy the maximum difference from the reference depth of 1.5 cm was related to the depth of 10 cm. Meanwhile, by increasing the absorbed dose, the response associated with the depth of 20 cm showed the maximum deviation from the reference depth. The variations of MN as the function of the depth for the absorbed dose of 2 Gy is shown in Fig. 1(b). The results indicated that MN varies as a bi-exponential function of the depth. The other remarkable point is the saturation of the dosimeter at lower doses with an increase in depth (for instance, the results related to the dose of 3.5 Gy at the depth of 20 cm); i.e. the saturation of the dosimeter (shoulder area in response dosimeter). In other words, the biological effects of the radiation on the depth of 20 cm are more than the rest of depths. Ślosarek et al. found that the dose rate can be effective in the biological response.[37] Moreover, Kwan et al. reported the effects of the radiation energy on the response of MN.[38] These findings reinforced the idea that the variations of response at different depths can be due to the changes in energy or dose rate. In order to investigate it precisely, the energy spectrum was simulated by MCNPX code[39] at different depths in a geometry quite similar to the experimental conditions. The results of the simulations are presented in Fig. 2.

The curve's slope in dosimetry represents the sensitivity of the dosimeter. In micronuclei dosimetry, the sensitivity of dosimeter can also be obtained by deriving the equation of dose–response variations. The sensitivity as a function of dose absorbed at different depths is shown in Fig. 3.

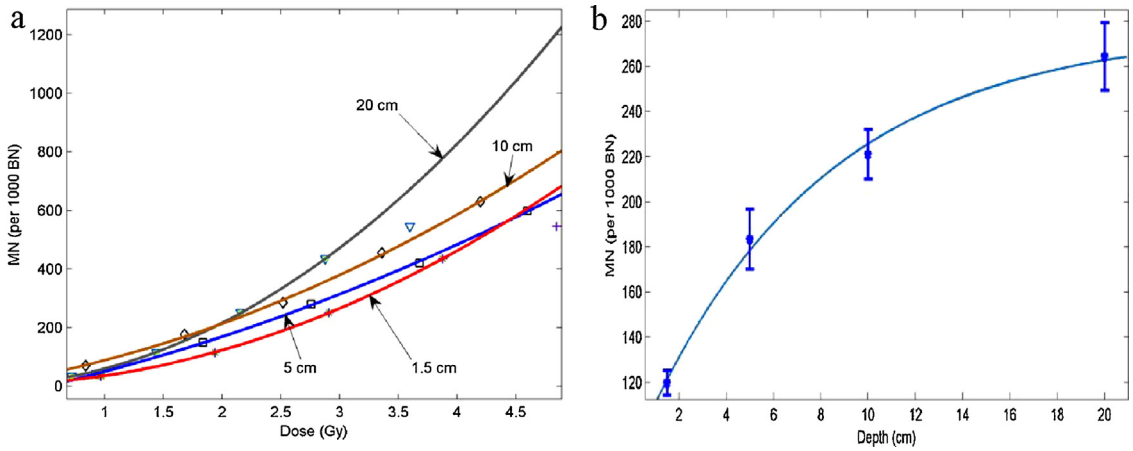

Fig. 1 – (a) The MN dose–response curve at different depths. (b) The variations of MN as a bi-exponential function of depth.

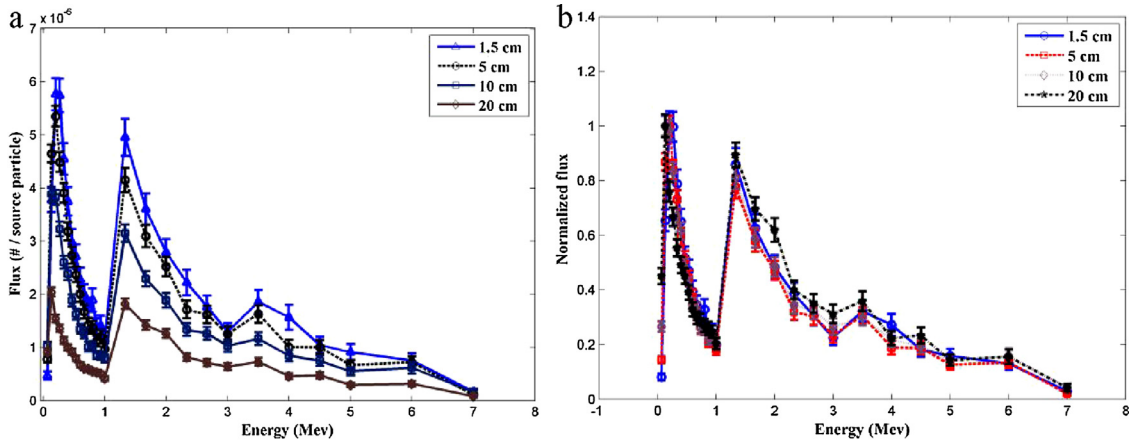

Fig. 2 – The energy spectrum simulated by MCNPX code in a geometry quite similar to the experimental conditions. (a) The spectrum per each particle emitted from the irradiation source (the information of the dose rate variations can be deduced from this figure). (b) The normalized values to the maximum responses (the energy changes with the depth can be deduced from this figure).

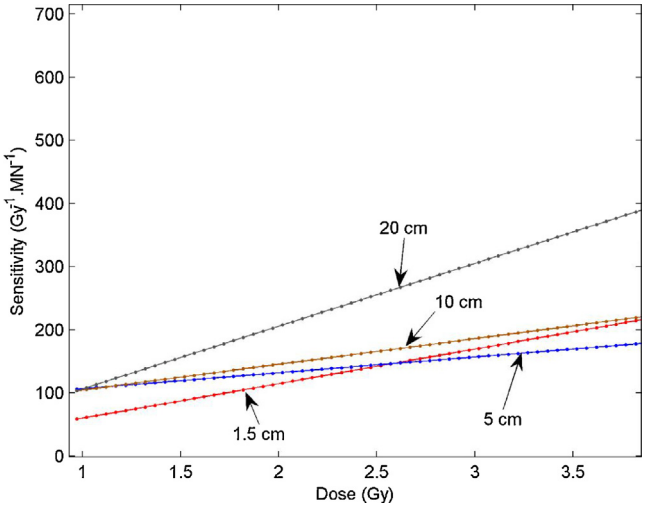

Fig. 3 – The sensitivity of the dose–MN curve as a function of dose absorbed.

According to the results, there was no significant change in the sensitivity by increasing the depth to 10 cm; however, a significant increase was observed in the sensitivity at the depth of 20 cm.

As displayed in Fig. 1, if the calibration curve was related to the maximum depth and the dose was absorbed at other depths, there would be obvious errors in the dose estimation. Therefore, it is necessary to apply a correction factor with regard to the depth of absorbed dose. The maximum depth of the absorbed dose is 1.5 cm for the energy of 6 MeV. If the calibration is performed at this depth (which is usually the case) and the dose at other depths is estimated, the correction factors will be as shown in Fig. 4. For instance, if the dose of 2 Gy was absorbed at the depth of 10 cm, it would create 284 micronuclei. However, the same number of MN is created at the depth of 1.5 cm if the dose absorption equals 3.11 Gy. Thus, if the dose related to the depth of 10 cm is obtained through the calibration curve associated with the depth of 1.5 cm, an overestimation will occur in dose estimation. However, if the corresponding correction factor (i.e. 0.81) is applied, the correct amount of dose is obtained.

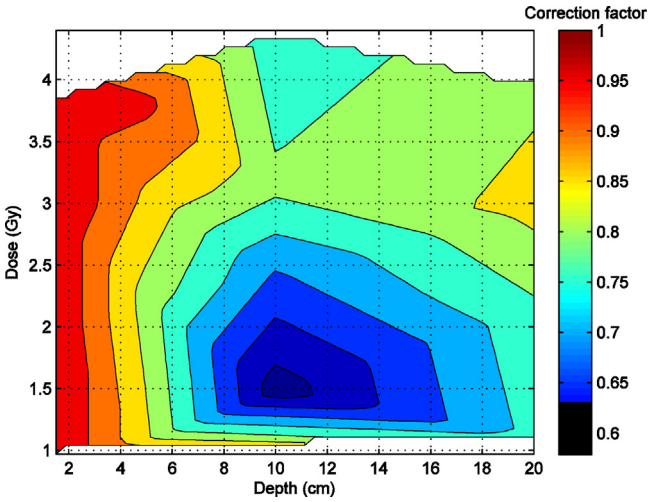

Fig. 4 – The map of correction factor as a function of dose absorbed and the depth of radiation absorption for the irradiation energy of 6 MeV. On the vertical axis, the absorbed dose, obtained by counting MN before applying the correction factor, is shown. The horizontal axis shows the depth of the absorbed dose.

As illustrated in Fig. 4, the values of dose without applying the correction factor can be significantly different from the actual value. This difference results in the highest value at the depth of 10 cm.

In order to confirm the result of the experiments, the response to the dose of 2 Gy at different depths (1.5 cm, 5 cm, 10 cm, and 20 cm) was investigated in a separate test. The results of statistical analysis between the mean of MN at different depths showed that the mean of MN had the minimum and maximum values at the depths of 1.5 cm and 20 cm, respectively. Furthermore, using the one-way ANOVA, there was a significant difference between the mean amounts of MN at all four depths in 2 Gy dose. Furthermore, the significance of pairwise difference of MN at different depths was investigated using the Tukey test. The Tukey test also showed that the number of MN at all depths in 2 Gy shows a significant pairwise difference.

5. Discussion

In the present study, the mean MN significantly increased by increasing the dose at all the observed depths (*P*-value = 0.05). The dose–response curves had the best fitness with the linear–quadratic model at all depths, which were consistent with previous studies.[40] Prosser et al. reported a linear–quadratic relationship between the frequency of micronuclei and the dose at the energy of 250 keV X-ray.[35] Also, Silva et al. intended to obtain a dose–response relationship for the micronuclei method after being irradiated with Cobalt-60 gamma ray. They found that a dose–response relationship for MN is consistent with a linear–quadratic model.[36] Furthermore, Huber et al. examined the creation of micronuclei in the lymphocytes of different individuals after irradiation with the combination of neutron-gamma beam resulted from nuclear fission, 220 keV X-ray and Cobalt-60 gamma ray. Accordingly, the dose–response relationship was quadratic linear for frequency of micronuclei obtained for both types of irradiation with low and high LET.[41] The current study, which also examined the response of MN at the energy of 6 MeV, approved the quadratic changes for MN-dose.

In the next step, the dose of 2 Gy was given to the vials at different depths to assay the effect of depth on the same dose. The results indicated that, by increasing the depth, the average frequency of MN increases; so that the mean of MN shows the minimum and maximum amounts at the depths of 1.5 cm and 20 cm, respectively. This result has also been confirmed in previous studies.[42] Given that an increase in the depth may change the energy spectrum and the dose rate, the changes in the number of MN can be attributed to the changes in these two factors, i.e. energy spectrum and dose rate, by increasing the depth. Many studies have investigated the effects of energy on the biological damage, such as damages to micronuclei, which showed the increase of MN frequency with energy reduction.[38,43–45] However, according to the results of simulation in the present study, there is an inconsiderable change in energy even at the depth of 20 cm. On the other hand, the simulation results showed a dose-rate reduction with an increase in the depth. Thus, the MN increase, due to an increase in the depth, was attributed to a decrease in the dose rate in the present study. The MN increase as the result of dose rate reduction has also been approved in previous studies. In the study of Ślosarek et al., the cells were irradiated at the depth of 3 cm and 15 cm with different dose rates (100 MU/min and 300 MU/min), using linear accelerator with the energy of 6 MeV.[37] Their results indicated that the cells located in the irradiation field incurred more cell damage at the dose rate of 100 MU/min than 600 MU/min.

In a review paper, Gordon Steel et al. investigated the effect of dose rate on the range of 200 cGy/min to 1 cGy/min.[46] Generally, three areas were considered for the doserate effect on the biological effect within this range: A) when the dose rate decreases from 200 cGy/min to 5 cGy/min, the biological effect decreases due to the repair of sub-lethal damages and B) when the dose rate decreases from 5 cGy/min to 1 cGy/min, sometimes the cellular damage increases due to the decrease in the dose rate which is justified by the phenomenon of the cell cycle progression; so that the cells move toward the radiosensitive phase of cellular cycle by decreasing the dose rate. The dose rate of 5 cGy/min and higher is sufficient to stop the cellular cycle. C) In the dose rate under 2 cGy/min, the cells are set for division to compensate for cellular death. That is, the cellular death decreases by reducing the dose rate. Gordon Steel et al. stated that in ultra-high dose rates of about 10^7 Gy/min, obtained in the pulsar accelerators, a reduction is observed in radiation sensitivity of aerobic cells due to oxygen depletion of the location by a fast pulse of irradiation. This process is applicable for dose rates higher than Gy/min.[46] On the contrary, under this range, the radiation sensitivity remains constant at the maximum limit. Localized oxygen depletion and radiation sensitivity reduction in ultra-high dose rates was also described by Hall et al. in 1991.[47] It is noteworthy that the radiation dose rate affects not only the biological dosimeters but also different types of dosimeters.[48–50]

When the beam interacts with the tissue, its energy is transferred to the tissue at 10^{-8} s and creates ions at 10^{-16}–10^{-17} s. In the nucleus of mammal's cells, 1 Gy X-ray creates 10^5 ionization events that are mainly H_2O^+ and e^-. The combination of H and OH radicals produces radiochemical products such as H_2 and H_2O_2.[51] These radiochemical events are centralized through the beam path, especially near the Bragg peak of the primary and secondary electrons. The radical–radical combination occurs basically in this small volume; nevertheless, they also react with intracellular components such as DNA. So that 1 Gy of X-ray causes 900 Single Strand Breaks and 25 Double Strand Breaks in DNA, caused by the direct and indirect actions of the beam, i.e. by OH radicals which have the emission distance of about 4 nm.[52]

It has been proved that about 60% of cell deaths is caused by water Radiolytic products (mainly OH radicals) through irradiation.[52] Therefore, a reduction in the free radicals, due to the radical–radical recombination, may decrease the cell death per dose unit. The probability of such recombination is affected by the amount of dose, delivery speed and cell volume in which the ions and free radicals are created.[53] As a consequence, the increase in the number of MN, resulted from an increase in the depth at the same dose, can be attributed to a decrease in the recombination due to the reduction of dose rate.

6. Conclusions

The present study intended to investigate the dependence of MN dose response on the depth of absorbed dose. The results indicated that the frequency of Micronuclei in lymphocytes, caused by irradiation at the energy of 6 MeV, was dependent on the dose received by these cells. It was concluded that there was no significant change in the slope of the dose–response curve by increasing the depth to 10 cm. However, the sensitivity increased significantly for the depth of 20 cm. Furthermore, the response of the micronuclei assay was dependent on the depth at all doses. The response variations, resulting from the changes in the depth, were attributed to the dose rate variations.

Conflict of interest

None declared.

Financial disclosure

None declared.

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