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CYTOGENETIC DETECTION TOOLS FOR EFFECTS OF IONIZING RADIATION ON HUMAN

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Abstract

Everyday people are constantly exposed to a background dose of ionizing radiation which comes from the rocks and from outer space. Soldiers on mission could be exposed to ionizing radiation more frequently than it would be expected. In case of a terror attack, a radiological accident or industrial disaster people can receive much higher dose than in everyday life. People can get a dose even unperceived, as humans have no specialized sensing organ for ionizing radiation. Tumors can develop years after the irradiation due DNA damage caused by ionizing radiation. In cases when affected people didn't wear any personal dosimeter, the received dose can be estimated with different biodosimetry tools in order to decide about the appropriate medical treatment or even the compensation. In this article the author gives a short review about the recently used biodosimetry methods.

Keywords: *biodosimetry, micronucleus, chromosome aberrations, dicentrics.*

Absztrakt

Ionizáló sugárzásnak folyamatosan ki vagyunk téve, ez a háttérsugárzás elsősorban a kőzetekből és a világűrből származik. Ennek sokszorosát szenvedhetjük el katasztrófahelyzetben, például egy nukleáris baleset vagy támadás után. A Magyar Honvédség állománya nagyobb valószínűséggel kerülhet kapcsolatba ionizáló sugárzással, mint a civilek. Tekintve, hogy nincs az ionizáló sugárzás érzékelésére specializálódott érzékszervünk, a sugárzás észrevétlenül érhet minket. Az ionizáló sugárzás által kiváltott DNS károsító hatás következményeként évekkel a besugárzás után is keletkezhetnek a sérülteken daganatos megbetegedések. A személyi dozimétert nem viselő személyeknél a sugársérülés mértékét különböző biodozimetriai eljárásokkal tudjuk megbecsülni, így kiválasztható a megfelelő kezelés. Jelen közleményben a szerző ismerteti, majd röviden összehasonlítja ezeket az eljárásokat.

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Kulcsszavak: *biodozimetria, mikronukleusz, kromoszóma aberráció, dicentrikus kromoszóma*

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INTRODUCTION

Biodosimetry is a monitoring system which reveals whether the examined animal or human has been exposed to ionizing radiation or not and how much dose of radiation was received. Biodosimetry always gives an estimation, because each living organism always reacts to the ionizing radiation on his own way, but the huge amount of knowledge and experimental data accumulated during the last few decades helps us to assign the past radiation burden to the outcome.

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As during the evolution there is no a single organ has specialized for perception of radiation, though it could reach us, it remains unnoticed in our environment. This information would important for a soldier, who was around a radiation source; he may need medical treatment even though he doesn't have any symptoms. Another time the possibility of radiation exposure is emerging due to paradox clinical symptoms of the soldiers getting back from missions.

Basically we can have two different reasons for carrying out a dosimetry test:

1. We know about the radiation event – we need to evaluate the received dose
2. We have to elucidate, whether suspicious clinical symptoms are caused by a former radiation (e.g. events around Alexander Litvinenko's death, although his polonium poisoning was not a past rather an ongoing process)

The biodosimetric methods reviewed below are selected by – to combined with each other - be suitable to examination of people who were possibly exposed by ionizing radiation. These people can be industrial workers, casualties or soldiers getting back from missions.

These methods are also important tools in triage of a large scale incident or disaster. Triage is the process which makes ranking of the casualties by priority of service. In situations where ionizing radiation is involved can be many people who have no symptoms but they were potentially exposed to radiation, these people must be examined to minimalize the chance of cancerous diseases in the future.

The possibility that terrorism or a large-scale incident could result potential radiation exposure of hundreds of thousands of people is a real threat in our days. Emergency preparedness to these kinds of scenarios includes the usage of biodosimetry tools to make retrospective dose estimation. Biodosimetry is an important tool in any radiological event since the estimation of the received dose makes the triage and the medical treatment of the affected people easier.

Biodosimetry would help:

- −estimate the number of people received doses that did not require acute care
- −classify patients who need further evaluation into treatment-level categories
- −guide the actual treatment
- −help handle the long-term consequences of exposures to ionizing radiation (including planning for treatment and patient compensation) [1]

Every biodosimetry tools by definition assess changes in the examined person's cells or tissues which happen in response to ionizing radiation and whose resulting measurements can be reliably attributed to the level of dose received. [2]

Much of the available biodosimetry methods are „biology based", i.e. they detect the direct or indirect biological response to the irradiation in living cells, [3] however changes can be detected in non-living materials of the human body as well (hair, teeth, bones). [4]

Most part of biologically-based biodosimetry methods measures changes in white blood cells. Besides these methods there are such techniques assess biological markers of DNA damage and repair, gene activation, metabolomics or proteomics. Generally, these responses involve such biological systems whose normal function is to respond to pathophysiological processes or physical injuries, thus, these systems are not specific to ionizing radiation. [2]

Most of the available techniques are not specific to ionizing radiation exposure, and their results can be confounded by a variety of factors (e.g. age, disease status, stress, lifestyle, and gender) [5]

The ideal biodosimetry method is radiation specific, has a low background and dispersion, the dose-effect can be calibrated, detects a long-term effect, sampling is easy, can give result quickly and the false positive and false negative results can be distinguished. [6]

Not a single technique fulfils all the criteria of an ideal dosimeter, however an integrated approach using multiple techniques tailored to the exposure scenario can cover most requirements. [4]

As regards in most cases these methods have significantly different characteristics (e.g. time requirement, throughput) it's important to set them together, this way one can select the most suitable method or methods in case of disaster situations.

The comparison also important in planning the methodology, and the proper instruments.

The aim of this article is to review the most commonly used cytogenetic biodosimetry techniques and compare their major parameters.

An important aspect in the view of mass events the automation of the test or at least a high throughput semi-automated solution and it is also worthy of note that biodosimetry methods are also suitable to study radioprotective substances and their mechanism of action.

CYTOGENETIC TECHNIQUES

Besides chemical substances which can cause various genetic damages, ionizing radiation causes several kind of changes in DNA of the exposed cells as well; it can alter the morphology of the chromosomes (e.g. chromosome shape alterations, minutes). For biodosimetric purposes it's common to examine the cytogenetic damage caused by ionizing radiation in peripheral blood lymphocytes. The application of certain methods depends on the stability of the examined chromosome aberration. The frequency of the dicentric chromosomes, PCC fragments and micronuclei decrease with the renewal of the lymphocytes, thus these methods can be applied mostly in the cases where irradiation happened in the recent past. If the irradiation happened years or decades earlier, the FISH method is the best choice due it detects stable translocations.

DICENTRIC CHROMOSOME ASSAY

The centromere is the part of a chromosome which has dual function: links a pair of sister chromatids and during mitosis, spindle fibers attach to the centromere via the kinetochore. [7] Ionizing radiation causes breaks in the chromosomes what makes the possibility to the fusion of two chromosome segments, each with a centromere, resulting acentric fragments and formation of dicentric chromosomes. [8] On rare occasions ring chromosomes can be formed. During the analysis the ration of dicentric chromosomes to normal ones is assessed. A dicentric chromosome are always accompanied by an acentric fragment which helps the investigator, because during the microscopic work the acentric fragments can be identify easier than its dicentric partner.

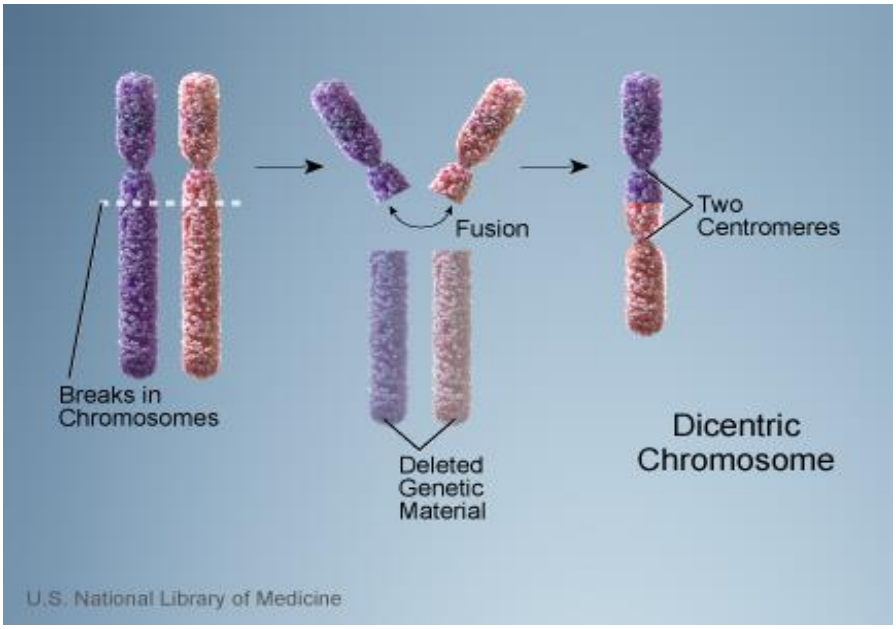

Figure 1. Formation of dicentric chromosomes: Fusion of the broken ends of two chromosomes. [9]

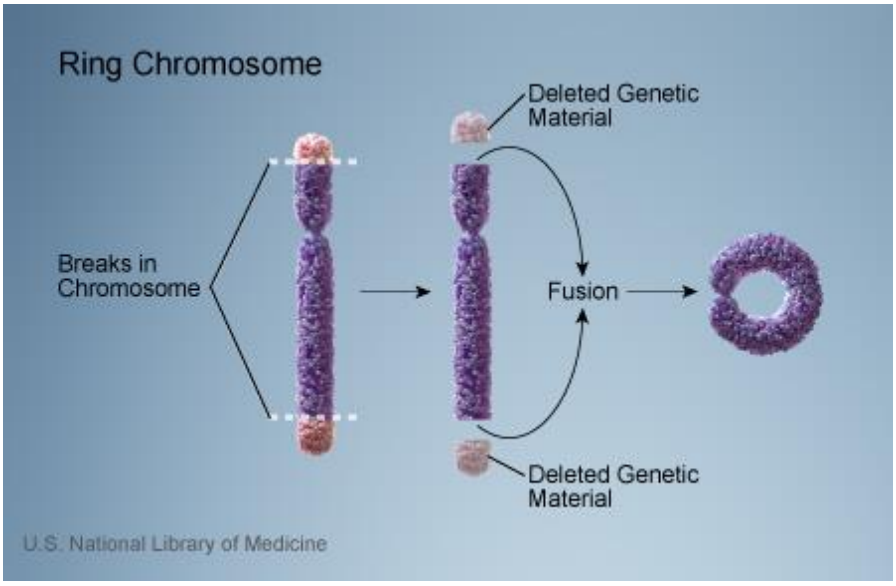

Figure 2. Formation of ring chromosomes: Fusion of the two broken ends of the same chromosome. [9]

Alterations resulted by double strand breaks (dicentric chromosomes, centric rings) are specific to ionizing radiation. The number of these alterations grows with the dose. [10] [11]

Double strand breaks hereby dicentric chromosomes arise nearly exclusively by the effect of ionizing radiation [8] thus this method is considered as „gold standard" [12]. The dose range and time frame make this method useful for biodosimetric purposes. However, it requires cell culturing and induction of cell division as chromosomes condensate only in dividing cells. Mature lymphocites in healthy people's peripheral blood are not dividing cells. In order to make the chromosomes visible, phytohemagglutinin and colcemid are added to the cells to trigger lymphocyte cell division and arrest them at metaphase, respectively. Mostly the CD4+ and CD8+ T cells are stimulated in vitro by phytohaemagglutinin thus are used for biological dosimetry. [13]

In case of acute irradiation, the frequency of the dicentric chromosomes in peripheral lymphocytes can be characterized by a linear-quadratic dose-response curve up to 5 Gray. In

healthy humans the background of dicentric chromosomes is low (~1 dicentric/1000 cells). Due to the low background the sensitivity of the method is good, a ~0,1 Gray whole-body radiation burden can be detected by examining 500-1000 metaphases. [13] [14]

The main disadvantage of this assessment besides its time consuming microscopic analysis is that dicentric chromosomes can be disappear by the renewal of the lymphocytes, as in the bone mellow. Thus in cases when irradiation happened many years ago the dicentric method can be applied with restrictions.

There are efforts to automatize the dicentric chromosome assay (e.g. LUCIA, Metafer) however these are semi-automatic techniques, the human verification is always necessary.

PREMATURE CHROMOSOME CONDENSATION (PCC)

Chromosome condensation happens normally in the prophase of cell division under physiological circumstances; however experimentally it can be caused before the synthesis phase. In vitro adding chemical substances or suitable regulator proteins via cell fusion, chromosomes appear earlier and they become analyzable.

PCC has two subtypes: fusion with Chinese hamster ovary (CHO) mitotic cells and chemically induced PCC. In the first case the rate of countable cells is lower and it requires much experience, but this is a method how to form chromosomes as fast as possible. The chromosomes, what we obtain by PCC, contains only one chromatid therefore – this feature and the smaller size help the investigator to distinguish between human and hamster chromosomes - we can investigate the centromeres only with additional labeling, but it is suitable to detects translocations as well. At the case of chemically induced version of PCC, we don't need CHO cell to fuse with, but is more time consuming due to the extended incubation.

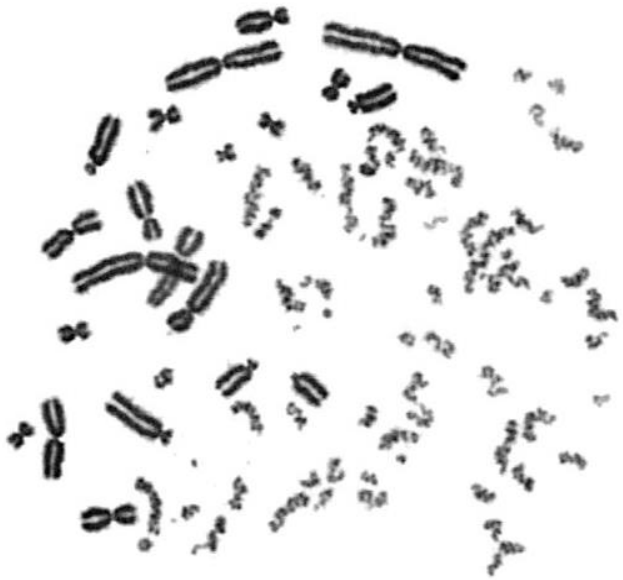

Figure 3. Human G_0 PCC [10]

Cell fusion PCC is also a potential biodosimetric method [15] if the fast and accurate dose evaluation is the main priority. [10] PCC allows of measure the chromosomal aberrations immediately after irradiation. The method needs fusing human lymphocytes with Chinese hamster ovary (CHO) mitotic cells in the presence of a fusing agent, polyethylene glycol

(PEG), this way there is no need for any mitogen stimulation or culturing. [16] Using polyethylene glycol-mediated cell fusion with mitotic cells or chemically induced PCC using calyculin A or okadaic acid chromosome condensation can be achieved without the completion of DNA replication. [4] This method was first described in 1984. [17]

In PCC assay the number of PCC fragments over the 46 chromosomes is counted. On average 4-5 extra fragments/cell/Gray are perceptible in case of low LET irradiation. The background of spontaneous PCC fragments is like dicentric chromosomes, 1-3/1000 cells. [4] The length of the fragments can be informative as well. [18] If the assay is combined with staining procedures, centromeres and dislocations can be measured as well.

Using proper calibration curves, Giemsa stained PCC fragments allow a fast dose evaluation [17] [19] especially if PCC is combined with C-banding, [20] FISH, specific DNA libraries, telomere-centromere staining or peptide nucleic acid (PNA) probes. In the latter case it's possible to detect not only the PCC fragments and translocations but the dicentric chromosomes as well, thus the method can provide fast and accurate dose evaluation. [21] [22] [23]

The assay is useful either to determine low dose exposure or following life threatening high acute doses (both low and high LET radiation). PCC can discriminate accurately between total and partial body exposures. PCC can be a useful tool "for triage of a population after a mass exposure event" especially if it's combined with TC staining or computerized automation. [21]

The cells contain PCC fragments are searched and identified manually but semi-automated systems (e.g. MetaSystems) make scoring much easier. The evaluation makes scoring of light stained chromatids necessary, they can be easily distinguished from dark stained CHO mitotic chromosomes. In unirradiated lymphocytes there are 46 PCC fragments. In irradiated samples the number of PCC fragments is registered, the evaluation made by the number of PCC fragments is correlated to number of PCC fragments in unirradiated samples.

Time after irradiation affects the result of dose evaluation. After irradiation the lymphocytes sampling must be happens as soon as possible in order to make them fusion with the Chinese hamster ovary cells. If the sampling delays, we must calculate with the DNA repair mechanisms during the dose assessment. Studies revealed that the number of PCC fragments 4 hours after irradiation was double as after 1 and 7 days after irradiation, while there wasn't significant difference in numbers between the 1 and 7 days samples. [24]

The assay has several recognized problems, namely radiation induced mitotic delay and cell death during the two day assay culture (especially after high doses), can cause considerable underestimation of the radiation exposure dose. [13]

FLUORESCENT IN SITU HYBRIDIZATION (FISH)

FISH assays are the most important methods in cases where the exposure happened a long time ago. Translocations detected by FISH techniques are alterations which don't affect centromeres and don't perturb the cell division process. This assures the alterations to pass during the cell divisions and to accumulate through life.

FISH techniques are used for assessment of past exposures for many years. The most commonly used version of the technique is single color FISH (sFISH), which makes the detection of inter-chromosomal exchanges - such as dicentrics and translocations - possible. Multi-color FISH and for whole genome analysis M-FISH have been also developed in order to assess induced translocations among different labelled chromosomes. Using pancentromeric and telomeric probes combined with chromosome paint probes enable to discriminate accurately between translocations and dicentrics, as well as between two-way and one-way translocations between chromosomes. In case of protracted exposure (e.g.

occupational doses, or for historic exposure assessment), translocations are the aberration of choice.

In circulating lymphocytes translocation frequencies have been shown persistent for many years, particularly when the analysis is restricted to stable lymphocytes, [25] [26] [27] [28] however background frequencies show significant increase with age [29] [30] and can be greatly variable between individuals of similar age and dose history. Gender or race have been observed have no significant effects on the translocation frequencies but smoking has been suggested to be of significance. [30]

Due to these aforementioned confounding factors the lower detection limit is around 0.5 Gy cumulative lifetime dose for individual dose assessment. [28] In younger non-smoking individuals the detection limit may lower to 0.2 Gy. In partial-body exposures, translocation containing cells are often unstable thus the frequency is reduced with time. [28] First results by FISH are available only ~5 days after receipt of a blood sample, because the method needs for mitotic lymphocytes and lengthy hybridisation protocols.

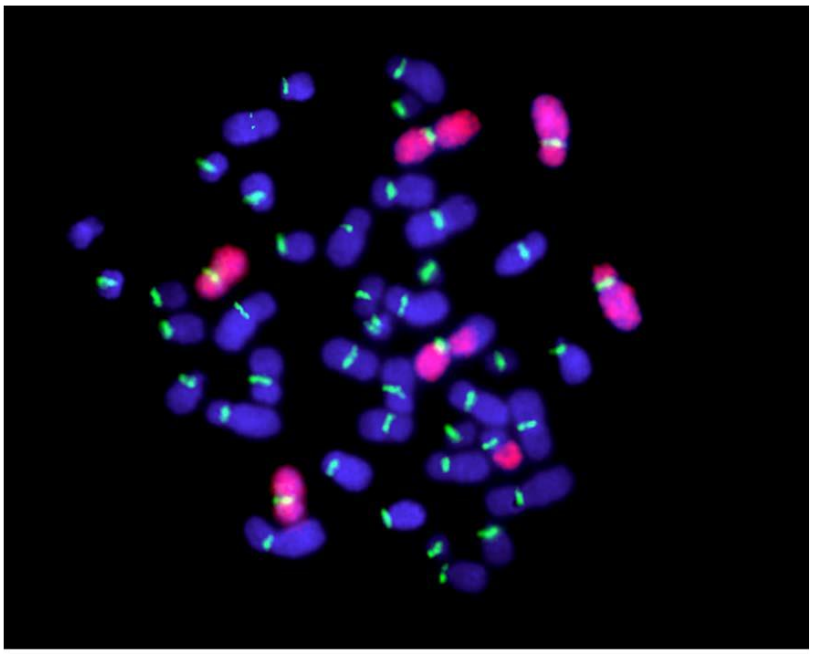

Figure 4. Human metaphase with monocoloured painted chromosomes. Cromosomes 1, 4 and 11 labelled with Cy3 (red), centromeres highlighted with a pancetromeric probe labelled with FITC (green), and the rest counterstained with DAPI. [10]

Using fluorescence microscope with proper staining protocols improves the specificity of every cytogenetic assay, however FISH assays are based only on fluorescence microscopy.

Most retrospective dosimetry has been undertaken on individuals exposed to low LET radiation (reviewed in [4]). FISH techniques have also been used to retrospectively assess chromosome damage in individuals with exposure to high LET radiation. In plutonium workers many years after the exposure increased translocation frequencies have been observed. [4] However, their situation is confounded by significant external gamma irradiation, making the interpretation of results difficult. Other aberrations, such as insertions, intra-chromosomal and complex aberrations have also been suggested as biomarkers of high LET exposure.

Two EU concerted actions aimed at standardizing sFISH concluded that only ‘complete’ cells (i.e. those with all ‘painted’ material present and 46 chromosomes) should be used and frequencies calculated using stable cells only. For population-based studies it is recommended

to analyze at least ~300 genome equivalent cells per individual. Accurate individual dose assessment requires at least ~1000 genome equivalent cells." [4] Automated scoring systems available e.g. LUCIA control-system. [31]

MICRONUCLEUS ASSAY

In vitro cytokinesis blocked micronuclei method is also a cytogenetic biodosimetry assay. By the definition of IAEA: "Ionizing radiation induces the formation of acentric chromosome fragments and to a small extent malsegregation of whole chromosomes. Acentric chromosome fragments and whole chromosomes that are unable to interact with the spindle lag behind at anaphase, and as a result they are not included in the main daughter nuclei. A lagging chromosome fragment or whole chromosome forms into a small separate nucleus; hence the term micronucleus."

In methodology aspect it resembles to the dicentric chromosome assay however it's simpler thus more popular as well.

It requires a lower magnification (~40-60X) than dicentric chromosome analysis, latter needs ~100X magnification. Usually you do not need a fluorescence microscope to perform the micronucleus assay, however fluorescent centromere staining improves the specificity of the assay since micronuclei containing centromere are uncharacteristic to ionizing radiation.

As peripheral lymphocytes are non-dividing cells, the induction of cell division in ex vivo samples requires phytohemagglutinin just as in dicentric chromosome assay. The postmitotic nucleus can be investigated in telophase, so the division is stopped with cytochalasine-B what makes the cell culturing one day longer than in case of dicentric chromosomes. [32] Cytochalasine-B inhibits the actine polymerization thus prevents the separation of the daughter cells resulting binuclear cells. Binuclear cells already have two nuclei but they have only one cytoplasm yet, so that the freshly divided cells are clearly distinguishable from other ones, which are not divided. Usually the micronuclei are formed by those acentric fragments or chromosomes which can't migrate to the poles during the cell division, [33] however they can be formed even from whole chromosomes in case of mitotic spindle or centromere attachment damage [10] These are recognizable spherical bodies in the binuclear cell's cytoplasm which are smaller and have a similar morphology and staining properties as nuclei. [4]

The micronucleus formation is not radiation specific; they can be influenced by many clastogenic and anegenic agents.

The number of micronuclei increases because of exposure to ionizing radiation, [10] age, [32] untreated tumorous diseases, [34] smoking, harmful environmental and occupational factors [35].

The CBMN assay is a well validated and standardized assay to evaluate the exposure of occupationally, medically and accidentally exposed individuals. [4] Like dicentric chromosomes, micronuclei are unstable cytogenetic aberrations, which disappear with time after exposure, and thus their use is limited for exposures that occurred many years ago.

The lower limit of the dose detection is 0,2-0,3 Gray [4] what caused by the relatively high and variable spontaneous MN yield. This yield is more pronounced in females and increases with age. [36]

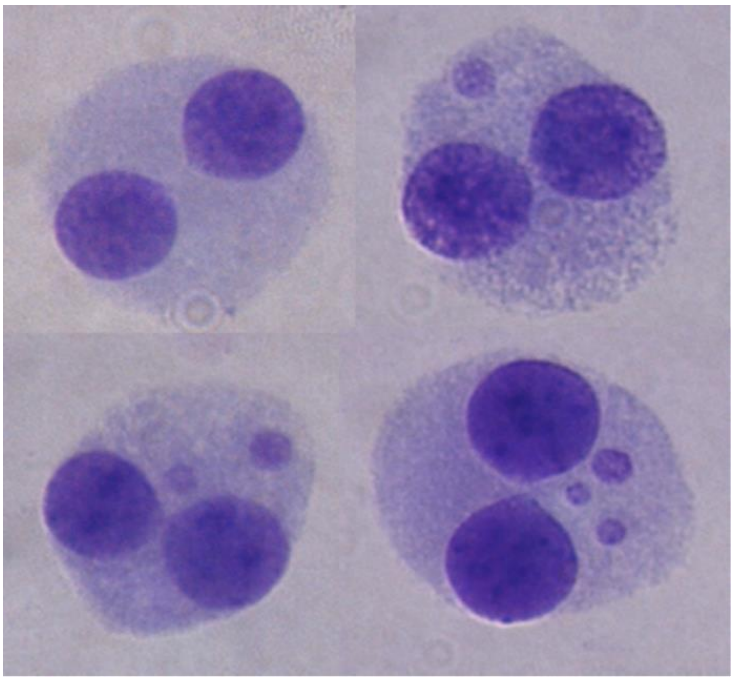

Figure 5. Human Lymphocytes with 0, 1, 2 and 3 micronuclei respectively (our workgroup's own preparations. Thanks to Sandor Papp for contribution).

Compared to the dicentric chromosome assay, although the cell culturing is one day longer, scoring CBMN assay is easier and requires less time consuming microscopic work.

In case of CBMN assay there are some automation efforts as well (e.g. LUCIA, [31] RABiT, [37] Metafer [38]). The main problem of older systems in this field is the shortage of cytoplasm detection. As the binuclear cells are identified on the basis of the distance between the nuclei, they work with a high dilution of cells to minimize this error.

CONCLUSIONS

After a terror attack or an industrial accident with numerous casualties the first steps of the triage is to identify the clinical symptoms and a complete blood count / haemogram determination. These are routine clinical dosimetry processes. The decline of the white blood cells number alludes to radiation exposure.

Numerous biodosimetric tests may require cooperation of several laboratories. These times those laboratories come to the fore where automated biodosimetry systems are available.

The most plausible solution is the micronucleus assay due it has the highest throughput amongst the aforementioned techniques if it's made manually and it can be automated. However the method is not clearly specific to ionizing radiation, if the involvement of ionizing radiation is known, the method is suitable to dose evaluation.

The sensitivity and specificity of the dicentric assay is considerably good, it can be partly automatized and can be carried out in any laboratory.

Fortunately, mass casualty radiation event is rarely occurs, more often we search the answer to a different kind of question with biodosimetry. Examination of soldiers getting back from missions can reveal whether they were exposed by ionizing radiation or not. It's especially important if one has medical problems. In these situations, there are enough time and opportunity for an accurate medical check-up. It's advisable to apply several different biodosimetry tests at the same time; for example, comparing the results of micronucleus and dicentric assays can be informative, because if only the micronucleus count shows alteration, then the received harmful impact most likely was not ionizing radiation.

Detection of chromosome aberrations (e.g. dicentrics, acentric fragments) and cytogenetic damages by micronucleus assay is commonly used for dose assessment.

Translocations detected by FISH techniques are useful especially in exposure scenarios happened years before, but the results are questionable on occasion (mostly due the variable background).

This comparison of the above mentioned methods is shown in table 1.

Assay	Minimum time from exposure until marker is valid	Maximum time from exposure when marker remains valid	Total processing time (from receiving sample to get the result)	Lowest detection limit (Gy)
Cytogenetic dosimetry methods				
Dicentric chromosome	0–1 day	>6 months	4–9 days	0,1 Gy
Mikronucleus	0–1 day	1 year	4-6 days	0,2–0,3 Gy
PCC	0–1 day	7 days[5]	CHO-23 hours chemically induced–51 hours[5]	0,2 Gy[5]
FISH	0–1 day	years	5 nap[4]	0,5 (0,2) Gy
Routine laboratory test to take into account for triage				
Lymphocyte count decrease	12 hours	48 hours	1,5–2 days	~0,5 Gy

Table 1. Timeframes and detection limits of the cytogenetic biodosimetry assays. (After Flood et al. 2014. [39] reworked)

There is no method which is ideal for every scenario, thus it's expedient to select the method accordingly the actual situation. The best solution is to apply several methods at the same time, putting together all possible pieces of information from multiple sources, however in some cases (disasters, terror attack) it's cannot be accomplished due to the huge number of samples and the lack of time. It shell be considered how to get the more information with the less effort under minimal term.

The methods presented here were selected according to the consideration, that the tests, combined with each other, would be able to quickly and comprehensively examine persons suffering from a suspected casualty injury, whether they are casualties or a smaller or larger group of mission-returning soldiers. It is why the procedures in their most important parameters (thresholds of detection, time of completion, timely detection of effect) may differ significantly.

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