NATIONAL UNIVERSITY OF PUBLIC SERVICE
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Laboratory examination of low-dose radiation injuries using polymerase chain reaction, with particular attention to DNA damage

author’s presentation of doctoral (PhD) dissertation

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INTRODUCTION

A small amount of background radiation constantly hits our body, it consists of terrestrial radiation and cosmic radiation. (In this case, terrestrial radiation means the component of natural background radiation that affects living organisms as a result of the continuous decay of ancient radioactive isotopes (^{238}U, ^{235}U, ^{232}Th, ^{40}K) and their daughter elements occurring in the Earth's magmatic material and crust). In Hungary, the value of background radiation is 1.75 mSv/year. Many times this amount of radiation can reach the body during a radiation event, such as a nuclear or workplace accident, terrorist attack, or a large-scale industrial accident (Chernobyl, Fukushima).

Ionizing radiation can cause health damage in such a way that the fact of the radiation remains unnoticed. We don't have specialized organ to detect ionizing radiation, even though even the smallest exposure can leave a permanent damage in our cells. Radiation exposure can happen without the people concerned noticing it, and not being informed about it afterwards. One of the important tasks of disaster prevention is radiation protection. However, in the event of a disaster or an unexpected situation, the pre-planned protection cannot always be carried out, or it is only partially carried out, and since the Hungarian Defence Forces usually assumes a significant role in the tasks of disaster prevention, including nuclear accident prevention, both the commanding staff and the executive staff can stay in the in a dose range higher than natural. For persons not wearing a dosimeter, the amount of radiation dose can only be estimated afterwards, with the help of various biodosimetry methods.

Biodosimetry provides an answer to whether radiation exposure has occurred, how much radiation the people present have suffered and the resulting cell damage through the laboratory examination of those involved. In this way, the injured can receive appropriate medical treatment and legal remedies. The visible symptoms of radiation exposure - skin reaction, vomiting, headache, diarrhea - only appear in case of a larger absorbed dose of more than 1 Gray, depending on the situation, with a delay of several hours. These symptoms are nonspecific, they can easily be confused with symptoms of other diseases, so it is possible that patients will not receive the appropriate treatment if the doctor does not suspect radiation exposure (for example: Alexander Litvinenko's case). Radiation exposure can also occur in connection with a known radiation event, but even in this case we do not know exactly how much the effect was on individual people based on the terrain conditions and the time spent there, since, for example, radiation

sensitivity may differ from person to person. In a disaster situation, for example during a nuclear or workplace accident or terrorist attack, those present may be exposed to radiation, and in the case of a large-scale industrial accident, radiation may reach the human body even thousands of kilometers away, i.e. beyond the borders of the affected country (as happened in the case of Chernobyl).

In the case of every mass accident, the acting command must set up an order of emergency care among those involved, this process is called triage. During an event involving a large number of people, there will be many people who may not even manifest clinical symptoms, but in their case the risk of various cancers will increase significantly later, and it must also be taken into account that there will be people who do show symptoms, but they did not develop due to radiation exposure (stress, chemicals, trauma).

ACTUALITY AND IMPORTANCE OF THE RESEARCH TOPIC

The generally accepted "gold standard" methods in the field of biodosimetry examine chromosome breaks and their consequences for the microscopic morphology of cells. Despite their many advantages, in practice these procedures also have shortcomings, especially regarding their time requirements. Accordingly, there are many attempts worldwide to automate classical methods, which significantly increases the throughput, but does not reduce the cell culture time, nor does it change the subjectivity, since the evaluator must check the recordings. Therefore, more and more laboratories are looking for new targets (mRNA and protein expression studies) to detect the suffered radiation exposure, which is made possible by rapidly developing molecular biological methods.
Due to its function, the Hungarian Defence Forces must be prepared for the execution of tasks in a CBRN operational environment, or even for disaster prevention activities in a possible nuclear emergency in a domestic environment. This, of course, comes with the risk of suffering significant radiation exposure.

Using the methods mentioned above, the radiation sensitivity of the soldiers assigned to such a task can be determined, and by checking the persons returning with complaints or with greater radiation exposure, the appropriate therapy can be selected based on the knowledge of the determined radiation injuries.

The polymerase chain reaction (PCR) is a molecular biological procedure based on the multiplication of nucleic acid chains, which is also suitable for the unique identification of individual DNA/RNA sections. With the spread of the method, a new tool is offered for biodosimetry, as ionizing radiation damages DNA (both nuclear, i.e.,

chromosomal DNA and mitochondrial DNA), and also changes the expression pattern of some protein mRNAs. One of the advantages of molecular biological methods over microscopic methods is that these methods are more widely used, can be learned more quickly and require less practice than techniques based on microscopic counting. DNA damage caused by radiation can be detected using PCR technology, but a biodosimetry method based on this has not yet been developed. The necessary infrastructure and the operating molecular biologists are present in several institutions of the country, in a disaster situation it is easier to involve external laboratories and find help than in the case of difficult microscopic techniques.

THE FORMULATION OF THE SCIENTIFIC PROBLEM

The current "gold standard" methods for determining radiation exposure, although sensitive and accurate, are very time-consuming, requiring several days of cell culture, manual sample processing, meticulous microscopic work, and statistical processing. Data processing requires microscopic photography, storage of glass slides carrying stained histological samples, and archiving of large amounts of digital images. The implementation is difficult and expensive, because it requires many working hours and requires special practice from the evaluators, which makes it difficult to involve a larger number of staff and additional laboratories if necessary. The evaluation is very subjective, so the laboratories participating in the biodosimetry work must draw up their own dose-effect curve, which means work of several months, possibly years. These methods have a very low throughput, which is why they can only be used to a limited extent during triage.

During events involving large crowds, a procedure is needed during triage that enables the examination of potentially injured persons in a shorter time and with greater throughput.

RESEARCH HYPOTHESES

In accordance with the objectives of the research, based on the research questions discussed in detail in Chapters 4 and 5, I formulated the following research hypotheses:

1. The chromosome aberration method currently used as the "gold standard" method is suitable for the qualitative determination of radiation exposures below deterministic values, as well as their quantitative estimation.

2. Similar to the chromosome aberration method, the micronucleus assay is suitable for the qualitative and quantitative analysis of analog radiation exposures, taking into account chemical-biochemical interference effects as well.

3. In the case of a DNA sequence that can be easily measured and changes as a result of radiation, preferably present in many copies, a procedure based on the PCR technique can be set up, which can be suitable both for biodosimetry tests and for pre-screening and follow-up in the case of large crowds for the purpose of triage. Based on these, a procedure can be developed by in vitro testing of blood cellular components, which can be used to determine the qualitative and quantitative characteristics of deterministic or sub-deterministic radiation exposures by examining lymphocytes, platelets, or the whole blood itself.

4. With the examination of mitochondrial DNA using irradiation, such DNA damage can be revealed, based on which dose values below the deterministic threshold can be detected with acceptable significance even after a longer period of time using PCR.

RESEARCH OBJECTIVES

The aim of the research is to map the observations related to the mechanism of radiation effect, as well as the methods suitable for testing materials that further strengthen (radioprotective) or weaken (radiosensitizing) the level of individual radiation sensitivity. In light of all of this, my aim is to establish the foundation for setting up a faster, cost-effective, high-throughput, objective method than the currently used methodologies using the modern PCR technique, which can be suitable for biodosimetry purposes after selecting the appropriate target, and can significantly shorten the time spent on the examination, thus suitable for the subsequent determination of personal radiation exposure, and in a disaster situation as a pre-screening examination and/or for follow-up during the triage.

The purpose of my PhD work is to establish this molecular biological method, to find a target sequence necessary for this, and to carefully examine the applicability of opportunities to further develop the test. Based on all this, I formulated the following research objectives:

1. Review the literature on biodosimetric procedures and possible directions for development.
2. Conducting targeted experiments on the applicability of dicentric chromosome analysis, with particular reference to the sensitivity of the method towards radiomimetic agents.
3. Conducting targeted experiments on the applicability of the micronucleus assay, including examination of a numerical distribution of micronuclei.
4. Based on the results of the literature research, to review the possibility of the development of DNA changes that have not yet been investigated in relation to radiation, which can be measured with a PCR device, as well as to examine with laboratory experiments the possibilities of setting up a biodosimetry procedure based on the PCR technique.
5. Examine whether the procedure described in point 4 can be used in each form of blood cellular components or in some of their cultures.
6. To examine the possibilities of whether the procedure described in point 4 can be used in case of the mitochondrial DNA, carrying out laboratory experiments to prove this.
7. To examine the specificity of the procedure to be set up through exposure to a radiomimetic agent.

RESEARCH METHODS

The aim of the thesis is to lay the foundation for the development of a new test method which is based on the detection of the DNA-damaging effect of ionizing radiation using the PCR technique. Accordingly, I chose theoretical and practical research methods to achieve my objective.

During the research I examined the structure and operation of other similar systems. I examined the applicability of traditional cytogenetic procedures. This was partly literature research, on the other hand it included cell culturing, microscopic work, the use of traditional image processing methods, and use of molecular biology methods.

During the experiments, I modeled the radiation exposure to the body by exposing the blood samples to X-rays. I performed the tests discussed in detail later on the samples obtained in this way.

I report on the detailed experiences gained during the setup of the new molecular biological methods in the last chapter of my dissertation. In addition, I also describe in detail the procedures used for each examination.

REVIEW OF RELEVANT LITERATURE

During the preparation of my dissertation, I studied the relevant literature on the topic and related subtopics. In my review of the background literature, I examined the experiences of numerous books, journal articles, and previous doctoral dissertations. In addition to the sources from mainly international research, I also tried to review the available domestic literature and incorporate it into my research. The literature on the topic is so diverse that I summarized it by topic in the dissertation. The first 4 chapters and the referenced bibliography at the end of the dissertation contain all the publications and their detailed source data that I used during my research.

BRIEF DESCRIPTION OF THE STUDY CARRIED OUT BY CHAPTER

In my dissertation I first described the basic properties of ionizing radiation. I presented in detail the types of radiation exposures from different sources, their relative ratios and I tried to give a clear picture of the actual extent of these radiation exposures.

In the second chapter I reviewed the biological effects that the radiation exposures described in the previous chapter causes in the human body. Several interdependent levels of this are known, which I have examined and illustrated in detail. In the chapter, I discussed the individual differences in the above-mentioned damages, as well as the description of agents that cause changes in the body similar to radiation.

In the third chapter I presented the tools that are suitable for determining individual radiation exposure. These are mostly tools based on physical methods. I described the biodosimetry methods that are suitable for this purpose and can give a more detailed picture of the extent of the actual biological damage. I wrote about the applicability of each technique, and briefly reviewed the parameters and relative advantages of each procedure.

In the fourth chapter I described the mitochondrion and the structure of its DNA. In connection with this I presented its characteristics that can make it suitable for biodosimetry tests. In connection with these properties I examined the biological targets and procedures that, when organized into a system, make it possible to develop a new, faster and more objective biodosimetry procedure than before.

In the fifth chapter I described in detail the laboratory tests I carried out during the research. As a first step, I examined the two most commonly used cytogenetic biodosimetry methodss, during which I analyzed their feasibility and evaluation from a practical point of view. I then performed molecular biological tests with mitochondrial DNA, which I presented in chronological and logical order in the thesis, from the setting of the basic parameters of the method to the detailed results of the tests performed on different sample types. The obtained results show that the method can be a promising tool in the toolbox of biodosimetry, especially in the case of a large number of samples, so it can be a significant help for disaster prevention.

Based on the tests I carried out, I made a recommendation for the use of the results, as well as explained the directions and limitations of the practical use of the results.

SUMMARIZED CONCLUSIONS

The "gold standard" method based on the examination of dicentric chromosomes is suitable for the detection of radiation effects despite its limitations regarding quantitative analysis, however, the time required for cell culturing and microscopic examination casts strong doubt on its suitability for triage purposes. This justifies the development of a method that can be used not only for the quantitative analysis of radiation effects, but whose time requirement is suitable for the purpose of triage, which is particularly important in the implementation of both the national defense and general disaster prevention tasks.

The manual processing of the micronucleus assay is simpler and requires less practice than in the case of the dicentric method, so it is more objective and provides fairly quick results manually. A very valuable feature of the micronucleus assay is that we can deduce the level of radiation exposure based on the distribution of the micronucleus number, this method can be especially useful if someone has been partially irradiated.

The mitochondrial tests carried out on the blood resulted in unexpected and extremely useful results, as they confirmed my assumption that radiation damage in the

mitochondrial DNA not only clearly proves the doses suffered, even below the deterministic threshold, but also provides information characterized by an acceptable significance for their quantity. It is worth noting that the effect of the radiomimetic compound I examined (bleomycin) can be sharply separated from the effect of radiation, thus the method has a kind of specific character in relation to radiation. Of course, it is still necessary to research the interference effect of other chemical reagents on the given assays.

NEW SCIENTIFIC FINDINGS

1. Through laboratory tests and data analysis I *proved* that the fact of radiation exposure can be confirmed or exclude with good accuracy based on the presence of dicentric chromosomes using the chromosome aberration method, with the caveat that the possibility of dicentric chromosomes caused by possible radiomimetics must also be taken into account. Furthermore, I *verified* that the dicentric chromosome analysis can only make an approximate estimate of the degree of radiation damage, taking into account several external factors and the results of other tests. Despite the fact that the procedure is used as a "gold standard" method and recommended by the International Atomic Energy Agency and other international professional organizations method, in the case of relatively small ($D<1$ Gy) deterministic doses, according to the experience of my work, it can only be applied conditionally.

2. Using a traditional, manual microscopic method I *showed* that different micronucleus number distributions correspond to individual irradiation doses. Based on this, if the distribution does not correspond to the dose value calculated on the basis of the micronucleus number, we can assume that the radiation exposure was not uniform, so the examination may be suitable for recognizing the fact of partial body irradiation. The obtained results are an important addition to the treatment of radiation-injured patients.

3. During laboratory experiments, I *verified* that both the culture of Jurkat cells and the platelet-rich plasma fraction are suitable for setting the basic parameters of the PCR measurement system to be performed later on whole blood. Based on the

experimental results obtained during the development of the test method, I *found* that the separation of the certain blood cellular components is not justified, and whole blood is the appropriate sample type for performing the test.

4. During my irradiation experiments I was the *first to prove* on whole blood that the "common" deletion marker can be measured between 3-24 hours, and it changes under the influence of ionizing radiation in the dose range I used (between 0-2 Gy). After 24 hours, the "common" deletion marker was detectable in the platelet-rich plasma preparation and in whole blood, but no significant change was detected. With a shorter incubation of 3 hours, the CD marker shows a significant maximum in whole blood at 0.5 Gy. There are still no clinical symptoms in this dose range, so the result provides a particularly valuable clue for test development. In this regard, I was the *first to establish* that bleomycin, although it behaves similarly to ionizing radiation in terms of creating dicentric chromosomes, does not induce an increase in "common" deletions in mtDNA in the same dose range in whole blood.

5. During my laboratory experiments, I was the *first to investigate and prove* that an approx. 200 base tandem duplication in mtDNA is more likely to be found in irradiated blood. Based on my measurements, this duplication is more likely to be found in irradiated blood. Since I considered this phenomenon suitable for the long-term detection of radiation damage, I started refining the system with a more specific, fluorescent probe-based procedure, thus developing the basics of a procedure applicable in radiation biology for the long-term detection of radiation damage. Until now, no one has investigated the relationship between tandem duplication and ionizing radiation, so the result has scientific novelty value.

I summarized the relationship between the individual hypotheses, the corresponding research objectives and finally the theses in the table below.

H1	O1, O2	T1
H2	O1, O3	T2
H3	O4, O5	T3
H4	O6, O7	T4, T5

PRACTICAL APPLICABILITY OF RESEARCH RESULTS

The literature review section of my research comprehensively summarizes the impact of ionizing radiation on the human body, as well as its detection possibilities. From the results of the literature research, it is clear that due to the biological and physical characteristics of radiation exposure, there are many areas where the test systems should and can be refined, and this research is a good starting point for this.

A detailed description and investigation of biological targets for the detection of radiation exposure is essentially beyond the scope of this research due to the large number of such targets, but the present work may be a useful contribution to the foundation of future biodosimetric research and the development of new analytical methods.

As a result of this research, I have developed a procedure that could be the basis of a PCR-based biodosimetry method which is faster and more objective than traditional cytogenetic procedures. The development of the technology into a concrete biodosimetry method requires further studies, but my research can be a perfect starting point for this.

My research has highlighted the importance of a new target, tandem duplication.

RECOMMENDATIONS

I recommend the results presented in my PhD thesis primarily to those who conduct research in the field of radiation biology and biodosimetry. This thesis contains a lot of useful information for starting future research.

I recommend the detailed literature review and my summary papers on the subject to those who are just getting involved in the research of mitochondrial DNA damage, the effect of radiomimetics and biodosimetry.

Due to its practical applicability, my thesis can be a useful aid for the development of a high-throughput and objective biodosimetry method, which can be an important tool in the toolbox of the Hungarian Defence Forces and civilian laboratories, as it could significantly speed up the triage process and shorten the time leading to care in a disaster situation involving radiation exposure or in operational conditions.

I recommend my thesis for further research in the field of radiobiology, biodosimetry and radiochemistry.

LIST OF PUBLICATIONS PREPARED BY THE PHD CANDIDATE

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PROFESSIONAL-SCIENTIFIC BIOGRAPHY OF THE DOCTORAL CANDIDATE

Name: Gábor Deli

Place and time of birth: Berettyóújfalu, December 8, 1987.

Studies:

In 2009 he obtained a certificate in the biology department of the University of Debrecen, majoring in laboratory operator, and in 2011, he also obtained his MSc qualification with molecular, immunological and microbiological specialization at the biology department of the University of Debrecen.

Professional career:

Between 2016 and 2022 he was a senior research fellow of the Radiobiology Laboratory of the Medical Center of the Hungarian Defence Forces. His work mainly included research tasks. It was here that he became involved in radiobiological and biodosimetric research, which later became the main thrust of his research. From January 2023, he is an employee of the Forensic Genetics Laboratory of the Clinical Center of the University of Debrecen.

Language skills:

He has a state-recognized intermediate "C" language exam in English and German.

Qualification:

Since October 2016, he has had an active, comprehensive radiation protection exam.

Memberships:

Member of the Society of Hungarian Military and Disaster Medicine since 2016.

Budapest, 2023.03.28

Gábor Deli