2007

Chemical Modification of Direct and Bystander Effects Induced by Radiation and Laser Light

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CHEMICAL MODIFICATION OF DIRECT AND BYSTANDER EFFECTS INDUCED BY RADIATION AND LASER LIGHT

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For the Degree of Doctor of Philosophy to the Dublin Institute of Technology

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January 2007

ABSTRACT

In April 2006, 20 years have passed since the explosion of the fourth block of the Chernobyl nuclear power plant (CNPP). This accident affected millions of people, and large territories were contaminated by radionuclides. As a result, background radiation levels increased, and people from contaminated territories are living constantly in low dose radiation conditions. The aim of this study was to assess the direct and bystander effects from low level γ-radiation and from serum samples from CNPP accident victims on human blood lymphocytes and keratinocytes *in vitro*. The possibility to modify these effects using radioprotective substances was also investigated. Bystander effects induced by laser radiation was also studied. The results have shown that melanin, melatonin and α-tocopherol were able to decrease direct and bystander radiation effects. Protection against direct irradiation was more effective than protection against bystander effects. Melatonin showed the best protective effect, whereas α-tocopherol showed the least protection. Serum samples from people affected by the Chernobyl accident, even 20 years after the accident, could induce micronuclei formation and decrease the viability of human keratinocytes. A direct correlation was shown between the frequency of aberrations in human peripheral blood lymphocytes from victims of the Chernobyl accident and the level of bystander factors in their blood serum. The most affected group were the Chernobyl liquidators, who were exposed to the highest radiation doses. Radioprotective substances were not able to protect cells from these bystander factors. In general, the present study has aided in our understanding of the nature of bystander effects.

DECLARATION

I certify that this thesis which I now submit for examination for the award of Ph.D., is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work. The cytogenetic test in Chapter 5 was performed in cooperation with Dr. Sergey MELNOV.

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Pavel MAROZIK

ACKNOWLEDGEMENTS

First of all, I'd like to thank my supervisors Fiona LYNG, Irma MOSSE and Carmel MOTHERSILL for giving me the opportunity to do a PhD. Thank you, Fiona, for your patience, spending days and weeks correcting all my reports and thesis and teaching me different assays and techniques. Thank you to Carmel and Irma for giving me the possibility to study at DIT and for your help, encouragements and brilliant ideas. Without your help, this work would never be realised. Big thanks to Colin SEYMOUR for his help and especially irradiations in St. Luke's during this research.

I'd like to acknowledge and thank Prof. Sergey MELNOV, head of Department of Ecological and Molecular Genetics in International Sakharov Environmental University, Minsk, for his advices and help in realisation of this research. He is an excellent organiser and thanks to him I received work space and access to equipment and biological materials in Republican Scientific and Practical Centre of Radiation Medicine and Human Ecology in Gomel (Belarus), where a large part of the present work has been carried out.

Thank you to Dr. James MURPHY who provided me with brilliant experience in PCR and oxygen electrode assays. Thank you to all the staff from Radiation and Environmental Science Centre, DIT, for their help and patience, especially Niamh and Rocky, our past and present lab managers. Thanks to everybody for great nights out.

Thank you to all my best friends Yuri, Ales, Shura, Viktor, Igor, Sergey and Vitaly and others. Special thanks to Olga for her love and understanding.

And a special thanks to all my family for comprehension, encouragement and willingness to help me. Especially I'd like to thank my father who did so much for me and supported me during all my studies. Thanks, everybody!!!

LIST OF ABBREVIATIONS

AB	Alamar Blue dye
AIF	Apoptosis Inducing Factor
Bq	Becquerel
Cdk	Cyclin-dependent kinase
CHO	Chinese Hamster Ovary cell line
Ci	Curie
CNPP	Chernobyl Nuclear Power Plant
CSF	Cerebrospinal Fluid
DI	DNA Index
FSH	Follicle Stimulating Hormone
FU	Fluorescent Units
GH	Growth Hormone
GSH	Glutathione
Gy	Gray
HPV-G	Human Papillomavirus G cell line
IAEA	International Atomic Energy Agency
ICCM	Irradiated Cell Conditioned Medium
IL	Interleukin
ITP	Inosine triphosphate
LET	Linear Energy Transfer
LH	Luteinising Hormone
LIBE	Laser Induced Bystander Effect
MDA	Malondialdehyde

Ml	Melanin
MN	Micronuclei
Mt	Melatonin
OH	Hydroxyl Radical
PBS	Phosphate Buffered Saline
PE	Plating efficiency
Q	Quality factor of radiation
R	Roentgen
RIBE	Radiation-Induced Bystander Effect
RNMDR	Russian National Medical Dosimetric Registry
RSH	Sulphydryl compound
SCN	Suprachiasmatic nucleus
SF	Surviving Fraction
Sv	Sievert
TGFβ1	Tumour Growth Factor β1
TNFα	Tumour Necrosis Factor α
Tp	α-Tocopherol
UV	Ultraviolet
WHO	World Health Organisation

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GENERAL INTRODUCTION

1.1. HISTORY OF RADIATION

The history of radiation is connected with three great discoveries at the end of the 19th century: the discovery of X-rays by Wilhelm Conrad Roentgen, the discovery of radiation emitted by uranium by Henri Becquerel and the discovery of the radioactive properties of polonium and radium by Marie and Pierre Curie.

Soon after this, it was realized that the biological effect of such radiation could be either beneficial or harmful, depending on how it was used.

Roentgen had been experimenting in his laboratory at the University of Würzburg with the discharge of electricity in "vacuum tubes". On the evening of November 8, 1895, Roentgen covered the tube completely with black cardboard. The laboratory was completely dark. Several feet away from the tube was a piece of paper, used as a screen, covered with barium-platinum cyanide. In the darkened room, Roentgen noticed the screen fluorescing, emitting light.

Roentgen experimented with these "new rays" in his laboratory for many weeks. He found that objects were transparent to these rays in different degrees, photographic plates were sensitive to X-rays.

On December 28, 1895, Roentgen delivered a preliminary paper, which also included X-ray photographs of hands, to the secretary of the Physical-Medical Society of Würzburg. The response to this new discovery was tremendous. Roentgen received the first Nobel Prize for physics in 1901.

The discovery of radioactivity followed the discovery of X-rays by only 2.5 months. Henri Becquerel, conjectured that other fluorescing materials might also emit X-rays. He worked for many days, testing a number of phosphorescent and fluorescent substances without success, until he tried uranium salt. The uranium salt emitted rays capable of penetrating black paper.

Becquerel found that the "Becquerel Rays" ionised gases. Thus it was possible to measure the activity in a sample simply by measuring the ionisation that it produced. Becquerel shared the 1903 Nobel Prize in physics and is known as the discoverer of radioactivity.

In September of 1897, Marie Curie found that thorium also emits rays. Then she investigated natural ores. The radioactivity of some of the ores was three to four times greater than could be accounted for on the basis of just their uranium and thorium content.

Together with Pierre Curie, Marie Curie found new elements they called polonium and radium. They also found that polonium disappeared spontaneously reducing itself to one half in a characteristic time.

In 1903, the Curies shared the Nobel Prize in Physics with Henri Becquerel. In 1911 Marie Curie received a second Nobel Prize, this time in chemistry, for her purification of radium metal. She is the first person ever to have won the prize twice.

Ernest Rutherford, a physicist from New Zealand, was working at the Cavendish Laboratory in 1898-1899. Through a long series of experiments he realized that there were two kinds of radiation emitted from Uranium. He called them alpha and beta. In a few years it was concluded that beta rays were cathode rays, that is, electrons.

In 1900, P. Villard in France had also found an additional penetrating radiation from Uranium. This radiation was not alpha nor beta, but more penetrating than the beta

particles; it was given the name gamma (these effects were observed by Becquerel when he developed his photographic plates).

In 1931, Irene Curie and François Joliot discovered that radioactivity could be induced artificially, and in 1935 they received the Nobel prize for this discovery. In 1932, James Chadwick discovered the neutron.

All these discoveries have been put to good use in medicine and industry. The medical use of X-rays continues to be the diagnosis of many forms of disease and the treatment of cancer. Artificial radioactive isotopes are also used for diagnosis and sometimes for treatment, as is the natural isotope radium. In industry, X-rays are used for quality control and for sterilization of food and medical equipment.

The studies of biological effects of ionising radiation started after the discovery of X-rays in 1895. The observed effects were skin damage (erythema, dermatitis), hair loss, lung cancer. At the same time, the first attempts to treat cancer with radiation appeared. According to data from these investigations, in 1906, Bergonie and Tribondeau reported that rapidly proliferating cells are more sensitive to the effects of radiation than cells which divide more slowly. Understanding of the processes of the cell cycle increased and it became apparent that rapidly dividing cells exhibited radiation damage quickly explaining their observed sensitivity.

Muller demonstrated the effect of X-rays on the mutation rates of the *Drosophila* fruit fly in 1927. This was the first work which outlined a dose-response relationship between radiation administered and an increase in mutation rate.

In 1935 Mottram noted the effect of oxygen on radiosensitivity of *Vicia faba* roots and postulated its importance to radiotherapy.

In 1940, Gray and his colleagues described some of the biological effects of fast neutrons, and in 1953 showed the influence of oxygen concentration on the response of tissues to radiation.

Patt in 1949 discovered the first substance – cysteine – able to decrease biological effects of radiation. And later, in 1951, Russell reported the hereditary effects of radiation on mice.

In 1956 Puck and Marcus explored the dose-response relationship by exposing cells in culture to radiation and demonstrated the effects of this radiation on proliferating cell populations.

In 1962, for the first time the dose-rate effect in cells *in vitro* was demonstrated by Hall and Bedford. At the same time, Terasima and Tolmach for the first time observed variation of radiosensitivity through the cell cycle.

In 1968, Casarett suggested the classification of tissue radiosensitivity.

All these and many others were important stages in the development of radiobiology, which is the scientific study of the biological effects of radiation.

1.2 IONISING RADIATION

From the beginning of time, mankind has been exposed to some form of radiation. This is because radiation is the process by which a body emits radiant energy, and we are all exposed to radiant energy, emitted by the Sun. According to the electromagnetic spectrum, only radiations with shorter wavelengths affect living organisms, when ionisation occurs.

Ionising radiation is a type of radiation in which an individual particle, such as a photon, electron, or helium nucleus, carries enough energy to ionise an atom or molecule, that is, to completely remove an electron from its orbit. If the individual particles do not carry this amount of energy, it is essentially impossible for even a large number of particles to cause ionisation. These ionisations, if enough occur, can be very destructive to living tissue, and can cause DNA damage and mutations.

The composition of ionising radiation can vary. Electromagnetic radiation can cause ionisation if the energy per photon, or frequency, is high enough, and thus the wavelength is short enough. Far ultraviolet, X-rays, and gamma rays are all ionising radiation, while visible light, microwaves, and radio waves are non-ionising radiation. Ionising radiation may also consist of fast-moving particles such as electrons, positrons, or small atomic nuclei.

There are two classes of ionising radiation:

- Corpuscular radiation – consists of streams of particles, atomic or subatomic, moving at high velocity, carrying energy which is dependent on their mass and velocities (α-, β-particles, protons, neutrons).
- Electromagnetic radiation – consists of vibrating electromagnetic fields (infra-red, visible light, ultra-violet, X-rays, γ rays).

All forms of radiation have similar features. They all travel in straight lines and their intensity falls off with distance travelled. This intensity observes the inverse square law; radiation will have a quarter of its intensity after it has travelled twice the distance. Interactions between ionising radiation and atomic particles occur at random and it is impossible to predict if any particular electron or nucleus will respond to the radiation and in what way it will respond. All ionising radiations can eject orbital electrons from some atoms of the material through which they pass, producing positive and negative ions. These chemically active ions are probably responsible for most of the significant radiobiological damage. Also, all the energy absorbing events depend on interactions between photons or particles of radiation and the subatomic particles of the absorbing matter but only the atomic composition of the absorbing matter is important.

The various types of radiation differ in their ability to penetrate into matter and in the spatial distribution of the ionisations and excitations that they produce. Electromagnetic radiations penetrate deeply whereas charged particles have a definite range which depends on the type of particle, its energy and the kind of material it passes through.

Radioactive materials release alpha particles which are the nuclei of helium, beta particles, which are fast moving electrons or positrons or gamma rays. Alpha and beta particles can often be shielded by a piece of paper or a sheet of aluminium, respectively. They cause most damage when they are emitted inside the human body. Gamma rays are less ionising than either alpha or beta rays, but protection against them requires thicker shielding. They produce damage similar to that caused by X-rays such as burns, and cancer through mutations.

1.2.1 Radiation units

Quite a number of pioneer radiologists suffered severe injuries, and some even died, as a result of prolonged exposure to dangerously high intensities of X-rays. These

early workers in the field had no means of measuring the harm caused by radiation accurately and depended on unreliable effects such as the degree of skin reddening caused by the exposure, or on timing the exposure from a certain type of X-ray machine to establish quantity.

One of the earliest observed properties of X-rays was their ability to ionise air and thereby create electric charges which can be collected and measured. In 1928 the International Congress of Radiology specified this property as a means of measuring the amount of X-radiation, and defined a unit which was named the roentgen (R). The R is a unit of exposure and defined as the quantity of X or γ-radiation such that the associated secondary electrons emitted produce ions of 2.58×10^{-4} Coulombs per kilogram of air. The gray is the unit of absorbed dose and is defined as the absorption of one joule of radiation energy per kilogram of material ($1\ Gy = 1\ Jkg^{-1}$).

The activity of a radioactive source is measured in Becquerels (Bq), where one becquerel is equal to one nuclear disintegration per second. An older unit is the Curie (Ci). $1\ Ci = 3.7 \times 10^{10}$ Bq.

To obtain the biologically effective dose for different types of radiation, the term "dose equivalence" is used. The unit of dose equivalence, the sievert (Sv), is numerically equal to a dose in grays multiplied by the quality factor (Q) of the radiation. The quality factor takes into account the relative biological effectiveness of the type of radiation being used. For alpha particles Q may be as high as 20. For beta particles, x-rays and γ-radiation (which was used in this study) the quality factor is taken as 1, so that the gray and sievert are equivalent for those radiation sources.

1.2.2 γ-radiation

In this study, cells were irradiated with γ rays from a radioactive cobalt source with an atomic mass of 60 g per mole (^{60}Co).

γ rays are an energetic form of electromagnetic radiation produced by radioactive decay or other nuclear or subatomic processes such as electron-positron annihilation. They form the highest-energy end of the electromagnetic spectrum and are often defined to begin at an energy of 10 keV. The wavelength of the γ-rays depends on both the energy of the electrons and the atomic number of the isotope. Atoms on biological tissues which may be hit by ionising radiation have varying atomic number and would respond in different ways to the same radiation dose.

In terms of ionisation, γ radiation interacts with matter via three main processes: the photoelectric effect, Compton scattering, and pair production.

Photoelectric effect describes the case in which a γ-photon interacts with and transfers all of its energy to an orbital electron, ejecting that electron from the atom. The photoelectric effect is thought to be the dominant energy transfer mechanism for X-ray and γ-ray photons with energies below 50 keV, but it is much less important at higher energies.

Compton scattering is an interaction in which an incident γ photon loses enough energy to an orbital electron to cause its ejection, with the remainder of the original photon's energy being emitted as a new, lower energy γ photon with an emission direction different from that of the incident γ photon. The probability of Compton scatter decreases with increasing photon energy. Compton scattering is thought to be the principal absorption mechanism for γ rays in the intermediate energy range 100 keV to 10 MeV, an energy spectrum which includes most γ radiation present in a nuclear explosion. Compton scattering is relatively independent of the atomic number of the absorbing material.

By interaction in the vicinity of the Coulomb force of the nucleus, the energy of the incident photon is spontaneously converted into the mass of an electron-positron

pair. A positron is the anti-matter equivalent of an electron; it has the same mass as an electron, but it has a positive charge equal in strength to the negative charge of an electron. Energy in excess of the equivalent rest mass of the two particles (1.02 MeV) appears as the kinetic energy of the pair and the recoil nucleus. The electron of the pair, frequently referred to as the secondary electron, is densely ionising. The positron has a very short lifetime. It combines within 10^{-8} seconds with a free electron. The entire mass of these two particles is then converted into two γ photons of 0.51 MeV energy each.

γ rays are often produced alongside other forms of radiation such as α or β. When a nucleus emits an α- or β-particle, the daughter nucleus is sometimes left in an excited state. It can then jump to a lower level by emitting a γ ray in much the same way that an atomic electron can jump to a lower level by emitting ultraviolet radiation. An example of γ ray production:

First ^{60}Co decays to excited ^{60}Ni by β decay (creating electron and electron-type antineutrino):

$$^{60}Co \rightarrow {}^{60}Ni^{*} + e^{-} + \nu_e$$

then the excited ^{60}Ni drops down to the ground state by emitting a γ ray:

$$^{60}Ni^{*} \rightarrow {}^{60}Ni + \gamma$$

γ rays of 1.17 MeV and 1.33 MeV are produced.

Cobalt 60 emits γ rays with an energy of 1.25 MeV so most of the interactions with the cells are Compton interactions. These interactions generate many free radicals. Free radicals are atomic or molecular species with unpaired electrons on an otherwise open shell configuration. These unpaired neutrons are usually highly reactive, so radicals are likely to take part in chemical reactions. Radicals play an important role in combustion, atmospheric chemistry, polymerization, plasma chemistry, biochemistry, and many other chemical processes, including human physiology.

1.3 RADIOBIOLOGY

When cells are exposed to ionising radiation the standard physical effects between radiation and the atoms or molecules of the cells occur first and the possible biological damage to cell functions follows later. The biological effects of radiation result mainly from damage to DNA, which is the most critical target within the cell; however, there are also other sites in the cell that, when damaged, may lead to cell death. When directly ionising radiation is absorbed in biological material, the damage to the cell may occur in one of two ways: direct or indirect.

1.3.1 Direct action of radiation

In direct action the radiation interacts directly with the critical target in the cell. The atoms of the target itself may be ionised or excited through Coulomb interactions, leading to the chain of physical and chemical events that eventually produce the biological damage. Direct action is the dominant process in the interaction of high LET (linear energy transfer - a measure of the energy transferred to material when ionising particle travels through it; used to quantify the effects of ionising radiation on biological specimens or electronic devices) particles with biological material.

Target theory states that cells contain at least one critical site or target that must be hit by radiation if the cell is to be killed. Ionisation events outside the target do not cause cell death. Target theory strictly applies to the direct action of radiation. DNA is considered to be the main target for cell damage by radiation [Okada, 1970; Painter, 1980].

There is strong evidence to indicate that DNA is the principal target for the biological effects of radiation [Zirkle and Bloom, 1953; Munro, 1970]. If cells are irradiated with γ rays many breaks of a single strand of DNA occur. In intact DNA single strand breaks are of little consequence, as these are repaired readily using the opposite strand as a template. If a double strand break occurs where the breaks are

opposite each other this could result in mutation or cell death. The yield of double strand breaks following irradiation of cells is 0.04 times that of single strand breaks.

It is believed that the effects of radiation on DNA cause long-term cell damage. A protein has a short life span which means radiation damaged proteins have minimal impact on the cell as a whole. Proteins which are not hit by radiation but are merely present when the cell is irradiated are destroyed shortly afterwards. However, DNA damage can cause continued production of a damaged protein and thus long-term cell damage.

Damage to the nucleus is regarded as the main cause of radiogenic cell death [Zirkle and Bloom, 1953; Munro, 1970]. Nuclear and nucleolar lesions, invaginations of the nuclear envelope, increases in nuclear diameter, marginated chromatin, micronucleation, convolution of the nuclear envelope and nuclear fragmentation are effects that result from exposure to radiation [McClain *et al.,* 1990; Gasperin *et al.,* 1992; Cornforth and Goodwin, 1991). Such events left unchecked will lead to cell death.

1.3.2 Indirect action of radiation

In indirect action the radiation interacts with other molecules and atoms (mainly water, since about 80% of a cell is composed of water) within the cell to produce free radicals, which can, through diffusion in the cell, damage the critical target within the cell. In interactions of radiation with water, short lived yet extremely reactive free radicals such as H_3O^+ (water ion) and $OH^\bullet$ (hydroxyl radical) are produced. The free radicals in turn can cause damage to the target within the cell.

The free radicals that break the chemical bonds and produce chemical changes that lead to biological damage are highly reactive molecules because they have an unpaired valence electron.

About two thirds of the biological damage by low LET radiations (sparsely ionising radiations) such as X rays or electrons is due to indirect action.

Indirect action can be modified by chemical sensitizers or radiation protectors.

1.3.3 Effects of radiation

As ionising radiation passes through the body, it interacts with the tissues transferring energy to cells and other constituents by ionisation of their atoms. This phenomenon has been extensively studied in the critical genetic material, DNA, which controls the functions of the cells. If the damage to DNA is slight and the rate of damage production is not rapid, i.e. at low dose rate, the cell may be able to repair most of the damage. If the damage is irreparable and severe enough to interfere with cellular function, the cell may die either immediately or after several divisions.

At low doses, cell death can be avoided by the normal mechanisms that regulate cellular regeneration. However, at high doses and dose rates, repair and regeneration may be inadequate, so that a large number of cells may be destroyed leading to impaired organ function. This rapid cell death at high doses leads to early deleterious radiation effects which become evident within days or weeks of exposure, and are known as "deterministic effects".

Lower doses and dose rates do not produce these acute early effects, because the available cellular repair mechanisms are able to compensate for the damage. However, this repair may be incomplete or defective, in which case the cell may be altered so that it may develop into a cancerous cell, perhaps many years into the future, or its transformation may lead to inheritable defects in the long term. These late effects, cancer induction and hereditary defects, are known as "stochastic effects" and are those effects whose frequency, not severity, is dose dependent. Moreover, they are not

radiation-specific and, therefore, cannot be directly attributed to a given radiation exposure.

For this reason, low dose health effects in humans cannot be measured and, therefore, risk projections of the future health impact of low-dose ionising radiation exposure have to be extrapolated from measured high-dose effects. The assumption is made that no dose of ionising radiation is without potential harm, and that the frequency of stochastic effects at low doses is proportional to that occurring at high doses. This prudent assumption has been adopted to assist in the planning of radiation protection provisions when considering the introduction of practices involving ionising radiations. The ICRP has estimated the risk of fatal cancer to the general population from whole-body exposure to be 5% per Sv [ICRP, 1991].

1.3.4 Cell survival curves

A cell survival curve describes the relationship between the surviving fraction of cells (i.e. the fraction of irradiated cells that maintain their reproductive integrity (clonogenic cells) and the absorbed dose. Cell survival as a function of radiation dose is graphically represented by plotting the surviving fraction on a logarithmic scale on the ordinate against dose on a linear scale on the abscissa.

Cell surviving fractions are determined with *in vitro* or *in vivo* techniques. A plot of surviving fraction against dose describes the relation of radiation dose administered to the proportion of cells that survive. Example of survival curve for human squamous carcinoma cells irradiated by sparsely ionising (low-LET) radiation beams is shown in Figure 1.1. In this assay, cell survival means those cells that retained reproductive ability. If a cell is no longer reproductively viable it ceases to have any influence on the subsequent cell population. In the context of genetic proliferation such a cell is dead.

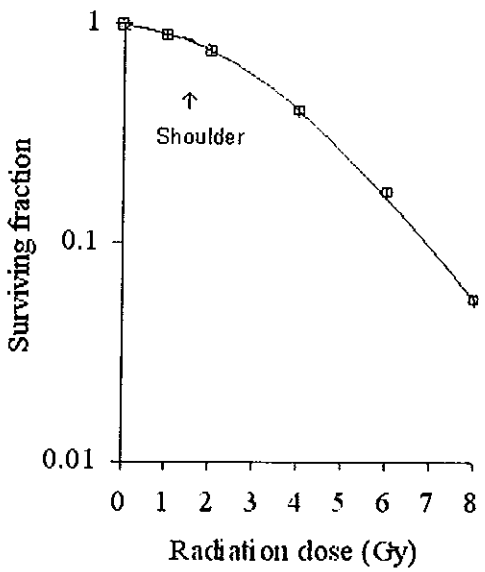

Figure 1.1. Typical cell survival curve for low LET radiation [Kriehuber et al., 1999]. This is a linear quadratic curve, with the shoulder region indicating repair at low doses.

1.3.4 Non-targeted effects

Ever since the discovery of X-rays was made by Roentgen more than a hundred years ago, it had been generally accepted that the important biological effects of ionising radiation in mammalian cells were a direct consequence of unrepaired or misrepaired DNA damage in the irradiated cells. The classical dogma of radiation biology, as narrowly interpreted from target theory, asserts that genetic damage occurs only during or very shortly after deposition of energy in nuclear DNA (targeted effects) and, is due only to the direct action of the irradiation or from very short lived oxy-radicals generated by it, and that the course of biological consequences is fixed within one or two cell generations.

However, recent experimental evidence, mainly from *in vitro* α-particle studies, indicates that ionising radiation can cause biological effects, including DNA damage, by mechanisms that are independent of nuclear traversals – "non-targeted" effects. Several studies have shown that genetic changes occur in a greater number of cells than expected when mammalian cell cultures are exposed to low fluencies of α-particles by which only a very small fraction of the cells is traversed by a particle track and thus directly exposed to radiation [Nagasawa and Little, 1992; Lehnert *et al.*, 1997; Azzam *et al.*, 1998]. These studies, along with others involving low linear energy transfer radiation from incorporated radionuclides [Bishayee *et al.*, 1999] and the transfer of growth media from irradiated to non-irradiated cell cultures [Mothersill *et al.*, 1997], and microbeam studies [Prise *et al.*, 1998] challenge the paradigm that radiation traversal through the nucleus of a cell is a prerequisite to produce genetic changes or a biological response. They indicate that cells in the vicinity of directly irradiated cells or recipient of medium from irradiated cultures can also respond to the radiation exposure.

Thus, the major feature of "non-targeted" effects is that direct nuclear (DNA) exposure is not required for their expression. Among these effects that cannot be explained by the classical dogma is the "bystander effect", defined as an effect elicited in cells that are not directly "hit" by radiation. There are other "non-targeted" phenomena including the radiation-induced adaptive response and long-lasting alterations in gene expression, transmissible genomic instability, low dose radio hyper-sensitivity, delayed reproductive death and radiation-induced long-lived radicals. The present studies were focused on the so-called "bystander effect".

1.3.4.1. Radiation-induced bystander effect

The radiation-induced bystander effect is a phenomenon whereby cellular damage (sister chromatid exchanges, chromosome aberrations, apoptosis, micronucleation, transformation, mutations and changes of gene expression) is expressed in unirradiated neighbouring cells, connected or not to an irradiated cell or cells (Figure 1.2). In the last decades huge advances have been made in this area, and quite a lot of information about these "bystander factors" has been discovered, although the specific nature of them has yet to be elucidated [Morgan *et al.*, 2005].

The radiation-induced bystander effect has been broadly defined as referring to the occurrence of biological effects in unirradiated cells as a result of exposure of other cells to ionising radiation. Several protocols have been used to detect radiation induced bystander effects: cultures consisting of sparse or density-inhibited cells were exposed to low fluencies of α-particles generated from conventional broad- or micro-beam irradiators; radiolabelled cells were mixed with non-labelled cells and assembled in multicellular clusters; growth medium was harvested from irradiated cells and added to non-irradiated cultures [Mothersill and Seymour, 2001; Little *et al.*, 2002].

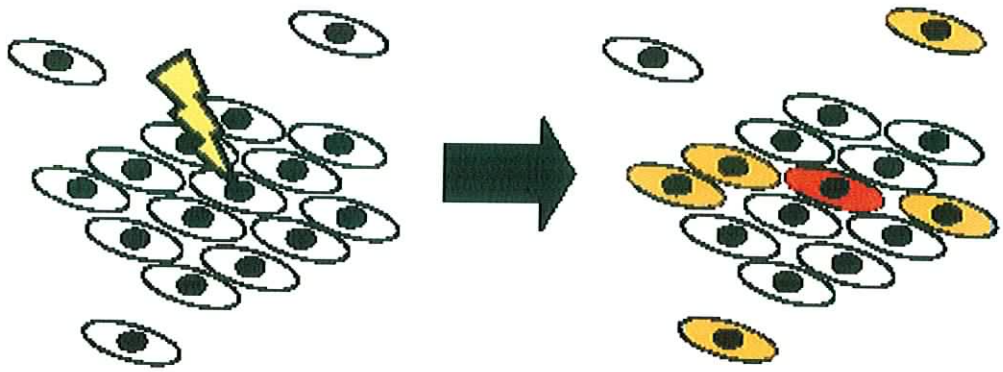

Figure 1.2. Scheme of bystander effect. Directly damaged cell is highlighted dark; bystander damaged cells are highlighted light.

In 1954 Parsons showed that children that were receiving radiation to the spleen for treatment of leukaemia, had damage to their bone marrow. This then was the first evidence to suggest that damage from radiation is not confined to the tissue that had been exposed.

A bystander effect induced in cell cultures exposed to α-particles was first described by Nagasawa and Little [1992]. An enhanced frequency of sister chromatid exchanges in 20-40% of Chinese hamster ovary cells was observed in cultures exposed to fluencies by which only 0.1-1% of the cell's nuclei were actually traversed by a particle track. These results indicated that the target for genetic damage by α- particles is much larger than the nucleus or in fact than the cell itself. This was subsequently confirmed by others for the same endpoint in human fibroblasts [Deshpande *et al.*, 1996]. Since then, it has been shown that an enhanced frequency of specific gene mutations can also occur in bystander cells present in cultures exposed to very low fluencies of α-particles [Nagasawa *et al.*, 1999; Zhou *et al.*, 2000]. Also, an enhanced frequency of micronucleus formation and apoptosis in bystander cells was observed [Prise *et al.*, 1998; Azzam *et al.*, 2002], and *in vitro* neoplastic transformation

experiments have shown that bystander cells neighbouring irradiated cells are also at risk [Sawant *et al.*, 2001]. The latter studies thus suggest that, under some conditions, mutations and chromosomal aberrations induced in bystander cells may lead to tumourigenesis.

Bystander effects were also observed in studies using other radiation sources. With relevance to the study of nonuniform distribution of radioactivity, cytotoxic effects were observed in bystander cells when cells labelled with short-range radiation emitters were mixed with unlabeled cells and assembled in a three-dimensional architecture [Bishayee *et al.*, 1999; Howell *et al.*, 2002]. In studies with low-LET radiations, growth medium harvested from γ-irradiated cultures containing epithelial cells reduced the clonogenic survival of unirradiated control cells present in a different culture dish [Mothersill and Seymour, 1997]. Highlighting radiation induced epigenetic effects, conditioned medium harvested from cells derived from a clone that had previously survived exposure to ionising radiation possessed a persistent and potent death inducing effect on bystander cells [Nagar *et al.*, 2003].

Bystander effects are observed in a number of different cell types irrespective of the type of radiation exposure. The mechanisms of the bystander effect are not yet known. However, there is evidence that the bystander effect may have at least two separate pathways for the transfer of damage from irradiated cells to unirradiated neighbours: through gap junctions or by cell culture mediated factors. Both high LET α-particles [Nagasawa and Little, 1992; Deshpande *et al.*, 1996; Lorimore *et al.*, 1998] and low LET γ-irradiation [Mothersill and Seymour, 1997; 1998] have been shown to induce this effect; however, it remains unclear whether the same signal is involved for both types of radiation.

Induction of the bystander effect with alpha particles may involve cell-cell communication [Zhou *et al.*, 2000] and there is evidence for the involvement of gap junction mediated intercellular communication [Azzam *et al.*, 2001] in confluent cultures of primary human diploid fibroblasts exposed to low fluencies of α-particles. They showed that *p53* and *p21* mediated pathways are activated. Hickman *et al.* [1994] reported that a *p53*-mediated signalling pathway could be activated in the bystander effect. They studied effects of low-dose α-particle irradiation of rat lung epithelial cells. Flow cytometry analysis of the fraction of cells with elevated levels of *p53* protein detected an increased expression in a higher proportion of cells than were hit by a α-particle. While direct evidence for the involvement of gap junction mediated intercellular communication in the bystander effect has been demonstrated, the nature of the factors communicated through gap-junctions has not been identified. However, the size would have to be small (≤2000 Da, e.g. ions, second messengers).

In contrast, the low LET radiation-induced bystander effect seem to be independent of cell-cell contact and are a consequence of secreted factors into the culture medium [Mothersill and Seymour, 1998]. A series of studies [Lehnert and Goodwin, 1997; Narayanan *et al.*, 1999] suggest a mechanism in which the irradiated cells secrete cytokines or other factors that act to increase intracellular levels of reactive oxygen species in unirradiated cells. Lehnert and Goodwin [1997] demonstrated that the culture medium harvested from the cells irradiated with low fluencies of α-particles could induce an increase in sister chromatid exchanges when incubated with unirradiated test cells. Experiments from *in vitro* studies are reproducible and several investigators have reported an increase in genetic damage and a reduction in plating efficiency either upon co-culture or exposure to medium from irradiated cells [Nagasawa and Little, 1992, 1999; Azzam *et al.*, 2001; Sawant *et al.*, 2001; Mothersill

and Seymour, 1997, 1998]. In this scenario it was hypothesized that irradiated cells release cytotoxic factors into their growth medium which may induce signal transduction pathways leading to cell death in unirradiated cells [Mothersill and Seymour, 1998]. Although a specific factor or signal has not been identified to date, a potential mechanism may involve production of cytokines such as IL-8, which is implicated in the alpha-particle mediated bystander effect [Deshpande *et al.,* 1996; Narayanan *et al.,* 1999]. Barcellos-Hoff and Brooks [2001] have also hypothesized that TGFβ1, an extracellular sensor of damage, may also be involved in the bystander effect. Another possible mediator of the bystander effect is the apoptosis inducing factor (AIF), secreted by mitochondria in response to oxidative stress [Kroemer, 1997]. Although it acts intracellularly, AIF may signal the downstream release of additional extracellular cytotoxic factors in the culture medium.

Recently more data about factors, possible mechanisms of RIBE and their relationship with other processes have appeared.

Bystander-induced mutations usually are almost entirely point mutations, whereas deletions are predominately found in directly irradiated cells [Nagasawa *et al.*, 2003]. Also, in bystander cells chromatid-type aberrations are predominant, arising from single strand breaks or DNA damage, while in directly irradiated cells, chromosome-type aberrations (double strand breaks) are predominant [Nagasawa *et al.*, 2005]. At the same time, in other investigations in bystander cells high levels of MN were observed, which arise primarily from double strand breaks [Shao *et al.*, 2005; Konopacka and Rzeszowska-Wolny, 2006]. An elevated fraction of cells exhibiting histone γH2AX foci, phosphorylation of which is one of the earliest cellular responses to ionising radiation, or an increased focus number/cell occurs in bystander cells [Hu *et al.*, 2006; Burdak-Rothkamm *et al.*, 2007].

There is evidence that increased expression of p53 and p21Waf1 proteins is observed in bystander recipient cells, leading to transient arrest in G1 phase of the cell cycle [Azzam *et al.*, 2002]. It was demonstrated that TGF-α and TNF-α, secreted from irradiated cells, could activate extracellular signal-regulated kinase 1/2 (ERK) in bystander cells, which is also responsible for bystander-induced survival reduction and mutagenesis and is a member of mitogen-activated protein kinase (MAPK) superfamily [Hagan *et al.*, 2004; Murphy *et al.*, 2005; Zhou *et al.*, 2005].

Also in recent studies it was shown that reactive oxygen species (ROS) participate in bystander responses [Lyng *et al.*, 2006a; Yang *et al.*, 2005; Burdak-Rothkamm *et al.*, 2007]. Recent evidence emerged for the persistent increase in intracellular ROS levels in bystander cells [Lyng *et al.*, 2002; 2006a, 2006b]. In these studies, the importance of oxidative stress and calcium signalling in γ-radiation-induced bystander effects was shown. Apoptosis was found to be a contributor to bystander induced cell death in certain cell types [Lyng *et al.*, 2002; 2006a, 2006b; Liu *et al.*, 2006]. ROS were involved in decreased mitochondrial membrane potential and increased intacellular calcium levels in bystander recipient cells. It was shown that c-JUN NH2-terminal kinase (JNK) and ERK were activated in bystander cells, but not p38 (another member of MAPK superfamily). Chelating of calcium and inhibition of voltage-dependent calcium channels resulted in suppression of apoptosis induction in bystander cells, suggesting that calcium signalling mediates RIBE. Inhibition of ERK pathway appeared to increase bystander-induced apoptosis, while inhibition of the JNK pathway appeared to decrease apoptosis [Lyng *et al.*, 2006a]. Shao *et al.* [2006] also reported modulation of α-particle induced bystander response by calcium fluxes.

Not only nuclear, but also cytoplasmic irradiation has been shown to induce gene mutations in bystander-recipient cells [Zhou *et al.*, 2006]. In this process nitric

oxide was involved, which has previously been shown to be induced in bystander cells [Matsumoto *et al.*, 2001; Shao *et al.*, 2006].

Several studies have shown a relationship between RIBE and genomic instability. Sowa Resat and Morgan [2004] found that bystander factors from chromosomally unstable progeny of irradiated cells are able to induce cell death and chromosomal instability in recipient cells. Bystander factors were able to induce ROS, increase intracellular calcium levels and decrease mitochondrial membrane potential in bystander cells after 35 population doublings [Lyng *et al.*, 2002]. Also, delayed chromosomal instability has been shown in the progeny of bystander cells in *in vivo* [Lorimore *et al.*, 2005] and *in vitro* [Moore *et al.*, 2005; Bowler *et al.*, 2006] studies.

Radiation-induced bystander effects have not been exclusive to tissue culture analyses. There is increasing evidence indicating that the bystander effect may be found *in vivo* as well.

In vivo experiments performed as early as 1974, have also demonstrated their existence. Brooks *et al.* [1974] have shown that when α- particle emitters are concentrated in the liver of Chinese hamsters, all cells in the liver are at the same risk for the induction of chromosome damage even though a small fraction of the total liver cell population were actually exposed to α-particles. Using a bone marrow transplantation protocol, in which a mixture of irradiated and non-irradiated bone marrow cells that were distinguishable by a cytogenetic marker were transplanted, the chromosomal instability in the progeny of non-irradiated hemopoetic stem cells have been demonstrated [Watson *et al.*, 2000].

The bystander effect induced *in vivo* by radioactive decay was demonstrated when mice were injected with a mixture of radiolabelled (at a lethal concentration) and unlabeled tumour cells. A distinct inhibitory effect ensued in the growth of tumours

derived from unlabelled cells [Xue *et al.*, 2002]. Lorimore *et al.* [2001] observed inflammatory type responses after exposure of hemopoetic cells to ionising radiation *in vivo*, attributable to a bystander factor, which may contribute to leukaemogenesis. In the clinic, physicians now treat solid tumours with a spatially fractionated radiotherapy regimen that results in reduction in tumour size, presumably mediated by a bystander-like effect [Mohiuddin *et al.*, 1999]. Results from these and other studies clearly prove that the bystander effect is not present solely in tissue culture systems but also exists *in vivo*.

Recent *in vivo* mouse experiments have shown that the *p53* protein is a mediator of the radiation-induced abscopal effect [Camphausen *et al.*, 2003]. *p53* was previously shown to have a role in the secretion of stress-induced growth inhibitors [Komarova *et al.*, 1998]. The secretion of factors capable of inhibitory abscopal/bystander effects when *p53* wild type tumours are irradiated would potentiate the effect of radiation in eradicating tumours. Following irradiation of the base of a rat lung, an increased frequency of micronuclei was observed in the unirradiated upper lung, with attenuation occurring by pre-treatment with superoxide dismutase [Khan *et al.*, 1998]. These abscopal effects of radiation have also been described in patients with chronic leukaemia [Nobler, 1969], and in the bone marrow of children with chronic granulocytic leukaemia after irradiation of their spleen [Parsons *et al.*, 1954].

There have been a large number of studies of internally deposited radioactive materials where the radionuclide and the dose are limited to specific tissues of the body. In all of these studies, the site of the cancer is the same as the site of the deposition of the radioactive material [Stannard, 1988]. Such studies suggest that if a soluble factor is produced and released into the blood stream, it has had little impact on risk for the development of cancer in other tissues. The observed difference between the production

of clastogenic factors, which are thought to increase tumour risk, and the failure of internally deposited radioactive materials to increase the frequency of cancers outside the dose field may have resulted from differences associated with the dose-rate of the exposure. After acute exposure, the release and damage from these factors may be large and occur within a short time frame. For the chronic, low dose-rate exposure, the amount of such factors released into the blood stream at any one time may result in a very low concentration that could not cause cellular damage outside the organ or tissue of interest. These studies suggest that at low dose-rates, clastogenic effects would not have any influence outside the tissue where the dose is delivered and therefore would not be important *in vivo*.

The importance of bystander effects to fractionated radiotherapy has been emphasized [Mothersill and Seymour, 2002]. Growth medium harvested from cultured cells receiving fractionated irradiation resulted in greater cytotoxicity when added to bystander cells than growth medium harvested from cultures receiving a single dose of irradiation. This cell killing effect of conditioned medium from irradiated cultures is contrasted with the split dose recovery observed in cultures directly exposed to fractionated irradiation.

Since the bystander effect can be induced after doses as low as 5mGy γ rays [Mothersill and Seymour, 2002] or 1 alpha particle traversal *in vitro* [Sawant *et al.*, 2002], it can be concluded that it has relevance for low dose radiation exposure. The bystander signal may lead to the accumulation of aberrant cells that are genomically unstable and may progress towards carcinogenesis. Also, radioadaptive bystander cells may potentially manifest more complex types of DNA damage and be more susceptible to transformation after subsequent exposure to therapeutic doses of ionising radiation [Iyer and Lehnert, 2002]. An understanding of the effects of radiation as a coordinated

multicellular response that affects not just the irradiated cells but also unirradiated cells may help clarify the contribution of effects in unirradiated cells to radiation risk estimates. Carcinogenesis models will ultimately need to incorporate both targeted as well as epigenetic aspects when estimating such risk. Mothersill and Seymour [2002] have suggested that novel therapeutic strategies might involve restoring the tissue's ability to control and coordinate a response following radiation exposure.

In the present research, the influence of melanin, melatonin and alpha-tocopherol (Vitamin E) on bystander effects in HPV-G cells has been studied.

1.4 POPULATIONS AFFECTED BY THE CHERNOBYL ACCIDENT

For millions of inhabitants of the planet the explosion of the fourth block of the Chernobyl Nuclear Power Plant (CNPP) on the 26th of April 1986 divided their life into two parts: pre and post Chernobyl.

Twenty years after the Chernobyl disaster, the need for continued study of its far-reaching consequences remains as great as ever. The radioactive fallout from the Chernobyl accident contaminated large territories in Belarus, Russia and Ukraine affecting life in rural communities for decades to come. Agriculture and forestry are forbidden in wide areas. Many of those living in the affected areas are ignorant of the risks that they face, or have adopted an apathetic and fatalistic attitude.

Substantial doses of radiation were received by the general population and liquidators in the period immediately following the accident. The associated risks involved have already been incurred and cannot be influenced by environmental management measures. The main problem today relates to internal irradiation resulting from the consumption of contaminated foodstuffs, notably milk, meat and forest products such as game, berries and mushrooms.

Today several million people (by various estimates, from 5 to 8 million) still reside in areas that will remain highly contaminated by Chernobyl's radioactive pollution for many years to come. Since the half-life of the major (though far from the only) radioactive element released, caesium-137 (^{137}Cs), is a little over 30 years, the radiological (and hence health) consequences of this nuclear accident will continue to be experienced for centuries to come.

According to the most wildly accepted estimates, the most affected groups of population are the following:

1. accident liquidators, including civilian and the military personnel drafted to carry out clean-up activities and construct the protective cover for the reactor;

2. evacuees from dangerously contaminated territories inside the 30-km zone around the power plant;

3. people living in contaminated areas of the former Soviet Union;

4. populations outside the former Soviet Union – the radioactive materials of a volatile nature (such as iodine and caesium) that were released during the accident spread throughout the entire Northern hemisphere. The doses received by populations outside the former Soviet Union are relatively low, and show large differences from one country to another depending mainly upon whether rainfall occurred during the passage of the radioactive cloud. These doses range from a lower extreme of a few mSv or tens of mSv outside Europe, to an upper extreme of 1 or 2 mSv in some specific areas of some European countries.

1.4.1 Chernobyl Liquidators

Liquidators were hundreds of thousands of workers and military personnel, who were involved in the emergency actions on the site during the accident and the subsequent clean-up operations which lasted for a few years. A restricted number, of the order of 400 people, including plant staff, firemen and medical aid personnel, were on the site during the accident and its immediate aftermath, and received very high doses from a variety of sources and exposure pathways. The doses to these people ranged from a few Gy to well above 10 Gy to the whole body from external irradiation and comparable or even higher internal doses, in particular to the thyroid, from incorporation of radionuclides. The largest group of liquidators participated in clean-up operations for variable durations over a number of years after the accident. Although they were no longer working in emergency conditions, and were subject to controls and

dose limitations, they received significant doses ranging from tens to hundreds of millisieverts.

Chernobyl liquidators are decontamination workers who were in the 30 km zone in 1986-1989. The total number of liquidators has yet to be determined accurately, since only some of the data from some of those organizations have been collected so far in the national registries of Belarus, Russia, Ukraine and other republics of the former Soviet Union. The most realistic estimation is given by Ilyin [1995] – about 300,000 – 320,000 people. This data is confirmed by the data from the All-Union distribution register (USSR, 1986-1989) – in 1990 it included 316,553 people. The workers were all adults, mostly males aged 20-45 years.

Most of the liquidators can be divided into two groups: (1) the people who were working at the Chernobyl power station at the time of the accident - the staff of the station, the firemen and the people who went to the aid of the victims, they number a few hundred persons; (2) the workers, who were active in 1986-1989 at the power station or in the zone surrounding it for the decontamination, sarcophagus construction and other recovery operations.

The first group received doses of radiation ranging from 1 to 16 Sv according to biological dosimetry estimation [UNSCEAR, 1988]. The second category of liquidators were initially subjected to a radiation dose limit for one year of 250 mSv. The registry data show that the average recorded doses in the three national registries decreased from year to year, being about 170 mSv in 1986, 130 mSv in 1987, 30 mSv in 1988 and 15 mSv in 1989 [Sevan'kaev *et al.,* 1995].

A large number of papers concerning health effects in liquidators have been published in various scientific journals so far. The health impact of the Chernobyl accident can be classified in terms of acute health effects ("deterministic effects") and

of late health effects ("stochastic effects"). Moreover, there are also social and psychological effects which can influence health.

1.4.1.1 Acute health effects

Liquidators received doses above 0.5 Sv were considered to receive a high dose of radiation and to have acute effects of radiation. These effects become apparent immediately or within few days. The immune system is weakened, changes in blood count occurred, and the digestive tract, lungs, other internal organs and the central nervous system all damaged.

1.4.1.2 Late health effects

Because of the often long delay between exposure to radiation and the appearance of its effects, lengthy follow up times are necessary before definite conclusions can be drawn.

Cancer effects

Until recently, publications from Russian National Medical Dosimetric registry (RNMDR) [Ivanov *et al.,* 1997a] and a separate paper, dealing only with thyroid cancer [Ivanov *et al.,* 1997b] reports that there was a greater level of thyroid morbidity among liquidators than was expected (47 cancer cases within a cohort of 114,504 Chernobyl liquidators). Analyses showed that there was an excess relative risk of thyroid cancer per Gy of 5.31 (95% confidence intervals 0.04 and 10.58) and an excess absolute risk of thyroid cancer per 10^4 person-years per Gy of 1.15 (95% confidence intervals 0.08 and 2.22). But no similar increase has been reported by other groups.

Ivanov *et al* [1997d] in their study of Russian cleanup workers suggest that one of every two leukaemias diagnosed in emergency workers today could be radiation-induced. They also point out that the incidence of leukaemia in the Russian liquidators is consistent with the incidence predicted from the atomic bomb survivors.

No significant increase in the incidence of solid cancers (defined as all cancers excluding haematological malignancies) was seen in a cohort of over 55,000 Russian liquidators [Ivanov *et al.*, 2004].

Using data for liquidators from the Belarus National Cancer Registry, Okeanov *et al.* [2004] compared baseline incidence rates for overall cancer and various cancers between 1976-1985 with those between 1990-2000. The cohort numbered 71,840 people. An average 40% increase in cancer incidence was observed in all regions with the most pronounced increase in the most contaminated region. The 56% increase between the two time periods was statistically significant. In 1997-2000, male liquidators had a statistically significantly raised risk of cancers of all sites, colon, lung and bladder cancer compared with adults in the least contaminated region.

Non-cancer effects.

A cohort of 68,145 liquidators from State Register of Ukraine who worked between 1986 and 1987 were studied annually from 1988-2003 to ascertain the extent of non-cancer mortality and morbidity. Analysis revealed consistent adverse changes in health status of these liquidators. The study showed that during the period from 1988 to 2003 there was a 9.3-fold increase in non-tumour diseases [Tereshchenko *et al.*, 2003], mainly attributable to cardiovascular diseases, alimentary system diseases, diseases of the nervous system and sensory organs, and to diseases of the respiratory, skeletal, muscular and endocrine systems.

These findings are supported by the data from the RNMDR [Ivanov and Tsyb, 1996a; Ivanov and Tsyb, 1996b], which shows an increase in nearly all general classes of non-cancer diseases among Russian liquidators in comparison with national average figures.

The most common causes of mortality among liquidators include diseases of the blood circulation system (63%) as well as malignant neoplasm (26.3%). Remarkably few deaths among liquidators, however, have been caused by diseases of the gastrointestinal tract (7%), lung diseases (5%), injuries and poisoning (5%), or tuberculosis (3%) [Khrisanfov and Meskikh, 2001].

1.4.1.3 Hereditable effects

There is an international consensus that radiation can induce genetic effects. This has been shown in a wide range of studies on plants and animals from the contaminated territories [Kovalchuk *et al.,* 1998].

Livshits *et al.* [2001] and Kiuru *et al.* [2003] found exposure to radiation had no significant effect on minisatellite mutations in the children of Chernobyl liquidators compared with the children of control families from the Ukraine. Slebos *et al* [2004] also examined DNA from lymphocytes in the children of liquidators and found no significant difference in mutation frequency between children conceived before their father's exposure and those conceived after.

1.4.1.4 Psychosocial Effects

Many papers about liquidators have noted the possible involvement of strong psycho-emotional tension during clean-up work which could lead to the development of a whole spectrum of diseases. The liquidators are under the highest risk of neuro-psychiatric disorders due to their greatest exposure to both the radiation and the other factors of the disaster aftermath.

In a considerable proportion of the personnel, especially those who continued their work in the 1990s, pathology met the criteria of Chronic Fatigue Syndrome (CFS), leading to suggestions that CFS may be developing under the combined impacts of low

or very low doses and psychological stress [Loganovsky *et al.,* 1999a, 1999b; Loganovsky, 2005].

According to data from the RNMDR, the incidence of psychiatric disease takes 5th place among liquidators [Ivanov *et al.,* 1999]. However, according to data from the Expert Councils which investigate the connection between illness and exposure to Chernobyl-derived radiation, the portion of psychiatric disorders is even higher [Khrisanov and Meskih, 2001; 2004].

Another sad consequence in the Chernobyl liquidators has been found during the Estonian Chernobyl Liquidators project [Tekkel *et al.,* 1997; Vanchieri, 1997]. The diseases directly attributable to radiation appear to be of relatively minor importance when compared with the substantial excess of deaths due to suicide. A total of 144 deaths were observed in the Estonian liquidators' subgroup consisting of 4,742 people during an average of 6.5 years of follow-up. Twenty-eight deaths (19.4%) were suicides.

1.4.2 Residents of contaminated territories

Twenty years after the Chernobyl accident, several million people still reside in areas that will remain highly contaminated by radioactive pollution for many years to come (defined as those with ^{137}Cs deposition levels greater than 37 kBq/m^2).

Although efforts have been made to limit their doses, 4,400,000 inhabitants were living in areas with a ^{137}Cs contamination ranging from 37 to 185 kBq/m^2, 580,000 in areas 185-555 kBq/m^2.

About 270,000 people continue to live in contaminated areas with radio caesium deposition levels in excess of 555 kBq/m^2, where protection measures still continue to be required. In these areas, preventive measures have successfully maintained an annual effective dose below 5 mSv.

The exposure of the population as a result of the Chernobyl accident resulted in two main pathways of exposure. The first is the radiation dose to the thyroid as a result of the concentration of radioiodine and similar radionuclides in the gland. The second is the whole-body dose caused largely by external irradiation mainly from radio caesium [ICRP, 1991].

For the total population of Belarus, the average dose to the thyroid is 0.9 to 1 Gy for 0-7 year-old children and 0.3 Gy for the total population giving collective doses of 34,000 and 134,000 man Gy respectively [Ilyin *et al.*, 1995]. For the populations of the three countries, the collective thyroid doses are roughly estimated to 550,000, 200,000-300,000, 390,000 man Gy for Belarus, Russian Federation and Ukraine respectively [UNSCEAR, 2000].

Total collective thyroid doses in Belarus, the Russian Federation, and the Ukraine, respectively, were estimated to be 550,000, 250,000 and 740,000 man Gy.

1.4.2.1 Health effects

No members of the general public received such high whole-body doses as to induce Acute Radiation Syndrome [IAEA, 1986]. This was confirmed in Belarus, where, between May and June 1986, 11,600 people were investigated without the discovery of any cases of acute radiation sickness. Thus, only late health effects were observed in affected populations.

Cancer effects

Between 1990 and 2000, a 40% increase in all cancers in Belarus was documented, with higher increases (52%) in the highly contaminated Gomel region than in the less contaminated regions of Brest (33%) and Mogilev (32%). In Russia, cancer morbidity in the highly contaminated Kaluga and Bryansk regions was higher than across the country as a whole. In contaminated areas of the Zhytomir region of Ukraine, the number of adults with cancer increased almost threefold between 1986 and 1994, from 1.34% to 3.91%.

The Chernobyl catastrophe released enormous quantities of radioactive iodine, which becomes concentrated in the thyroid glands of exposed individuals. Iodine-131 leads to pathological cell modifications in thyroid. This process is exacerbated in areas - such as Belarus, southwest Russia and Ukraine - where iodine deficiency is endemic.

The main health effect of radiation from the accident observed to date is a dramatic increase in the incidence of thyroid cancer in persons exposed as young people. This increase was observed first in the early 1990s in Belarus and continues until now in the most contaminated areas of Belarus, Ukraine and the Russian Federation [Jacob *et al.*, 2006, Kazakov *et al.*, 1992, Stsjazhko *et al.*, 1995, UNSCEAR 2000]. To illustrate this, Figure 1.3 shows the temporal trends of childhood (0–14 years), adolescent (15–18 years) and adult (19–34 years) thyroid cancer in the general

population of Belarus following the accident. By 1995, the incidence of childhood thyroid cancer had increased to four per 100,000 per year compared to 0.03–0.05 cases per 100,000 per year prior to the accident. As those who were children at the time of the accident have aged (by 2002, even the very youngest had reached adulthood), the childhood thyroid cancer rates have declined to near zero and parallel increases in the incidence of thyroid cancer in adolescents and slightly later in young adults have been seen.

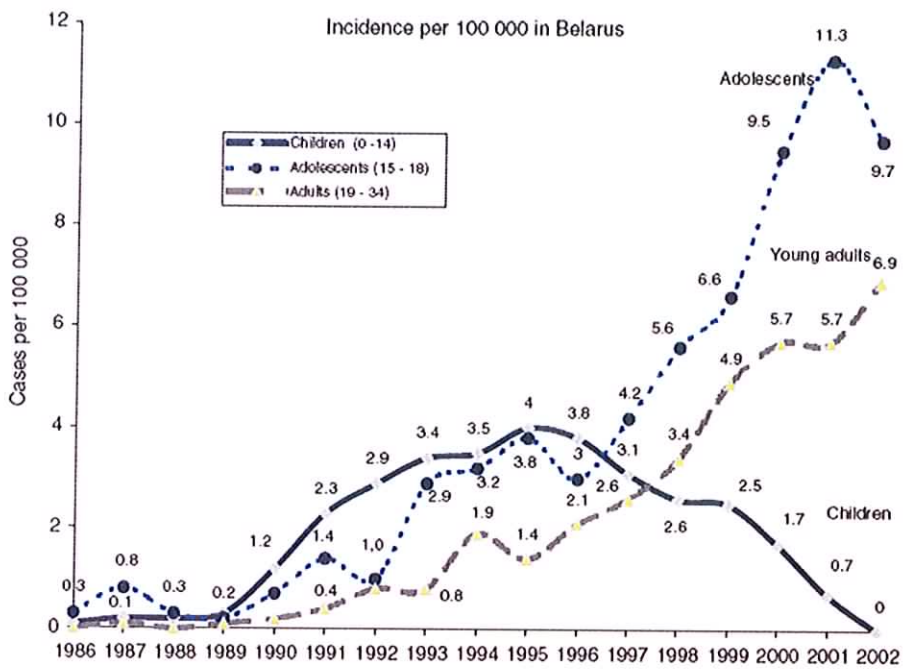

Figure 1.3. Annual incidence of childhood, adolescent and adult thyroid cancer in Belarus [Demidchik *et al.*, 2006].

Altogether close to 5000 cases of thyroid cancer were observed in Belarus, Ukraine and in the four most contaminated regions of Russia during 1986–2002 among those who were children (<15) or adolescents (15–17) at the time of the Chernobyl accident. Of these, 15 are known to have been fatal up to now.

The German specialist in radiation medicine and Chernobyl expert, Professor Edmund Lengfelder, which has been running a thyroid centre in Belarus since 1991, warns of up to 100,000 additional cases of thyroid cancer in all age groups [Lengfelder *et al.*, 2000]. In view of the pattern of cases reported to date, the World Health Organization (WHO) predicts that one third of all the children from the area around Gomel aged between 0 and 4 at the time of the accident will develop thyroid cancer during their lifetime - a total of 50,000 children in this group alone.

From 1992, significant increases in the incidence of all forms of leukaemia were detectable in the adult population of Belarus as a whole. In the Gomel region an increase in leukaemia cases of about 50% compared to the period before the disaster was recorded in both children and adults, according to the clinics responsible [Lengfelder *et al.*, 2000].

Childhood leukaemia in the Tula region in the post-Chernobyl period significantly exceeded the average rates for Russia, especially in children aged 10-14. In Lipetsk, cases of leukaemia increased 4.5 times from 1989 to 1995. Some data suggest increased risk of leukaemia even for children exposed in the womb [Ushakov *et al.*, 2001].

Although ionising radiation has been shown to increase the risk of cancers at many sites, data from Chernobyl on cancers other than thyroid cancer are very sparse [UNSCEAR, 2000].

No significant increase in the incidence of solid cancers was found among residents of the contaminated region of Kaluga in Russia [Ivanov *et al.*, 1997c].

Between 1987 and 1990, the number of referrals to the Eye Microsurgical Centre in Minsk for retinoblastoma (cancer of the retina) doubled [Birich *et al.*, 1994]. Among 32,000 men evacuated from the 30-km zone, the level of lung cancer

morbidity was 4 times higher than the average throughout Belarus [Marples, 1996]. In the Gomel region, a noticeable increase in morbidity from intestinal, rectal, breast, urinary bladder, kidney and lung cancer was observed. The incidence of these cancers was correlated with the level of land contamination caused by the Chernobyl catastrophe [Okeanov *et al.*, 1996, Goncharova, 2000].

In December 2004 the «Swiss Medical Weekly» published findings from the Clinical Institute of Radiation Medicine and Endocrinology Research, Minsk, Belarus showing a 40% increase in cancer between 1990 and 2000. The researchers used data from the National Cancer Registry, established in 1973. They compared the post Chernobyl period with rates before the accident on April 26, 1986 [Okeanov *et al.*, 2004].

Non-cancer effects

Over the last twenty years, a large number of health effects have been attributed to the Chernobyl accident, including reduced fertility, increased incidence of stillbirths, birth defects, Down's syndrome and infant mortality. Evaluation of the many reports and claims is extremely difficult, given the prevailing context of political changes, adverse economic circumstances and the apparent deterioration of many health and well-being indices. The problems associated with many of these reports of adverse health effects are different diagnostic criteria, insufficient control groups and presence of confounding factors (notably smoking and alcohol).

It is widely recognized that a general weakening of the immune system can be observed in the population affected by Chernobyl [Sahm, 1999]. The report from UNDP and UNICEF on the consequences of the Chernobyl accident also leaves no room for doubt that the health of the people in the regions affected by the accident is very poor [UNDP, 2002].

Amongst people living in contaminated zones examined in Belarus, cases of respiratory morbidity almost doubled. Such morbidity accounted for around a third of the problems observed in evacuees and in those adults and adolescents who continued to dwell in the contaminated territories. Ten years after the Chernobyl accident, blood diseases generally increased, with a greater increase reported in the contaminated areas.

An analysis of Belarusian health statistics by the United Nations Children's Fund (UNICEF) showed that between 1990 and 1994 disorders of the nervous system increased by 43%, cardiovascular diseases by 43%, gastrointestinal diseases by 28%, disorders of bone, muscle and connective tissue by 62%, and diabetes by 28% [UNDP, 2002].

1.4.2.2. Hereditable effects

A study by the United Nations Scientific Committee on the Effects of Atomic Radiation in 2001 concluded that "No radiation-induced genetic diseases have so far been demonstrated in human populations exposed to ionising radiation" [UNSCEAR, 2001]. The IAEA [2005] comes to a similar conclusion: because of the relatively low dose levels to which the population of the Chernobyl-affected regions was exposed, there is no evidence or any likelihood of observing decreased fertility among males or females in the general population as a direct result of radiation exposure.

A statistical record of the ten most common hereditary defects observed among newborns in Belarus since 1982 has been prepared by the Belarusian geneticist Gennady Lazjuk. He compared the number of birth defects before (1983 to 1985) and after (1987 to 2000) the nuclear accident. According to his data, the incidence of defects rose in proportion to the radioactive contamination of the areas [Lazjuk *et al.*, 2003].

In other papers scientists presented evidence that even the children of parents who were exposed to Chernobyl radiation exhibit significantly more mutations in the human minisatellite genome than would be expected. A correlation was found with the level of soil contamination in the areas in which the parents had lived. The rise in the mutation rate among the children of parents exposed to radiation was found to be dose-dependent [Dubrova, 2003].

1.4.2.3 Psychosocial Effects

Symptoms such as headaches, depression, sleep disturbance, inability to concentrate, and emotional imbalance have been reported and seen to be related to the difficult conditions and stressful events that followed the accident [Lee, 1996a; 1996b].

Repeated adverse experiences since the nuclear disaster have created a feeling of helplessness in the people affected. In their report «The human consequences of the Chernobyl nuclear accident» UNDP and UNICEF suggest that people feel they are victims of developments over which they have no influence. They have little confidence in their own ability to improve their situation [UNDP, 2002].

The sense of victimization was further reinforced by the nature of the aid provided in the past by the state and the international community. A complex system of social privileges and benefits was supposed to make the suffering and the health consequences more bearable. In practice, according to UNDP and UNICEF, this aid simply reinforced people's sense of helplessness.

Efficient healthcare will remain important in the future. Furthermore, projects should be designed to encourage and support people. The objective must be to restore a sense of purpose in people's lives by "helping them to help themselves" [UNDP, 2002].

For this reason, the IAEA calls for the population to be released from its "victim role", since a large part of the exposure to radioactivity is limited to "only" around 100,000 people remaining in the strongly contaminated areas [IAEA, 2005].

Clearly the overall body of evidence concerning human health impacts of the radiation released by the Chernobyl accident is highly diverse and complex but of great significance.

Many uncertainties remain. In particular there are still very few estimates of non-cancer mortalities attributed to Chernobyl, while long latency periods for development of cancers (in some cases greater than 40 years) inevitably mean that new cases are likely to emerge well in to the future. The health impacts on the children of the exposed are evident and will continue throughout their lifetimes, and possibly through those of their own children.

1.5 RADIOPROTECTIVE SUBSTANCES

A radioprotector is a chemical or biological compound capable of modifying the normal response of a biological system to radiation-induced toxicity or lethality. Long-term exposure of large population groups to low doses of environmental radiation has stimulated the search for radioprotective substances of a new type capable of reducing the effects of long-term irradiation. Various chemical agents were examined for their radioprotective capability to prove partial protection against radiation injury over the past 50 years. Conventional radioprotectors are known to be effective only during a single dose and to display their radioprotective properties only when applied in high, toxic concentrations. Application of the most effective radioprotectors to reduce the genetic effects of irradiation has shown that they are either unable to protect hereditary structures or are less effective than in protection against radiation death [Mosse, 1990].

An ideal radioprotector should offer significant protection against lethality from acute and long-term effects of radiation exposure; be suitable for oral administration and be rapidly absorbed and distributed throughout the body; cause no significant toxicological effects, particularly those on behaviour; be readily available and affordable; and be chemically stable to permit easy handling and storage.

Mechanisms of radiation protection

The radioprotectors can elicit their action by various mechanisms, such as: 1) by suppressing the formation of reactive species; 2) detoxification of radiation induced species; 3) target stabilization; 4) enhancing repair and recovery processes.

As a corollary to the oxygen effects, protectors were initially thought to bring about a reversal of the oxygen effect. Pharmacological agents, capable of altering hemodynamics *in vivo*, were found to be radioprotective by interfering with the delivery

of oxygen into irradiated tissues. Physical blockage of blood perfusion by microsphere embolisation of the intestine or kidney and perfusion with deoxygenated dextran haemoglobin decreased the sensitivity of the tissues [Holt, 1975] by altering the distribution of blood supply, and thereby the delivery of oxygen. Biogenic amines, histamine, serotonin, nor-epinephrine and epinephrine also manifest their radioprotective action by inducing local hypoxia.

The chemical or biochemical consumption of oxygen can bring about hypoxia in cells and tissues. This may be one of the mechanisms by which sulphydryl compounds (RSH), which can undergo an oxidation reaction with molecular oxygen, manifest radioprotection. Metal ions, such as Fe^{+++}, can catalyze the oxidation reaction [Halliwell Gutteridge, 1999].

$$2\ RSH + 2\ O_2 \quad RSSR + H\ _2O_2 + O_2$$

The removal of radiation-induced reactive aqueous free-radical species with short lifetimes in the nanosecond range can significantly mitigate radiation damage. Several free-radical scavengers are known to interact with aqueous free radicals and to prevent the radiation-induced lethality of cells. The radioprotectors may also react with water radicals or radicals of bio molecules (X') by donating hydrogen atoms to repair the radical species:

$$2\ X^{\cdot} + 2\ RSH \quad 2\ XH + RSSR$$

$$2\ RSH + 2OH \quad RSSR \quad + 2H_2O$$

Radioprotectors can also interact with cellular targets, like DNA, by forming mixed disulfides and prevent radiation damage by stabilizing the target. Several amino thiol radioprotectors, such as cysteamine, guanidoethyldisulfide and glutathione disulfide, bind to DNA and their DNA binding paralleled their radioprotective potency.

However, diamines like cadaverine, pentamethylene diguanidine bind well to DNA, but offer no radioprotection [Kollman *et al.*, 1973].

The radioprotective activity of a number of thiol compounds (RSH, R'SH *etc*) has been correlated with the ratio and extent of mixed disulfide (RSSR') formation [Eldjarn and Phil, 1958]. The regeneration of native proteins can be achieved by a thiol disulfide exchange with reduced GSH, possibly catalyzed by thiol transferase, and subsequent action of GSH redox system coupled with GSH reductase and NADPH.

$$\text{Protein-S-S-R} \quad \text{Protein} - \text{S} + {}^{\bullet}\text{SR}$$

$$\text{RSH} + \text{R'SSR'} \quad \text{RSSR'} + \text{R'} - \text{SH}$$

$$\text{RSSR'} + \text{RSH} \quad \text{RSSR} + \text{R'SH}$$

The mixed disulfide hypothesis is limited to the protection of enzymes and proteins, and fails to explain the radioprotection of nucleic acids, because the SH group is restricted only to proteins.

DNA repair and cell recovery processes

The cellular recovery or repair processes have been found to be enhanced by radioprotectors in several studies. The most important vital target damaged by radiation is the genomic DNA of a cell. The types of damage suffered by DNA due to ionising radiation include strand breaks of single and double-strand types, base damage and sugar damage. The frequencies of double-strand breaks in DNA is inversely correlated with cell survival [Bryant, 1985; Radford, 1985]. Base damage and DNA single-strand breaks may also hold deleterious consequences for cells [Kohn, 1979].

All of these DNA lesions could lead to cell death, mutagenicity or altered function, or may be repaired and cells may recover from radiation-induced effects [Orr, 1984]. Endogenous radioprotective substances have been investigated with respect to their role in cellular recovery from radiation and chemical onslaughts [Bump *et al.*,

1982; Roizin Towle *et al.*, 1984; Bertsche *et al.*, 1986]. Thiols, such as GSH, may be involved in the repair of DNA single-strand breaks. Cells genetically deficient in GSH synthesis or cells in which GSH deficiency is produced by DL-Buthionine-sulfoxime or by hypoxia or misonidazole show a lack of DNA single-strand break repair [Clark *et al.*, 1984; Edgreen, 1983]. Cells genetically deficient in GSH synthase have an increased amount of gamma glutamyl cysteine, cystine and other low molecular weight thiols, but these cannot substitute GSH in supporting DNA single strand break repair. Several compounds that stress cells are reported to induce DNA repair enzymes. These include H_2O_2, methyl methane sulfonate, nickel compounds, and dinitropyrene. When cells are irradiated and immediately treated with inhibitors of protein synthesis or cell division, they exhibit increased radiation resistance. This phenomenon is called potential lethal damage recovery. Holding cells under conditions that are sub-optimal for growth could also lead to similar results [Belli and Shelton, 1969]. The mechanism and molecules involved in this recovery process are not well understood.

The cellular defence mechanisms against radiation and chemical stresses elicit an early SOS response to damage and subsequent adaptation. The SOS response is for eliminating lesions in DNA and an adaptation response is for restoring cellular metabolism and normal functioning. In protecting the vital targets, SOS repair has a very important role, which comprises the activation or synthesis of several proteins, DNA precursor synthesizing enzymes and DNA precursors [Pulatova *et al.*, 1999]. Mammalian ribonucleotide reductase has been reported to be a DNA-damage inducible enzyme having a role in excision repair, because the concentration of deoxyribonucleotides is important for repair synthesis [Filatov *et al.*, 1996; Hurta and Wright, 1992; Tanaka *et al.*, 2000]. Drugs and chemicals, which stimulate or increase the activity of DNA precursor-synthesizing enzymes, such as ribonucleotide reductase,

could function as radioprotectors. The administration of the drugs indralin and indometaphen, prior to radiation exposure, to animals (mice and dogs) resulted in a higher survival of animals from lethal doses of gamma-radiation [Pulatova *et al.,* 1999]. An increase in the activity of the enzyme, ribonucleotide reductase, occurred in several organs of these animals. As a result of the activation of the DNA precursor-synthesizing enzymes increase in the extent of DNA repair could be expected. Further, higher cellular pools of DNA precursors could prevent the formation of new lesions. The stimulated synthesis of deoxyribonucleotides as well as DNA and protein synthesis in irradiated animals has been reported to be the mechanism underlying radioprotection by these drugs [Pulatova *et al.,* 1999].

1.5.1. Melanin

There is some evidence for a radioprotective action of melanin. This pigment is widespread in the living world. It gives our hair, skin and eyes their colour.

Among the numerous pigments found in animals and plants, melanin occupies a unique position because of its prominent appearance in mammalian tissues. In humans, almost all normal pigmentation is due to varying amounts, types, and distribution of melanin throughout the epidermis. The eventual colouration is modified by reflectance and diffraction, by subcutaneous vasculature, and by the occasional presence in the skin of other pigments, such as bile pigments, during foetal life and disease.

1.5.1.1. Melanin functions

Melanin has many different functions, only one of which is radioprotection [Hill, 1992]. Melanin is a dark compound that is called a photoprotective pigment. In nearly all mammals and in most other vertebrates, melanin has two important functions: it increases the optical efficiency of the eye, and it is responsible for the production of colour patterns, usually of adaptive significance, in the hair and the superficial epidermis. The major role of melanin pigment in the skin is to absorb the ultraviolet (UV) light that comes from the sun so that the skin is not damaged. Sun exposure normally produces a tan which is an increase in melanin pigment in the skin. The role of melanin in photoprotection is that pigmented skin is by far less susceptible than white skin to sunburn and phototoxic reactions.

Perhaps the most relevant from the physiological viewpoint is the ability to scavenge oxygen-derived radicals, such as superoxide anion and hydrogen peroxide, which are normally formed during biochemical and photochemical processes.

Potentially harmful free radicals that would otherwise initiate a chain of damaging oxidation reactions in the retina are neutralised by melanin. Unfortunately we lose this valuable protection to our retina during the ageing process.

All types of melanin contain enormous amounts of bound water, which appears to be essential to preserving the structure of the pigment in a solvent-swollen state. Natural and synthetic melanins exhibit intrinsic absorption throughout the UV and visible regions. Published spectra of melanin are characterized by a monotonic increase in the absorbance with decreasing wavelength, with a barely detectable shoulder between 290 and 320nm [Jacques *et al.*, 1996].

Melanin also could act as an intrinsic semiconductor because of an exceptional electron-accepting ability arising from extension of the lower empty band in the bonding energy region.

Many drugs and xenobiotics are taken up and retained for a long period of time in melanin-containing tissues, such as the eye, inner ear, skin. Examples of such substances are chlorpromazine, chloroquine, haloperidol, cocaine, polycyclic aromatic hydrocarbons, aflatoxins, etc.

Melanin pigment is present in the retina, and the area of the retina called the fovea does not develop correctly if melanin pigment is not present during development. The other areas of the retina develop normally whether or not melanin pigment is present. The fovea is the area of the retina which allows sharp vision, such as reading. Also the nerve connections between the retina and the brain are altered if melanin pigment is not present in the retina during development.

1.5.1.2. Melanin synthesis

Melanin forms in special cells called melanocytes. These cells are found in the skin, in the hair follicle, and in the iris and retina of the eye. Surprisingly, all humans

have roughly the same number of pigment cells in their skin. Melanocytes are present in the basal layer of the epithelium and have a round nucleus with a double nuclear membrane and clear cytoplasm lacking desmosomes or attachment plates.

The amount and type of melanin formed in melanocytes are genetically determined, but may be influenced by a variety of hormonal and environmental factors, including inflammation, sex, age, and exposure to UV light, which causes the familiar phenomenon of sun tanning.

There are many steps in the process of converting the amino acid tyrosine to melanin pigment. Two types of melanin forms: black-brown eumelanin and red pheomelanin (Figure 1.4). Eumelanin is the predominant form in humans, and acts as the primary photoprotectant in our skin and eyes.

There is a number of genetic or acquired developmental abnormalities which are accompanied by a decrease (hypomelanosis) or an increase (hypermelanosis) in melanin formation. Some of these pigmentary disorders are well known and include albinism, vitiligo, Mongolian spot, and melanoma (tumour with a high propensity to metastasize).

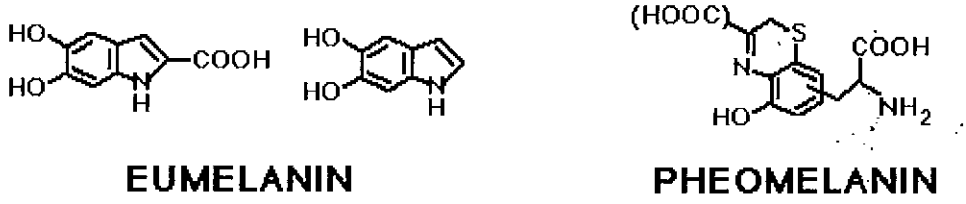

Figure 1.4. The melanin chemical structure.

1.5.1.3. Melanin as radioprotective substance

It has been found that melanin under some conditions increases the survival of fungi after a lethal dose of ionising radiation [Baraboi, 1984]. Dark pigmented fungi live in areas of high radiation background due to radionuclide contamination. Some of

these organisms have survived even after irradiation of the soil with 6400 Gy [Shilds and Durell, 1961]. It has been shown that intensively pigmented plants increase in areas contaminated with ^{90}Sr [Krivolutzky, *et al.,* 1972]. Wallace and King [1958] reported that primarily melanin-containing fungi survived on the Bikini atoll after the atomic bomb explosion.

Also it was found that white and grey mice became more pigmented after long-term irradiation with low doses [Quevedo and Grain, 1958]. Lukievich [1976] discovered that Chinese hamster melanoma cells with melanin were twice as resistant to the lethal action of ionising irradiation than such cells without melanin.

Brunst [1965] reported that irradiation of young *Axolotis* with 5, 15, and 30 Gy, respectively, stimulated the melanisation process in the liver, head and eyes of these animals. In Brunst's opinion, such hyperpigmentation was a protective reaction against irradiation. Later an analogous effect was found in frogs [Hollaender *et al.,* 1953].

Various attempts have been made to use melanin to increase biological radioresistance. Berdishev [1964] found increased survival and longer life expectancy of irradiated white mice due to intraperitoneal injection of melanin before irradiation with 8 Gy.

Melanin has a very effective radical scavenging capacity, and it possesses a high ability to accept and donate electrons. Melanin has a polymerous structure, which ensures the capture, stabilization, and inactivation of emerging free radicals. The complicated netted structure of melanin molecules makes them ideal "molecular sieves", in which active molecule fragments, radicals, and other products of irradiation are trapped [Baraboi, 1984]. Also it is very important that melanin can absorb all types of physical energy and convert it into heat [Hill, 1992].

Melanin exerts its protective action at the initial stage of irradiation, preventing DNA damage and not affecting the biochemical repair system. It is effective against low radiation doses, but not against high doses because if the energy input is too great, the output can be expressed in the form of activated chemical species that can damage cellular macromolecules.

All these investigations of radioprotective ability of melanin concern its influence on lethal radiation effects. Also, the antimutagenic activity of this pigment has been studied and it was found that it significantly decreased the frequencies of different types of mutations induced by radiation in germ cells of *Drosophila* and mice [Mosse, 1990; Mosse *et al.*, 1996]. Melanin's ability to reduce the number of genetic lesions inherited from generation to generation that accumulate in populations as a "genetic load" is especially valuable [Mosse and Lyach, 1994].

1.5.2 Melatonin

Melatonin is another substance famous for its radioprotective properties.

Melatonin was discovered as a result of the observation that bovine pineal extracts caused blanching of the skin of tadpoles when it was added to swimming water. Aaron Lerner, an American dermatologist, isolated and characterized the hormone from beef pineal extracts in 1958, naming it melatonin based on its ability to lighten melanocytes [Lerner *et al.*, 1958].

Melatonin is present in a number of organisms such as bacteria, algae, fungi, plants, insects and vertebrates, including humans. Melatonin is also found in foodstuffs such as vegetables, fruits, rice, wheat and herbal medicines [Tan *et al.*, 2003].

Early research involving melatonin was conducted on animals and examined its effects on gonadal maturation and circadian systems. These early animal experiments provided evidence for chronobiologic and sleep-inducing effects of melatonin [Wurtman, 1985], suggesting a role for this hormone in sleep and behaviour in humans. The first experiments of melatonin on humans were conducted in the early 1970s, which provided evidence of a sleep inducing effect of melatonin in humans [Cramer *et al.*, 1974]. The first study involving administration of chronic small doses of melatonin in human volunteers was conducted in 1984 and this study found that melatonin increased self-rated tiredness. Sedative-hypnotic effects of melatonin were also noted in a study examining the behavioural effects of melatonin [Lieberman *et al.*, 1984]. In 1984, melatonin was tested for its ability to alleviate the symptoms of jet lag, and this stimulated further trials of melatonin for the treatment of sleep disorders [Arendt *et al.*, 1984].

1.5.2.1. Melatonin functions

Melatonin plays important role in thermoregulation [Heldmaier and Hoffman, 1974; Heldmaier *et al.,* 1981]. Seasonal physiological adjustment to the food supply is mostly assumed to be mediated via seasonal variations of melatonin synthesis.

Melatonin has a stimulating effect on brown adipose tissue [Heldmaier and Hoffman, 1974; Guerrero *et al.,* 1990]. The main advantage of this type of heat generation is the direct transformation of stored metabolic energy into heat. In contrast, heat gain through shivering is an indirect and thus energy-wasting type of heat production.

Also melatonin has been shown to regulate "diurnal" and "circadian" rhythms, and exogenous melatonin is capable of significantly reducing the effects of jet lag [Comperatore *et al.,* 1996]. The hypothesis is that by taking melatonin the biological clock is "reset" and thus more quickly adjusted to the new "right" time [Lewy *et al.,* 1995].

Melatonin was shown to be an oncostatic agent. It is well known that the pineal organ *in vivo* may have an inhibitory effect on the growth of malignant tumours, that is, an oncostatic effect [Conti and Maestroni, 1995]. Apart from the often examined breast cancer and melanoma, this concerns a number of other cancer types, among them: colon cancer [Barni *et al.,* 1995], lung cancer [Conti and Maestroni, 1995], and leukaemia [Dilman *et al.,* 1979]. For a long time, the reason for these findings has been unknown. However, now it is assumed that melatonin's characteristics as a potential scavenger of free radicals are of crucial significance. But also peptides from the pineal organ have been implicated [Dilman *et al.,* 1979].

These effects not only occur *in vivo* but also when cancer cells are treated with melatonin *in vitro* [Hill *et al.*, 1988]. Effects on tumour growth with up to 80% growth inhibition have been observed.

Interestingly, melatonin here is often found to have effects only within a concentration window of about the same order of magnitude as the concentration of the hormone in the blood during the night (about 5 x 10^{-10} M). Further, there is evidence for a continuous presence of melatonin being less effective than one imitating physiological variations [Cos and Sanchez-Barcelo, 1994].

Melatonin may augment the anti tumour activity of IL-2 (Interleukin 2) by inhibiting tumour growth factor production. A pilot study was carried out using low dose IL-2 plus melatonin in 14 patients with untreatable endocrine tumours. The results suggest that low dose IL-2 and melatonin may be a well tolerated therapy for advanced endocrine tumours [Lissoni *et al.*, 1995]. Melatonin was added to IL-2 therapy for advanced solid neoplasms resistant to IL-2. Melatonin may enhance IL-2 antitumour immune effect. Melatonin was given every day, starting 7 days prior to IL-2. Objective tumour regression was noted in 3/14 cases (lung, kidney and liver tumours) [Lissoni *et al.*, 1994].

The field of oncostatic effects of the pineal hormone is currently one of the most interesting areas of melatonin research. In this context, quite a number of melatonin analogues have already been developed to intensify melatonin effects [Ying *et al.*, 1996].

1.5.2.2. Melatonin synthesis

Melatonin (N-acetyl-5-methoxytryptamine) (Figure 1.5) is a neurohormone that is primarily produced especially at night by the pineal gland, located behind the third ventricle in the brain [Gordon, 2000]. The pineal gland is now recognized as a key

element in the maintenance of the body's endocrine regulation (hormone balance), immune system integrity, and circadian rhythm (daily metabolic balance). Melatonin secretion is stimulated by the dark and inhibited by light. In the synthesis of melatonin, tryptophan is hydroxylated to 5-hydroxytryptophan, which in turn is decarboxylated to 5-hydroxytryptamine (serotonin). Serotonin is converted to the melatonin precursor and metabolite N-acetylserotonin by the enzyme N-acetyl transferase [Leone *et al.*, 1984]. N-acetylserotonin is methylated via the enzyme hydroxyindole-o-methyltransferase to produce melatonin, which is an indole. Approximately 90 percent of melatonin is cleared in a single passage through the liver. Microsomal enzymes of hepatic cells metabolize melatonin to 6-hydroxymelatonin. The majority of the latter compound is subsequently conjugated with sulphate to produce 6-sulfoxymelatonin, while a smaller proportion is conjugated to glucuronide, prior to excretion in the urine. A small proportion of unmetabolised melatonin is also excreted in the urine [Vijayalaxmi *et al.*, 2002]. The suprachiasmatic nuclei (SCN) of the hypothalamus have melatonin receptors and melatonin may have a direct action on SCN to influence "circadian" rhythms. [Weaver *et al.*, 1993].

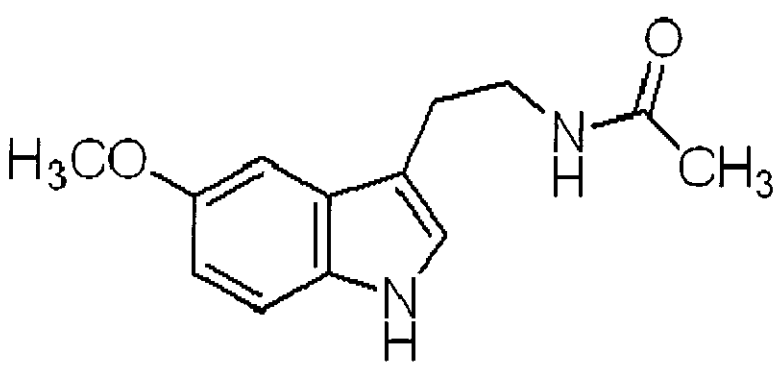

Figure 1.5. Chemical structure of melatonin

Melatonin secretion follows a circadian rhythm and is entrained to the light/dark cycle; light suppresses the production of melatonin, and with the onset of darkness,

melatonin is produced and secreted from pinealocytes [Reiter, 2003]. Light input is transmitted from the light receptors in the retina through the retinohypothalamic tract to the SCN, which is located in the anterior hypothalamus and functions as the central circadian pacemaker of the body [Geoffriau *et al.,* 1998]. During the dark period, the SCN stimulates the release of norepinephrine from the superior cervical ganglion; activation of pinealocyctes by norepinephrine results in production and release of melatonin [Brzezinski, 1997].

Melatonin is not stored in the pineal gland, but is secreted upon production. The hormone is likely secreted into the bloodstream before entering the cerebrospinal fluid (CSF) of the third ventricle, although it may also be secreted directly into cerebrospinal fluid [Tricoire *et al.,* 2002]. Evidence for direct secretion of melatonin into CSF has been provided by findings that melatonin levels in CSF are substantially higher than in plasma [Skinner and Malpaux, 1999]. Melatonin can also be measured in saliva, where levels are about 70% of plasma levels. The onset of melatonin secretion occurs at approximately 22.00-23.00 hours and maximal plasma concentrations occur at about 3.00-4.00 hours for a regular sleep cycle. The offset of melatonin secretion occurs at approximately 7.00-9.00 hours [Karasek, 1999]. The levels of metabolite in urine correlate positively with plasma levels of the hormone and provide a non-invasive method of measuring melatonin levels in the body [Kennaway *et al.,* 1992].

Although melatonin is present in plasma of newborns, the circadian rhythm of melatonin does not exist at birth, but appears at 9-12 weeks of age and is fully established by 5-6 months of age [Kennaway *et al.,* 1992]. Melatonin reaches high values at 1-3 years of age, with plasma levels peaking at approximately 250 pg/ml. Melatonin levels in plasma begin to decrease just prior to puberty to peak values of less than 100 pg/ml in adulthood [Waldhauser *et al.,* 1988]. There are, however, marked

individual differences in the levels of melatonin that are produced by the pineal gland [Reiter, 2003].

There is some evidence for melatonin affecting synthesis or functions of other hormones in humans, such as steroids (e.g., estrogens, testosterone, progesterone), prolactin, gonadotropines (LH and FSH), growth hormone (GH) [Waldhauser *et al.*, 1988; Jan and Espezel, 1995; Shanahan and Czeisler, 2000; Valcavi *et al.*, 1993; Webley *et al.*, 1988].

In particular, there seems to exist a phase relation between melatonin and the synthesis of prolactin and growth hormone. For the time being, it is still not known to what extent this relationship is endocrinically relevant.

1.5.2.3. Melatonin as a scavenger of radicals and radioprotective substance

The insight that melatonin is a natural and potent scavenger of oxygen-derived radicals was unexpected [Poeggeler *et al.*, 1993; Tan *et al.*, 1993].

Melatonin has been shown to act as an antioxidant by scavenging the hydroxyl radicals, peroxynitrite anions, peroxyl radicals, and the superoxide anion radical [Poeggler *et al.* 1993; Marshall *et al.* 1996] through a non-receptor mediated action. Additionally, the activation of a nuclear melatonin binding site increases mRNA levels of superoxide dismutase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase, all of which are antioxidative enzymes [Reiter 1998]. These antioxidant actions of melatonin are hypothesized to decrease the signs of aging by protecting DNA and mitochondria from oxidative damage [Tan *et al.* 1993].

The concentrations of the hormone required to neutralise these radicals are substantially smaller than those of known antioxidants (for example, GSH). Apart from artificial administration of relatively high doses of melatonin, it has been shown that physiological concentrations of melatonin are capable of considerably diminishing the

harmful effects of certain hydroxyl-radical (OH) – forming substances [Reiter, 1998]. It is of substantial relevance for the antioxidative effects of melatonin that the hormone as an extremely lipophilic substance which can easily cross the blood brain barrier and accumulate in nerve cells. Thus, melatonin is assumed to be of relevance for protecting the nervous system [Reiter, 1997]. Vitamin E (alpha- tocopherol in particular) and vitamin C (ascorbate) aid in protecting the brain from oxidative stress by directly scavenging toxic radicals. *In vitro* melatonin is more effective than GSH in scavenging the highly toxic (OH) radical and also more efficient than vitamin E in neutralising the peroxyl radical. It also stimulates the main antioxidant enzyme of the brain, glutathione peroxidase.

Melatonin is also reported to activate several cellular enzymatic antioxidant defence mechanisms. In mammalian tissues, both endogenously produced and exogenously administered melatonin is reported to be more highly concentrated in the nucleus than in the cytosol. Thus, melatonin may be present at highest concentrations in the portions of the cell that contains the most sensitive target molecule (nuclear and mitochondrial DNA) for the action of ionising radiation.

The radioprotective ability of melatonin was examined in several investigations. In an *in vivo* study, it has been demonstrated that the lymphocytes in the blood samples (from human volunteers) collected at 1 and 2 hours after a single oral dose of 300 mg of melatonin and exposed *in vitro* to 1.5 Gy gamma radiation exhibited a significant decrease in the extent of primary DNA damage in the form of single strand breaks (20-30%), and in the incidence of chromosomal aberrations and micronuclei (60-70%), as compared with similarly irradiated lymphocytes from the blood sample collected before melatonin ingestion [Vijayalaxmi *et al.*, 1996b, 1998a]. From these data, it is hypothesized that:

1. melatonin in the nucleus bestows a direct protection in reducing primary DNA damage by scavenging the radiation-induced free radicals,

2. melatonin may be acting at the cell membrane and in the cytosol by generating "signals" that trigger the activation of one or more of the existing DNA repair enzymes, and/or activation of a set of genes that lead to *de novo* protein synthesis associated with the repair of damaged DNA. The newly synthesized and/or activated DNA repair enzymes provide the cells with additional repair of damaged DNA, thereby reducing the incidence of chromosomal aberrations and micronuclei determined after 48 and 72 hours, respectively [Vijayalaxmi *et al.*, 1998b]. Whole-body irradiation studies have also demonstrated that pre-treatment of mice with melatonin significantly increased their survival from lethal effects of acute LD50/30 dose of gamma radiation [Vijayalaxmi *et al.*, 1999a], and significantly reduced the extent of ionising radiation-induced genetic damage in their peripheral blood and bone marrow cells [Vijayalaxmi *et al.*, 1999b].

Later Badr *et al.* [1999] performed a study to evaluate the radioprotective effects of melatonin. Male adult albino mice were treated (intraperitoneal) with 10 mg/kg melatonin either 1 h before or 1/2 h after exposure to 1.5 Gy of gamma-irradiation. Control, melatonin, irradiated and melatonin plus irradiation groups were sacrificed 24 h following treatment. The incidence of micronuclei in bone marrow cells was determined in all groups. The results showed that melatonin caused a significant reduction in micronuclei frequency when animals were treated with melatonin before and not after exposure to radiation. Mitotic and meiotic metaphases were prepared from spermatogonial and primary spermatocytes, respectively. Examination and analysis of metaphases showed no mutagenic effect of melatonin on chromosomal aberration frequency in spermatogonial chromosomes. Administration of one single dose of melatonin to animals before irradiation lowered total chromosomal aberration from 46 to 32%. However, no significant effect was

observed when melatonin was given after irradiation. Similarly, the frequency of chromosomal aberration in meiotic metaphases decreased from 43.5% in the irradiated group to 31.5% in the irradiated group treated with melatonin 1 h before irradiation, but no change was observed when melatonin was administered after irradiation.

Badr *et al.* [1999] suggested that melatonin radioprotection is achieved by its ability as a scavenger for free radicals generated by ionising radiation as melatonin administration confers protection against damage inflicted by radiation when given prior to exposure to irradiation and not after.

Koc *et al.* [2003] reported that melatonin was able to decrease oxidative damage effects in plasma and erythrocytes of rats after total-body gamma-irradiation at 0.5 Gy.

Undeger *et al.* [2004] investigated the melatonin effect on DNA strand breakage and lipid peroxidation induced by radiation in the rat brain in order to clarify its radioprotective ability. And it was shown that pre-treatment of rats with melatonin provided a significant decrease in the DNA strand breakage and lipid peroxidation, and melatonin can protect brain cells from oxidative damage induced by ionising radiation.

Monobe *et al* [2005] examined the protective effects of melatonin on intestinal damage induced by gamma-rays. They reported that melatonin can be a potential protector against intestinal damage associated with radiotherapy, and its radioprotective effect correlated with dosage.

Yilmaz and Yilmaz [2006] recently studied the effects of treatment with Vitamin E and melatonin and radiation-induced lipid peroxidation and its association with antioxidant enzymes in the total bone (bone and bone marrow) and skeletal muscle of rats subjected to total body irradiation. They concluded that melatonin was able to protect the total bone from the damaging effects of irradiation, and its actions protected total bone from oxidative stress. Protective effects of Vitamin E were not observed.

1.5.3. Alpha-Tocopherol

α-Tocopherol (commonly known as Vitamin E, Figure 1.6) is a natural component of mammalian cell membranes, a fat-soluble vitamin in eight forms and is considered the main line of defence against lipid membrane peroxidation. Vitamin E is often used in skin creams and lotions because it is believed to play a role in encouraging skin healing and reducing scarring after injuries such as burns.

Natural vitamin E exists in eight different forms or isomers, four tocopherols and four tocotrienols. All isomers have a chromanol ring, with a hydroxyl group which can donate a hydrogen atom to reduce free radicals and a hydrophobic side chain which allows for penetration into biological membranes. There is an alpha, beta, gamma and delta form of both the tocopherols and tocotrienols, determined by the number of methyl groups on the chromanol ring. Each form has its own biological activity, the measure of potency or functional use in the body.

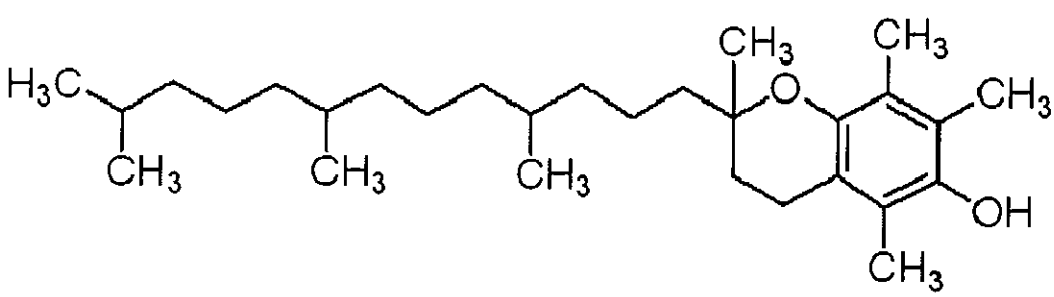

Figure 1.6. Chemical structure of α-Tocopherol

Alpha-tocopherol is traditionally recognized as the most active form of vitamin E in humans, and is a powerful biological antioxidant.

1.5.3.1. Tocopherol functions

Since its discovery, vitamin E has been mentioned as an essential nutrient for all animal species. It has been used in the treatment of diabetes mellitus, autoimmune diseases, and genetic disorders [Packer and Landvik, 1989]. Epidemiological studies have reported that a high vitamin E intake correlates with a reduced risk of cardiovascular diseases and vitamin E also plays specific roles beyond that of its antioxidant function [Stampfer *et al.,* 1993]. In addition to the free phenolic form, oxidation-protected forms such as α-tocopheryl acetate and α-tocopheryl succinate are also employed.

Alpha-Tocopherol is the most important lipophilic radical-chain-breaking antioxidant in living tissues. It also participates in the stabilization of biological membranes. The absence of vitamin E in membranes could make them highly permeable and therefore vulnerable to degradation. Vitamin E seems also to influence other important biophysical membrane characteristics, such as fluidity, in a manner similar to that of cholesterol [Fryer, 1992].

Preliminary research has led to a widely held belief that vitamin E may help prevent or delay coronary heart disease. Researchers are fairly certain that oxidative modification of LDL-cholesterol promotes blockages in coronary arteries that may lead to atherosclerosis and heart attacks. Vitamin E may help prevent or delay coronary heart disease by limiting the oxidation of LDL-cholesterol. Vitamin E also may help prevent the formation of blood clots, which could lead to a heart attack. Observational studies have associated lower rates of heart disease with higher vitamin E intake. A study of approximately 90,000 nurses suggested that the incidence of heart disease was 30% to 40% lower among nurses with the highest intake of vitamin E from diet and supplements. A 1994 review of 5,133 Finnish men and women aged 30 - 69 years

suggested that increased dietary intake of vitamin E was associated with decreased mortality (death) from heart disease.

Antioxidants such as vitamin E help protect against the damaging effects of free radicals, which may contribute to the development of chronic diseases such as cancer. Vitamin E also may block the formation of nitrosamines, which are carcinogens formed in the stomach from nitrites consumed in the diet. It also may protect against the development of cancers by enhancing immune function.

Some evidence associates higher intake of vitamin E with a decreased incidence of prostate cancer and breast cancer. Some studies correlate additional cofactors, such as specific vitamin E isomers, e.g. γ-tocopherol, and other nutrients, e.g. selenium, with dramatic risk reductions in prostate cancer [Helzlsouer *et al.*, 2000].

1.5.3.2. Tocopherol synthesis

In foods, the most abundant sources of vitamin E are vegetable oils such as palm oil, sunflower, canola, corn, soybean and olive oil. Nuts, sunflower seeds, sea buckthorn berries, and wheat germ are also good sources. Other sources of vitamin E are whole grains, fish, peanut butter, and green leafy vegetables. Although originally extracted from wheat germ oil, most natural vitamin E supplements are now derived from vegetable oils, usually soybean oil.

The synthesis of α-tocopherol from 2,3-dimethylphytylquinol and Sadenosyl-L-methionine was achieved using *Capsicum annuum* (yellow pepper) fruit chromoplasts. The enzymes involved in the cyclisation (2,3-dimethyl-phytylquinol cyclase) and methylation (S-adenosyl methionine:γ-tocopherol methyltransferase) are both localized in the chromoplast membrane fraction (Figure 1.7).

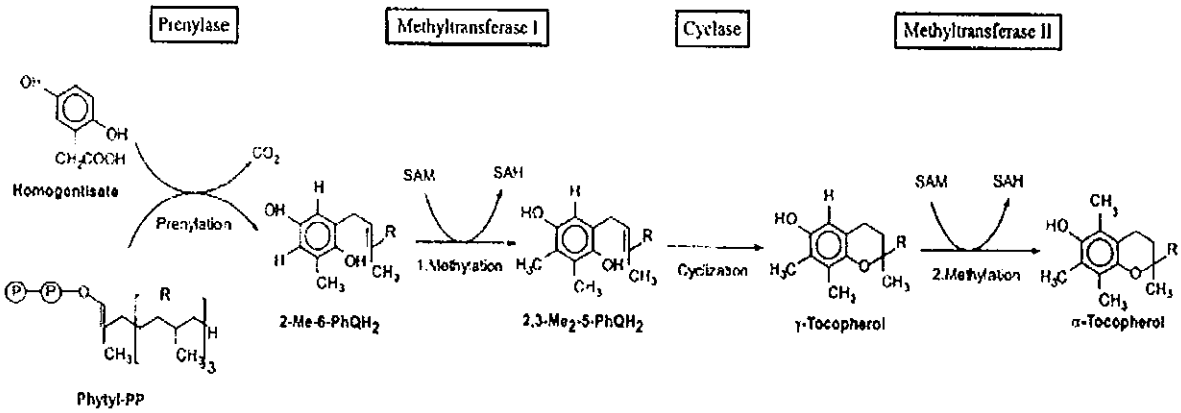

Figure 1.7. Alpha-Tocopherol synthesis

1.5.2.3. Tocopherol as a scavenger of radicals and radioprotective substance

Vitamin E is a naturally occurring free radical scavenger and its most widely accepted biological function is its antioxidant property. Vitamin E is well accepted as nature's most effective lipid-soluble, chain-breaking antioxidant, protecting cell membranes from peroxidative damage [Oski, 1980]. In addition to its antioxidant function, vitamin E influences the cellular response to oxidative stress through modulation of signal- transduction pathways [Azzi *et al.*, 1992].

Antioxidants such as vitamin E act to protect cells against the effects of free radicals, which are potentially damaging by-products of the body's metabolism. Free radicals can cause cell damage that may contribute to the development of cardiovascular disease and cancer. Vitamin C and other antioxidants recycle vitamin E end-products back into effective suppressors of free radicals. Studies are underway to determine whether vitamin E might help prevent or delay the development of those chronic diseases.

It has been suggested that vitamin E prevents lipid peroxidation chain reactions in the cell membrane in two ways: by interaction with unsaturated fatty acids, and by protecting the polypeptide chains of proteins [Pascoe *et al.*, 1987].

Tocopherol can scavenge molecular oxygen, peroxide and hydroxyl radicals and atomic oxygen radicals [Beilsk, 1982; Hemila *et al.,* 1985], while vitamin C can scavenge molecular oxygen and hydroxyl radicals and atomic oxygen radicals [Bump *et al.,* 1992; Awad *et al.,* 1992].

It has been observed that vitamin E exerts protective effects against cancers caused by chemical agents and radiation, and it has been shown in animal experiments that it blocks the formation of carcinogenic nitrous amines [Knekt *et al.,* 1991]. Both clinical and experimental findings suggest a correlation between serum levels of vitamin E and cancer [Knekt *et al.,* 1991]. Vitamin E exerts its protective effects in all cell membranes, in the nucleus, endoplasmic reticulum and mitochondria [Burton and Foster, 1995]. Glutathione is effective in the cytoplasm and mitochondria and shows its effects by scavenging the free radicals, thereby maintaining cell homeostasis [Floersheim, 1984].

Vitamin E fed at 3 times the normal mouse requirement for 1 week before and for 30 days following an 8.5- Gy dose of ^{60}Co radiation led to 60% 30-day survival, whereas 100% of control animals succumbed. At 7.5 Gy, the control survival was 10% and 100% of the vitamin E-treated animals survived [Srinivasan, 1983]. It was observed that subcutaneously administered vitamin E provided 79% survival in mice exposed to the supralethal dose of 10.5 Gy; oral administration was ineffective [Kumar, 2002]. In mice exposed to 1 Gy of whole-body ^{60}Co radiation 2 hr before or 2 hr after oral administration of vitamin E, both bone marrow polychromatic erythrocyte micronucleus formation and chromosome aberrations were significantly suppressed [Sarma and Kevasan, 1993].

França *et al.* [2003] analyzed the radioprotective effect of tocopherol in the ileum of mice by estimating the number of apoptotic cells. It was shown that tocopherol caused a significant reduction in the number of apoptotic cells.

Songthaveesin *et al.* [2004] reported that administration of Vitamin E prior to gamma-irradiation protects spermatogenic cells of mice from radiation.

Laurent *et al.* [2005] showed that the combination pentoxifylline and alpha-tocopherol was highly efficient in reducing late radiation-induced skin damage.

1.6. CELL CYCLE

All cells reproduce by dividing in two, with each parental cell giving rise to two daughter cells on completion of each cycle of cell division. These newly formed daughter cells can themselves grow and divide, giving rise to a new cell population formed by the growth and division of a single parental cell and its progeny. In the simplest case, such cycles of growth and division allow a single bacterium to form a colony consisting of millions of progeny cells during overnight incubation on a plate of nutrient agar medium. In a more complex case, repeated cycles of cell growth and division result in the development of a single fertilised egg into the more than 10^{13} cells that make up the human body.

The division of all cells must be carefully regulated and coordinated with both cell growth and DNA replication in order to ensure the formation of progeny cells containing intact genomes. In eukaryotic cells, progression through the cell cycle is controlled by a series of protein kinases that have been conserved from yeasts to mammals. In higher eukaryotes, this cell cycle machinery is itself regulated by the growth factors that control cell proliferation, allowing the division of individual cells to be coordinated with the needs of the organism as a whole. Not surprisingly, defects in cell cycle regulation are a common cause of the abnormal proliferation of cancer cells, so studies of the cell cycle and cancer have become closely interconnected, similar to the relationship between studies of cancer and cell signalling.

The division cycle of most cells consists of four coordinated processes: cell growth, DNA replication, distribution of the duplicated chromosomes to daughter cells, and cell division. In bacteria, cell growth and DNA replication take place throughout most of the cell cycle, and duplicated chromosomes are distributed to daughter cells in association with the plasma membrane. In eukaryotes, however, the cell cycle is more

complex and consists of four discrete phases. Although cell growth is usually a continuous process, DNA is synthesized during only one phase of the cell cycle and the replicated chromosomes are then distributed to daughter nuclei by a complex series of events preceding cell division. Progression between these stages of the cell cycle is controlled by a conserved regulatory apparatus, which not only coordinates the different events of the cell cycle but also links the cell cycle with extracellular signals that control cell proliferation.

1.6.1 Phases of the eukaryotic cell cycle

A typical eukaryotic cell cycle is illustrated by human cells in culture, which divide approximately every 24 hours. As viewed in the microscope, the cell cycle is divided into two basic parts: mitosis and interphase. Mitosis (nuclear division) is the most dramatic stage of the cell cycle, corresponding to the separation of daughter chromosomes and usually ending with cell division (cytokinesis). However, mitosis and cytokinesis last only about an hour, so approximately 95% of the cell cycle is spent in interphase – the period between mitoses.

During interphase, the chromosomes are decondensed and distributed throughout the nucleus, so the nucleus appears morphologically uniform. At the molecular level, however, interphase is the time during which both cell growth and DNA replication occur in an orderly manner in preparation for cell division.

The cell grows at a steady rate throughout interphase, with dividing cells doubling in size between one mitosis and the next. In contrast, DNA is synthesized during only a portion of interphase. The timing of DNA synthesis thus divides the cycle of eukaryotic cells into four discrete phases: presynthetic G_1, synthetic S, postsynthetic G_2 and mitosis M. The M phase of the cycle corresponds to mitosis, which is usually followed by cytokinesis. This phase is followed by the G_1 phase (gap 1), which

corresponds to the interval (gap) between mitosis and initiation of DNA replication. During G_1, the cell is metabolically active and continuously grows but does not replicate its DNA. G_1 is followed by S phase (synthesis), during which DNA replication takes place. The completion of DNA synthesis is followed by the G_2 phase (gap 2), during which cell growth continues and proteins are synthesized in preparation for mitosis. In addition, the term G_0 is used to describe cells that have exited the cell cycle and become quiescent [Murray, 1993].

During the gap phases, the cell monitors the internal and external environments to ensure that conditions are suitable and preparations are complete before it commits itself to the major upheavals of S phase and mitosis. At particular points in G_1 and G_2, the cell decides whether to proceed to the next phase or pause to allow more time to prepare. During all of interphase, a cell continues to transcribe genes, synthesize proteins, and grow in mass. Together, G_1 and G_2 phases provide additional time for the cell to grow and duplicate its cytoplasmic organelles: if interphase lasted only long enough for DNA replication, the cell would not have time to double its mass before it divided and would consequently get smaller and smaller with each division.

The first readily visible sign that a cell is about to enter M phase is the progressive condensation of its chromosomes. Chromosome condensation marks the end of the G_2 phase.

Mitosis is the most dramatic period of the cell cycle, involving a major reorganization of virtually all cell components. Mitosis is conventionally divided into four stages – prophase, metaphase, anaphase, and telophase. During mitosis (nuclear division), the chromosomes condense, the nuclear envelope of most cells breaks down, the cytoskeleton reorganizes to form the mitotic spindle, and the chromosomes move to opposite poles [Earnshaw and Pluta, 1994].

The beginning of prophase is marked by the appearance of condensed chromosomes, each of which consists of two sister chromatids. The condensed chromatids are then held together at the centromere, which is a DNA sequence to which proteins bind to form the kinetochore – the site of eventual attachment of the spindle microtubules [Murray, 1998, Rieder and Salmon, 1998]. The centrosomes separate and move to opposite sides of the nucleus.

Then cell enters prometaphase – a transition period between prophase and metaphase: the microtubules of the mitotic spindle attach to the kinetochores of condensed chromosomes, the kinetochores of sister chromatids are oriented on opposite sides of the chromosome, so they attach to microtubules emanating from opposite poles of the spindle, the chromosomes align on the metaphase plate in the centre of the spindle. At this stage, the cell has reached metaphase [Staehelin and Hepler, 1996]. The transition from metaphase to anaphase is triggered by breakage of the link between sister chromatids [Page and Hieter, 1999], which then separate and move to opposite poles of the spindle. Mitosis ends with telophase, during which the nucleus reforms and the chromosomes decondense. Cytokinesis usually begins during late anaphase and is almost complete by the end of telophase, resulting in the formation of two interphase daughter cells.

The duration of these cell cycle phases varies considerably in different kinds of cells. For a typical rapidly proliferating human cell with a total cycle time of 24 hours, the G_1 phase might last about 11 hours, S phase about 8 hours, G_2 about 4 hours, and M about 1 hour. Other types of cells, however, can divide much more rapidly. In contrast to the rapid proliferation of embryonic cells, some cells in adult animals cease division altogether (e.g., nerve cells) and many other cells divide only occasionally, as needed to replace cells that have been lost because of injury or cell death. Cells of the latter type

include skin fibroblasts, as well as the cells of many internal organs, such as the liver, kidney, and lung [Pardee, 1989]. These cells exit G_1 to enter a quiescent stage of the cycle called G_0, where they remain metabolically active but no longer proliferate unless called on to do so by appropriate extracellular signals.

Analysis of the cell cycle requires identification of cells at the different stages. Although mitotic cells can be distinguished microscopically, cells in other phases of the cycle (G_1, S, and G_2) must be identified by biochemical criteria. Cells at different stages of the cell cycle can be distinguished by their DNA content. For example, animal cells in G_1 are diploid (containing one copy of DNA molecule), so their DNA content is referred to as 2n (n designates the haploid DNA content of the genome). During S phase, replication increases the DNA content of the cell from 2n to 4n, so cells in S phase have DNA contents ranging from 2n to 4n. DNA content then remains at 4n for cells in G_2 and M, decreasing to 2n after cytokinesis. Experimentally, cellular DNA content can be determined by incubation of cells with a fluorescent dye that binds to DNA, followed by analysis of the fluorescence intensity of individual cells in a flow cytometer or fluorescence - activated cell sorter, thereby distinguishing cells in the G_1, S, and G_2/M phases of the cell cycle.

1.6.2. Cell cycle checkpoints

The cell-cycle control system triggers the events of the cycle in a specific order. It triggers mitosis, for example, only after the entire DNA has been replicated, and it permits the cell to divide in two only after mitosis has been completed. If one of the steps is delayed, the control system delays the activation of the next steps so that the normal sequence is maintained. This self-regulating property of the control system ensures, for example, that if DNA synthesis is halted for some reason during S phase, the cell will not proceed into M phase with its DNA only half replicated. The control

system accomplishes this feat through the action of molecular brakes that can stop the cell cycle at specific checkpoints, allowing the cell to monitor its internal state and its environment before continuing through the cycle. There are 3 major checkpoints in the cell cycle: G_1/S, G_2/M and anaphase checkpoint.

For the most part, the molecular mechanisms responsible for stopping cell-cycle progression at checkpoints are poorly understood. In some cases, however, specific cyclin-dependent kinase inhibitor proteins come into play; these block the assembly or activity of one or more cyclin–Cdk complexes. One of the best understood checkpoint mechanisms halts the cell cycle in G_1 if DNA is damaged, helping to ensure that a cell does not replicate damaged DNA. DNA damage causes an increase in both the concentration and activity of a gene regulatory protein called *p53*, which activates the transcription of a gene encoding a Cdk inhibitor protein called *p21* [Elledge, 1996]. The *p21* protein binds to G_1/S-Cdk and S-Cdk, preventing them from driving the cell into S phase. The arrest of the cell cycle in G_1 allows the cell time to repair the damaged DNA before replicating it. If *p53* is missing or defective, the unrestrained replication of damaged DNA leads to a high rate of mutation and the production of cells that tend to become cancerous. In fact, mutations in the *p53* gene are found in about half of all human cancers [Levine, 1997].

Another cell-cycle checkpoint occurs in mitosis. At this point, the cell determines whether all of its chromosomes are attached appropriately to the mitotic spindle. The mitotic spindle is a cytoskeletal machine that physically pulls the duplicated chromosomes apart and segregates them into the two daughter cells. If the cell begins to segregate its chromosomes before all chromosomes are properly attached to the spindle, one daughter will receive an incomplete set of chromosomes, while the other daughter receives a surplus. Both situations can be lethal for the cell. Thus, a

dividing cell must be sure that every last chromosome is attached properly to the spindle before it completes mitosis. To monitor chromosome attachment, the cell makes use of a negative signal: unattached chromosomes send a "stop" signal to the cell-cycle control system [Russell, 1998]. Although the exact nature of the signal remains elusive, it inhibits further progress through mitosis by blocking the activation of anaphase-promoting complex. Without active anaphase-promoting complex, the duplicated chromosomes remain glued together. Thus, none of the duplicated chromosomes can be pulled apart until every chromosome is positioned correctly on the mitotic spindle.

The third checkpoint occurs during anaphase, triggering both the exit from mitosis and the beginning of cytokinesis.

1.6.3. G_0 phase

The most radical decision that the cell-cycle control system can make is to withdraw from the cell cycle entirely and stop the cell from dividing. This is a different matter from pausing in the middle of a cycle to cope with a temporary delay, and it has a special importance in multicellular organisms. In the human body, for example, nerve cells and skeletal muscle cells persist for a lifetime without dividing; they enter G_0, a modified G_1 state in which the cell-cycle control system is largely dismantled, in that many of the Cdks and cyclins disappear. Some cell types, such as liver cells, normally divide only once every year or two, while certain epithelial cells in the gut divide more than twice a day in order to renew the lining of the gut continually. Many of our cells fall somewhere in between: they can divide if the need arises but normally do so infrequently.

It seems to be a general rule that mammalian cells will multiply (proliferate) only if they are stimulated to do so by signals from other cells. If deprived of such signals, the cell cycle arrests at a G_1/S checkpoint and enters the G_0 state, where the

cells can remain for days or weeks, or for the lifetime of the organism. Most of the diversity in cell-division rates in the adult body thus lies in the variation in the time cells spend in G_0 or in G_1; once past the G_1/S checkpoint, a cell usually proceeds through the rest of the cell cycle quickly – typically within 12–24 hours in mammals. The G_1/S checkpoint is therefore sometimes called Start, because passing it represents a commitment to complete a full division cycle.

The starting and stopping of cell proliferation are fundamentally important in controlling cell numbers and bodily proportions in a multicellular organism. But controls on cell division are only half the story. On the other side of the balance sheet lie other, equally important, controls that determine whether a cell lives, or whether it dies by programmed cell death.

1.7 APOPTOSIS

The cells of a multicellular organism are members of a highly organized community. The number of cells in this community is tightly regulated – not simply by controlling the rate of cell division, but also by controlling the rate of cell death. If cells are no longer needed, they commit suicide by activating an intracellular death programme. This process is called apoptosis.

The term apoptosis (derived from the Greek word for a natural process of leaves falling from trees) first appeared in the biomedical literature in 1972 [Kerr *et al.*, 1972], to delineate a structurally-distinctive mode of programmed cell death responsible for cell loss within living tissues. Although such programmed deaths were described many decades ago, the significance of apoptosis had been largely overlooked, particularly its relevance to disease. The improved understanding of apoptotic signalling pathways, and the cloning and characterization of pro- or anti-apoptotic genes have attracted great interest and raised the possibility that therapeutic strategies which involve apoptotic pathways may be useful in the treatment of cancer, infectious diseases, degenerative syndromes and other pathological conditions.

This remarkable process is responsible for cell death in development, normal tissue turnover, atrophy induced by endocrine and other stimuli, negative selection in the immune system, and a substantial proportion of T-cell killing. It also accounts for many cell deaths following exposure to cytotoxic compounds, hypoxia or viral infection. It is a major factor in the cell kinetics of tumours, both growing and regressing. Many cancer therapeutic agents exert their effects through initiation of apoptosis, and even the process of carcinogenesis itself seems sometimes to depend upon a selective, critical failure of apoptosis that permits the survival of cells after

mutagenic DNA damage. Apoptosis probably contributes to many chronic degenerative processes, including Alzheimer's disease, Parkinson's disease and heart failure.

1.6.1 Apoptosis features

It is well known that normal organisms and all of its systems functions are supported by balanced tissue renovation, which is mediated in cell populations by processes of proliferation and physiological elimination of functionally incomplete cells. "Programmed cell death" plays a leading role in it.

Cell death may occur via at least two broadly defined mechanisms: necrosis or apoptosis.

There are many observable morphological biochemical differences between necrosis and apoptosis. Necrosis is a passive, catabolic, pathological cell death process which generally occurs in response to external toxic factors such as inflammation, ischemic or toxic injury, when cells are exposed to extreme variance from physiological conditions (e.g., hypothermia, hypoxia) which may result in damage to the plasma membrane. Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, the cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo*, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response.

In contrast, apoptosis is an active, metabolic, genetically encoded and evolutionarily selected death pathway and the cell is an active participant in its own demise ("cellular suicide"). It occurs under either physiological or pathological conditions. This suicidal pathway is characterized by membrane blebbing, chromatin

aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles which contain ribosomes, morphologically intact mitochondria and nuclear material and activation of an endonucleolytic process, which leads to the sequential cleavage of chromosomal DNA to a size of several hundred kilobases, then to 50 kb and eventually to 200 bp. As a result, cells shrink and condense into multiple small membrane-bound "apoptotic bodies" which display a particular propensity as targets for phagocytes. This removes apoptotic cells without leaking the cytoplasmic contents into the intercellular space, minimizing tissue inflammation, avoiding damage to neighbouring cells, and efficiently degrading host (or viral) DNA.

1.6.2 Apoptosis functions

Apoptosis is a fundamental biological process that is implicated in early development such as during metamorphosis in insects and amphibians, and organogenesis in virtually all multicellular organisms. As examples of this cellular altruism, apoptosis plays an active part in the removal of interdigital webs in fingers and toes and in the formation of T and B cell repertoires of the immune system by eliminating non-reactive or self-reactive cells.

Apoptosis also exerts a role opposite to mitosis in the maintenance of cell populations. As many as 10^{11} cells die in an adult human per day to ensure tissue homeostasis, and it is estimated that within a typical year, the mass of cells a person loses through cell death is almost equivalent to their entire body weight [Gilbert, 2000]. Such death therefore probably plays an important part in dynamic processes such as tissue remodelling and responses to stress. Apoptosis is also a protective mechanism, directing lysis of virus-infected cells, foreign cells or incipient neoplasm.

Apoptosis plays a central role in the immune system. Under physiological conditions, immature lymphocytes that bind to autoantigens are eliminated by apoptosis. This is thought to protect against immune recognition of "self".

Apoptosis is a significant physiological mechanism for establishing B-cell tolerance and shaping the B-cell repertoire. B cells are subject to death by apoptosis throughout most stages of their maturation and 60–70% cell loss has been calculated during the pre-B to B cell transition in bone marrow.

1.8 AIMS

As a result of the Chernobyl accident, in Belarus, Ukraine and Russia there are huge contaminated territories and populations which are constantly under the influence of low doses of ionizing radiation. Therefore, the study of the mechanisms of low dose radiation effects on human health has become increasingly important. The present research includes non-targeted effects of radiation such as bystander effects.

Therefore the general aims of the present work are:

1. To study both direct effects of low doses of radiation on human keratinocytes, immortalised with human papilloma virus and human peripheral blood lymphocytes and bystander effects *in vitro* using immortalised human keratinocytes. Melanin, melatonin and α-tocopherol were evaluated as radioprotective substances (chemical modificators) for both direct irradiation and bystander effects.

2. To study the effect of serum samples from people exposed to different levels of radiation (Chernobyl liquidators, people working and living in contaminated areas) on human keratinocytes using micronuclei and viability assays and radioprotective substances. Cytogenetic analysis was used as a measure of radiation effects *in vivo* in serum donors.

3. To study the effects of medium derived from laser irradiated human cell cultures on untreated cells – laser-induced bystander effect and its comparison with the radiation-induced bystander effect.

CHAPTER 2

EFFECTS OF RADIOPROTECTIVE SUBSTANCES ON IRRADIATED HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND HPV-G CELLS

2.1 Introduction

In the present research three radioprotective substances were used to study the direct and bystander irradiation of HPV-G cells and human peripheral blood lymphocytes.

The radioprotective effect of melanin *in vivo* has been shown previously [Mosse, 1990; Mosse *et al.*, 1994; 1999]. However, information about melanin's effects *in vitro* (in cultured cells) is controversial.

In some experiments, melanin was able to protect such cells against radiation. Thus, Hirobe [1982] found increased survival of irradiated cultured mouse cells due to the addition of melanin into the culture medium. It was also shown [Hill *et al.*, 1987] that melanin is able to protect DNA against the radiolysis of thymine and thymidine induced by gamma-radiation *in vitro*.

However, in other investigations, melanin was shown to be ineffective at protecting against radiation damage. The effect of melanin on the response of mammalian cells to gamma-radiation was investigated in Chinese hamster ovary cells [Hopwood *et al.*, 1985] and in mouse melanoma cells [Stephens *et al.*, 1986]. In these experiments, the variation of melanin content inside the irradiated cells did not influence their radiation sensitivity. The study of melanin action on the mammary epithelial cell response after low- and high-linear energy transfer radiation exposure has shown no significant effect on the cell survival at high radiation doses [Grossi *et al.*, 1998].

Thus, as melanin effects in cultured cells are somewhat controversial, the present study has been carried out.

Melatonin is known as another very popular radioprotective substance. And its ability to protect cells from the damaging influence of ionising radiation *in vivo* has been shown in many investigations. However, there are not many investigations examining its radioprotective properties in *in vitro* studies.

Human peripheral blood lymphocytes which were pre-treated with melatonin exhibited significantly reduced (60-70%) gamma radiation-induced genetic damage, determined from the incidence of chromosomal aberrations and micronuclei, as compared with irradiated cells which were not pre-treated with melatonin [Vijayalaxmi *et al.* 1995; 1996a].

Chun Kim *et al.* [2000] tried to clarify the role of melatonin as a radioprotective compound and to understand the mechanisms of its radioprotective action. In response to X-ray irradiation, they investigated the effects of X-ray irradiation and melatonin on cytotoxicity, lipid peroxidation and alteration of the cell cycle in cultured skin fibroblasts. An 8 Gy dose of X radiation resulted in cell death in 63% of irradiated cells, i.e. the cell viability was 37%. The damage was associated with lipid peroxidation of the cell membrane, as shown by the accumulation of malondialdehyde (MDA). By pre-incubation with melatonin (10^{-5} M), a significant preventive effect was noted as up to 68% of cells survived, and the levels of MDA were markedly decreased. These findings suggested a close correlation between an increased lipid peroxidation and cell death [Chun Kim *et al.*, 2001]. DNA flow-cytometry analysis revealed that X radiation increased pre-G1 apoptotic population by 7.6% compared to a very low level (1.3%) of non-irradiated cells. However, in the presence of melatonin, this apoptotic population decreased up to 4.5% at 10^{-5} M. The *p53* and *p21* protein levels of skin

fibroblasts increased 4 h after 8 Gy irradiation, but melatonin pre-treatment did not change those levels. This study suggested that melatonin pre-treatment inhibits radiation-induced apoptosis, and melatonin exerts its radioprotective effect by inhibition of lipid peroxidation and without any involvement of the *p53/p21* pathway.

α-tocopherol (Vitamin E) has a very different chemical structure from melanin and melatonin and is also a naturally occurring substance. The radioprotective properties of Vitamin E have been analyzed in different studies, and it was shown not always to protect cells against irradiation.

Nair *et al.* [2003] found that tocopherol monoglucoside, a water soluble derivative of vitamin E, offers protection against deleterious effects of ionising radiation, both under *in vivo* and *in vitro* conditions. Chromosome damage suppression by vitamin E has been demonstrated in mouse cells [Konopacka, 1998] and in human lymphocytes [Konopacka, 2001].

Kleveta *et al.* [2003] showed that additional administration of alpha-tocopherol resulted in a decrease of the first and end products of lipid peroxidation in enterocytes of the small intestine and blood cells after ionising irradiation.

Fantappie *et al.* [2004] demonstrated that Vitamin E protects DNA from oxidative damage in human hepatocellular carcinoma cell lines after ionising irradiation.

Ortmann *et al.* [2004] investigated the effect of alpha-tocopherol on radiation-induced apoptosis in cells in culture. Human T-lymphoblastic MOLT-3 cells were irradiated with a dose of 3 Gy 1 h after or immediately prior to the addition of vitamins in three concentrations (0.01 microM, 1 μM and 100 μM). Eight hours later, apoptosis was scored morphologically by staining the nuclear DNA. When given prior to irradiation, vitamin E reduced the amount of radiation-induced apoptosis significantly at

concentrations of 0.01 μM and 1 μM. When given immediately after irradiation, vitamin E showed a protective effect at 0.01 μM. In addition, the combination of alpha-tocopherol and vitamin C reduced radiation-induced apoptosis slightly when given at 1 μM.

Yilmaz and Yilmaz [2006] recently studied the effects of treatment with Vitamin E on radiation-induced lipid peroxidation and its association with antioxidant enzymes in the total bone (bone and bone marrow) and skeletal muscle of rats subjected to total body irradiation. They didn't observe any protective effects of Vitamin E.

In this chapter, the radioprotective effects of melanin, melatonin and vitamin E on gamma-irradiated human keratinocyte cells immortalised with HPV virus (HPV-G cells) and human peripheral blood lymphocytes were studied. HPV-G cells are lacking *p53* protein, which is very important in transfer of intercellular signals, and its absence may influence the radioprotective effect of melanin.

In this study primary lymphocyte cells and immortalised keratinocytes were used. Primary cells are cultured directly from *in vivo* tissue and are cytologically very similar to the cells found in that particular tissue. Such cultures, therefore, allow *in vitro* assays using cells which very closely approximate to *in vivo* conditions. However, blood lymphocytes cannot be maintained for a long time because of the destructive processes (apoptosis, necrosis), which are usually observed after 3-4 days.

Immortal cell lines are homogenous and are unlike primary cultures in that they are "immortalised" cells from a common ancestor that divide while retaining the same genotype.

The radiation-induced bystander effect has been studied extensively since 1992 by many researchers, but to date there have been no published reports on the use of

radioprotectors in modifying bystander responses. All studies are focused on understanding possible mechanisms of bystander effects and the nature of bystander factor. Meanwhile, modification of this phenomenon may help to understand its possible mechanisms, proceeding from the properties of the modifying substances.

2.2 Materials and methods.

2.2.1 Cell culture

HPV-G cells

The HPV-G cell line is a human keratinocyte line, which has been immortalised by transfection with the HPV virus, rendering the cells *p53* null. They grow in culture to form a monolayer, display contact inhibition and gap junction intracellular communication.

HPV-G cells were cultured in Dulbecco's MEM: F12 (1:1) medium supplemented with 10% Foetal bovine serum, 1% penicillin-streptomycin (1g per 100 ml), 1% L-glutamine and 1 μg/ml hydrocortisone. The cells were maintained in an incubator at 37 degrees Celsius, with 95% humidity and 5% carbon dioxide and routinely subcultured every 8-10 days.

When 80-100% confluent, the medium was poured from the flask and replaced with 1:1 solution of versene (1nM solution) to trypsin (0,25% in Hank's Balanced Salt Solution) (Gibco, Irvine, UK) after washing with sterile PBS. The flask was placed in the incubator at 37 degrees Celsius for about 11 minutes until the cells started to detach.

The flask was then shaken to ensure that all cells had been removed from the base of the flask. The cell suspension was added to an equal volume of DMEM F12 medium to neutralise the trypsin. From this solution new flasks could be seeded at the required cell density.

Human lymphocytes

Blood samples were collected by standard venous puncture and stored with heparin no longer than 2-3 hours before investigation. Blood lymphocytes of healthy

people were cultured in RPMI-1640 medium, containing foetal bovine serum (15%), phytohaemagglutinin (2%) (Sigma) and gentamycin (0.2%).

2.2.2 Radioprotective substances

2.2.2.1 Melanin

Melanin was isolated from animal hair by Belarus Pharmaceutical Association (Minsk). By analysis, it was determined to be eumelanin. Both ortochinoid and indolic fragments were present. According to the results of preliminary experiments, melanin was added to the cell medium at 10 mg/l concentration 30 min – 1 hour before irradiation for directly irradiated cells and 1 hour after irradiation to the irradiated cell conditioned medium (ICCM) before filtration for bystander recipient cells.

2.2.2.2 Melatonin

Melatonin (*N*-Acetyl-5-methoxytryptamine) was received from Sigma (Germany) as white powder, synthetic. According to the results of preliminary experiments, melatonin was added to the cell medium at 10 mg/l 30 min – 1 hour before irradiation for directly irradiated cells and 1 hour after irradiation to the ICCM before filtration for bystander recipient cells.

2.2.2.3 Vitamin E

α-Tocopherol (Vitamin E) water soluble analogue - Trolox was received from Sigma (Germany) as a brown liquid, synthetic. According to the results of preliminary experiments, tocopherol was added to the cell medium at 2 μg/ml concentration 30 min – 1 hour before irradiation for directly irradiated cells and 1 hour after irradiation to the ICCM before filtration for bystander recipient cells.

2.2.3 γ-Irradiation

HPV-G cells were γ-irradiated 12-24 hours after plating in culture flasks, size 25 cm^2, in densities appropriate to the assay. By this time they had attached to the bottom of the flask. Human lymphocytes were irradiated in glass tubes (Vacutainers) 24 hours after being taken. The dose was delivered at room temperature using a ^{60}Cobalt teletherapy source, delivering approximately 1.9 Gy per minute at a source-to cell distance of 80 cm. The control cultures were removed from the incubator and brought to the ^{60}Co teletherapy unit with the irradiated cultures but were not irradiated. All cells in the flasks received the same dose of 0.5 or 1 Gy. Once irradiated, the cells were immediately replaced in the CO_2 incubator und left undisturbed before analysis.

2.2.4 Clonogenic protocol for direct irradiation and bystander effect (HPV-G cells)

The "colony formation" assay or "clonogenic assay" is a classical test to evaluate cell growth after treatment. A clonogenic cell is defined as a cell with the capacity for sustained proliferation. Cells that can be shown to have undergone a minimum of 5-6 doublings to give rise to colonies containing at least 50 cells are said to be clonogenic.

The HPV-G cell suspension after dilution was counted using a Coulter counter (Coulter Z1). Appropriate cell numbers were plated according to the Puck and Marcus (1956) technique in 5 ml medium in 25 cm^2 NUNC flasks. There were 3 types of flasks: direct irradiation, bystander donor and bystander recipient. Bystander donor flasks were very heavily seeded with cells ($0.5x10^6$ cells per flask) in order to produce the bystander

factor into the medium after irradiation. Bystander recipient flasks were set up with the cloning number of 300 cells per flask and received no treatment except the bystander medium (ICCM) from the bystander donor flasks. The direct irradiation flasks were seeded with 300 cells per flask, after irradiation they received no further treatment. Each of 3 types of flasks had 4 sets in triplicate: control, melanin, irradiated cells and irradiated cells with melanin added. All clonogenic experiments using melanin were repeated 9 times when melanin was added before irradiation and 7 times – when melanin was added after irradiation. All experiments with melatonin and tocopherol were repeated 7 times.

After seeding cells, the flasks were left at 37°C in the incubator to attach for 12 hours. Then bystander donor and directly irradiated flasks after treatment were placed back in the incubator at 37 degrees Celsius for one hour. The medium from bystander donor flasks was removed and the radioprotective substance added in appropriate concentration for 30-60 min. Further, the medium was filtered through NALGENE 0.22 μm sterile syringe filters (to ensure that no cells were present in the medium) and used to replace the medium from bystander recipient flasks. Then all flasks were returned to the incubator and left for 9-10 days (until colonies were visible) and then stained with carbol fuchsin and colonies were counted and surviving fraction calculated.

The data is presented as mean ± standard error in all cases. Significance was determined using the t-test.

For the clonogenic assay experiments, plating efficiency is the proportion of *in vitro* plated cells that form colonies and it is calculated as a percentage of the final number of colonies counted over the initial number of cells plated.

$$\mathbf{PE} = \frac{\textbf{No. of colonies}}{\textbf{No. of cells plated}}.$$

Surviving fraction of cells is calculated from the plating efficiency of the irradiated cells divided by the plating efficiency of the control cells (it is expressed as a percentage of the control plating efficiency).

$$SF = \frac{\text{PE (Treated cells).}}{\text{PE (Control cells)}}$$

2.2.5 Micronuclei protocol for direct irradiation and bystander effect (HPV-G cells)

Micronuclei are structures, containing chromosome fragments or whole chromosomes (sometimes groups of chromosomes) situated in the cytoplasm. At mitosis they are not included in the nucleus because of the lack of a centromere (acentric fragments) or damage of the fibres of the mitotic spindle or centromere (whole centromere).

After irradiation micronuclei are formed in all cell types. The only condition of their formation is the passage of the cell through mitosis.

The main criteria for micronuclei detection are:

- micronuclei should have a similar structure to the nucleus;
- they should be smaller than the nucleus (less than 1/2 - 1/3 of the nucleus);
- they should be round and well separated from the main nucleus;
- the cell should have well separated cytoplasm.

The HPV-G cell suspension after dilution was counted using a Coulter counter (Coulter Z1). For direct irradiation and bystander recipient experiments, about 6000 cells were plated on glass coverslips (diameter 23 mm) in Petri dishes (diameter 60 mm) in 1 ml of medium for 6 hours to attach. Then another 5 ml of cell culture medium were added to the dishes. Bystander donor cells were plated in 5 ml of medium in 25 cm^2 NUNC flasks and were very heavily seeded with cells ($0.5x10^6$ cells/flask) in order to

produce the bystander factor into the medium after irradiation. Each of 3 types of flasks had 4 sets in triplicate: control, melanin, irradiated cells and irradiated cells with melanin added. All micronuclei experiments were repeated 5 times.

6 hours later, bystander donor and direct irradiation dishes were irradiated and replaced back in the incubator at 37 degrees Celsius for one hour. Later, the medium from bystander donor cells was removed and the radioprotective substance was added in appropriate concentration for 30 mins. Then ICCM was filtered through NALGENE 0,22 μm sterile syringe filter to ensure that no cells were present in the medium and no melanin added to recipient flasks thus showing the usual protective effect. This sterile filtered ICCM medium was used to replace the medium from bystander recipient cells. The direct irradiation cells were exposed to the radioprotective substance 30-60 min before irradiation and then left untouched as a direct irradiation test sample.

Then cells were moved back to incubator. After 1 – 1.5 hours cytochalasin B was added at 7 μg/ml concentration to block cell division and to form micronuclei, and the cells were incubated for 24 hours. After this the cell culture medium was removed, the cells were washed with PBS and fixed with chilled Karnua solution (1 part of glacial acetic acid and 3 parts of methanol, 10-15 ml 3 times for 10-20 min). Later coverslips were dried and stained by 10% Giemsa solution. Using mounting medium (Sigma), coverslips were plated on the microscope slides.

The micronuclei count was carried out on an inverted microscope (x400, Zeiss, Germany). Micronuclei were counted only in binucleated cells (1000 binucleated cells per flask).

All the data was recorded as micronuclei number per 1000 binucleated cells (micronuclei were analyzed only in binucleated cells).

2.2.6 Alamar Blue assay

Alamar Blue is a safe and stable in culture medium aqueous dye that is used to assess cell viability and cell proliferation. Its main advantages are that it is non-toxic and the cells under study can be returned to culture or used for other purposes including histological studies. Also this thechnique is easy to use and is not expensive. The continuous monitoring of cells in culture is therefore permitted. Using this assay, the innate metabolic activity of cells can be monitored. Specifically, Alamar Blue does not alter the viability of cells cultured for various times as monitored by Trypan Blue exclusion.

The oxidised indigo blue, non-fluorescing form of this chromogenic indicator dye (Biosource, Camarillo, CA, USA) is reduced by cellular dehydrogenases, to a reduced pink fluorescent form. Proliferation measurements with Alamar Blue may be performed either spectrophotometrically by monitoring the absorption of Alamar Blue-supplemented cell culture media at two wavelengths using a standard spectrophotometer, or alternatively, proliferation measurements with Alamar Blue may be performed fluorometrically using a spectrofluorimeter, or a microtiter well plate reader. Alamar Blue is supplied as a sterile indigo coloured liquid.

For the Alamar Blue assay, cell culture medium should not contain phenol red sodium salt, because it is fluorescent and will interfere with the Alamar blue dye.

Powdered Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (Sigma, Ireland), 1,56 g, was added to 100 mls of deionised water (15-20°C) and stirred until dissolved. To the solution, 0,12 g of sodium bicarbonate was added and dissolved.

After this, the pH of the medium was adjusted to 6.9 using 1N HCl or 1N NaOH. Then the medium was sterilized using 0,22 microns filter and dispensed into a sterile container and stored at 2-8°C in the dark. Before experiments, Alamar Blue dye was added to the medium to make a 5% solution.

Cells were seeded in 96-well microplates (NUNC, USA) at a density of $2x10^4$ cells/well. Bystander donor cells were seeded in 25 cm^2 flasks at a density of $0.5x10^6$ cells per flask in order to produce bystander signal to the culture medium. Microplates were set up in triplicate: non-irradiated (control and control with radioprotective substance added), directly irradiated (directly irradiated cells at 0.5 Gy and irradiated with radioprotective substance added) and bystander recipient (non-irradiated cells receiving only ICCM from bystander donor cells). After seeding, cells were incubated for 24 hours to attach to the bottom of the well. Then directly irradiated and bystander donor cells were irradiated at 0.5 Gy and moved back to the incubator. After 1 hour, medium from bystander donor flasks was removed, the radioprotective substance added, and 30 min later it was used to replace the medium from bystander recipient microplates. After 24 hours, medium was removed, cells rinsed with PBS and 5% Alamar Blue in phenol red free DMEM medium was added. After 3 hours, fluorescence was quantified. The data is expressed as fluorescent units (FU). All experiments were repeated 6 times.

2.2.7 Micronuclei protocol for human lymphocytes using flow cytometry

Sample treatment

Lymphocytes were isolated from whole blood using a centrifuge – for 5 min at 800g, and the top layer of supernatant was removed. Then the cells were resuspended in 50 µl of RPMI-1640 medium. Solution 1, containing 584 mg/L NaCl, 1000 mg/L Na-

citrate; 10 mg/L RNAse A and 0,3 ml Nonidet-P40 (NP40) was added to the mixed cell suspension. The number of cells did not exceed $1,5x10^6$. Then the cells were incubated for one hour, and 1 ml of solution 2, containing 15 mg/L citrate acid, 0,25 M sacharose and 10mg/ml of Ethidium bromide, was added. The cells were homogenized on a shaker. After an incubation period of 1 hour the samples were either analyzed immediately or stored for 1-2 weeks at 4-8°C. Before measurement, the suspension was checked under a fluorescence microscope.

Data analysis

The measurement was carried out using FACS Vantage (Becton Dickinson) flow cytometer, using 488 nm 1 W argon laser.

The principle of data collection is presented in Figure 2.1. The frequency of micronuclei is defined by computer software. In order to decrease possible mistakes, the flow cytometer analyses at the same time forward light scattering (cell size) and side light scattering (the size and shape of nuclei) from the fluorescent dye.

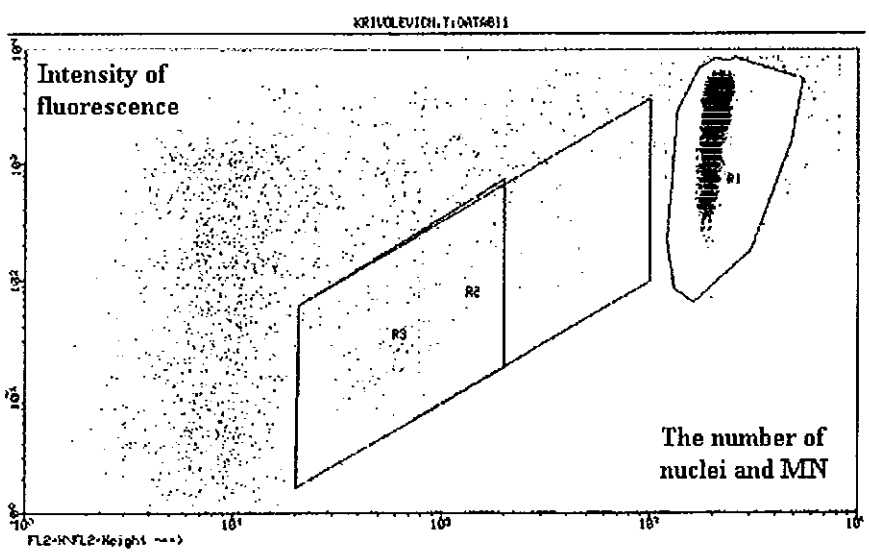

Figure 2.1 The principle of data analysis in micronuclei analysis using flow cytometry. On the figure, every point is a fluorescent nucleus, micronucleus or debris. Statistically by DNA content (intensity of fluorescence) and particle number

the main region consists of nuclei (R1). The region from R2 to R3 contains micronuclei (1-10% of all G_1-peak).

Excellent correspondence between the usual micronuclei assay using light microscopy and this micronuclei assay using flow cytometry has been found in the laboratory.

2.2.8 Cell cycle analysis of human lymphocytes using flow cytometry

Fixation

Human peripheral blood lymphocytes were isolated by centrifugation at 800g for 5 min and resuspended in 500 µl of PBS and then chilled at 4-8°C. After this, the cell suspension ($1x10^6$ cells) was fixed in 12x75 mm tube with 500 µl of 100% chilled ethanol and stored for 15 min in 4-8°C.

Debris removal

The cell suspension was transferred to 1 ml of FCS and centrifuged for 3 minutes at 300g. The upper layer of supernatant was removed and cells were resuspended in 2 ml of PBS.

DNA staining

The cell suspension was centrifuged for 3 minutes (300g) and the upper layer of supernatant was removed. Then 125 µl of RNAse solution (500 units/ml in 1,12% Na-citrate) was added to the cell suspension. After incubation for 15 min at 37°C, DNA stain (5 mg of propidium iodide in 100 ml of 1,12% Na-citrate) was added and cell suspension shaken. Before flow cytometry analysis the cells were stored at room temperature for 30 minutes. Then cells were washed in PBS and fixed with 70% ethanol. In such condition, cells were stored at 4-8°C for up to 1 week. Before analysis,

the cells were centrifuged at 2000g for 5 min, supernatant removed, and cells were resuspended in PBS. Flow cytometry analysis was performed at a wavelength of 488 nm.

Data analysis

For the DNA measurement program "CellFIT" was used. All cell populations (by DNA content) were divided into subpopulations according to cell cycle phases: G_0/G_1, S and G_2+M. The presented data, the proliferative activity (in %) is the proportion of the cells in G_2+M phase to G_0/G_1+S phase (cells with DNA Index DI=2 and DI=1).

2.2.9 "Genetic" apoptosis analysis of human lymphocytes using flow cytometry

The cell suspension was prepared as for cell cycle analysis (section 2.2.8)

Data analysis:

Data collection and analysis was performed by the program LYSYS II (Becton Dickinson). The data is presented as percent correlation of diploidic cells to hypodiploidic cells.

The main sign of apoptosis is DNA fragmentation. After staining with propidium iodide these cells, having decreased fluorescence, are observed as a sub-G_1-peak (Figure 2.2). The sub-G_1-peak is sometimes not well separated from the G_1-peak. It is analyzed statistically by computer software, which counts hypodiploid cells (cells having a chromosome number less than the diploid number).

The frequency of cells with DNA content less than 2n was analyzed.

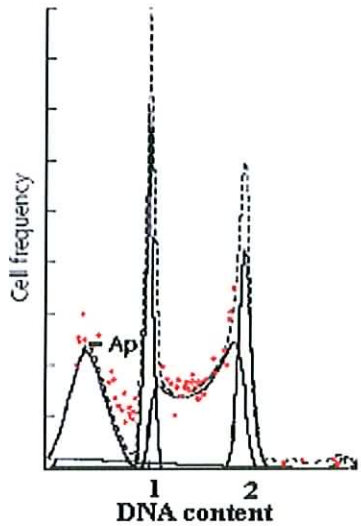

Figure 2.2 The principle of data analysis in cell cycle analysis using flow cytometry. All cell populations (by DNA content) were divided into subpopulations according to cell cycle phases: G_0/G_1, S and G_2+M. The presented data, the cell frequency is the proportion of the cells in G_2+M phase to G_0/G_1+S phase (cells with DNA Index DI=2 and DI=1). Sub-G_1-peak indicates the level of apoptotic cells.

2.2.10 Spectroscopy

The absorbance of intact filtered culture medium, culture medium with melanin added and filtered culture medium with melanin added has been analyzed using a Perkin Elmer Lamda 900 UV/VIS/NIR Spectrometer. This spectrometer is a double-beam, double monochromator ratio recording system with pre-aligned tungsten-halogen and deuterium lamps as sources. The wavelength is from 175 to 3300nm with an accuracy of 0.08 nm in the UV-visible region and 0.3nm in the NIR- region guaranteed.

2.2.10 Statistical analysis

Flow cytometry experiments were repeated three times, clonogenic – from 7 to 9 times, micronuclei – 5 times, and Alamar Blue – 6 times. Within each experiment

cultures were set up in triplicate. Results are expressed as the means +/- standard errors. Significance was determined using the paired *t* test, because the data distribution was normal. The level of trustworthiness was chosen 95%; at $t \geq 2.67$, the difference is highly significant with $P<0.01$, at $t \geq 1.96$, the difference is significant with $P<0.05$. At $P>0.05$, the difference is not significant.

2.3 Results

Study of the effect of radioprotective substances on survival of HPV-G cells

Melanin

Table 2.1 shows the results obtained for the clonogenic assay for direct irradiation and bystander recipient flasks: control, control with melanin, cells irradiated at 0.5 Gy and cells irradiated at 0.5 Gy with melanin added. Melanin was added 30 min before irradiation. The medium from irradiated bystander donor flasks containing HPV-G cells was removed 1 hour after irradiation and after being filtered was used to replace the medium from unirradiated bystander recipient flasks of the same cell type seeded at cloning densities.

Figure 2.3 shows the effect of melanin on direct and bystander irradiation of HPV-G cells at 0.5 Gy (controls were set at 100%). Data from Figure 2.3 show a significant decrease in the survival of directly irradiated or bystander recipient HPV- G cells irradiated at 0.5 Gy (the SF is 73.7%±1.0 and 81.2%±1.7, respectively) and irradiated at 0.5 Gy with melanin added (84.5%±2.6 and 88.5%±1.4) compared to controls (100%±2.3 and 100%±1.4). Addition of melanin to the medium of irradiated cells shows a significant increase in the number of colonies compared with cells irradiated without melanin in the medium ($t=3.86$, $P<0.01$). And addition of melanin to the ICCM before transfer to recipient cells shows not as significant an increase in the number of colonies compared with cells treated with ICCM without melanin ($t=3.30$; $P<0.01$). Melanin treatment alone was not found to alter the survival of HPV-G cells (98.0%±1.4 and 93.9%±1.8; $P>0.05$).

Table 2.1 The effect of animal hair melanin (Ml) on HPV-G cells survival (dose 0.5 Gy, melanin added before irradiation).

		Cells plated	Average No. colonies	P	PE, %
Direct irradiation	Control	300	146±3.2	-	49±1.1
	Melanin	300	143±1.2	> 0.05	48±0.4
	0,5 Gy	300	108±3.5	< 0.01	36±1.2
	0,5 Gy+ Ml	300	123±1.5	< 0.01	41±0.5
Bystander recipients	Control	300	147±2.2	-	49±0.7
	Melanin	300	138±1.2	> 0.05	46±0.4
	0,5 Gy	300	119±1.2	< 0.01	40±0.4
	0,5 Gy+ Ml	300	130±0.6	< 0.01	43±0.2

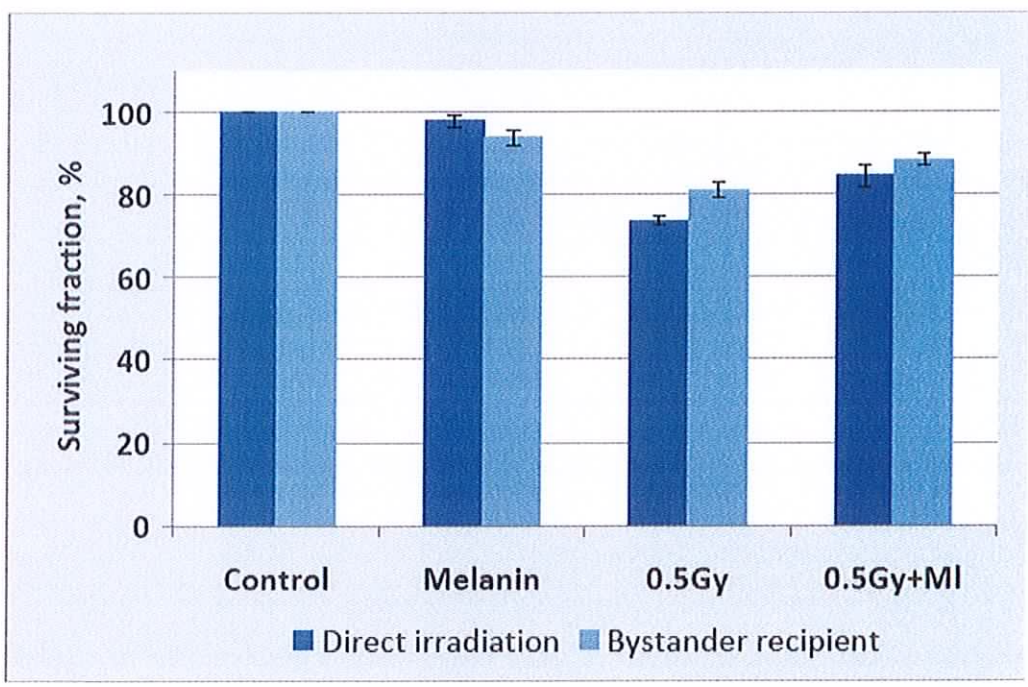

Figure 2.3 The surviving fraction of direct irradiated and bystander recipient HPV-G cells. Cells were irradiated at 0.5 Gy, melanin was added before irradiation. Melanin clearly reduces the effect of direct irradiation and to a lesser extent the bystander effect (P<0.01).

Table 2.2 present results of clonogenic assay for both direct irradiation and bystander recipient HPV-G cells. Cells were irradiated at 1 Gy, and melanin was added 30 min before irradiation.

Figure 2.4 shows effect of melanin on directly and bystander irradiated HPV-G cells (dose 1 Gy). There is a significant decrease in survival of HPV-G cells after direct irradiation and bystander donor medium transfer at a dose of 1 Gy compared with the control. Addition of melanin to the medium of directly irradiated cells significantly increased ($t=11.48$; $P<0.01$) survival in comparison to cells irradiated at 1 Gy without melanin added. And treatment of cells with irradiated medium with melanin added again decreased effects of bystander factors, but the effect was not as significant as for direct irradiation ($t=3.19$; $P<0.01$). Melanin alone was not found to be cytotoxic to HPV-G cells.

Figure 2.5 presents the absorbance spectra of three different HPV-G cell culture media: intact, with melanin added and with melanin added and filtered through 0.22 μm filter. As it can be seen from figure, the spectra of intact and filtered media is identical.

Table 2.3 presents data for both direct and bystander clonogenic study of the effects of melanin on HPV-G cells. Melanin was added before irradiation in direct irradiation flasks and 1h after irradiation to the ICCM from bystander donor flasks. 30 mins later, the ICCM was filtered and replaced the medium from bystander recipient cells.

Table 2.2 The effect of melanin after direct irradiation and bystander effect on HPV-G cells survival (dose 1 Gy, melanin added before irradiation).

		Cells plated	Average colonies No.	P	PE, %
Direct irradiation	Control	300	158±0.6	-	53±0.2
	Melanin	300	156±0.7	> 0.05	52±0.2
	1 Gy	300	111±1.2	< 0.01	37±0.4
	1 Gy+ Ml	300	129±1.2	< 0.01	43±0.4
Bystander recipients	Control	300	152±2.0	-	51±0.7
	Melanin	300	151±1.0	> 0.05	50±0.3
	1 Gy	300	121±2.5	< 0.01	40±0.8
	1 Gy+ Ml	300	133±1.8	< 0.01	44±0.6

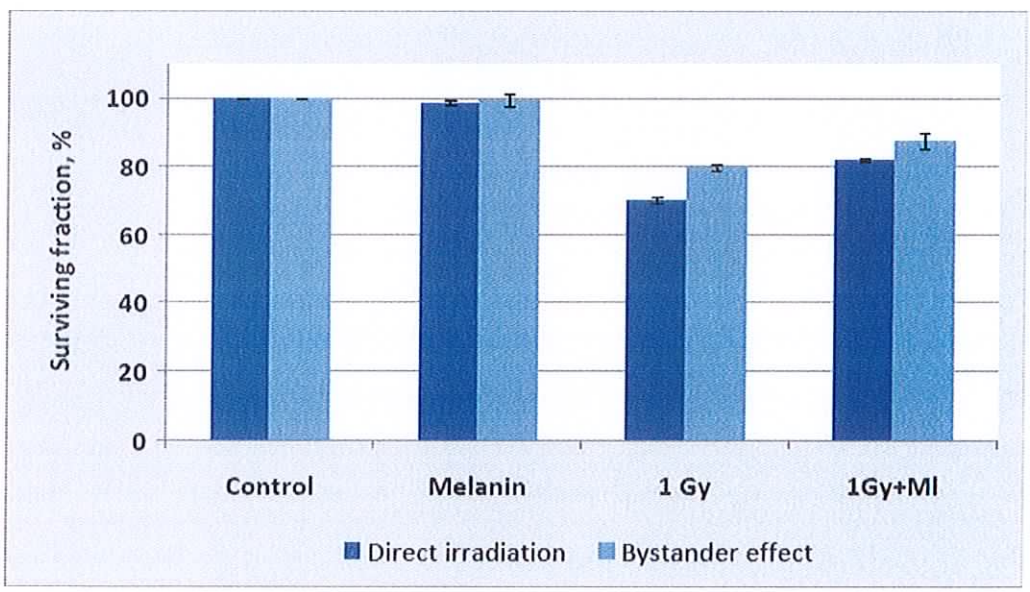

Figure 2.4 The surviving fraction of direct irradiated and bystander recipient HPV-G cells. Cells were irradiated at 1 Gy, melanin was added before irradiation. Melanin clearly reduces the effect of direct irradiation and to a lesser extent the bystander effect (compared to cells irradiated without melanin; $P<0.01$ in both cases).

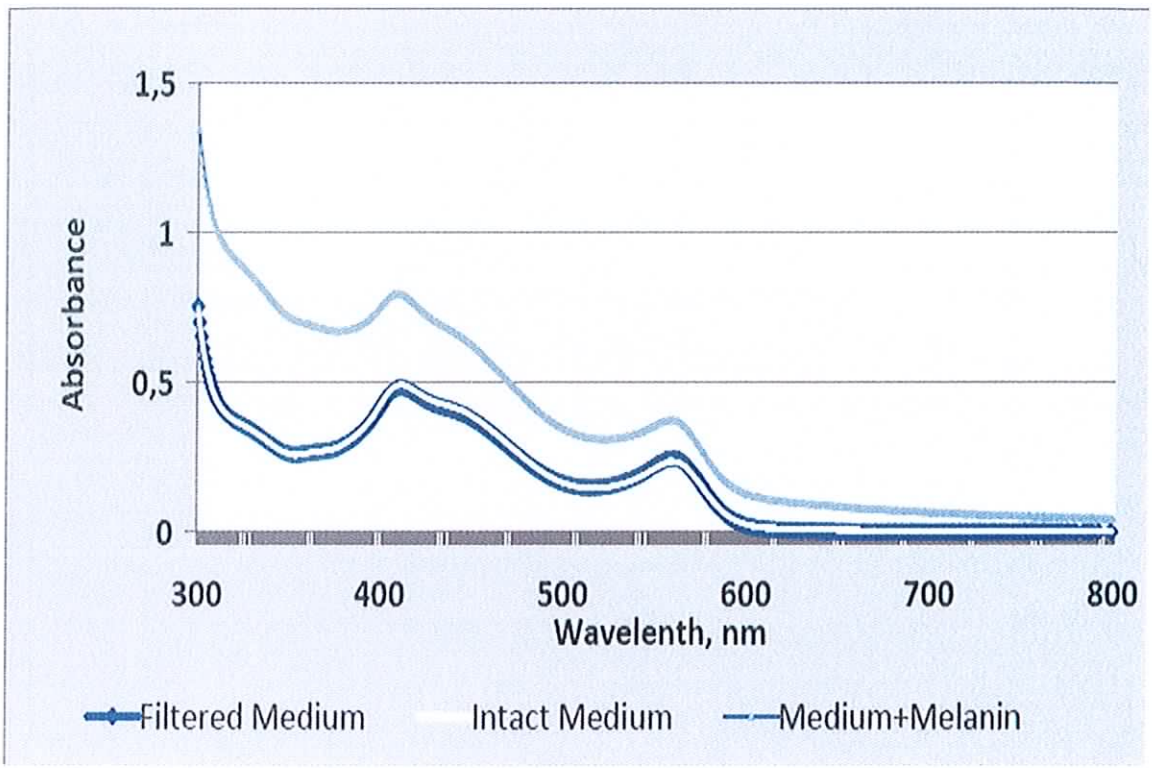

Figure 2.5 Absorbance spectra of HPV-G cell culture intact medium, medium containing melanin and filtered medium containing melanin. The absorbance spectra of intact and filtered media are similar, i.e. there is no melanin in the medium left after filtration. The absorbance spectrum of the medium containing melanin corresponds to the spectrum of melanin alone [Stark *et al.*, 2003].

Figure 2.6 presents the surviving fraction for experiments when melanin was added after irradiation. The data shows that direct irradiation and irradiated bystander medium clearly reduces the survival of HPV-G cells compared with controls. No cytotoxic effect of melanin on HPV-G cells was observed. Addition of melanin to directly irradiated cells clearly reduces the radiation effects, significantly increasing survival of HPV-G cells ($t=7.55$; $P<0.01$). Addition of melanin to the ICCM after irradiation increased the survival of HPV-G cells compared to cells treated with ICCM without melanin, but the effect was not as significant ($t=2.43$; $P<0.05$).

Figure 2.7 presents a comparison of surviving fractions of directly irradiated and bystander recipient HPV-G cells when melanin was added before irradiation and after irradiation. As can be seen from this figure, there is no dependence from time of melanin addition in directly irradiated cells with melanin added: the difference is non significant ($t=0.28$; $P>0.05$), because melanin was added to directly irradiated cells before irradiation. And in bystander recipient cells, there is dependence from time of melanin addition: the difference is significant ($t=1.94$, $P<0.05$), and the protection is more effective when melanin was added before irradiation.

Table 2.3 The effect of melanin after direct irradiation and bystander effect on HPV-G cells colony forming ability (dose 0.5 Gy, melanin added after irradiation).

		Cells plated	Average colonies No.	P	PE, %
Direct irradiation	Control	300	140±0.2	-	47±0.1
	Melanin	300	137±2.2	> 0.05	46±0.7
	0,5 Gy	300	102±0.9	< 0.01	34±0.3
	0,5 Gy+ Ml	300	117±2.0	< 0.01	39±0.7
Bystander recipients	Control	300	141±0.7	-	47±0.2
	Melanin	300	139±1.2	> 0.05	46±0.4
	0,5 Gy	300	114±2.7	< 0.01	38±0.9
	0,5 Gy+ Ml	300	121±0.7	< 0.01	40±0.2

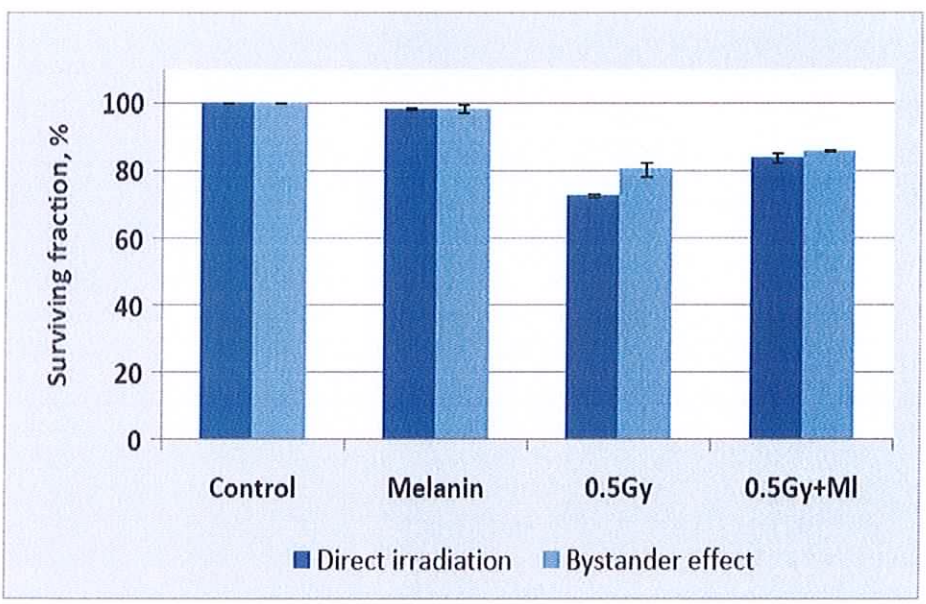

Figure 2.6 The surviving fraction of directly irradiated and bystander recipient HPV-G cells. Cells were irradiated at 0.5 Gy, melanin in bystander experiments was added after irradiation. Melanin reduces the effect of direct irradiation ($P<0.01$) and to a lesser extent the bystander effect (compared to cells irradiated without melanin; $P<0.05$).

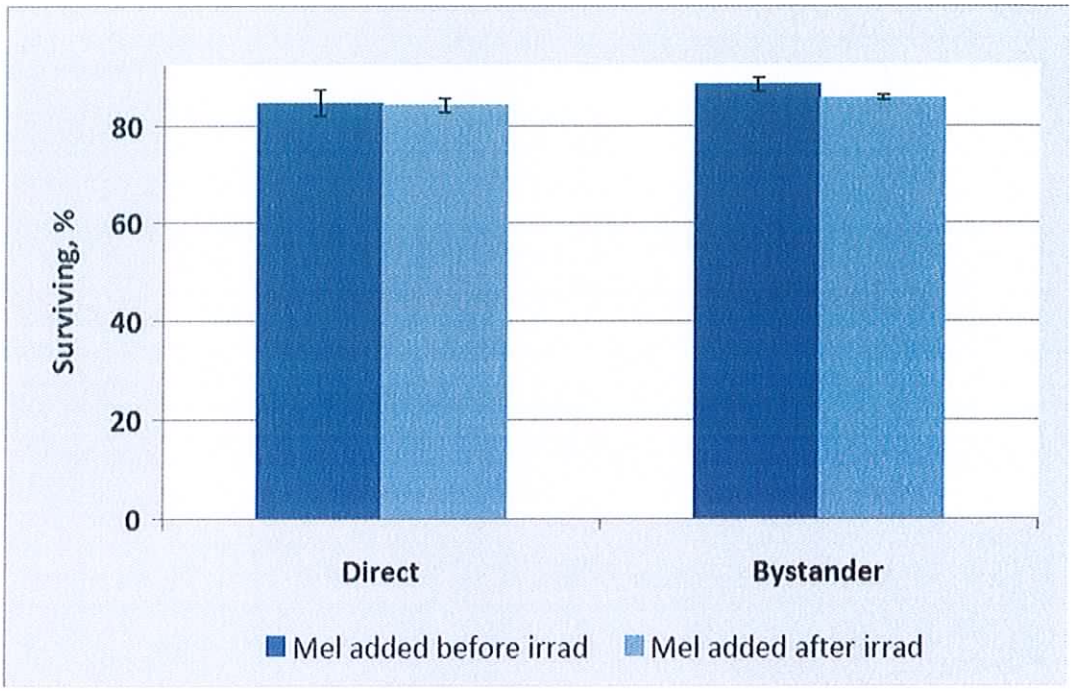

Figure 2.7 Comparison of the melanin protective effect on directly irradiated and bystander recipient HPV-G cells depending on the time of melanin addition. Cells were irradiated at 0.5 Gy. For directly irradiated cells, there is no statistically significant dependence on time of melanin addition. For bystander recipient cells, there is a statistically significant ($P<0.05$) dependence of the protective effect on the time of melanin addition as the protection is more effective when melanin was added before irradiation.

Melatonin

The radioprotective effect of melatonin was studied using HPV-G cells. Parametric methods of statistical analysis were used, as the distribution was normal. Melatonin was added before direct irradiation and to the ICCM in bystander experiments. Cells were irradiated at 0.5 Gy.

Table 2.4 presents the results of the effect of melatonin on survival of directly irradiated and ICCM exposed HPV-G cells. Melatonin was added before irradiation to directly irradiated cells and after irradiation to bystander donor cells.

Figure 2.8 presents surviving fraction for experiments with melatonin added to directly irradiated and bystander recipient HPV-G cells. The results show that there is no significant influence of melatonin on HPV-G cells ($P>0.05$). Thus, no cytotoxic or proliferation inducing effect of melatonin was observed – the survival of control cells with melatonin added is very close to controls.

The data shows a significant decrease in survival of HPV- G cells irradiated at 0.5 Gy (26% compared to control, $P<0.01$) and after bystander donor medium transfer (20%, $P<0.01$). Addition of melatonin to the medium of directly irradiated cells results in a significant increase in survival on 12% compared to cells irradiated without melatonin present in the medium ($t=4.84$; $P<0.01$), although it is still less than controls. And for bystander experiments, addition of melatonin also increased the survival of bystander recipient HPV-G cells, but not as significant as in direct irradiation experiments – on 9% ($t=2.16$; $P<0.05$).

Table 2.4 The effect of melatonin (Mt) on directly irradiated and bystander recipient HPV-G cells survival (dose 0.5 Gy, melatonin added after irradiation).

		Cells plated	Average colonies No.	P	PE, %
Direct irradiation	Control	300	149±2.3	-	50±0.8
	Melatonin	300	150±3.5	> 0.05	50±1.2
	0,5 Gy	300	97±3.2	< 0.01	32±1.1
	0,5 Gy+ Mt	300	123±2.3	< 0.01	41±0.8
Bystander recipients	Control	300	150±0.8	-	50±0.3
	Melatonin	300	150±2.9	> 0.05	50±1.0
	0,5 Gy	300	118±3.8	< 0.01	39±1.3
	0,5 Gy+ Mt	300	132±3.5	< 0.01	44±1.2

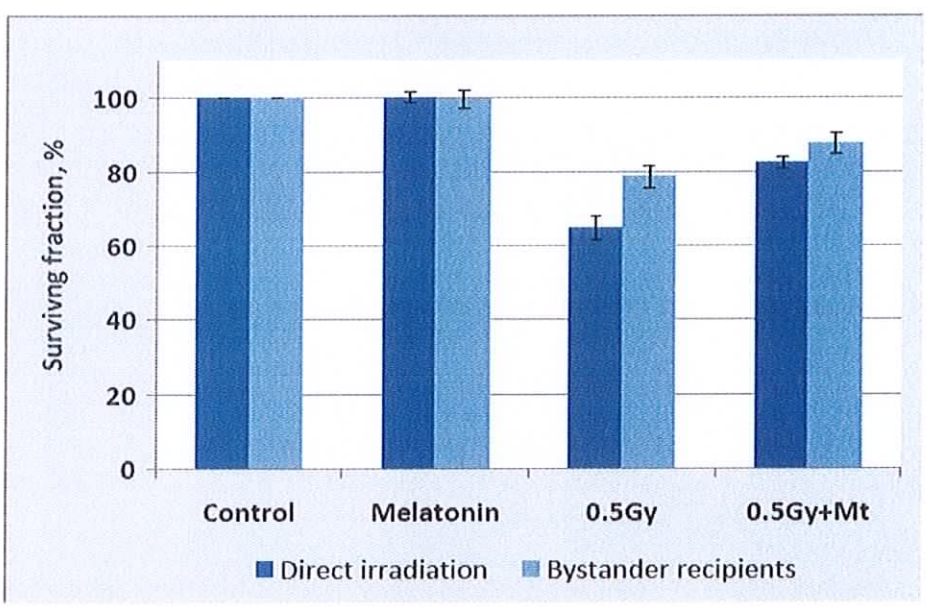

Figure 2.8 The surviving fraction of directly irradiated and bystander recipient HPV-G cells. Cells were irradiated at 0.5 Gy, melatonin was added after irradiation. Melatonin was very effective in protecting cells against direct irradiation (compared to cells irradiated without melatonin; P<0.01) and to a lesser extent against bystander effects (compared to cells treated with ICCM without melatonin; P<0.01).

Alpha tocopherol

Table 2.5 shows the results obtained for control cells, control cells with tocopherol, cells irradiated at 0.5 Gy and cells irradiated at 0.5 Gy with tocopherol added. Vitamin E was added to the medium of bystander donor cells after irradiation before filtration.

Figure 2.9 presents the surviving fraction for directly irradiated and bystander recipient cells treated with tocopherol (expressed as percent of control).

Data presented in Figure 2.9 shows the effect of tocopherol on the survival of HPV-G cells after irradiation. Cells irradiated at 0.5 Gy have clearly lower survival compared with control cells ($P<0.01$). Addition of tocopherol to the medium of non-irradiated cells does not have any cytotoxic effect.

The survival of irradiated cells with tocopherol added to the medium is lower, than controls, but higher, than the survival of cells irradiated without tocopherol ($t=4.92$; $P<0.01$). Addition of tocopherol to the ICCM increased the survival of bystander recipient HPV-G cells, but not as significantly as in direct irradiation experiments ($t=2.12$; $P<0.05$).

Figure 2.10 presents the efficacy of the radioprotective effect against direct irradiation and bystander factors of all three analysed radioprotective substances: melanin, melatonin and tocopherol. The efficacy was calculated as the difference between surviving fractions of cells irradiated at 0.5 Gy with radioprotective substances and SF of cells irradiated without radioprotective substances (in %).

As it can be seen from Figure 2.10, the best protective effect against both direct irradiation and bystander factors was melatonin. Tocopherol provided the least effective protection.

Table 2.5 The effect of α-tocopherol (Tp) on directly irradiated and bystander recipient HPV-G cells survival (dose 0.5 Gy, tocopherol added after irradiation).

		Cells plated	Average colonies No.	P	PE, %
Direct irradiation	Control	300	172±1.2	-	57±0.4
	Tocopherol	300	170±0.9	> 0.05	57±0.3
	0,5 Gy	300	127±0.3	< 0.01	42±0.2
	0,5 Gy+ Tp	300	136±0.9	< 0.01	45±0.3
Bystander recipients	Control	300	170±1.5	-	57±0.5
	Tocopherol	300	169±1.2	> 0.05	56±0.4
	0,5 Gy	300	133±0.9	< 0.01	44±0.3
	0,5 Gy+ Tp	300	138±1.5	< 0.01	48±0.5

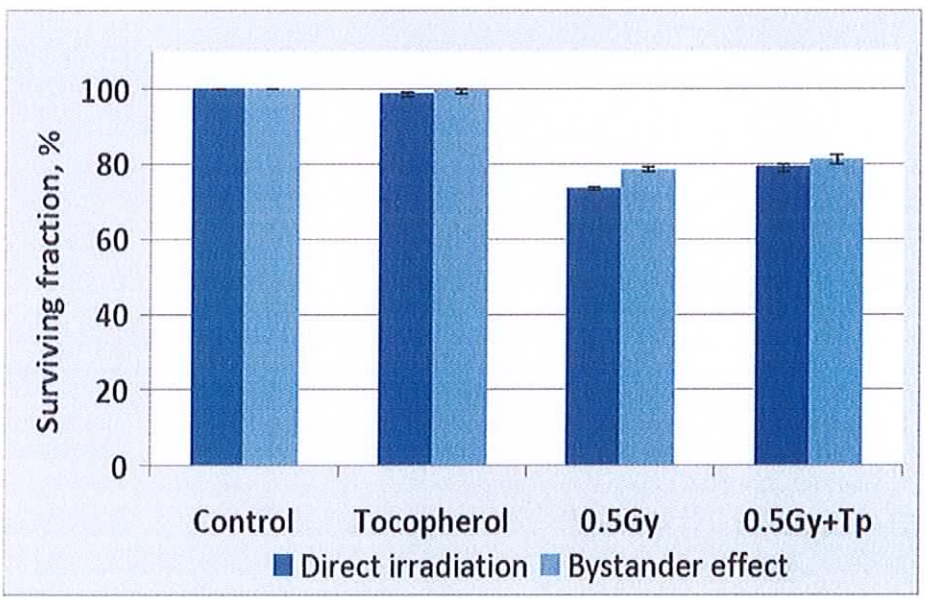

Figure 2.9 The surviving fraction of directly irradiated and bystander recipient HPV-G cells treated with α-tocopherol. Cells were irradiated at 0.5 Gy; tocopherol added after irradiation. Tocopherol decreased the effects of direct irradiation ($P<0.01$) and to a lesser extent bystander effects ($P<0.05$).

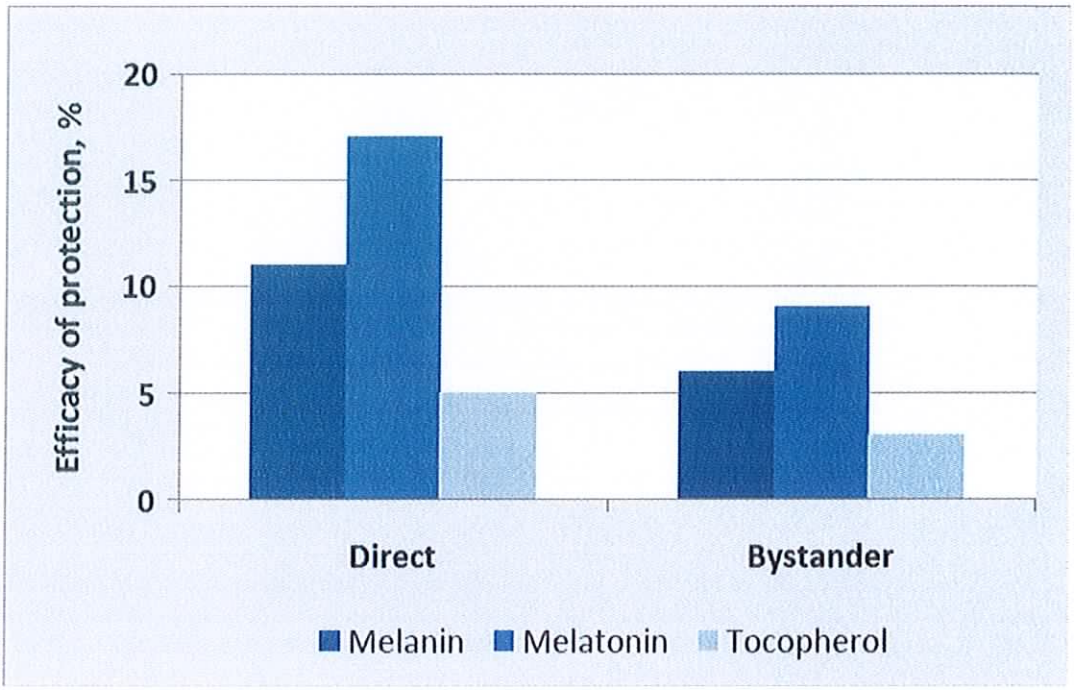

Figure 2.10 Comparison of the efficacies of radioprotective effect of melanin, melatonin and tocopherol against direct irradiation and bystander effect (clonogenic assay). Efficacy was calculated as a difference between SF of cells irradiated with radioprotectors and SF of cells irradiated without radioprotectors (in %). The best protective effect against both direct and bystander irradiations was melatonin, with tocopherol showing the least protective effect.

Study of the effect of radioprotective substances on HPV-G cells using the micronucleus assay

The micronucleus assay is widely used in genetic monitoring of populations and for evaluation of the mutagenic effects *in vitro*. This assay is also used to evaluate individual sensitivity to physical and chemical mutagens.

Melanin

The results of melanin influence on micronuclei frequency using cytochalasin B block in HPV-G cells irradiated at 0.5 Gy are presented in Table 2.6. Melanin was added before irradiation in direct irradiation flasks and 1h after irradiation in bystander donor flasks. 30 min later, the ICCM from bystander donor flasks was filtered and replaced the medium from bystander recipient cells.

The micronuclei frequency in the controls was comparatively low and indicates the low level of spontaneous mutagenesis. The number of the cells with 2 and 3 micronuclei was much lower than the number of the cells with 1 micronucleus.

As it can be seen from Table 2.6, the average micronuclei frequency increased in irradiated cells and after bystander donor medium transfer (230.00±10.87 and 149.67±9.21, correspondingly) compared with control (75.33±6.81 and 76.67±6.87). Melanin was not found to induce micronuclei – after addition of melanin to the non-irradiated cells the micronuclei frequency was very close to the control (78.00±6.92 and 72.00±6.67, correspondingly; the difference was not significant). This proves that melanin does not have any toxic effect on the cells and that it has no micronuclei inducing or suppressing ability.

After addition of melanin to the medium of the directly irradiated cells the micronuclei frequency (144.00±9.07) was higher, than in the controls, but much less than in irradiated cells ($t=6.08$; $P<0.01$). After addition of melanin to ICCM after

irradiation before filtration and transfer to bystander recipient cells the micronuclei frequency (114.01±8.21) was again higher, than in the controls, but less than in cells treated with ICCM without melanin ($t=2.89$; $P<0.01$).

The data shows highly significant total micronuclei frequency ($P<0.01$) for both direct and bystander effects of radiation. Looking at the total number of cells with micronuclei, for directly irradiated cells, the addition of melanin to the medium again significantly reduces the number of cells with micronuclei compared to cells irradiated without melanin ($t=5.35$; $P<0.01$). But the number of micronuclei cells in bystander recipient cells treated with ICCM with melanin added is not as significant if compared with cells treated with ICCM without melanin ($t=2.44$; $P<0.05$). The same situation occurs for the number of the cells with 1 micronucleus. For direct irradiation the difference is highly significant ($t=4.72$; $P<0.01$), but not as significant for bystander cells ($t=2.12$; $P<0.05$).

Table 2.6 The effect of melanin on micronuclei frequency of directly irradiated and bystander recipient HPV-G cells. Cells were irradiated at a dose of 0.5 Gy; melanin was added after irradiation. Melanin protected HPV-G cells against direct irradiation, significantly decreasing micronuclei frequency and the frequency of MN cells (compared to cells irradiated without melanin; $P<0.01$) and to a lesser extent against bystander effects (compared to cells treated with ICCM without melanin; $P<0.05$).

		Analysed cells	Frequency of cells with MN, ‰			Total number of MN cells, ‰	Total number of micronuclei, ‰	P comp. to control
			1 MN	2 MN	3 MN			
Direct irradiation	Control	1500	59.33±6.10	6.00±1.99	1.33±0.94	66.67±6.44	75.32±6.81	-
	Melanin	1500	62.67±6.26	6.67±2.10	0.67±0.67	70.00±6.59	78.00±6.92	> 0.05
	0,5Gy	1500	168.00±9.65	22.00±3.79	6.00±1.99	196.00±10.25	230.00±10.87	< 0.01
	0,5Gy+Ml	1500	108.67±8.04**	12.67±2.89*	3.33±1.49†	124.67±8.53**	144.00±9.07**	< 0.01
Bystander effect	Control	1500	62.00±6.23	5.33±1.88	1.33±0.94	68.67±6.53	76.65±6.87	-
	Melanin	1500	54.67±5.87	6.67±2.10	1.33±0.94	62.67±5.26	72.00±6.67	> 0.05
	0,5Gy	1500	109.67±8.07	14.00±3.03	4.00±1.63	127.67±8.62	149.67±9.21	< 0.01
	0,5Gy+Ml	1500	86.67±7.26*	10.67±2.65†	2.00±1.15†	99.34±7.72*	114.01±8.21*	< 0.01

Comparing to cells irradiated/treated with ICCM without melanin: ** $P<0.05$; * $P<0.01$; † $P>0.05$

Melatonin

Table 2.7 presents results for the effect of melatonin on the radiation-induced bystander effect using the micronuclei test. Melatonin was added to the ICCM after irradiation at 0.5 Gy.

Table 2.7 shows that radiation influence and irradiated bystander medium clearly induces micronuclei formation in HPV-G cells. As previously shown with melanin, the average total micronuclei frequency in control cells (75.33±6.81 and 76.67±6.87, respectively) is lower than the average total micronuclei frequency in irradiated cells (230.00±10.87 and 149.67±9.21, respectively). Compared to controls, no micronuclei inducing activity of melatonin was found. This means that melatonin has no cytotoxic effect on HPV-G cells.

The results indicate that the average micronuclei frequency of the cells irradiated with melatonin added (136.00±8.85) is higher than in the control, but lower, than in cells irradiated or treated with ICCM without melatonin added ($t=6.7$; $P<0.01$). After the transfer of bystander donor medium with melatonin added to recipient cells the micronuclei frequency (106.00±7.95) is also higher than in controls, but lower than in cells treated with ICCM without melatonin ($t=3.59$; $P<0.01$). Although the protective effect of melatonin is highly significant in both cases, it is again more effective for directly irradiated cells.

Looking at total number of cells with micronuclei, the addition of melatonin to directly irradiated and bystander recipient cells leads to a significant decrease in the micronuclei frequency compared to cells irradiated without melatonin ($t=6.07$ and $t=3.19$, respectively; $P<0.01$ in both cases).

Table 2.7 The effect of melatonin on micronuclei frequency of directly irradiated and bystander recipient HPV-G cells (dose 0.5 Gy; melatonin added after irradiation). Melatonin significantly decreased MN frequency and frequency of MN cells after direct irradiation and to a lesser extent against bystander effects compared to cells irradiated without melatonin (P<0.01 in all cases).

		Analysed cells	Frequency of cells with MN, ‰			Total number of MN cells, ‰	Total number of micronuclei, ‰	P comp. to control
			1 MN	2 MN	3 MN			
Direct irradiation	Control	1500	59.33±6.10	6.00±1.99	1.33±0.94	66.67±6.44	75.32±6.81	-
	Melatonin	1500	57.33±6.00	6.67±2.10	1.33±0.94	65.33±6.38	74.66±6.79	> 0.05
	0,5Gy	1500	168.00±9.65	22.00±3.79	6.00±1.99	196.00±10.25	230.00±10.87	< 0.01
	0,5Gy+Mt	1500	98.67±7.70**	14.67±3.10†	2.67±1.33†	116.01±8.27**	136.02±8.85**	< 0.01
Bystander effect	Control	1500	62.00±6.23	5.33±1.88	1.33±0.94	68.67±6.53	76.65±6.87	-
	Melatonin	1500	60.67±6.16	4.67±1.76	2.00±1.15	67.33±6.47	76.01±6.84	> 0.05
	0,5Gy	1500	109.67±8.07	14.00±3.03	4.00±1.63	127.67±8.62	149.67±9.21	< 0.01
	0,5Gy+Mt	1500	79.33±6.98**	9.33±2.48†	2.67±1.33†	91.33±7.44**	106.00±7.95**	< 0.01

Comparing to cells irradiated/treated with ICCM without melatonin: ** P<0.05; * P<0.01; † P>0.05

Alpha-tocopherol

Table 2.8 presents data for the effects of α-tocopherol on micronuclei frequency of directly irradiated and bystander recipient HPV-G cells.

The number of micronuclei in control cells (spontaneous micronuclei frequency) is comparatively low. The micronuclei frequency in control cells with tocopherol added is very close to controls (the difference is not significant). Thus, α-tocopherol doesn't have cytotoxic effect on HPV-G cells.

Irradiation at 0.5 Gy and ICCM transfer has a very high micronuclei inducing ability: the number of micronuclei in treated cells is 4 times higher than in controls. Addition of α-tocopherol to the medium before direct irradiation significantly reduces micronuclei frequency compared with cells irradiated without α-tocopherol ($t=5.05$; $P<0.01$). And micronuclei frequency in bystander recipient cells treated with ICCM with α-tocopherol added is higher, than in controls, but lower, than in recipient cells treated with ICCM without α-tocopherol added ($t=2.13$, $P<0.05$). Again α-tocopherol shows less effective protection against bystander factors compared with directly irradiated cells.

Table 2.8 The effect of α-tocopherol on micronuclei frequency of directly irradiated and bystander recipient HPV-G cells (dose 0.5 Gy; tocopherol added after irradiation). Tocopherol significantly protected cells against direct irradiation (P<0.01) and to a lesser extent against bystander effects (P<0.05).

		Analysed cells	Frequency of cells with MN, ‰			Total No of MN cells, ‰	Total No of micronuclei, ‰	P comp. to control
			1 MN	2 MN	3 MN			
Direct irradiation	Control	1500	67.67±6.49	6.00±1.99	1.33±0.94	75.00±6.80	83.66±7.15	-
	Tocopherol	1500	65.00±6.37	5.33±1.88	1.33±0.94	71.66±6.67	79.65±6.99	> 0.05
	0,5Gy	1500	239.33±11.02	35.00±4.75	9.00±2.44	283.33±11.63	336.33±12.20	< 0.01
	0,5Gy+Tph	1500	191.00±10.15**	24.33±3.98†	4.33±1.70†	219.66±10.69**	252.65±11.22**	< 0.01
Bystander effect	Control	1500	70.33±6.60	7.67±2.25	0.00±0.00	78.00±6.92	85.67±7.23	-
	Tocopherol	1500	65.67±6.40	6.33±2.05	1.33±0.94	73.33±6.73	82.32±7.10	> 0.05
	0,5Gy	1500	186.33±10.05	24.00±3.95	6.00±1.99	216.33±10.63	252.33±11.21	< 0.01
	0,5Gy+Tph	1500	169.94±9.70†	19.67±3.59†	3.33±1.49†	192.94±10.19†	219.27±10.68*	< 0.01

Comparing to cells irradiated/treated with ICCM without vitamin E: ** P<0.05; * P<0.01; † P>0.05

Study of the effect of radioprotective substances on HPV-G cells using the Alamar Blue assay

Melanin

Table 2.9 presents the Alamar Blue results for directly irradiated and bystander recipient HPV-G cells treated with melanin. The data are presented as fluorescent units (FU) and are an average of 5 independent experiments.

Figure 2.11 presents metabolic activity of directly irradiated and bystander recipient HPV-G cells treated with melanin (calculated in %; control is set at 100%).

The data presented in Figure 2.11 clearly shows that direct irradiation and transfer of irradiated medium reduces the viability of HPV-G cells compared to controls ($P<0.01$). No effect of melanin was found on viability of HPV-G cells: the average number of fluorescence units of control cells and non-irradiated cells with melanin is very close ($P>0.05$ - the difference is not significant). This again suggests that melanin has no cytotoxic effect on HPV-G cells.

The addition of melanin to the medium of directly irradiated cells clearly increased the viability of HPV-G cells compared to cells irradiated without melanin ($t=3.27$; $P<0.01$). The ICCM with melanin added before filtration and transfer to bystander recipient cells was shown to have a lower damaging effect (decrease in cell viability) compared to ICCM without melanin ($t=2.88$; $P<0.01$). But again, as for the clonogenic and micronuclei assays, the protective effect of melanin was more effective in directly irradiated cells than in bystander cells.

Table 2.9 The average fluorescent units in directly irradiated and bystander recipient HPV-G cells protected by melanin (dose 0.5 Gy).

		Average No. of FU, $x10^3$	P
Direct irradiation	Control	45.19±1.62	-
	Melanin	45.07±0.82	> 0.05
	0.5 Gy	29.07±1.18	< 0.01
	0.5 Gy +Ml	33.79±0.84	< 0.01
Bystander effect	Control	43.39±1.21	-
	Melanin	42.88±0.65	> 0.05
	0.5 Gy	33.07±1.02	< 0.01
	0.5 Gy +Ml	36.93±0.87	< 0.01

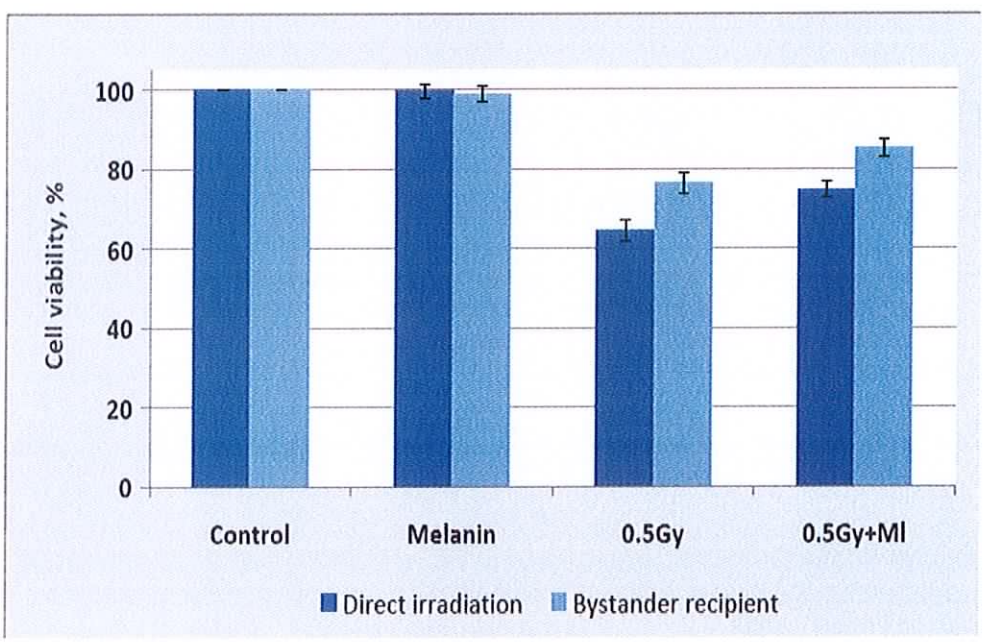

Figure 2.11 The effect of melanin on directly irradiated and bystander recipient HPV-G cells (dose 0.5 Gy, data presented as a percentage of unexposed cells). Melanin was added after irradiation. The data shows that melanin significantly increases viability of the irradiated cells ($P<0.01$) and to a lesser extent bystander recipient cells ($P<0.05$).

Melatonin

Table 2.10 presents results for directly irradiated and bystander recipient HPV-G cells treated with melatonin using the Alamar Blue assay. Melatonin was added to directly irradiated cells before irradiation and in bystander experiments after irradiation before filtration and transfer to recipient cells.

Figure 2.12 presents the results of metabolic activity in directly irradiated and bystander recipient HPV-G cells expressed as a percent of control.

As can be seen from Figure 2.12, direct irradiation and ICCM reduced the viability of HPV-G cells compared to control ($P<0.01$). No cytotoxic effect of melatonin on viability of HPV-G cells was found. Addition of melatonin to the medium of directly irradiated cells resulted in a decrease in the cytotoxic effects of the medium, the viability was higher than cells irradiated without melatonin ($t=3.75$; $P<0.01$). Addition of melatonin to ICCM also showed increased viability compared to cells treated with ICCM without melatonin ($t=3.23$, $P<0.01$). But again protection against direct irradiation was more effective than protection against bystander factors.

Table 2.10 The average fluorescent units for directly irradiated and bystander recipient HPV-G cells treated with melatonin (dose 0.5 Gy, melatonin added after irradiation).

		Average No of FU $x10^3$	P
Direct irradiation	Control	45.17±1.62	-
	Melatonin	44.53±0.92	> 0.05
	0.5 Gy	29.07±1.18	< 0.01
	0.5 Gy +Mt	35.01±1.06	< 0.01
Bystander effect	Control	43.39±1.21	-
	Melatonin	43.12±1.19	> 0.05
	0.5 Gy	33.07±1.02	< 0.01
	0.5 Gy +Mt	37.27±0.81	< 0.01

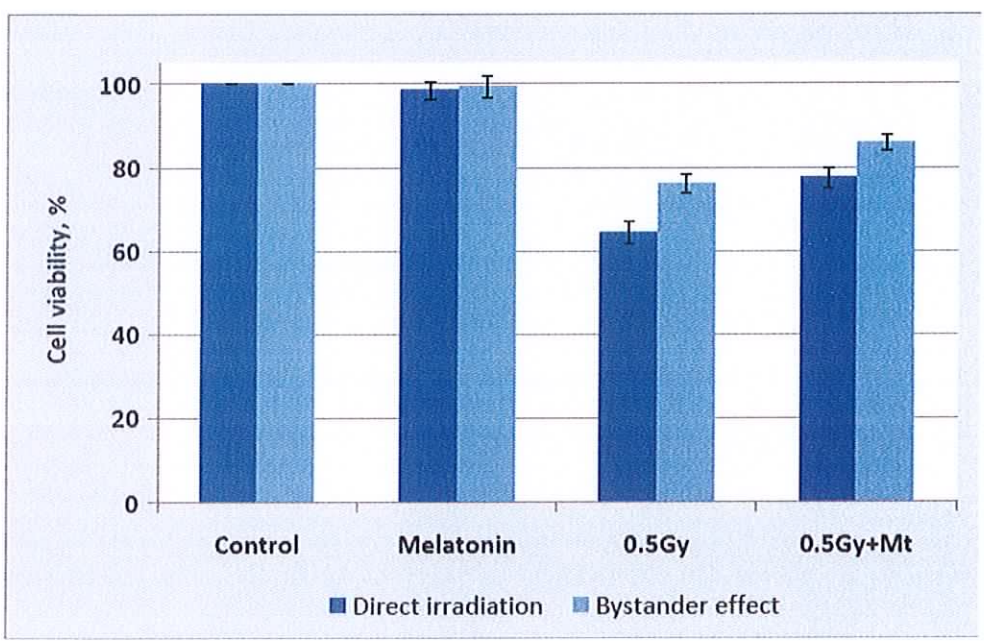

Figure 2.12 The effect of melatonin on directly irradiated and bystander recipient HPV-G cells (dose 0.5 Gy, data presented as a percentage of unexposed cells). Melatonin protected HPV-G cells from direct irradiation, significantly increasing their viability (P<0.01) and to a lesser extent from bystander factors (P<0.01).

Study of melanin effect on human lymphocytes using flow cytometric micronucleus assay

The results for cytofluorimetric micronuclei counts are presented in Figure 2.13. Melanin was added before irradiation. Cells were irradiated, and micronuclei frequency was analysed every 2 days after irradiation.

Analysis of micronuclei frequency using flow cytometry (Figure 2.13) reveals regularity in stage of physiologically active proliferation (2-4th day) following stimulation with PHA. The test loses its sensitivity in later stages because of the death of lymphocytes.

The micronuclei frequency of the irradiated cells was increased on the 2nd day after irradiation by 240% (1.9559±0.20 compared to controls 0.813%±0.13; $P<0.01$) and by 183% on 4th day after irradiation (0.477%±0.08 compared to controls 0.26%±0.07; $P<0.05$). On the later stages, the test loses its sensitivity because of the physiological death of the lymphocytes, and on the 6th, 8th and 10th days after treatment the difference between micronuclei frequency of irradiated cells and control cells is not significant ($P>0.05$ in all cases). Addition of melanin to the medium of non-irradiated cells decreased the micronuclei frequency to lower than the control value (2nd day – 0.762% compared to controls 0.813%±0.13; 4th day - 0.178%±0.04 compared with controls 0.26%±0.07), but the difference is not statistically significant; $P>0.05$ in all cases), possibly as a result of its antioxidant effect.

When added to the medium of irradiated lymphocytes before irradiation, melanin significantly decreased the micronuclei frequency (on the 2nd day – 1.323%±0.16; $P<0.01$ compared to cells irradiated without melanin). On the 4th day, the level of micronuclei was decreased up to control levels (0.228%±0.07; $P>0.05$ compared to control, $P<0.01$

compared to cells irradiated without melanin), thus showing radioprotective activity. On the later stages (6, 8, 10 days after irradiation) the level of micronuclei frequency was not significantly different between control, irradiated and irradiated with melanin cells, possibly again as a result of the cell death.

Thus, as can be seen from Figure 2.13, there is a high, statistically significant ($P<0.01$ on 2^{nd} day and $P<0.05$ on 4^{th} day after irradiation) increase in micronuclei frequency in the irradiated cells without melanin compared with control cells. At the same time, melanin added to irradiated cells blocks this effect, decreasing the frequency of micronuclei (2^{nd} day - $P<0.01$, 4^{th} day – $P<0.05$).

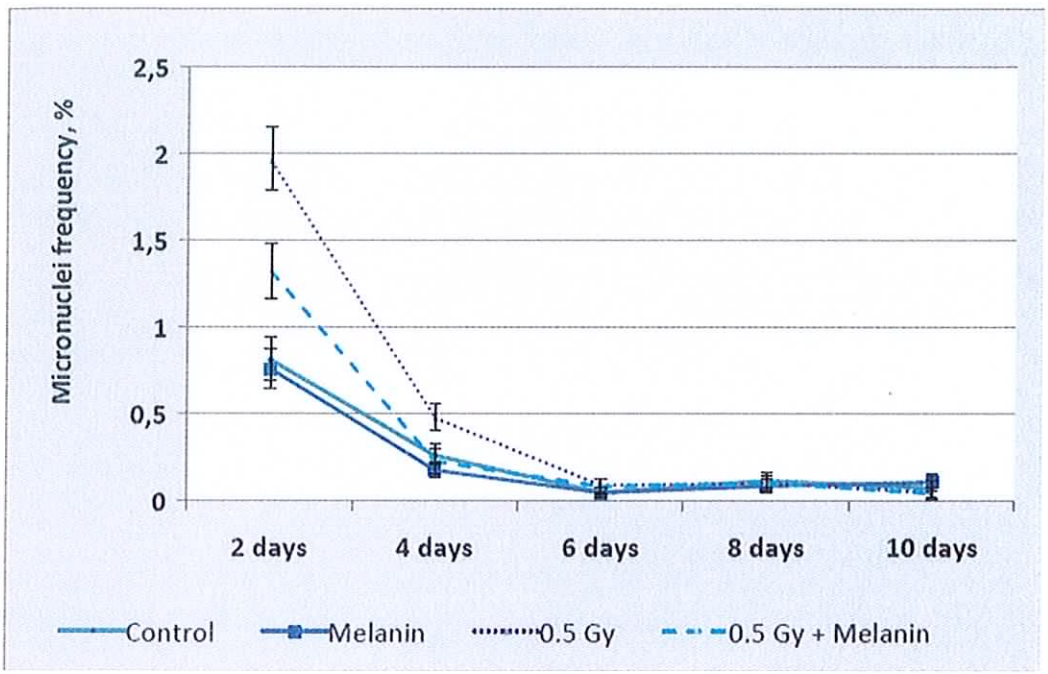

Figure 2.13 The effect of direct irradiation and melanin protection on micronuclei frequency of human peripheral blood lymphocytes analysed with flow cytometry. Melanin was added before irradiation, cells were irradiated at 0.5 Gy. On the 2nd day of experiments, melanin protected cells against irradiation, decreasing micronuclei frequency. On the later stages, the level of micronuclei is decreased because of the cell death.

Study of melanin effect on cell cycle of human peripheral blood lymphocytes

The cell cycle analysis of human peripheral blood lymphocytes, which was used in the present research, is the most commonly used approach that allows discrimination between cells that differ in DNA content, for instance, cells in G_0/G_1 versus those in G_2/M that have replicated (doubled) their DNA. The results of fluorescence measurements are displayed as cellular DNA content frequency histograms (Figure 2.14).

The content of DNA (DNA index, DI) doubles during S phase and therefore G_2 and M cells have twice as much DNA as G_1 cells (DI = 2.0). The cell's progression through S can be estimated based on the amount of replicated DNA (the increase in DI from 1.0 to 2.0). G_1 and G_2/M cells have a uniform DNA content (DI = 1.0 and 2.0, respectively) so that if their DNA could be measured with absolute accuracy these cell populations would be represented on the DNA content frequency histograms by single-channel (unit) bars. However, owing to the inaccuracy of DNA content measurements, their actual distributions are in the form of peaks whose width reflects the inaccuracy. The DNA-bound propidium iodide emits fluorescence when excited with blue light (488 nm). However, because ethidium bromide also stains RNA, the procedure includes incubation with RNAase to remove RNA.

The results of flow cytometric analysis of the distribution of human peripheral blood lymphocytes in cell cycle phases are presented in Figure 2.15.

The results presented in Figure 2.15 show that melanin on the 4^{th} day after irradiation does not have any significant effect on non-irradiated lymphocytes – the proliferation activity is very close to the control (30.50%±0.65 compared to controls

29.25%±0.64). But after 2 days after irradiation the micronuclei frequency of cells with melanin is even statistically significantly lower than controls (19.00%±0.50 compared to 22.62%±0.59; $P<0.01$). In the control sample, human peripheral blood lymphocytes reach their maximum proliferation activity *in vitro* on the 3-4th day of cultivation, which corresponds with data from the literature. In irradiated cells, the proliferation activity on the 2nd day (9.77%±0.42) and 4th day (15.5%±0.51) is almost half that of the controls (22.62%±0.59 and 29.25%±0.64, respectively). The addition of melanin to the culture medium before irradiation significantly increases the proliferation activity of the cells up to the control level (2nd day – 22.62%±0.59, 4th day 27.9%±0.63) compared to cells irradiated without melanin ($P<0.01$ in both cases).

Normally, in such cultivating conditions proliferation activity of lymphocytes reaches a maximum after 3-4 days and then decreases slowly, as the cells are not immortalised, but stimulated by PHA. Later (6, 8 and 10 days after irradiation) the level of cell death again does not allows the data to be analysed reliably.

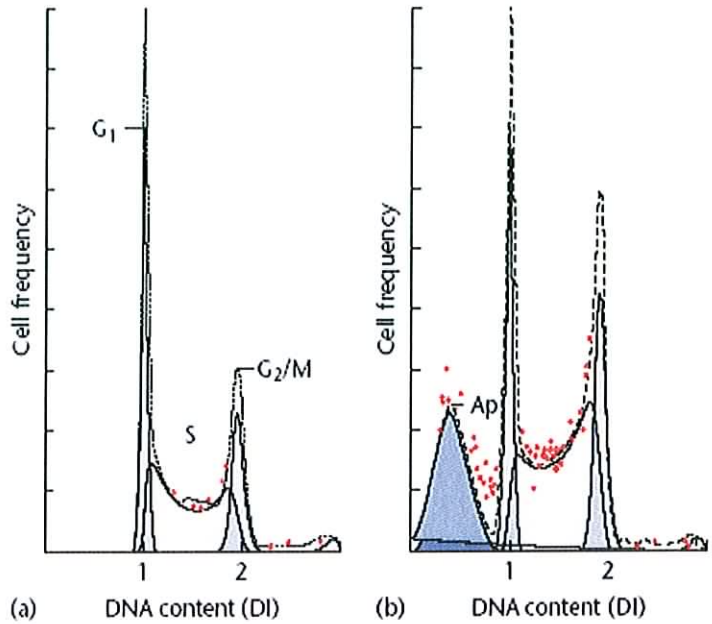

Figure 2.14 Relationship between the cell cycle position and DNA content. These typical histograms of the DNA content represent untreated cells (a) and irradiated cells (b). The cell cycle analysis allows discrimination between cells that differ in DNA content: cells in G_0/G_1 versus those in G_2/M that have replicated (doubled) their DNA. The content of DNA (DNA index, DI) doubles during S phase and therefore G_2 and M cells have twice as much DNA as G_1 cells (DI = 2.0). The cell's progression through S can be estimated based on the amount of replicated DNA (the increase in DI from 1.0 to 2.0). Cells undergoing through apoptosis have fragmented DNA. The frequency of such cells represents hypodiploid Ap-peak on figure (b).

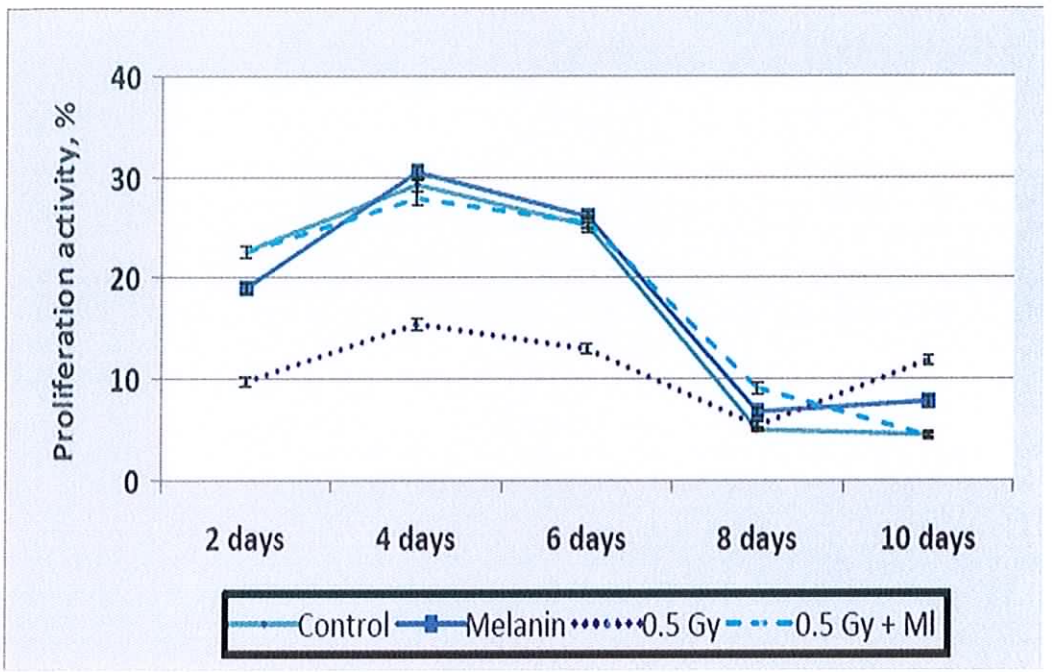

Figure 2.15 The level of proliferation activity of human peripheral blood lymphocytes after irradiation and protection with melanin. Melanin was added before irradiation. Melanin protected human lymphocytes, increasing the proliferation activity of irradiated lymphocytes back to control levels compared to cells irradiated without melanin. On the 8th day of analysis, the proliferation potential of cells activated by PHA decreased because of the lymphocyte cell death.

Study of melanin effect on the level of "genetic apoptosis" in human peripheral blood lymphocytes

The most widespread method of apoptosis level determination is based on its ability to lose genetic material, i.e. decreasing DNA quantity and formation of hypodiploid cells. This apoptosis hypodiploid peak is shown on Figure 2.14.

Figure 2.16 show the results for apoptosis levels in human lymphocytes after irradiation and treatment with melanin. Melanin was added before irradiation. Cells were analysed 2, 4, 6 and 8 days after irradiation (later because of the high level of destructive processes all the cells were dead).

The data presented in Figure 2.16 show that the level of genetic apoptosis increased every 2 days (with every cell division) even in the controls. This is the result of a decrease in proliferation activity and an increase in destructive processes.

The addition of melanin to the culture medium of human peripheral blood lymphocytes statistically decreased the apoptosis level (19.89±0.57) compared to controls (23.85±0.6) on the 4^{th} day of cultivation ($P<0.01$). On the second day after irradiation, the level of apoptosis in cells with melanin was again lower than controls, but not significant (4.27%±0.29 vs. 4.86±0.30; $P>0.05$).

Irradiation of the cells increased the level of apoptotic cells by 154% on the 2^{nd} day (7.51%±0.37, $P<0.01$) and by 123% on the 4^{th} day (29.24±0.64, $P<0.01$) compared with control levels. The addition of melanin to the lymphocyte medium before irradiation promoted a decrease in the number of apoptotic cells 4 days after irradiation by 15% (24.94±0.61) compared with irradiated cells without melanin in the medium ($P<0.01$); but the level of apoptotic cells was 5% higher compared to control levels and 25% higher

($P<0.01$) compared to control cells with melanin added to the medium. A similar situation was observed 2 days after irradiation.

Thus, the data shown in Figure 2.16, indicates a significant increase in apoptosis levels in populations of irradiated cells ($P<0.01$). At the same time, addition of melanin to the medium of irradiated cells decreases apoptosis level compared to cells irradiated without melanin (2 days – $P<0.01$, 4 days – $P<0.01$; 6 days – $P<0.05$).

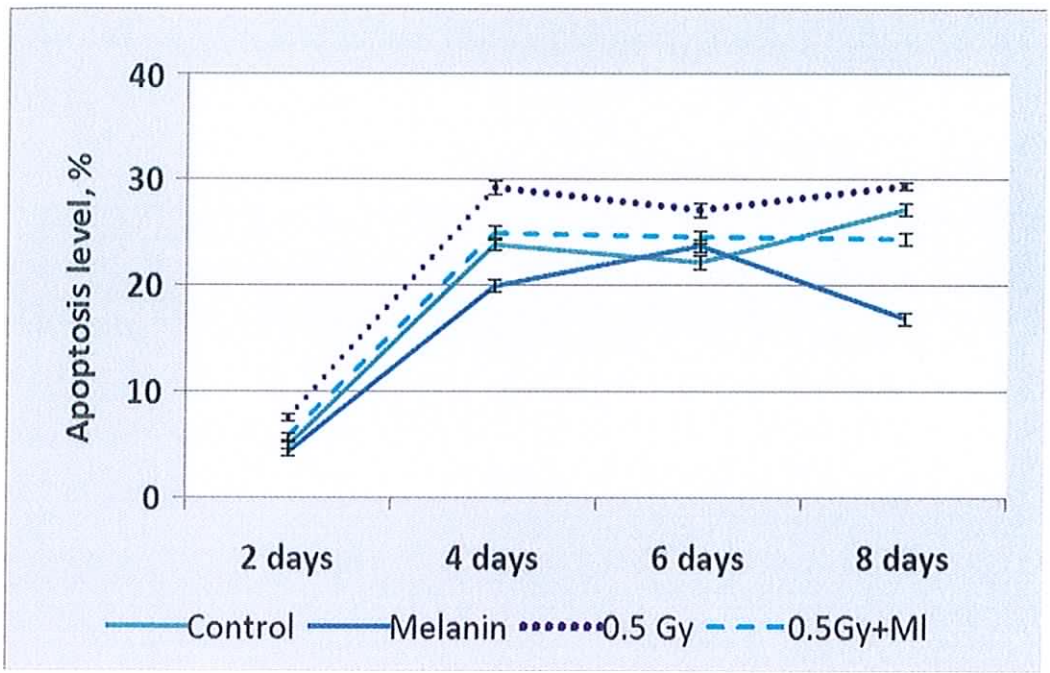

Figure 2.16 The level of apoptosis expression in human peripheral blood lymphocytes after irradiation at 0.5 Gy and protective effect of melanin. The level of apoptosis in irradiated cells increased with time. Addition of melanin protected cells during all experiments. Because of the lymphocyte cell death, the level of apoptosis even in control cells increased after 4^{th} day of analysis.

2.4 Discussion

Study of the effect of radioprotective substances on survival of HPV-G cells

The data presented in this chapter suggest that melanin when added before irradiation is able to decrease the effects of radiation on human cells. At the same time, melanin has no toxic or stimulating effect on HPV-G cells. Thus, it is possible to conclude that melanin is able to decrease the effects of radiation, not influencing cell survival.

In previous studies using γ-irradiated cell cultures, melanin was shown to have controversial effects. In some studies [Hirobe, 1982; Hill *et al.*, 1987] melanin showed very effective protection on cell cultures. In other studies [Grossi *et al.*, 1998; Stephens *et al.*, 1986; Hopwood *et al.*, 1985] melanin was shown to be ineffective in protecting cells against direct γ-irradiation. In the present study, melanin statistically significantly decreased the effects of direct irradiation.

At the same time, melanin significantly decreased the effects of bystander factors on HPV-G cells ($P<0.01$). To date, there is no published data on possible modification of radiation-induced bystander effect with radioprotective substances. However, the protection against bystander factors is not as effective as against direct irradiation.

Earlier it was shown [Mothersill and Seymour, 1997] that medium alone, irradiated in the absence of cells had no effect on survival of unirradiated cultures. This would seem to exclude the possibility that hydrolysis of medium to give radicals is involved. The time over which the effect persists also excluded any possibility that short-lived species are causing the cell death.

When cells were irradiated at 1 Gy, the effect of melanin (added before irradiation) was almost the same as after irradiation at 0.5 Gy: it was able to decrease both direct irradiation and ICCM effects, but again the protection was more effective against direct irradiation.

Comparing the difference between cells irradiated at 0.5 and 1 Gy (both direct and bystander with and without melanin), a statistically significant difference ($t=2.74$; $P<0.01$) was observed only between directly irradiated cells without melanin added, i.e. the higher the dose, the higher the effect. In bystander experiments without melanin, the difference is again not significant ($t=0.79$, $P>0.05$). Thus, the irradiation dose does not influence the level of expression and effect of bystander factor. This has been shown previously in a number of studies [Mothersil and Seymour, 1997].

For directly irradiated cells with melanin added, the difference between doses is not significant ($t=0.99$, $P>0.05$). However, a previous study [Mosse *et al.,* 1999] showed that the lower the dose, the better the melanin protection. A possible explanation may be that the difference of 0.5 Gy between doses is not big enough to observe difference. In bystander recipient cells with melanin added, the difference is also non-significant ($t=0.33$; $P>0.05$), showing that protection against bystander effects does not depend on the dose of the radiation inducing the bystander factors.

Thus, as the dose of radiation did not significantly influence the effects of melanin in protecting against direct irradiation and bystander effects, in all further experiments only a dose of 0.5 Gy was used.

In these experiments, melanin was added to directly irradiated and bystander donor cells before irradiation and filtration. It was important to ensure that melanin was not

present in the medium after filtration so that it could not influence bystander recipient cells, thus showing the usual radioprotective effect. The absorbance of intact culture medium, medium with melanin added and filtered medium with melanin added was compared. The data from Figure 2.5 shows that absorbance spectra of filtered medium with melanin is identical to intact culture medium, in contrast to the non-filtered medium with melanin. Thus, the data indicates that melanin does not pass through 0.22 μm filter and was not in direct contact with bystander recipient cells.

But also there is a possibility that melanin added before irradiation, influences bystander donor cells and in such a way protects them. As a result, bystander donor cells, irradiated with melanin, induce a decreased quantity of bystander factors into the ICCM, causing a decreased bystander effect in recipient cells, which is not connected with melanin's ability to neutralise bystander factors.

To show that melanin could decrease bystander effects in recipient cells, melanin was added to the ICCM 1 hour after irradiation (when the bystander factor was produced) before filtration and transfer to recipient cells.

On the first sight, when added after irradiation, melanin shows the same protective effect as when added before irradiation: it increased the survival of directly irradiated cells and cells treated with ICCM from bystander donor cells. Again, protection against direct irradiation was more effective compared with protection against bystander effects.

But when added before irradiation of bystander donor cells, the difference between cells, treated with ICCM with and without melanin, was highly significant ($P<0.01$), and when added after irradiation before filtration – less significant ($P<0.05$). Thus, when

melanin was added before irradiation, the protection was more effective compared to when it was added after irradiation.

This is evident from the comparison of the surviving fractions of directly irradiated and bystander recipient cells with melanin added before and after irradiation. In directly irradiated cells, there is no dependence on the time of melanin addition (the difference is not significant). And in bystander recipient cells, there is a dependence on the time of melanin addition: the protection was more effective when melanin was added before irradiation.

The fact that melanin was in the cell culture medium before and during irradiation may explain the increased protection. Any free radicals produced may have been scavenged by the melanin, thus preventing secondary damaging processes to the cells. Also, melanin is able to convert all types of physical energy, including ionising radiation, into heat [Mosse *et al.*, 1999]. Thus, it is possible to suggest that cells, protected by melanin, received less damaging bystander factors compared to bystander recipient cells, treated by the medium from cells irradiated without melanin.

When melanin was added after irradiation to ICCM before filtration, the bystander factors were already induced and melanin could not influence bystander donor cells. The observed protective effect may be due to neutralisation of bystander factors by melanin.

The data suggests that melanin clearly decreases direct irradiation effects and not as clearly bystander effects. In bystander recipient cells, the effect of melanin depends on the time of its addition to the medium. When added before irradiation, melanin decreased the bystander effect more significantly than if it was added after irradiation before filtration.

The radioprotective action of melanin was not as effective for bystander cells as for directly irradiated cells.

To confirm the result, the coefficient of difference K_{diff}, which shows the difference between surviving fractions of directly irradiated and bystander recipient cells with and without melanin added, was calculated:

$$K_{diff} = (SF_{Direct} - SF_{Direct+Ml}) - (SF_{Bystander} - SF_{Bystander+Ml}).$$

For the cells irradiated at 0.5 Gy and 1 Gy with melanin added before irradiation, the coefficient of difference was 3% and 1%, respectively (i.e. after irradiation at 1 Gy the protective effect against direct irradiation and bystander effect is almost the same) . And for experiments when melanin was added after irradiation, K_{diff} was 5%. These calculations again suggest that when added after irradiation, melanin shows the lowest protective effect against bystander effect compared to protection against direct irradiation.

In general, the experiments proved that melanin decreased not only the effect of direct irradiation, but also the effect of bystander medium influence. As melanin can absorb all types of physical energy, the result indicates that the bystander signal may have a physical nature (component).

Melanin has multiple properties which could be responsible for the protective effect against bystander factors, such as antioxidant properties, ability to take up and retain for a long period different xenobiotics and to convert all types of physical energy into heat [Mosse *et al.,* 1999]. In order to further define the possible nature of bystander effects, the influence of another radioprotective substance with antioxidant properties, but another mechanism of action was studied. Melatonin was shown in different studies to have very

effective radioprotective effect *in vivo*, but its effect *in vitro*, in cultured cells, has not been studied extensively.

As shown in the present study, there was no significant dependence of the protective effect against bystander factors on the dose of radiation (0.5 Gy or 1 Gy). In all further studies, cells were irradiated only at a dose of 0.5 Gy.

Also, as shown, the most objective conditions to study the protective effects of different substances are when they are added to the ICCM isolated one hour after irradiation from bystander donor cells before transfer to bystander recipient cells.

The effect of melatonin on directly irradiated and bystander recipient HPV-G cells has been studied using the clonogenic assay. Melatonin does not influence the survival of non-irradiated cells. Thus, this substance does not have any cytotoxic or stimulating effect on these cells. Melatonin was very effective in protecting cells against direct irradiation. But, as for melanin, melatonin was not as effective in protecting cells against bystander factors. Comparing the effects of melanin and melatonin, melatonin was more effective in protection against both direct irradiation and bystander effects.

Melatonin, like melanin, has very strong antioxidant effect. But melatonin is able to neutralise more types of different free radicals compared to melanin [Poeggler *et al.* 1993]. This is, possibly, the main reason for better protection against bystander factors.

Thus, melatonin can reduce the damaging effect of the bystander factor after its production, but was not as effective as the protection against the damaging effect of direct irradiation.

Both melanin and melatonin are able to decrease the effects of direct irradiation and bystander factors, protecting HPV-G cells. These natural substances have very effective antioxidant and radioprotective properties. But these substances both have a very similar chemical structure, and this is possibly the reason why they have a similar effect. It was important to study the effect of another substance on RIBE, which is natural, has antioxidant and radioprotective properties but a completely different chemical structure from melanin and melatonin. α-Tocopherol's radioprotective properties were shown in many investigations, although some of them are controversial.

The effect of tocopherol on directly irradiated and bystander recipient HPV-G cells has been studied using the clonogenic assay. Tocopherol was added after irradiation and before filtration. Cells were irradiated at a dose of 0.5 Gy.

The data indicates that tocopherol does not affect the survival of non-irradiated HPV-G cells. Tocopherol was able to protect directly irradiated and bystander recipient cells, but again the protection was more effective against direct irradiation.

The data presented in Figure 2.10 presents a comparison of the efficacy of the radioprotective effect of all three substances: melanin, melatonin and tocopherol. For both, direct irradiation and the bystander effect, α-tocopherol was found to be the least effective protector against radiation damage. And melatonin was found to provide the most effective protection for HPV-G cells against radiation. At the same time, all the substances showed more effective protection against direct radiation damage compared with bystander damage.

Study of the effect of radioprotective substances on HPV-G cells using the micronucleus assay

In the clonogenic assay experiments it was shown that all three radioprotective substances were able to increase the survival of HPV-G cells, decreasing the damaging effects of direct irradiation and bystander effect with different efficacy. All substances were able to increase survival after direct irradiation more effectively than after ICCM transfer.

The micronucleus assay was also used to study the radioprotective ability of melanin, melatonin and tocopherol. It has been shown that the micronucleus assay can determine the effect of radiation at a dose of 0.05 Gy (if the test was performed not later than few days after irradiation) [Melnov, 2002]. The micronucleus assay currently is widely used to evaluate individual sensitivity to physical and chemical mutagens.

The effect of radioprotective substances on micronuclei frequency after direct irradiation and bystander effects has been studied using HPV-G cells. Cells were irradiated at 0.5 Gy. Substances were added before direct irradiation and one hour after irradiation to isolated ICCM before filtration and transfer to recipient cells.

All three radioprotective substances had no effect on non-irradiated HPV-G cells, the micronuclei frequency in such cells was very close to control levels (the difference was not significant). And again, all three substances decreased micronuclei frequency after direct irradiation more effectively than protection against bystander effects. As for the clonogenic assay results, melatonin showed the best protective effect against both direct irradiation and bystander effect, while tocopherol showed the least protective effect.

Thus, it is possible to conclude that radioprotective substances are able to protect cells from direct irradiation and bystander effects by increasing their survival and decreasing micronuclei frequency.

Study of the effect of radioprotective substances on HPV-G cells using Alamar Blue assay

Using the Alamar Blue Assay, the effect of melanin and melatonin on the metabolic activity of directly irradiated and bystander recipient HPV-G cells has been studied. Cells were irradiated at 0.5 Gy and substances were added after irradiation.

The data shows that both melanin and melatonin increased the metabolic activity of directly irradiated and bystander recipient HPV-G cells. As for the clonogenic and micronucleus assays, protection against direct irradiation was better than protection against bystander effects. Both melanin and melatonin showed no effect on non-irradiated cells, i.e. the observed effect appears to be only due to the protection from radiation, but not the stimulating effect of the substances.

The analysis of the effects of all three radioprotective substances on survival, micronuclei frequency and metabolic activity of HPV-G cells after direct and bystander low dose radiation leads to the following conclusions:

1. None of the radioprotective substances have any cytotoxic effect on HPV-G cells.

2. Melanin, melatonin and α-tocopherol significantly increased survival and metabolic activity and decreased micronuclei frequency of HPV-G cells *in vitro* after direct low dose radiation.

3. When added to ICCM before filtration the radioprotective substances decreased bystander effects, but not as effectively as the protection against direct irradiation.

^{60}Co irradiation source emits γ-rays with an energy of 1.25 MeV. Thus, most of the interactions of these rays with cells are Compton interactions. These interactions generate many free radicals, which play a very important role in cell damage following irradiation.

All three radioprotective substances used in the present research were shown to have antioxidant effects and can neutralise free radicals. This is possibly the main mechanism of their radioprotective action.

However, many studies reported that these substances did not show any radioprotective effects in cultured cells [Hopwood *et al.*, 1985; Grossi *et al.*, 1998; Yilmaz and Yilmaz, 2006]. Moreover, to date, there is no published data on studies of radioprotective effects of melanin, melatonin and α-tocopherol on immortalised cell cultures, especially such as HPV-G cells which are *p53* null. *p53* participates in the process of formation of free radicals by converting Fe^{3+} to $Fe^{2+\bullet}$. Also, the first checkpoint of the cell cycle (G_1/S) is *p53* dependent.

A possible explanation of different protective effects against direct and bystander irradiations could be that in direct irradiation experiments the radioprotective substances were directly in contact with cells since irradiation and were able to protect them against all kinds of damaging factors, starting from energy input (some of these substances may convert all types of energy into heat) and concluding with emerging free radicals. In bystander experiments, the substances were not in contact with the cells until added to the

ICCM 1 hour after irradiation when the damaging factor was already produced. So melanin/melatonin/tocopherol could neutralise only some of the damaging factors (for example, long-living free radicals) and are unable to protect against the main damaging factors (bystander factors), the nature of which is still unknown.

The possible mechanisms by which they reduce bystander effects - the neutralisation of free radicals or another mechanism or factor – is not known. All three substances are natural, have antioxidant and radioprotective properties. But they have different mechanisms of action.

Melanin is able to take up and retain for a long period different substances and to convert all types of physical energy into heat [Mosse *et al.*, 1999].

Melatonin can neutralise a large number of free radicals. It is highly lipid and somewhat aqueous soluble and is able to scavenge free radicals in both lipid and aqueous environments within cell membranes. Melatonin may concentrate against a gradient, and may be in the highest concentration in the portion of the cell (the nucleus), that contains the most sensitive target molecule to ionising radiation, DNA. Also, melatonin protects lipids in membranes and proteins in cytosol. It may diffuse easily and quickly pass through all biological membranes to enter cells and subcellular components. Its transport may be receptor-dependent or independent. Also, melatonin is able to activate enzymes, involved in antioxidant protection of the cell, thus providing secondary effects.

Tocopherol effects include the influence on repair processes. Thus, it acts mostly after damage of DNA, and this is possibly the reason that tocopherol showed the least protective effect.

It is definitely clear that all three substances were not toxic or stimulating to the cells and did not increase their survival or viability or decrease micronuclei frequency.

Study of melanin effect on human lymphocytes using flow cytometric analysis of micronuclei frequency, cell cycle parameters and apoptosis levels

Using flow cytometry, the effect of melanin on directly irradiated human peripheral blood lymphocytes was studied. The cells were irradiated at a dose of 0.5 Gy.

Human peripheral blood lymphocytes were not immortalised but activated by PHA. Thus, usually such cells are suitable for analysis only during the first 3-4 days. Although in the present research, cells were analysed up to 10 days, only data for the first 4 days was analysed due to cell death after this time.

In the present experiments, three different parameters of human peripheral blood lymphocytes were analysed: micronuclei frequency, cell proliferation and apoptosis levels.

Melanin did not have any significant effect on non-irradiated lymphocytes. On the second day of analysis melanin clearly decreased the micronuclei frequency in human lymphocytes compared to cells irradiated without melanin. But at later stages (4^{th} day and later) because of cell death the level of micronuclei was almost similar to controls. Thus, melanin added before irradiation was able to protect human lymphocytes from the damaging effect of ionising radiation.

The maintenance of cell and tissue homeostasis is based on balanced renewal of the cells, which is determined by the activity of cell division. So, the ratio of the frequencies of phases of the different stages of the cell cycle can be a marker of the normal condition of the cell population. At the same time, it is well known that after radiation

influence, there are delays of cell division [Krishnaja *et al.*, 2004; Melnov, 2004]. The main target of radiation is DNA (replication, transcription and translation), that leads to changes in proliferation. Thus, cell cycle analysis can be very informative in studying mechanisms of radiation-induced DNA damage at the cellular level.

Flow cytometry is an important tool for biomedical research that provides a way to distinguish the physical and chemical characteristics of individual cells within a heterogeneous population. This technique has great importance for cell cycle analysis, and various assays using flow cytometry have been developed for research in this field.

In a study of cell cycle parameters, irradiation of cells resulted in a two-fold decrease in proliferation activity compared to controls 2 days after irradiation. This continued for 6 days, and only on the 8^{th} day the level of proliferative activity decreased because of cell death. The proliferation activity of the cells irradiated with melanin added was the same as controls during all experiments. Thus, surprisingly, melanin was able to completely inhibit the radiation effect on cell proliferation.

Apoptosis plays very important role in mediation of consequences of radiation. In the present study the level of "genetic" apoptosis has been analysed. This method is based on the analysis of detection of fragmented DNA as a result of apoptosis.

On the second day after irradiation, the level of apoptosis in irradiated cells was slightly higher than in control cells. The level of apoptosis in cells irradiated with melanin was higher than in control cells, but lower than in cells irradiated without melanin.

The level of apoptosis in irradiated cells increased from the 4^{th} day and reached a maximum level on the 8^{th} day. The level of apoptosis in cells irradiated with melanin was

consistently higher than controls but lower than cells irradiated without melanin at all time points.

Thus, melanin decreased the level of apoptosis in irradiated human peripheral blood lymphocytes.

The flow cytometric analysis of melanin effects on irradiated human peripheral blood lymphocytes *in vitro* leads to the conclusion that melanin shows a radioprotective effect, decreasing the effect of low dose radiation by:

- decreasing micronuclei frequency;
- decreasing the apoptosis level;
- increasing the proliferation ability.

In general, it is possible to conclude that all three substances showed radioprotective effect in the order melatonin>melanin>tocopherol. The protection against direct irradiation is more effective compared to protection against bystander effects. Melanin protected human peripheral blood lymphocytes against direct irradiation and decreased micronuclei and apoptosis level and increased their proliferation ability.

CHAPTER 3

STUDY OF A RADIATION-INDUCED BYSTANDER EFFECT IN POPULATIONS AFFECTED BY THE CHERNOBYL ACCIDENT.

3.1. Introduction

It is now well accepted that cells, in response to radiation exposure, may release certain transmissible factors. These transmissible factors, known as bystander factors [Mothersill *et al.*,1997; 1998], have been reported to induce a variety of effects, including genomic instability in cells that have not been exposed to radiation. Previously known as clastogenic factors, they were first described in the plasma of persons who had been irradiated accidentally or therapeutically [Goh *et al.*, 1968; Hollowell *et al.*, 1968] and also observed in A-bomb survivors, where they persisted for many years after irradiation [Pant *et al.*, 1977].

Clastogenic or bystander factors are now thought to be mixtures of prooxidants and other substances with chromosome damaging properties and not single factors, as thought by the first observers [Goh *et al.*, 1968; Hollowell *et al.*, 1968; Faguet *et al.*, 1984]. Biochemical analysis identified peroxidation products of arachidonic acid, released from membrane phospholipids, cytokines such as tumour necrosis factor alpha (TNFα), and unusual nucleotides, such as inosine di- and triphosphate (ITP). The clastogenic properties of these components of clastogenic factors were confirmed by cytogenetic studies of the corresponding commercial standards [Emerit, 1994b]. The relationship between these and bystander phenomena is unknown.

As it was shown in previous chapters, factors, inducing bystander effects, can be transferred through cell culture medium *in vitro* from irradiated donor cells to non-irradiated recipient cells. As blood provides interactions between all organs and tissues of an organism, it is possible to propose that blood serum may transfer bystander factors and produce bystander effects *in vivo*.

It has been reported that radiation exposure can result in the release of soluble factors into the circulating blood that are capable of producing chromosome damage in cultured cells [Hollowell *et al.*, 1968]. These factors may play a role in carcinogenesis [Emerit *et al.*, 1994a] or could indicate protective or healing processes. They could be similar to the soluble factors described in the bystander effects measured using culture media transfer experiments [Mothersill *et al.*, 1997]. The evidence for these soluble factors both *in vitro* and *in vivo* has been published following acute radiation exposures. Such studies provide evidence of a bystander effect *in vivo*. Bystander effects may have important biological consequences within an organ and the transfer of soluble substances may play a role in this *in vivo* clastogenic effect following large, acute radiation exposures.

As bystander effects may lead to an increase in frequency of chromosome aberrations [Lorimore *et al.*, 1998], there is a huge interest in its study for victims of the Chernobyl accident. Earlier it was shown for different groups of such populations that the total number of non-specific aberrations decreases from the moment of the accident. At the same time, there is an increase in general genomic instability (single and double fragments) [Melnov, 2003]. The level of specific marker aberrations (dicentric and ring chromosomes), induced by radiation, decreases with time, as their appearance is connected with the loss of

genetic material (or its substantial reorganization), significantly reducing the viability of the cells.

Emerit *et al.* [1994b; 1995; 1997b] reported the presence of clastogenic factors in the plasma of workers and of children exposed as a consequence of the Chernobyl reactor accident. Monitoring of these transmissible factors as overall indicators of biological response in human tissues/fluids would reflect levels of radiation-induced damage regardless of the specific targets being exposed.

In her research, Emerit studied the influence of blood serum samples from Chernobyl liquidators on patients own lymphocyte aberration frequency. In the present research, we used a human keratinocyte cell line, immortalized by human papilloma virus as a test system, which we treated with serum blood samples from different population groups (Chernobyl liquidators, people affected after the Chernobyl accident, Polessky state radiation and environmental reserve (PSRER) workers, liver cirrhosis patients from contaminated territories of Gomel region).

Polessky State Radiation and Environment Reserve (founded in 1988) is a territory of the Gomel region, where humans cannot live because of the very high levels of radiation contamination. The territory of this reservation is 215.5 hectares. While the level of radiation in some areas of this reservation is 40 Ci, in others, it can be up to 1000 Ci! There are people – foresters, workers, scientists – who work there. The reservation is always supervised and controlled in order to exclude further distribution of radiation. All the people were evacuated from these territories after the Chernobyl accident.

3.2 Materials and methods

3.2.1 Cell culture

HPV-G cells

The HPV-G cell line is a human keratinocyte line, which has been immortalised by transfection with the HPV virus, rendering the cells *p53* null. They grow in culture to form a monolayer, display contact inhibition and gap junction intracellular communication.

HPV-G cells were cultured in Dulbecco's MEM: F12 (1:1) medium supplemented with 10% Foetal bovine serum, 1 % penicillin-streptomycin (1g per 100 ml), 1 % L-glutamine and 1 μg/ml hydrocortisone. The cells were maintained in an incubator at 37 degrees Celsius, with 95% humidity and 5% carbon dioxide and routinely subcultured every 8-10 days.

When 80-100% confluent, the medium was poured from the flask and replaced with 1:1 solution of versene (1nM solution) to trypsin (0,25% in Hank's Balanced Salt Solution) (Gibco, Irvine, UK) after washing with sterile PBS. The flask was placed in the incubator at 37 degrees Celsius for about 11 minutes until the cells started to detach.

The flask was then shaken to ensure that all cells had been removed from the base of the flask. The cell suspension was added to an equal volume of DMEM F12 medium to neutralise the trypsin. From this solution new flasks could be seeded at the required cell quantity.

Human lymphocytes

Blood samples were collected by standard venous puncture and stored with heparin (20 units/ml) no longer than 2-3 hours before investigation. Blood lymphocytes were cultured in RPMI-1640 medium (100 ml), supplemented with foetal bovine serum (20%), and gentamycin (0,2%). 4.5 ml of medium was aliquot into 50 ml culture flasks and frozen at -20°C.

3.2.2 Sample donors

The victims of the Chernobyl accident included 3 main categories: Chernobyl liquidators 1986-1987 (22 persons, all males), workers from Polessky State Radiation and Environment Reserve (PSRER workers - 21 persons, all males) and people living in territories of Gomel region contaminated by radionuclides (15 persons, 4 females). Also, analysed group included 4 patients-residents from contaminated territories with acute virus infection (flu). All other patients were clinically healthy. The control group (clinically healthy people corresponding to other groups in age and sex aspects) included 71 persons for cytogenetic assay and 11 persons (8 males + 3 females) – for micronuclei and AB assays.

3.2.3 Blood serum extraction

The blood samples were taken and placed in Vacutainers for serum extraction (Becton Dickinson), centrifuged at 2000g for 10 minutes, and the serum was frozen and stored at -20°C before use. Before freezing, the sera were filtered through Nalgene 0,22 μm filters in order to remove all residual cell components of the blood.

3.2.4 Radioprotective substances

3.2.4.1 Melanin

Melanin was isolated from animal hair by Belarus Pharmaceutical Association (Minsk). By analysis, it was determined to be eumelanin. Both ortochinoid and indolic fragments were present. Melanin was added to the cell medium at 10 mg/l concentration 30 min – 1 hour before irradiation for directly irradiated cells and 1 hour after irradiation to the irradiated cell conditioned medium (ICCM) before filtration for bystander recipient cells.

3.2.4.2 Melatonin

Melatonin (*N*-Acetyl-5-methoxytryptamine) was received from Sigma (Germany) as white powder, synthetic. Melatonin was added to the cell medium at 10 mg/l 30 min – 1 hour before irradiation for directly irradiated cells and 1 hour after irradiation to the ICCM before filtration for bystander recipient cells.

3.2.5 Micronuclei protocol for clastogenic effect (HPV-G cells).

After seeding, cells were left at 37°C in the CO_2 incubator to attach for 12 hours. The blood serum from affected populations was added to 25 cm^2 flasks (NUNC, USA) (6000 cells per flask) 1-2 days after seeding, and cells were replaced in the incubator for 1-2 h. Then cytochalasin B was added (7 μg/ml concentration) and the cells were incubated for 24 hours.

After this the cell culture medium was removed, the cells were washed with PBS and fixed with chilled Karnua solution (1 part of glacial acetic acid and 3 parts of methanol,

10-15 ml 3 times for 10-20 min). Later flasks were dried and stained with 10% Giemsa solution.

The micronuclei count was carried out under inverted microscope (x400). Micronuclei were counted only in binucleated cells (1000 binucleated cells per flask).

All the data is calculated as the micronuclei number recorded per 1000 binucleated cells (micronuclei were analyzed only in binucleated cells).

3.2.6 Alamar Blue assay

Cells were seeded in 96-well microplates (NUNC, USA) at a concentration of $2x10^4$ cells/well. After seeding, cells were incubated for 24 hours to allow attachment to the bottom of the well. Then medium was removed, cells rinsed with phosphate buffered saline (PBS) and the blood serum from the Chernobyl accident populations was added to the cells together with melanin and melatonin as appropriate. Microplates were moved back to the incubators. 24 hours later, serum was removed, cells rinsed with PBS and 100 µl of a 5% solution of Alamar Blue prepared in phenol red free DMEM media was added. Microplates were moved back to the incubators. 3 hours later, fluorescence was quantified using a microplate reader (TECAN GENios, Grödig, Austria) at the respective excitation and emission wavelength of 540 and 595 nm. Wells containing medium and Alamar Blue without cells were used as blanks. The mean fluorescent units for the 3 replicate cultures for each exposure treatment were calculated and the mean blank value was subtracted from these results.

3.2.7. Routine cytogenetic test.

On the day of analysis flasks with cells were thawed, 0.5 ml of blood was added and 0.13 ml of PHA (Sigma). Flasks were stored in incubators for 48 hours at 37°C.

3 hours before the end of cultivation 30 µl/5 ml of colchicine was added. Lymphocytes were centrifuged for 5 min at 1500g, supernatant was removed and cells washed with warm 0.55% KCl solution (at 37°C) for 20-25 min. Cells were fixed using Karnua solution (1 part of glacial acetic acid and 3 parts of methanol, 5 ml of solution per flask). Fixator was changed 3 times.

Cells were mounted on microscope slides (3-4 drops of sample per glass) and dried at 40-42°C. Cells were stained using 10% Giemsa solution for 8 mins and after drying analyzed under light microscope. Only cells with the chromosomes number between 44 and 47 were analyzed. The following chromosome aberrations were counted:

Single fragments are formed after damage of single chromatid.

Double fragments are formed as a result of one or two breaks of chromosomes at G_1 phase.

A **ring chromosome** is a chromosome that is formed when the ends have been lost, and the arms fuse together forming a ring.

Dicentric chromosome is an aberrant chromosome after asymmetric translocation and having two centromeres.

Atypical chromosome is a chromosome formed after exchange type aberrations (translocations and inversions), resulted in its atypical morphology. Inversion is a chromosome rearrangement in which a segment of a chromosome is reversed end to end. An inversion occurs when a single chromosome undergoes breakage and rearrangement

within itself. Chromosome translocation is the interchange of parts between non-homologous chromosomes.

Also **polyploid** cells were analysed - cells that contain more than two copies of each of its chromosomes. Polyploid types are termed according to the number of haploid chromosome sets in the nucleus: triploid (three haploid sets; 3n), tetraploid (four haploid sets; 4n) and so on. Usually somatic cell of human organism is diploid and has two copies of chromosomes (2 haploid sets).

Number of aberrant cells is a percent of cells with aberrations.

Frequency of aberrations (%) is a total number (sum) of aberrations per 100 cells. Exchange aberrations (inversions and translocations) are counted for 2 aberrations.

2.2.10 Statistical analysis

All experiments were repeated at least three times, and within each experiment cultures were set up in triplicate. Results are expressed as the means +/- standard errors.

t-test

When the distribution was normal, significance was determined using the *t* test. The *t-test* is the most commonly used method to evaluate the differences in means between two groups. The groups can be independent or dependent. Theoretically, the *t-test* can be used even if the sample sizes are very small (e.g., as small as 10; some researchers claim that even smaller n's are possible), as long as the variables are approximately normally distributed and the variation of scores in the two groups is not reliably different

The level of trustworthiness was chosen 95%; at $t \geq 2.67$, the difference is highly significant with $P<0.01$, at $t \geq 1.96$, the difference is significant with $P<0.05$. At $P>0.05$, the difference is not significant.

Mann-Whitney U-test

The Mann-Whitney U test is a nonparametric alternative to the t-test for independent samples. The interpretation of the test is essentially identical to the interpretation of the result of a t-test for independent samples. The U test is the most powerful (or sensitive) nonparametric alternative to the t-test for independent samples; in fact, in some instances it may offer even greater power to reject the null hypothesis than the t-test. With samples larger than 20, the sampling distribution of the U statistic rapidly approaches the normal distribution. Hence, the U statistic will be accompanied by a z value (normal distribution variate value), and the respective P-value.

3.3 Results

Study of the effects of blood serum on HPV-G cells using micronuclei assay.

The bystander effect, induced *in vivo* in blood samples from people affected by the Chernobyl accident, was analysed *in vitro* on HPV-G cells using the micronucleus assay. Serum samples, containing bystander factors, were added to the cell culture medium.

Table 3.1 presents individual data of the effects of blood serum samples from different population groups on the total micronuclei frequency in HPV-G cells. Control blood serum samples were taken from healthy people of the same age and sex.

Table 3.2 presents average data, including frequency of cells with 1, 2 and 3 MN, total number of cells with MN and total micronuclei frequency for all groups of affected populations.

The micronuclei frequency in the controls indicates the level of spontaneous mutagenesis (it is comparatively low). The data from Table 3.2 shows that the number of the cells with 2 and especially 3 micronuclei is very low compared with the number of cells with one micronucleus.

The data presented in Table 3.1 shows a wide spread in individual values within each group. But within groups of PSRER workers and liquidators, all values of micronuclei frequency were higher than in control. Only in the group of residents of contaminated territories some values were lower than the average control value.

As it can be seen from Table 3.1, people exposed to chronic radiation (Polessky State Radiation and Environmental Reserve workers) have an increased mutagenic pressure, expressed as a considerable increase in micronuclei frequency (almost 3 times

higher than the control level – 248.03‰±20.77 compared with 80.30‰±13.14, P<0.01). At the same time an increase in the number of the cells with more than one micronucleus was observed.

Similar results were observed after comparative analysis of the micronuclei frequency between the control group and the liquidators group (people exposed to acute radiation). The total micronuclei frequency 273.7‰±22.4 and the frequency of cells with micronuclei 235.6‰±14.0 in cells treated with serum from liquidators is significantly higher than the control group (in both cases P>0.01). Also, an increase in the number of cells with more than one micronucleus was observed (Table 3.2).

The level of micronuclei induction by serum samples from the residents of contaminated areas of Gomel region is statistically significantly different from the control (156.47‰±11.22 vs. 80.30‰±13.14 P<0.01), but much lower than in people, exposed to additional radiation influence (compared to liquidators and PSRER workers in all cases P<0.01).

At the same time, the level of the micronuclei frequency induced by serum samples from the residents with acute virus infection in the active stage is higher than in all previous cases – the micronucleus frequency induced by the serum from these patients is 435.6‰±8.4, and the number of cells with micronuclei is 301.2‰±7.8. These figures are much higher than for liquidators (273.7±22.4 and 235.6±14.0, respectively; in both cases P<0.01).

Table 3.1 Cytotoxic effect of serum samples from different population groups on HPV-G cells (individual data for healthy people, Chernobyl liquidators (all males), PSRER workers (all males), residents of contaminated areas of Gomel region; residents with acute virus infection are not included). The level of background MN frequency of HPV-G cells without added serum was 78.10±6.92. The highest level of micronuclei induction has serum from liquidators and PSRER workers, the lowest – residents from contaminated areas (compared to control). The data varies significantly within each group.

Group	Healthy		Chernobyl Liquidators	PSRER	Residents	
		Sex				Sex
Total MN frequency, ‰	31.01±10.79	M	247.23±17.17	301.45±14.26	132.55±10.59	F
	70.37±15.57	M	313.95±25.02	241.41±13.41	73.34±8.21	M
	71.69±15.44	M	278.02±17.09	209.89±14.89	146.55±10.95	F
	90.91±14.25	F	315.79±22.74	211.23±14.92	183.95±12.12	F
	67.92±12.18	M	278.20±15.86	304.99±14.39	146.83±10.96	M
	103.57±18.2	M	212.62±12.75	292.99±14.10	171.04±11.74	M
	66.42±15.13	F	295.88±13.97	271.97±14.39	188.24±12.24	M
	55.35±13.89	M	266.67±28.54	227.06±14.37	194.20±12.30	M
	112.71±8.82	M	302.18±25.63	300.00±31.62	169.26±11.70	M
	107.82±9.58	F	255.87±21.14	343.48±31.31	122.17±10.28	M
	105.47±9.69	M	206.35±22.80	285.00±22.57	192.98±12.32	F
	-		339.42±28.60	198.11±17.31	-	
	-		243.24±22.30	246.15±26.72	-	
	-		353.91±30.68	262.50±28.40	-	
	-		299.12±24.80	210.00±23.52	-	
	-		295.65±30.09	295.29±22.72	-	
	-		228.57±28.98	175.56±17.93	-	
	-		223.08±25.82	262.95±27.79	-	
	-		291.19±28.12	229.39±25.17	-	
	-		278.88±15.32	222.22±26.67	-	
	-		262.47±15.94	116.98±19.74	-	
	-		232.56±20.37	-	-	
Average	80.30±13.14		273.67±22.44*†	248.03±20.77*†	156.47±11.22*	
* P<0.01 compared to control; † P<0.01 compared to residents						

Table 3.2 The effect of blood serum samples from different groups of population on HPV-G micronuclei frequency (average data is presented). The highest level of MN frequency and cells with MN showed serums from patients – residents of contaminated territories with acute virus infection (flu). Serum samples from PSRER workers, liquidators and residents from contaminated territories significantly increased levels of micronuclei frequency compared to control populations.

Group	№ cells in analysis	MN cells frequency, ‰			Cells with MN, ‰	MN frequency, ‰
		1 MN	2 MN	3 MN		
Control	11000	69.5±11.1	5.0±1.1	0.3±0.1	74.8±12.4	80.3±13.1
Liquidators	22000	200.6±12.2	30.6±2.6	4.6±1.1	235.6±14.0*†	273.7±22.4*†
PSRER Workers	21000	196.5±10.9	18.8±3.8	4.4±1.1	219.7±18.3*†	248.0±20.7*†
Residents	11000	130.5±7.7	11.8±1.8	0.8±0.3	143.1±9.3*	156.5±11.2*
Acute virus infection patients	3500	190.2±13.2	87.7±9.6	23.3±5.1	301.2±15.5*†	435.6±16.5*†
*** P<0.01 (compared to controls); † P<0.01 (compared to residents)**						

Comparative analysis of cytogenetic and micronuclei parameters in populations affected by the Chernobyl accident

The effect of serum samples from the victims of the Chernobyl accident (Chernobyl liquidators, PSRER workers and residents of contaminated territories) on the induction of genomic instability in human lymphocytes and bystander effects in HPV-G cells, and correlation between these parameters, has been studied.

The evaluation of genomic instability included analysis of 7 different parameters: frequency of single fragments, double fragments, dicentric and ring chromosomes, atypical chromosomes, polyploidic cells, aberrant cells and total number of aberrations and aberrant cells. Cell with normal chromosomes number (46) without aberration is presented in Figure 3.1. Figures 3.2-3.5 present cells with different types of chromosome aberrations and polyploidic cells.

The results of the effects of serum samples on HPV-G cells (micronuclei test) and on the lymphocytes of the donors (cytogenetic test) are presented in Table 3.3.

As it can be seen from Table 3.3, people affected by the Chernobyl accident have significantly higher levels of chromosome aberrations compared to the controls ($P<0.05$) as a result of a high level of non-specific aberrations (single and double fragments, $P<0.05$ and $P<0.01$ compared to control, respectively).

At the same time, the frequency of specific marker aberrations (dicentric and ring chromosomes) has no statistically significant difference between control and affected populations.

Summary analysis of micronuclei frequency in HPV-G cells after treatment with blood serum samples indicates that activity of serum samples from people affected by the Chernobyl accident was almost 3 times higher than in the controls (251.18 ± 22.96 and 80.29 ± 13.14, respectively; $P<0.01$).

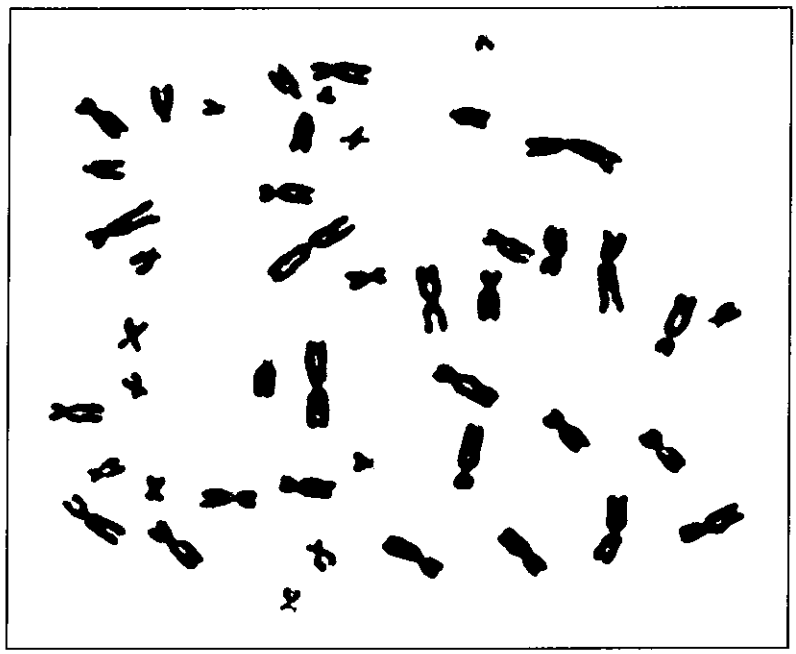

Figure 3.1 Standard human lymphocyte cell karyotype (46 chromosomes without aberrations).

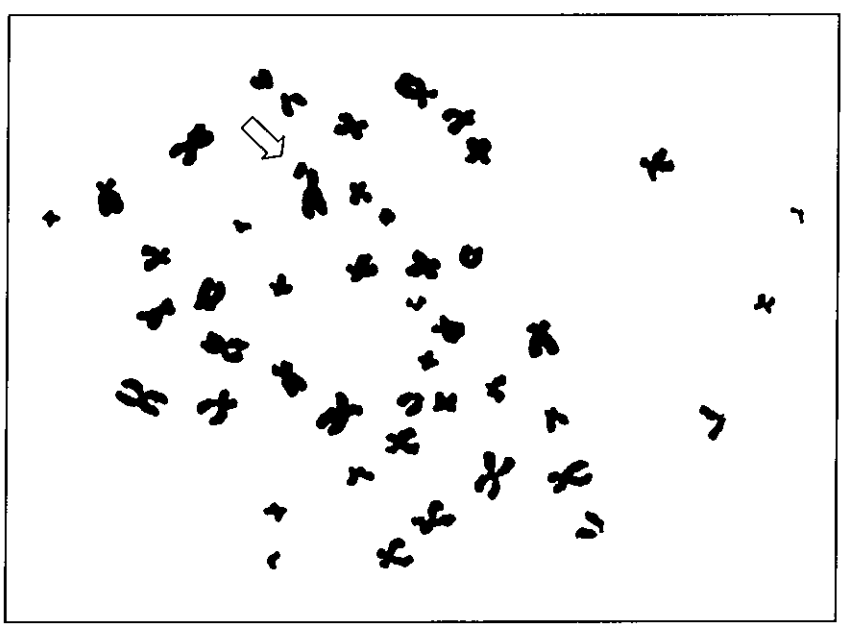

Figure 3.2 Cell with single fragment (white arrow), formed after damage of single chromatid.

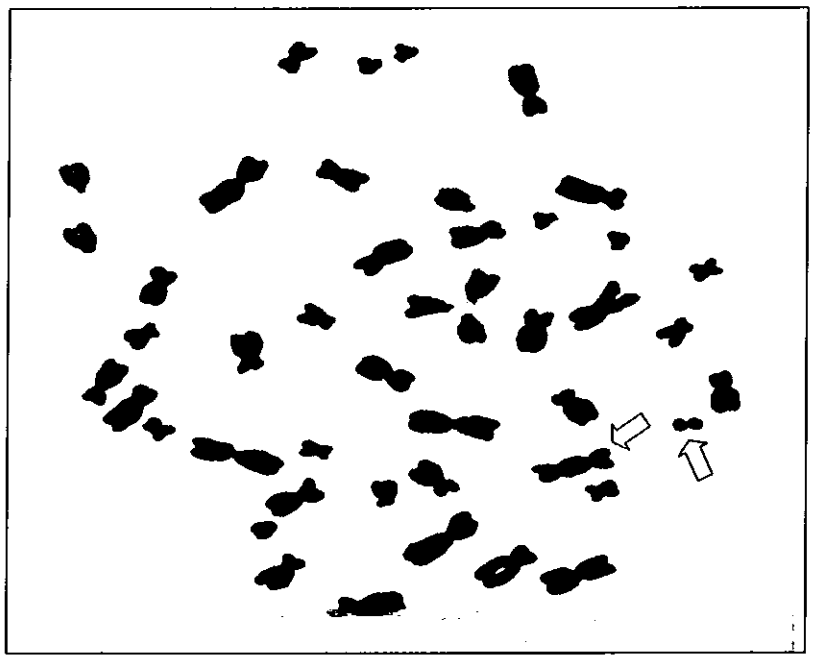

Figure 3.3 Cell with dicentric chromosome (having two centromeres - grey arrow) and double fragment (chromosome breaks - white arrow).

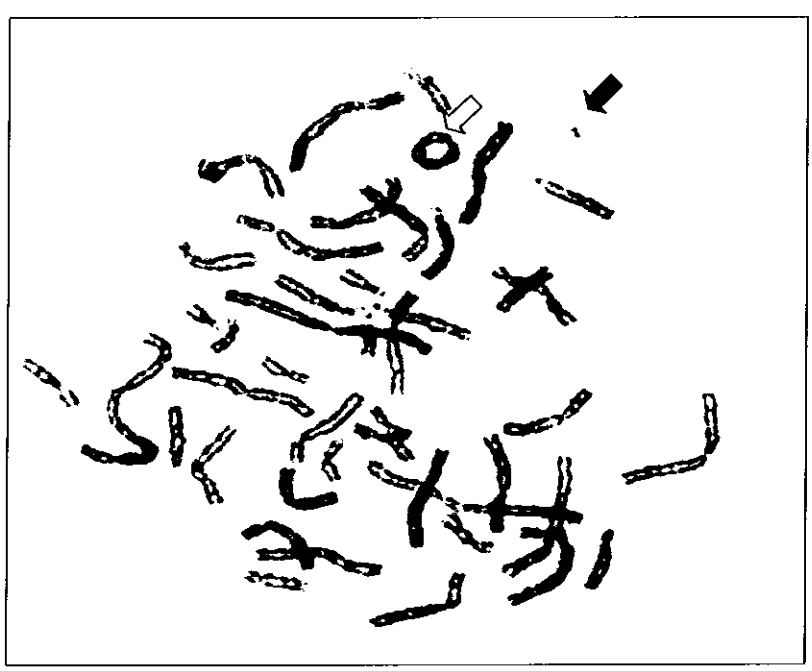

Figure 3.4 Cell with ring chromosome (white arrow) and double fragment (black arrow).

Figure 3.5 Polyploid cell (contains more than two copies of each of its chromosomes).

Table 3.3 Cytogenetic status of lymphocytes and micronuclei frequency in HPV-G cells after treatment with serum samples (average data). The level of chromosome aberrations and micronuclei frequency in control cells is significantly lower compared to victims of Chernobyl accident. The level of specific aberrations in lymphocytes of people affected by Chernobyl accident is 3 times higher compared to control, but not statistically significant.

Parameters		Groups of populations		P
		Control	Victims of Chernobyl accident	
Results of cytogenetic analysis				
People analyzed		61	32	-
Average age (years)		37.66±1.5	41.74±0.98	-
Cytogenetic status	Total number of metaphases	11179	8739	-
	Single fragments, %	1.67±0.12	5.78±1.97	P<0.05
	Double fragments, %	0.81±0.08	1.89±0.1	P<0.01
	Dicentrics and rings, %	0.11±0.03	0.31±0.22	P>0.05
	Atypical chromosomes, %	0.02±0.01	0.02±0.01	P>0.05
	Polyploidic cells, %	0.08±0.03	0.22±0.19	P>0.05
	Aberrant cells, %	2.48±0.14	7.59±2.37	P<0.05
	Total number of aberrations, %	2.63±0.15	8.59±2.94	P<0.05
Results of micronuclei test				
Micronuclei frequency in cells	Total number of binucleated cells	5554	14045	-
	Number of cells with 1 MN, ‰	69.50±12.21	189.66±20.74	P<0.01
	Number of cells with 2 MN, ‰	5.00±3.08	24.51±8.05	P<0.05
	Number of cells with 3 MN, ‰	0.26±0.21	4.56±3.14	P>0.05
	Total number of cells with MN, ‰	74.76±12.69	218.69±21.90	P<0.01
	Total frequency of micronuclei, ‰	80.29±13.14	251.18±22.96	P<0.01

Before further statistical analysis, parameters of the main and control groups were checked for normality of distribution using Kolmogorov-Smirnov and Chi-Square criteria.

For the parameter "total micronuclei frequency, ‰" using criteria Kolmogorov-Smirnov d=0.09231, criteria Chi-Square test = 1.20578, df = 1 (adjusted), p = 0.27217. Thus, the hypothesis that distribution is not normal is not accepted, and distribution is normal. For parameter "total number of cells with micronuclei, ‰" for criteria Kolmogorov-Smirnov d=0.10848, for criteria Chi-Square test = 0.77357, df = 1 (adjusted), p = 0.37912. Thus, the distribution is normal, and parametric methods of statistics can be used.

For parameter "total aberrations frequency, %" using criteria Kolmogorov-Smirnov d = 0.22164, criteria Chi-Square test = 15.77936, df = 1 (adjusted), p = 0.00007, the distribution is not normal. For parameters "total frequency of aberrant cells, %", "frequency of single fragments, %", "frequency of double fragments, %", "frequency of dicentric and ring chromosomes, %", "frequency of atypical chromosomes, %" and "frequency of polyploidic cells, %" the distribution is also not normal. Non-parametric analyses of statistics were used for these data.

In order to understand interactions between parameters (total number of cells with aberrations and micronuclei and total frequency of aberrations and micronuclei) the dependence between them using polynomial analysis has been studied.

The results of polynomial statistical analysis of individual data (correlation between the analysed parameters) are presented in Figures 3.6 and 3.7. The strongest dependence is observed between total micronuclei frequency and total frequency of aberrations. Also direct dependence, but not as evident, between parameters of total number of cells with micronuclei and total number of aberrant cells was found.

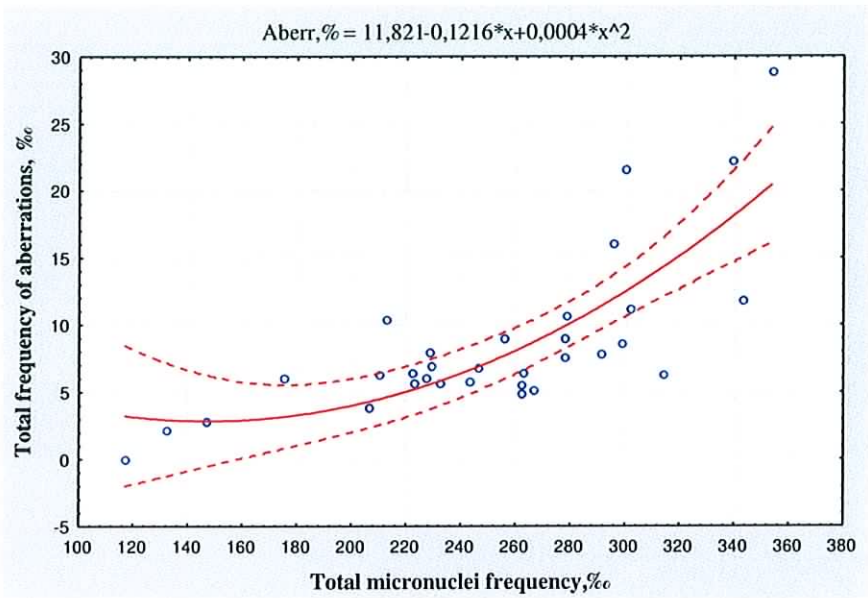

Figure 3.6 Dependence of total frequency of micronuclei on total frequency of aberrations (polynomial fitting).

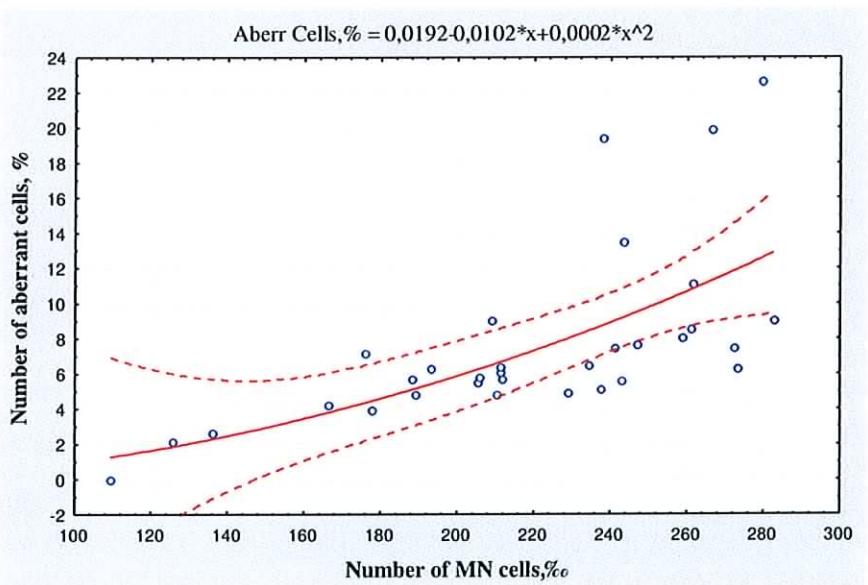

Figure 3.7 Dependence of total number of cells with micronuclei on total number of aberrant cells (polynomial fitting).

Figure 3.8 presents cluster analysis showing linkage between parameters "micronuclei frequency, ‰" and number of single, double fragments, dicentric and ring chromosomes, atypical chromosomes, polyploidic cells, aberrant cells frequency, aberrations frequency.

As it can be seen from Figure 3.8, the closest linkage is observed between parameters "total number of aberrations, %" and "total number of aberrant cells, %" (linkage distance d = 0.02). Parameter "total micronuclei frequency, ‰" has closest linkage with parameters "number of aberrant cells, %" (d = 0.28) and "aberrations frequency, %" (d = 0.24).

Using Spearman correlation analysis, a correlation matrix for cytogenetic parameters compared with bystander (micronuclei) parameters was built.

As it can be seen from Table 3.4, the strongest dependence is observed between total micronuclei frequency and total frequency of aberrations (Spearman coefficient of correlation r = 0.84; P<0.005). Also direct dependence, but not as evident, is observed between parameters "total number of cells with micronuclei" and "total number of aberrant cells" (r = 0.65; P<0.005). The level of single and double fragment have statistically reliable correlation with the level of micronuclei.

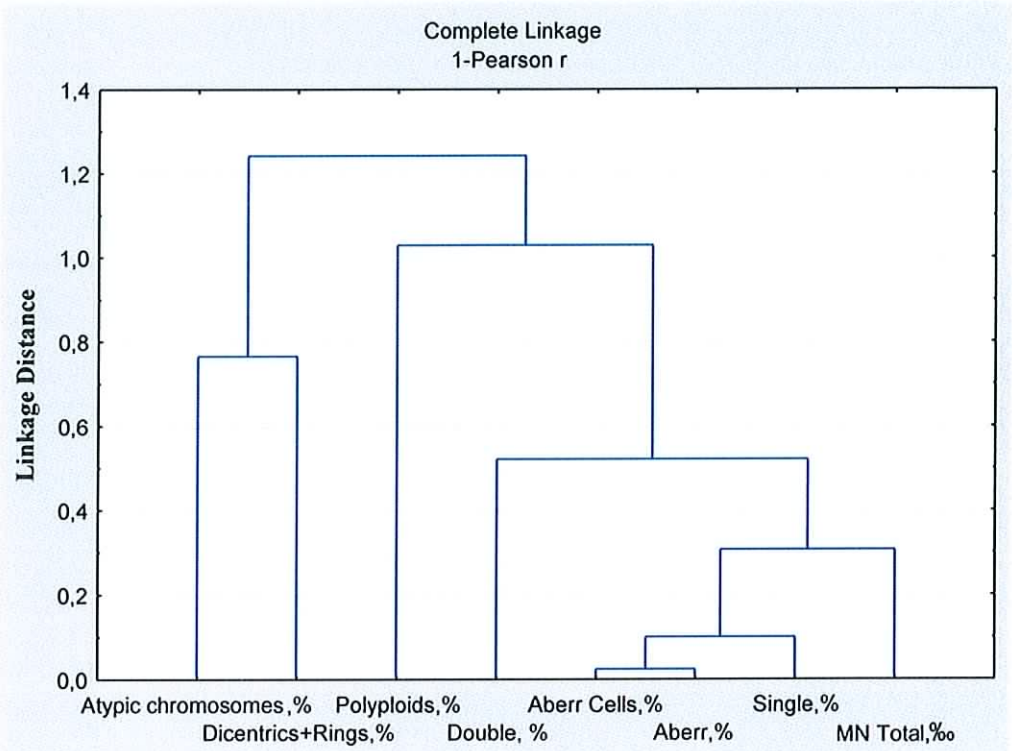

Figure 3.8 Joint tree (cluster analysis) of comparison of cytogenetic status and bystander serum effect (MN induction). The lower the linkage distance (d) between cytogenetic status parameters and bystander-inducing effect of blood serum samples from patients affected by the CNPP accident, the bigger the correlation between parameters. The highest correlation is between parameters "frequency of aberrations" and "aberrant cells". High correlation is observed between parameters "MN frequency" and "frequency of aberrations".

Table 3.4. Correlation matrix (Spearman correlation) of cytogenetic status versus bystander micronuclei induction effect. The strongest dependence is observed between parameters "total MN frequency" and "total aberrations frequency". Also high correlation is observed between these parameters and "frequency of MN cells" and "frequency of aberrant cells" ($P<0.01$ in all cases).

Parameters of comparison	Number of cells with micronuclei, ‰	Total number of micronuclei, ‰
Single fragments, %	0.50*	0.62*
Double fragments, %	0.49*	0.55*
Dicentric and ring chromosomes, %	0.07**	0.08**
Atypic chromosomes, %	-0.24**	-0.24**
Polyploid cells, %	0.13**	0.10**
Number of aberrant cells, %	0.65*	0.71*
Total number of aberrations, %	0.76*	0.84*
		* $P<0.005$; ** $P>0.1$

Table 3.5 presents comparative analysis of the groups of populations (liquidators, PSRER workers and residents from contaminated territories), performed using the Mann-Whitney U-test to reveal differences between groups.

Results presented in Table 3.5 suggest that there is no statistically significant difference between cytogenetic parameters of liquidators and PSRER workers. But there is a tendency ($P>0.05$, but <0.1) that PSRER workers have a lower micronuclei level (243.15±25.43 compared to 267.71±23.51 in liquidators) and less expressed genetic instability (aberrations frequency 10.15±2.50 and 7.93±1.50, respectively).

A much more significant difference is observed between liquidators and residents of contaminated areas of Gomel region. The liquidators group has increased genetic instability (aberrations frequency 7.93±1.50 compared to 3.24±1.00, respectively) and increased production of bystander factors in serum (micronuclei frequency 267.71±23.51 in liquidators compared to 184.54±15.25 in residents).

Similar, but less expressed changes are observed in comparative analysis of PSRER workers and residents of contaminated territories of Gomel region. PSRER workers have more expressed genetic instability (aberrations frequency 10.15±2.50 compared to 3.24±1.00, in Gomel area residents) and increased production of bystander factors (micronuclei frequency 243.15±25.43 compared to 184.54±15.25, respectively).

Table 3.5 Comparative analysis of individual cytogenetic and micronuclei parameters of 3 independent groups of populations (Mann-Whitney U-test). Bold figures are significant at P<0.05, for underlined figures, P>0.05 but ≤ 0.1. The highest difference is observed between Chernobyl liquidators and PSRER workers compared to residents of contaminated by radionuclides areas of Gomel region

Group of populations / Parameter of comparison	Liquidators vs. PSRER workers		Liquidators vs. Affected populations		PSRER workers vs. Affected populations	
	Z	P	Z	P	Z	P
MN Cells, ‰	1.57	> 0.1	0.68	**<0.01**	1.70	**<0.05**
Micronuclei frequency, ‰	1.70	<u>≤ 0.1</u>	0.51	**<0.001**	1.47	**<0.05**
Single fragments, %	0.09	> 0.1	1.80	<u>≤ 0.1</u>	1.38	> 0.1
Double fragments, %	1.66	<u>≤ 0.1</u>	1.62	<u>= 0.1</u>	0.39	> 0.1
Dicentrics & rings, %	-0.17	> 0.1	-0,12	> 0.1	-0.20	> 0.1
Atypical chromosomes, %	-0.41	> 0.1	0.33	> 0.1	0.20	> 0.1
Polyploidic cells, %	0.67	> 0.1	0.84	> 0.1	0.59	> 0.1
Aberrant cells, %	1.33	> 0.1	0.34	**< 0.01**	1.78	**<0.05**
Aberrations frequency, %	1.03	<u>≤ 0.1</u>	0.16	**< 0.01**	1.08	**<0.05**

Study of the effects of blood serum samples using the Alamar Blue assay

The blood serum samples for Alamar Blue analysis were taken from people affected by the Chernobyl accident. The study was run to understand if there are any bystander factors observed in serum samples of these population groups which may affect cell metabolic activity.

Table 3.6 presents individual data obtained in a study of the effect of serum samples from different groups of Chernobyl accident (Chernobyl liquidators and residents of contaminated territories) and healthy populations using the Alamar Blue assay. The individual values of Chernobyl liquidators varies over a wide range, but is always lower than in control or in healthy populations. And some individual values from residents group are higher than in control.

Figure 3.9 represents average data for all these groups calculated as percent of control. As it can be seen from Figure 3.9, the viability of the cells treated with serum samples from healthy people is very close to control (non-treated) levels – lower or higher ($t=0.33$ – the difference is not significant). It means that the metabolic activity of these cells is not damaged by serum samples from healthy people, not increasing or decreasing significantly the viability of HPV-G cells.

Treatment of the cells with serum samples from Chernobyl liquidators clearly reduces the viability of HPV-G cells more than 1.5 times – from 24.89 ± 0.25 $x10^3$ FU (control) and 24.67 ± 0.62 $x10^3$ FU (healthy people) to 15.65 ± 0.82 $x10^3$ FU (liquidators); $P<0.01$ in both cases ($t=10.79$ and 8.77, respectively).

Treatment of HPV-G cells with serum samples from residents of contaminated territories also reduces the viability of cells (average viability is 19.16±0.71 $x10^3$ FU), but not as significantly as serum samples from liquidators (t=7.62 compared to controls and t=5.84 compared to serums from healthy people).

Table 3.6 The effect of blood serum samples from healthy people and victims of Chernobyl accident on the viability of HPV-G cells. Serum samples from Chernobyl liquidators decreased metabolic activity of HPV-G more significantly compared to residents from contaminated territories. Serum samples from healthy people did not significantly influence the viability of HPV-G cells compared to control (untreated) cells.

Group	Control	Healthy people	Chernobyl Liquidators	Residents
Number of FU, $x10^3$	25.93±0.24	23.38±0.36	13.94±0.96	16.70±0.64
	24.64±0.23	22.78±0.61	14.78±1.09	16.35±0.73
	25.69±0.26	26.45±1.25	16.36±0.65	17.24±0.64
	25.19±0.25	25.60±0.35	13.62±0.91	21.37±0.99
	22.61±0.22	24.87±0.46	17.65±1.21	17.54±0.87
	24.89±0.29	24.93±0.70	16.49±1.04	23.61±0.48
	23.56±0.21	-	13.75±0.70	19.51±0.92
	26.61±0.28	-	13.90±0.68	20.47±0.79
	-	-	17.31±1.27	23.93±0.84
	-	-	15.50±0.92	22.61±0.74
	-	-	14.48±0.37	17.57±0.52
	-	-	12.84±0.66	15.33±0.41
	-	-	17.40±0.49	16.90±0.64
	-	-	17.18±1.31	-
	-	-	19.65±0.43	-
	-	-	15.60±0.39	-
Average	24.89±0.25	24.67±0.62*	15.65±0.82**,†	19.16±0.71**,†
* P>0.05; ** P<0.01 (compared with control); † P<0.01 (compared with healthy)				

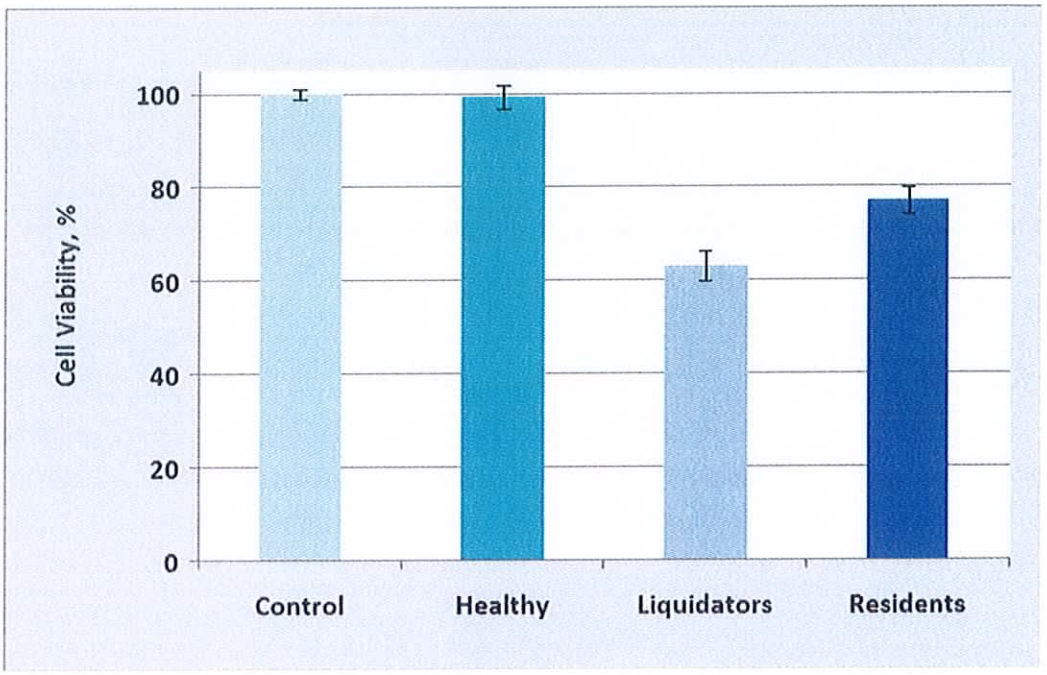

Figure 3.9 Cytotoxic effect of serum samples from healthy people, Chernobyl liquidators and residents from contaminated territories by the Chernobyl accident on HPV-G cells (control as non-treated cells, average data for all groups of populations as percent of control). Serum samples from liquidators and residents of contaminated territories significantly decrease viability of HPV-G cells compared to controls.

Table 3.7 presents the results of a comparison of all groups using the Mann-Whitney U-test. All population groups are of the same age and sex. The control is the viability of the HPV-G cells not treated with blood serum. As it can be seen from the table, there is statistically significant difference (with at least $P<0.005$) between all groups excluding control vs. healthy populations ($P>0.05$). The highest difference is observed between liquidators vs. control group and liquidators vs. group treated with serum samples from healthy populations.

Table 3.8 presents the results of an attempt to modify the effects of blood serum samples from Chernobyl liquidators on metabolic activity of HPV-G cells using melanin and melatonin.

As can be seen from Table 3.8, individual data for every category (liquidators, melanin and melatonin) is not significantly different from each other and significantly lower than in control wells ($t=11.21$, $t=8.35$ and $t=8.81$, respectively, compared to control).

As it can be seen from Figure 3.10, where the average data is presented as a percent of control, addition of melanin and melatonin to the medium together with serum samples from Chernobyl liquidators doesn't have any protective effect. The viability of cells treated with only serum samples ($16.24\pm0.73 \times 10^3$ FU) is almost the same as viability of cells treated with serum samples and melanin ($15.21\pm1.13 \times 10^3$ FU) and melatonin ($15.45\pm1.04 \times 10^3$ FU) – the difference is not significant ($t=0.77$ and $t=0.62$, respectively; $P>0.05$ in both cases).

Table 3.7 Comparison of 4 independent groups (control, healthy people, liquidators and residents of contaminated territories) on difference using Mann-Whitney U-test. Between control and healthy populations group, there is no difference observed. The highest difference is observed between liquidators vs. control and healthy people.

Groups of comparison	Z	P
Control vs. Healthy people	0.39	>0.6
Control vs. Liquidators	3.92*	<0.00001
Control vs. Residents	3.40*	<0.001
Healthy people vs. Liquidators	3.54*	<0.0005
Healthy people vs. Residents	3.07*	<0.005
Liquidators vs. Residents	3.03*	<0.005
*** Statistically significant at P<0.01**		

Table 3.8 The effect of melanin and melatonin on viability of HPV-G cells treated with serum blood samples (individual and average data for Chernobyl liquidators). Melanin and melatonin does not have any significant protective effect against bystander factors from serum samples from Chernobyl liquidators.

Group	Control	Chernobyl Liquidators	Melanin	Melatonin
Number of FU, $x10^3$		14.78±0.11	17.26±0.75	12.76±0.59
		12.84±0.66	13.27±0.50	15.60±1.91
		17.40±0.49	16.12±0.40	14.89±0.34
		17.18±0.13	15.30±0.25	17.54±0.73
		19.65±0.43	14.35±0.52	15.20±0.46
		15.60±0.39	14.94±0.74	16.71±2.21
Average	24.89±0.25	16.24±0.73*	15.21±1.13*†	15.45±1.04*†
*** P<0.01 (compared to control); † P>0.05 (compared to Chernobyl liquidators)**				

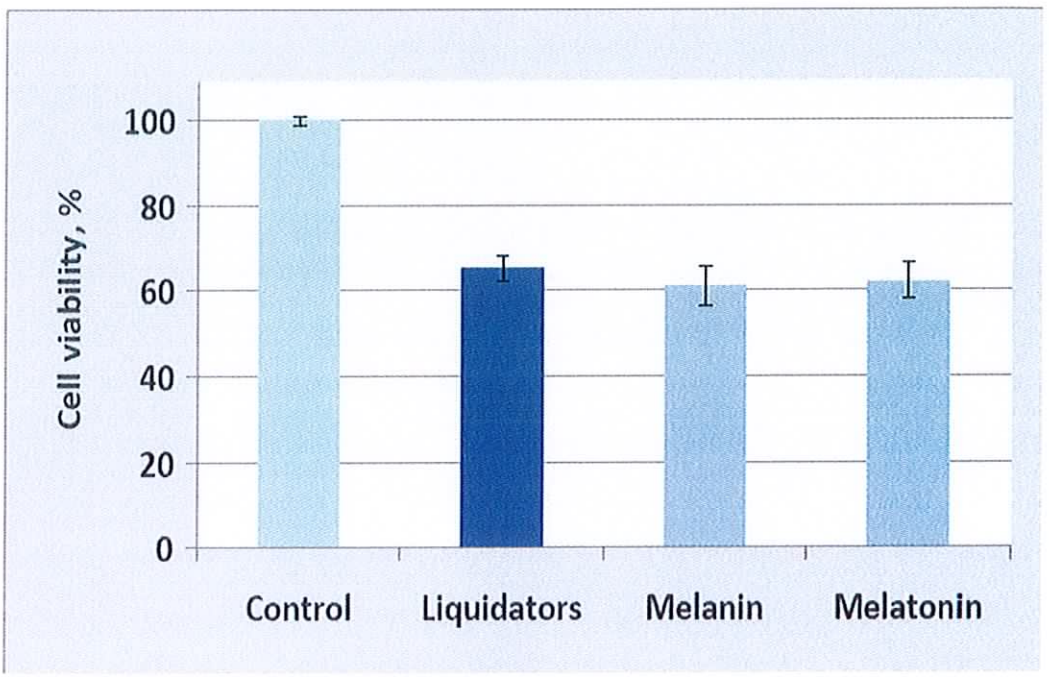

Figure 3.10 Average data obtained in study of the effect of melanin and melatonin on HPV-G cells treated with blood serum samples from Chernobyl liquidators. Cells treated with serum samples from liquidators alone, with melanin or melatonin added show very similar viability and significantly different from control.

Figure 3.11 presents an analysis of correlation between the parameters micronuclei frequency and viability in HPV-G cells treated with serum samples from people affected by the Chernobyl accident. As it can be seen from Figure 3.11, there is an inverse correlation between these parameters ($r = -0.73$).

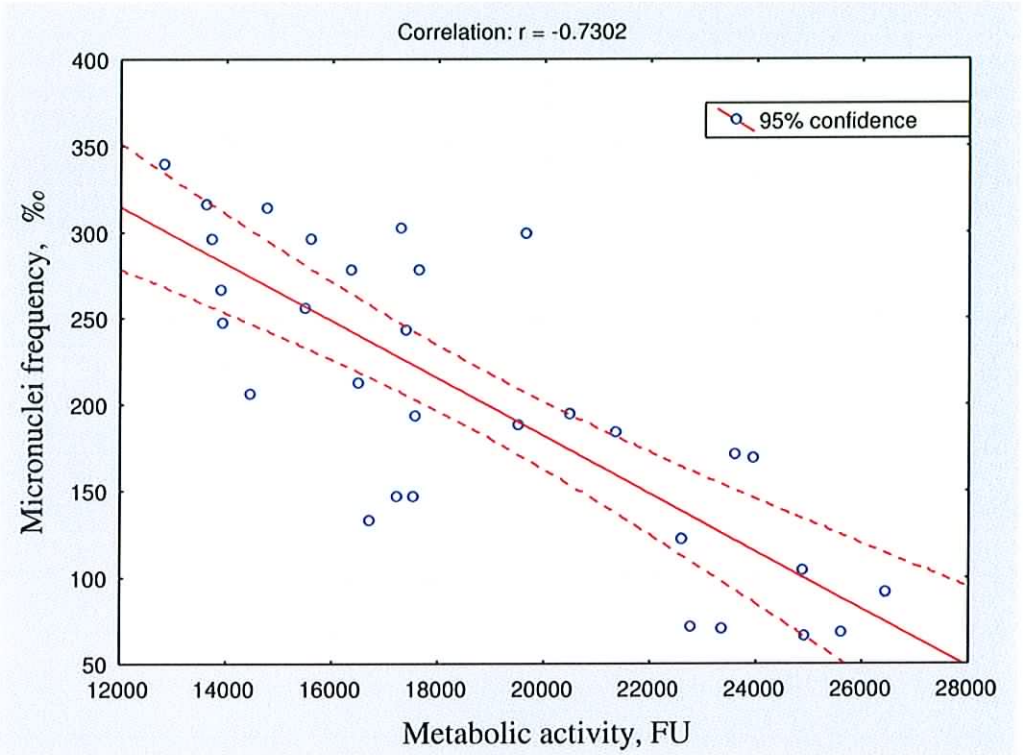

Figure 3.11 Multiple linear regression analysis of bivariate correlation between parameters micronuclei frequency and viability in human keratinocytes after treatment with serum samples from people affected after Chernobyl accident (r – coefficient of Pearson). An inverse dependence is observed between analysed the parameters.

3.4 Discussion

Study of the effects of blood serum on HPV-G cells using micronuclei assay.

In previous studies, human peripheral blood lymphocytes have been used as a model for the evaluation of the transfer of clastogenic factors [Emerit, 1990]. It was shown that so-called clastogenic factors may be present in serum samples from people exposed to radiation. But this method is complicated, because the level of spontaneous micronuclei frequency varies widely (from 2 to 60‰), and it is not very sensitive. Also, lymphocytes activated by PHA cannot be analysed later than 4 days from the start of cultivation.

Therefore in the present experiments human keratinocyte cells, immortalised by HPV-virus, were used. As a result of virus immortalisation, these cells have blocked first G_1 checkpoint of the cell cycle (*p53*-dependent), and are more sensitive to mutagenic factors - the level of spontaneous micronuclei frequency is about 80‰. Moreover, this parameter is similar in different series of experiments, thus significantly facilitating interpretation of results.

The data shows that serum samples from PSRER workers significantly increases micronuclei frequency of HPV-G cells compared to control cells treated by serum samples from healthy people. Thus, the data clearly indicates that chronic intensive radiation exposure for PSRER workers significantly influences the level of bystander factor accumulation in blood.

Similar data was received in analysis of serum samples from Chernobyl liquidators. Significant increase in micronuclei frequency in HPV-G cells was also observed. Importantly, these serum samples induced elevated levels of polymicronuclei

cells (cells with 2 and 3 MN). In the control group, cells with 3 micronuclei were not observed. In analysis of individual parameters of PSRER workers and Chernobyl liquidators, the serum samples from each individual induced increased levels of micronuclei compared to the controls. No significant difference was observed between results for liquidators and PSRER workers groups. This suggests that the effects of previous radiation exposure can be fixed for up to 19 years after radiation exposure. Thus, it is necessary to continue further thorough control and check up of the Chernobyl accident populations.

These results correlate well with the data from the Hiroshima population [Goh and Sumner, 1968]. The data on the bystander effect of the blood serum from PSRER workers confirms an earlier hypothesis [Melnov, 2002] on the possible effects of chronic exposures to low doses of radiation.

In the investigation of serum samples from residents of contaminated territories of Gomel region, there also was observed a significant increase in micronuclei frequency compared to control. But this increase was much lower compared to treatment with serum samples from PSRER workers and Chernobyl liquidators. Also, looking at individual data, in some cases serum samples from residents induced the same micronuclei frequency as control populations.

The highest level of micronuclei induction was observed in cells treated with serum samples from patients – residents of contaminated territories with acute virus infection (flu). Such high level of micronuclei induction could be possibly the result of extremely high levels of oxidants in the active stage of acute virus infection.

In another study [Melnov *et al.*, 2006] the micronuclei frequency in cells treated by serum samples from patients with acute virus infection has been compared with patients

having chronic virus infection (Hepatitis C). The level of micronuclei induction of serum samples from patients with hepatitis C was much lower compared to patients with acute virus infection. But 2 weeks later after recovery from acute virus infection the level of clastogenic factors in blood serum from patients became very similar to the level of residents from contaminated territories. In the serum samples from patients with chronic virus infection (hepatitis C) these factors continued to exist.

The similarity of clastogenic factor profiles for people affected by radiation and virus infection could be rooted in the similar role and higher induction of free radicals. It is well-known that free radicals are generated in excess in a diverse array of microbial infections. Free-radical induced pathogenicity in virus infections is of great importance, because evidence suggests that NO and oxygen radicals such as superoxide are key molecules in the pathogenesis of various infectious diseases and have a radio mimetic effect. Although oxygen radicals and NO have an antimicrobial effect on bacteria and protozoa, they have opposing effects in virus infections such as influenza virus pneumonia and several other neurotropic virus infections. The unique biological properties of free radicals were confirmed by the evidence showing accelerated viral mutation by NO-induced oxidative stress [Akaike, 2001].

Neutrophil host defence mechanisms are categorized as oxidative and non-oxidative. Oxidative mechanisms rely upon the production of superoxide, primarily by the multisubunit enzyme NADPH oxidase. Reactive oxygen species are also implicated in the activation of transcriptional factors (NF-kB and activator protein 1) leading to the transcription of genes that accentuate the inflammatory process [Swaun *et al.*, 2004]. Expression microarray analysis performed on lung tissues isolated from the infected

animals showed activation of many genes involved in the inflammatory response, including cytokine, apoptosis and lymphocyte genes that were common to different infection groups.

The results show that the level of bystander activity of serum blood samples from people affected by the Chernobyl accident may correspond to the level of activity of pathological processes, and that powerful oxidative stress from the acute virus infection may substantially influence the level of bystander factors, thus creating a possibility of temporary destabilisation of the genome of the somatic cells.

Comparative analysis of cytogenetic and micronuclei parameters in populations affected by the Chernobyl accident

In a further study of the effects of serum samples from people affected by the Chernobyl accident, the micronuclei frequency, induced by these serum samples, was compared with cytogenetic parameters of human peripheral blood lymphocytes from the same persons.

The data showed that the level of non-specific aberrations (single and double fragments) and total number of aberrations in human lymphocytes from people affected by the Chernobyl accident are significantly higher than in control. The level of specific aberrations (dicentric and ring chromosomes) is almost 3 times higher than in control, but not significantly ($P>0.05$). This data is confirmed by data from Sevan'kaev *et al.* [2005], showing that with time, a significant number of these non-stable marker aberrations is lost, decreasing the effectiveness of biological evaluation of radiation doses.

The preliminary comparative analysis of data shows that blood serum samples from people affected by the Chernobyl accident, even after a prolonged period (19 years to analysis date), may be reviewed as prolonged effects of radiation influence.

The data presented suggests that elevated levels of chromosome aberrations may be stimulated by clastogenic factors. If such idea is correct, there should be direct correlation between the clastogenic effect of serum samples and the level of aberrations in the lymphocytes from the same blood samples.

The analysis of data distribution has shown that for micronuclei test results, parametric methods of statistics can be used, and for cytogenetic test results – only non-parametric methods of analysis (as the distribution was not normal).

The dependence between parameters using polynomial analysis has been studied in order to understand interactions between them. According to analysis, the level of damaging bystander factors in blood serum samples (the level of MN frequency) from people affected by the Chernobyl accident is closely correlated with the level of genome damage (the level of aberrations frequency). By the cluster analysis it was shown that parameter "total micronuclei frequency, ‰" has very close linkage with parameters "number of aberrant cells, %" (d = 0.28) and "aberrations frequency, %" (d = 0.24). This data was also confirmed by Spearman correlation analysis, when the strongest dependence was observed between micronuclei frequency and frequency of aberrations. This data again suggests that there is a high level of correlation between the level of bystander factors in human peripheral blood and the level of aberrations in human peripheral blood lymphocytes.

Thus, the data allows the conclusion that serum samples from victims of the Chernobyl accident produce bystander factors. These factors are able to cause genomic instability. The level of bystander factors in serum samples significantly correlates with the frequency of chromosome aberrations in human lymphocytes.

As a consequence, the data suggests that the total level of genomic instability may correspond to both the level of radiation influence and to individual radiosensitivity.

Therefore, it was very interesting to analyse groups of populations (liquidators, PSRER workers and residents from contaminated territories) and reveal differences between them using comparative Mann-Whitney U-test analysis.

The analysis showed that there is no significant difference between cytogenetic parameters of liquidators and PSRER workers lymphocytes, but there is a tendency ($P>0.05$, but less than 0.1) that PSRER have lower micronuclei frequency and lower levels of chromosome aberrations. Between groups "liquidators" vs. "residents of contaminated areas" and "PSRER workers" vs. "residents", there is a high statistically significant difference.

Recently, the dynamics of micronuclei frequency of the Chernobyl liquidators group has been analysed [Melnov *et al.*, 2006]. According to this data, with time the sum of aberrations has increased constantly since 1991, and maximal level has been fixed in 2004-2005. At the same time, there were no fixed prominent dynamics for markers, dicentric and ring chromosomes, the main radiation specific cytogenetic markers. It means that the main increase in the total number of aberrations took place because of the elevated levels of unspecific aberrations (predominantly single and double fragments). In other words, the investigated group manifested the presence of genomic instability symptoms in somatic cells.

In the control group statistically reliable dynamics for both total number of aberrations and for the level of marker aberrations were absent. So, basing on this one can conclude that even 20 years after the Chernobyl accident liquidators showed cytogenetic effects in peripheral blood lymphocytes.

Thus, we may conclude that the most affected group of populations are the liquidators of the consequences of the Chernobyl accident 1986-1987, exposed to acute irradiation (the most expressed genomic instability and sharp increase in micronuclei and aberrations of different types).

Less affected are PSRER workers, who continue to stay on contaminated territories and are exposed to chronic radiation influence. The least expressed effects are observed in residents of contaminated areas of Gomel region (dose load is minimal).

The level of bystander factors production in serum has direct correlation with genomic instability. Thus, even in remote period after acute and chronic low-dose radiation influence in human blood *in vivo* there is accumulation of specific factors, transferring through serum and able to induce increased levels of chromosome aberrations in intact somatic cells, providing genomic instability in tissues of the irradiated organism.

Study of the effects of blood serum samples using the Alamar Blue assay

The effect of serum samples from patients affected by the Chernobyl accident were analysed using the Alamar Blue assay in order to define the presence of bystander factors in serum, which could affect metabolic activity of reporter cells. The blood serum samples for Alamar Blue analysis were taken from Chernobyl liquidators and residents of contaminated territories of Gomel region.

The analysis showed that treatment of HPV-G cells with serum samples from healthy people does not significantly influence the viability of the cells compared to control non-treated cells. The effect of serum samples from Chernobyl liquidators significantly decreases the viability of the cells. Treatment of HPV-G cells by serum samples from

residents of Gomel region also significantly decreased viability level compared to controls, but not as much as serum from liquidators. The analysis of the difference between all these groups showed that there is statistically significant difference between all groups, except when non-treated cells were compared with cells treated with serum samples from healthy people. The highest difference is between Chernobyl liquidators group and non-treated cells and cells treated with serum samples from healthy populations.

These data confirm our previous study with micronuclei and cytogenetic tests, showing that bystander factors are accumulated in serum of Chernobyl liquidators or individuals living in the areas with radiation.

The nature of these damaging bystander, or clastogenic, factors from blood serum samples is not known. The bystander factors were transferred from irradiated donor cells to non-irradiated recipient cells and still showed damaging effects after freezing at -20°C.

In previous studies it was shown that these transmissible clastogenic factors are mixtures of prooxidants with damaging properties [Faguet *et al.*, 1984]. The various components of these factors exert their effects by different mechanisms. Certain of these components may be harmful due to their superoxide-stimulating properties [Emerit, 1990]. Added to cell cultures, these components will induce further superoxide-mediated production of clastogenic factors. Given that superoxide generation leads to the formation of substances themselves generating superoxides, the system is self-sustaining and may be responsible for long-lasting genotoxic processes. Superoxide dismutase and other superoxide scavenging substances protect by interrupting this vicious circle [Emerit *et al.*, 1996]. The relationship between these clastogenic and bystander phenomena is unknown.

Emerit *et al.* [1995] in their studies on liquidators from Armenia observed that only 42% of liquidators had an increased level of clastogenic factors (chromosome aberrations) compared to the level of spontaneous mutations. In our experiments 100% of liquidators, 95% of PSRER workers and 82% of residents from contaminated areas had an increased level of factors compared to control levels. A possible explanation of such differences could be, as mentioned above, that HPV-G cells are much more sensitive for mutagenic factors compared to peripheral blood lymphocytes. Also, the micronuclei assay itself is more sensitive than cytogenetic assays. Another explanation could be that the cohort from Emerit *et al.* [1995] included liquidators of 1986-1988, and liquidators from 1986 expressed higher levels of clastogenic factors compared to liquidators of 1987-1988. In the present study, liquidators of the consequences of the Chernobyl accident in 1986-1987 were analysed, which were the most affected group and received the highest doses of radiation (higher than 250 mGy). Only after 1987 the dose limits for people engaged in clean-up works were reviewed and decreased. Moreover, all analysed liquidators were from the Gomel region and continue to live in territories that are much more contaminated by radionuclides compared to Armenian liquidators.

In another study [Emerit *et al.*, 1997b], a group of Russian liquidators were analysed. In this cohort, 62% of patients had increased level of clastogenic factors. This group also consisted of liquidators who participated in clean-up works from 1986 to 1988.

Since the liquidators from Russia and Armenia did not return to Chernobyl and were living in a non-contaminated environment, the persistence of clastogenic factors cannot be explained by persisting radiation exposure. Liquidators from the Gomel region were also from an environment with levels of contamination corresponding to norm (on which it is allowed to live), but higher compared to Russia and Armenia.

Emerit *et al.* [1994b] showed that irradiation of blood *in vitro* at a radiation dose of 500 mGy results in significant clastogenic activity and that cells incubated in fresh culture medium continue to produce factors in culture. The doses received by the Chernobyl liquidators from the Gomel region were sufficient for initiation of factor formation. This is possibly the main reason for the fact that the bystander factors persist in the blood 19 years after the Chernobyl accident.

In another study Emerit *et al.* [1997a, 1997b] compared the frequency of the increased level of clastogenic factors in children living or evacuated from areas of Belarus and Ukraine, contaminated by radionuclides. 52% of patients emigrated from the Gomel region to Israel showed an increased level of clastogenic factors. The percentage of these patients with increased levels of factors was higher compared to children emigrated from "clean" cities of the former Soviet Union and healthy Israeli children and people coming from Kyiv [Emerit *et al.*, 1997a].

The second group of patients were residents of contaminated regions of Ukraine. The frequency of people with increased levels of bystander factors varied between 14 and 100% as a function of their origin. The percentage of people with increased level of clastogenic factors was the same (30%) for patients emigrated from Kyiv to Israel and people still living in Kyiv. The highest values were observed in 27 patients, who had been evacuated from a zone with a highest level of contamination [Emerit *et al.*, 1997b].

In the present study, 82% of residents from Gomel regions had an increased level of bystander factors. As the emigration to Israel from Kyiv does not influence the frequency of people with increased level of bystander factors, the data (compared with 52% of "positive" immigrants from Gomel region) again suggests that the method of bystander factors detection used in present work is more sensitive.

The data of the atomic bomb survivors from Hiroshima and Nagasaki studies has some controversies with the study of Chernobyl victims.

Kyoizumi *et al.*, [1996] studied the relationship between radiation dose and mutations in blood cells, among persons exposed to the atomic bombings in Nagasaki and Hiroshima. The mutant frequency of the gene that produces a protein called erythrocyte glycophorin A increased with the radiation dose. Considering that the life span of erythrocytes in the human body is about 120 days, it is surprising that many mutations are still found in erythrocytes from atomic-bomb survivors 50 years after the atomic bombings. This is because mutations remain in the genes of the bone marrow cells that produce erythrocytes, i.e., the blood stem cells. Because these blood stem cells produce red and white blood cells throughout a person's lifetime, mutations produced by atomic-bomb radiation can be detected even 50 years after irradiation.

Ban *et al.* [1993] studied the radiosensitivity of atomic bomb survivors using the micronuclei assay. They suggested that if the atomic bomb survivors include a disproportionately large number of either radioresistant or radiosensitive persons, the surviving population would provide a biased estimate of the true risk of radiogenic cancer. To test this hypothesis, the *in vitro* X-ray sensitivities of peripheral blood lymphocytes obtained from 937 A-bomb survivors were measured with a cytokinesis-blocking micronucleus assay. Background frequencies (no irradiation *in vitro*) of micronuclei showed a wide distribution. Frequencies in both males and females tended to increase with increasing donor age. Frequencies in females were significantly higher than those in males. There was no effect of donor's sex on *in vitro* radiation sensitivity. Atomic bomb radiation and cigarette smoking had no significant effect on background and X-ray-induced micronuclei frequencies. Thus, the authors concluded that there were no difference in

radiosensitivity of peripheral blood lymphocytes between proximally and distally exposed survivors.

Recently Kodama *et al.* [2005] showed that clonally expanded human lymphocytes from atomic bomb survivors 60 years after irradiation did not show excess levels of chromosome aberrations.

In previous chapters it was shown that the bystander effect may be partially reduced by radioprotectors with radical scavenging capacities. Emerit *et al.* [1996] showed that superoxide-mediated clastogenesis is characteristic for various chronic inflammatory diseases with autoimmune reactions and probably plays a role in radiation-induced clastogenesis and in congenital breakage syndromes and may be prevented by exogenous superoxide dismutase (SOD).

In the present chapter, we also tried to prevent the damaging effect of bystander factors formed *in vivo* in serum samples from people affected by the Chernobyl accident using radioprotectors melanin and melatonin. These substances were added to the wells containing cells together with serum samples (serum sample from the same person was used in all cases as appropriate).

The data shows that melanin and melatonin are not able to protect HPV-G cells, neutralising bystander factors from serum samples. This data is in contrast to our previous studies (chapter 2), when radioprotective substances were able to partially neutralise bystander factors. Bystander factors from serum samples were induced *in vivo* and persisted in the blood stream for 19 years (for liquidators) or less (for other groups). As melanin and melatonin are natural and always present in human organism, it is possible to suggest that radioprotective substances are able to neutralise only some specific factors, but not all of

them. And the factors from which melanin and melatonin are able to protect, were already neutralised *in vivo* in blood stream by these or other antioxidants.

In general, the results from the study of the effects of serum samples on viability of HPV-G cells confirmed the data from previous experiments that there are some kind of damaging factors, able to induce chromosome aberrations and micronuclei and decrease viability of cells.

A very interesting question in this field is whether in some situations cells affected by bystander factors die and are thus removed from the population. Mothersill *et al.* [2005] showed that in mice irradiated in vivo, a clear difference could be seen between the CBA cancer prone strain and the C57 Bl6 strain which respond to radiation by inducing apoptotic pathways. Such sectoring along genetic lines could be important in human populations exposed to radiation. Since genomic instability is thought to be a "life with damage" option for irradiated cells, it was decided to compare individual patient serum samples for their ability to induce death (loss of viability in the Alamar blue assay) versus micronucleus formation (taken as an indicator of genomic instability).

The data showed that there is an inverse correlation between these parameters: increased genomic instability in an individual was associated with decreased viability, suggesting that cells were not making a choice between death and genomic instability at this level.

The results of the bystander experiment with serum blood samples from people affected by the Chernobyl accident allows the following conclusions:

1. The factors in serum samples from people affected by the Chernobyl accident are capable of causing cell death or genomic instability in HPV-G cells and can persist even for 20 years.
2. The level of bystander factor production in serum shows a direct correlation with genomic instability.
3. Melanin and melatonin are not able to neutralise damaging bystander factors from serum samples from Chernobyl victims.
4. The most affected group of populations are Chernobyl liquidators: even 20 years after the accident they have the highest level of bystander factors in serum. The least affected group are people from contaminated areas of Gomel region – they received the lowest doses of radiation. There is also preliminary evidence that acute virus infection (flu) may increase the frequency of clastogenic effects.

CHAPTER 4

STUDY OF LASER-INDUCED BYSTANDER EFFECT IN HUMAN KERATINOCYTES (HPV-G CELLS)

4.1 Introduction

In all previous experiments, only ionising or non-coherent UV radiation has been used to induce bystander effects. Today laser irradiation is becoming more and more popular in different areas of science, techniques and especially medicine.

A laser (from the acronym Light Amplification by Stimulated Emission of Radiation) is an optical source that emits photons in a coherent beam. Laser light is typically near-monochromatic, i.e., consisting of a single wavelength or colour, and emitted in a narrow beam. This is in contrast to common light sources, such as the incandescent light bulb, which emit incoherent photons in almost all directions, usually over a wide spectrum of wavelengths.

Laser action is explained by the theories of quantum mechanics and thermodynamics. Many materials have been found to have the required characteristics to form the laser gain medium needed to power a laser, and these have led to the invention of many types of lasers with different characteristics suitable for different applications.

Although the foundation for the invention of the laser was laid by Einstein in 1916 in a ground-breaking re-derivation of Max Planck's law of radiation based on the concepts of spontaneous and induced emission, the laser was proposed as a variation of the maser principle in the late 1950s, and the first laser was demonstrated in 1960. Since that time, laser manufacturing has become a multi-billion dollar industry, and the laser has found

applications in fields including science, military, industry, medicine and consumer electronics.

In medicine, the laser scalpel is used for laser vision correction and other surgical techniques. Lasers are also used for dermatological procedures and cosmetic surgery including removal of tattoos, birthmarks, scars and hair. They are also now used in dentistry for caries removal, tooth whitening and oral surgery procedures.

The biological influence of laser irradiation on individuals is not well studied, although it was started since the discovery of lasers. It is impossible to extrapolate data from radiation-induced studies of bystander effects to predict laser-induced bystander effects. The method of inducing bystander effects in human cells using laser irradiation helps in evaluating the risk of laser influence on individual health.

4.2. Materials and methods

4.2.1 Cell culture

HPV-G cells

HPV-G cells were cultured in Dulbecco's MEM: F12 (1:1) medium supplemented with 10% Foetal bovine serum, 1 % penicillin-streptomycin, 1 % L-glutamine and 1 μg/ml hydrocortisone. The cells were maintained in an incubator at 37 degrees Celsius, with 95% humidity and 5% carbon dioxide and routinely subcultured every 8-10 days.

When 80-100% confluent, the medium was poured from the flask and replaced with 1:1 solution of versene (1nM solution) to trypsin (0,25% in Hank's Balanced Salt Solution) (Gibco, Irvine, UK) after washing with sterile PBS. The flask was placed in the incubator at 37 degrees Celsius for about 11 minutes until the cells started to detach.

The flask was then shaken to ensure that all cells had been removed from the base of the flask. The cell suspension was added to an equal volume of DMEM F12 medium to neutralise the trypsin. From this solution new flasks could be seeded at the required cell quantity.

4.2.2 Laser irradiation

Cells were irradiated in the culture flasks at room temperature using a nitrogen laser with non-therapeutic wavelength (λ=337 nm) with frequency of 50 and 100 Hz for 1–2 min so that all the cells in the flasks received the same dose. According to electromagnetic spectrum, the wavelength corresponds to UV A. After irradiation, the cells were returned to the incubator.

4.2.3 Clonogenic protocol for HPV-G cells

The cell suspension after dilution was counted using haemocytometer under light microscope and seeded at the same quantity in 5 ml medium in $25cm^2$ NUNC flasks. There were 3 types of flasks: direct laser irradiation, bystander donor and bystander recipient. Bystander donor flasks were very heavily seeded with cells ($0.5x10^6$ cells per flask) in order to produce the bystander factor into the medium after irradiation. Bystander recipient flasks were set up with the ordinary cloning number (300 cells per flask) and received no treatment except the bystander medium from the bystander donor flasks. The direct laser irradiation flasks were ordinary survival measurement (seeded with 300 cells per flask), after irradiation they received no further treatment. Each of 3 types of flasks had 2 sets in triplicate: control cells and irradiated cells.

After seeding cells, the flasks were left at 37°C in the incubator to attach for 12 hours. Then bystander donor and directly irradiated flasks were treated and replaced back in the incubator at 37 degrees Celsius for one hour. The medium from bystander donor flasks was removed, filtered through NALGENE 0.22 μm sterile syringe filters (to ensure that no cells were present in the medium) and used to replace the medium from bystander recipient flasks. Then all flasks were returned to the incubator and left untouched for 9-10 days (until colonies were visible) and then stained with carbol fuchsine and colonies were counted and surviving fraction calculated.

The data is presented as mean ± standard error in all cases. Significance was determined using the t-test.

4.3 Results

The effect of laser radiation with different frequencies and time of exposure on HPV-G cells using the clonogenic assay is presented in Table 4.1 and Figure 4.1.

Table 4.1 presents the survival of both directly irradiated and bystander recipient HPV-G cells.

Figure 4.1 presents surviving fractions of directly laser-irradiated and bystander recipient HPV-G cells (controls were set to 100%).

The results presented in Figure 4.1 show that laser irradiation for both directly irradiated and bystander recipient cells significantly decreases the survival of HPV-G cells after 1 and 2 minutes of exposure time, for the frequency of 50 and 100 Hz. Depending on time of exposure and frequency, the survival of directly irradiated HPV-G cells is 42.5-57% lower than in control ($P<0.01$ in all cases). At the same time, recipients of the bystander medium from laser-irradiated donor cells showed 37.7-47.4% decrease in survival compared to control ($P<0.01$ in all cases).

In general, this effect is similar to the one induced by ionising radiation – directly irradiated cells show lower survival compared to cells treated by ICCM. Only after irradiation for 1 min at 50 Hz frequency the survival is 4.4% lower in bystander recipient cells compared to directly irradiated cells ($P>0.05$). In all other cases, the survival is 3.8-23.6% higher in bystander recipient cells compared to directly irradiated cells ($P<0.01$ for 50Hz 2' and 100Hz 1'; $P>0.05$ for 100Hz 2').

For both directly irradiated and bystander recipient cells, there is a non-significant dependence of the survival on the frequency of laser irradiation – the survival is higher with

lower frequency (Figure 4.1). For directly irradiated cells no dependence was observed on the time of exposure. But for bystander recipient cells, there is again a non-significant dependence of survival on the time of exposure – the longer the exposure, the higher the survival.

Table 4.1. The survival of HPV-G cells after direct laser irradiation and LIBE using the clonogenic assay. Both direct irradiation and ICCM treatment significantly decreased survival of HPV-G cells compared to the control. There was no statistically significant dependence on frequency of irradiation or time of exposure.

		Cells plated	Average colonies No.	P	PE, %
Direct irradiation	Control	300	207±5.20	-	69.00±1.73
	50 Hz 1'	300	119±5.29	< 0.01	39.67±1.76
	100 Hz 1'	300	89±3.46	< 0.01	29.67±1.15
	50 Hz 2'	300	108±4.04	< 0.01	36.00±1.35
	100 Hz 2'	300	105±1.53	< 0.01	35.00±0.51
Bystander effect	Control	300	207±8.74	-	69.00±2.91
	50 Hz 1'	300	114±3.46	< 0.01	38.00±1.15
	100 Hz 1'	300	110±4.04	< 0.01	36.67±1.35
	50 Hz 2'	300	129±2.52	< 0.01	43.00±0.84
	100 Hz 2'	300	109±4.58	< 0.01	36.33±1.53

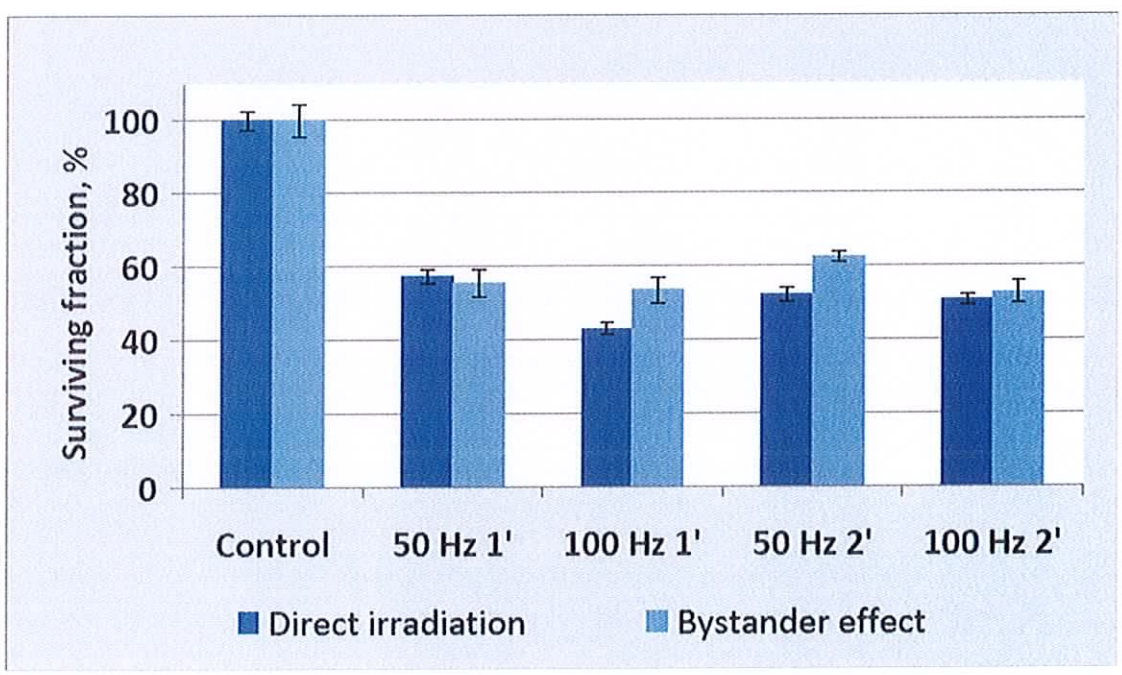

Figure 4.1 The surviving of HPV-G cells after direct and bystander laser irradiation (frequency 50 and 100 Hz, time of exposure 1 and 2 min). Laser irradiation significantly decreased survival of HPV-G cells. The effect of direct irradiation in most cases was more damaging compared to the bystander effect.

4.4 Discussion

In all previous experiments, only ionising or non-coherent UV radiation has been used to induce bystander effect. Today laser irradiation is becoming more and more popular in different areas of science, techniques and medicine. And the biological effects of laser irradiation on individuals needs further study.

In the present study the induction of bystander effect using a non-therapeutic laser in human keratinocytes has been studied.

The results clearly show that a laser-induced bystander effect can be observed after laser irradiation with different frequency (50 and 100 Hz) and time of exposure (1 and 2 min) if compared to control non-irradiated cells. But there was no statistically significant dependence of the effect on exposure time or irradiation frequency. As for ionising radiation, direct laser irradiation resulted in a higher decrease of survival of HPV-G cells compared to bystander effects.

The wavelength of laser irradiation used in present work (337 nm) is not used for therapeutic purposes. According to electromagnetic spectrum, this wavelength corresponds to ultraviolet A. As UV is a part of solar radiation, the data indicates that bystander effects may be induced by over-exposure to the sun.

Previously there were some studies on non-coherent UV-induced bystander effects. The present data corresponds well with these results. Dahle *et al.* [2005a] recently reported that non-coherent UV-light may induce delayed mutations via a bystander effect. The bystander effect was 5-fold higher for UV B radiation than for UV A radiation. The factors mediating the bystander effect were not identified. In a further study, Dahle *et al.* [2005b] found that these UV-induced bystander factors were strongly inhibited by

antioxidants and suggested that a persistent increase of the reactive oxygen species or their downstream products are involved in a bystander-induced formation of delayed mutations. This bystander effect was mediated both via the medium and via gap junction intercellular communication or transmitted from mother to daughter cells. In this work, as in the present research, UV-induced bystander factors significantly reduced the survival of the cells compared to control cells.

Previously Ghosh *et al.* [1995] studied the effect of supernatant from UV-irradiated CHO cells. The results of their experiments indicated that factors secreted after UV-irradiation into the supernatant showed a protective effect when added to non-treated cells before their UV- or gamma-irradiation. The authors suggested that these factors were probably involved directly or they triggered repair process(es), related to oxidative stress. Non-coherent UV A radiation acts primarily in the presence of oxygen and endogenous photosensitisers by generating reactive oxygen species in exposed cells [Arimoto-Kobayashi *et al.*, 2002].

As mentioned previously, in the present study, in contrast to previous studies, coherent laser irradiation in UV-range of 337 nm wavelength has been used. This laser gives a steady wavelength, fixed in a small range. Moreover, irradiation does not result in heating of the biological samples, which takes places after the influence of "non-laser" UV or after laser irradiation in the infrared spectrum [Dudarev, 1992]. Thus, the data indicates that observed effect is clearly physical effect of energetic quanta of laser irradiation. The laser spot was 5 cm in diameter and was able to cover several cells.

Today lasers are becoming more and more popular in all areas of science, including medicine. The wavelength influences the penetration of the laser radiation. The therapeutic lasers have wavelength of 600-1000 nm, which allows penetration through

epidermis to the tissue or organ. The level of energy in these lasers corresponds to red and infrared range, usually followed by heating. These lasers in many studies were shown to be safe for use on humans. But there are lasers with wavelength of 310 nm (eximer xenon-chloride laser), which are used in dermatology and also considered to be safe for humans. According to the data presented in this study, it is necessary to further study the effects of these lasers, as they may induce bystander effects and damage human cells.

Also the data presented has expanded our knowledge on biological effects of laser irradiation in people working with lasers or undergoing laser therapy. The data needs to be further investigated and compared with ionizing radiation-induced bystander effect.

Thus, it is possible to conclude that:

1. Laser radiation may induce bystander effects in human keratinocytes
2. As for ionising radiation, the effect of laser-induced bystander factors is less damaging compared to direct irradiation.
3. The effect of laser irradiation does not depend on the time of exposure or the frequency of irradiation.

CHAPTER 5

GENERAL DISCUSSION

The aims of this study were to evaluate the direct and bystander effects of γ-radiation on human cells and their modification using radioprotective substances, the effect of serum samples from people affected by the Chernobyl accident (bystander effect *in vivo*) and the laser-induced bystander effect. After the CNPP accident in 1986, the radiation background of the planet increased considerably. As a result, millions people are constantly exposed to chronic low doses of radiation. Under such conditions, it is very important to protect populations from the damaging action of radiation. One of the possible ways could be the use of non toxic and natural radioprotective substances which are able to protect against acute or chronic radiation exposure.

This study, as a whole, is divided into three parts. The first part (Chapter 2) concerns the study of radiation-induced bystander effects *in vitro* using different radioprotectors – melanin, melatonin and α-tocopherol. The second part (Chapter 3) is a study of bystander effect induced by ionising radiation *in vivo* – the influence of serum samples from the blood of victims affected by the Chernobyl accident on HPV-G cells and modification of these effects using melanin and melatonin. And the third part (Chapter 4) is a study of laser-induced bystander effects.

In Chapter 2, human peripheral blood lymphocytes and human keratinocytes immortalised with the human papilloma virus were exposed to direct and bystander γ-radiation and treated with the radioprotective substances: melanin, melatonin and tocopherol. Post direct irradiation, decreases in survival, viability and proliferation activity

and increases in micronuclei frequency and apoptosis were observed in these cells. Addition of melanin resulted in a significant decrease in the effects of radiation compared to cells irradiated without melanin. After ICCM transfer (in bystander experiments), decreases in survival and viability and increases in micronuclei frequency were observed, but these were not as significant as after direct irradiation. Addition of melanin to the ICCM before and after irradiation resulted in a slight increase in survival and viability and decrease in micronuclei frequency in HPV-G cells compared to cells treated with ICCM without melanin, but much less significant than in direct irradiation experiments. As melanin had no toxic or stimulating influence on cells, the observed effect allows us to suggest that melanin is able to protect against bystander and especially direct radiation effects. Melanin has been shown previously to be a very effective antioxidant substance and able to convert all types of physical energy into heat [Mosse *et al.*, 1996]. Since melanin both decreases the direct effect of the radiation dose and also influences the bystander effect itself, it is possible that there are two mechanisms of action: the absorption of energy during irradiation and subsequently the neutralisation of secondary biochemical processes connected with the formation of free radicals. These radicals appearing as a result of secondary or tertiary reactions may define the biological effects of ionising radiation. As melanin can absorb all types of physical energy, the result may indicate that the bystander signal has a physical component.

In a study of the radioprotective effect of melatonin on direct and bystander irradiated HPV-G cells among radiation effects, decreases in survival and viability and increases in micronuclei frequency were noted following direct γ-irradiation. Melatonin had no effect on untreated cells and significantly protected directly irradiated cells, increasing the survival and viability and decreasing micronuclei frequency compared to cells

irradiated without melatonin. Treatment of HPV-G cells with ICCM and melatonin also led to increases in survival and viability and decreases in micronuclei frequency compared to cells treated with ICCM without melatonin, but not as significant as after direct radiation influence. Melatonin also is known as very effective antioxidant substance, and it has shown even better protection against direct and bystander irradiation compared to melanin.

The experiments concerning the influence of α-tocopherol on HPV-G cells after direct and bystander γ-radiation showed that α-tocopherol can decrease the effects of direct and bystander irradiation, increasing the survival and decreasing the micronuclei frequency compared to cells irradiated without α-tocopherol. But the protection against direct irradiation, as for melanin and melatonin, was much more significant compared to protection against the bystander effect. α-tocopherol was found to be the least effective protector against radiation damage whereas melatonin appeared to provide the most effective protection. This data is confirmed by a recent study [Yilmaz and Yilmaz, 2006], where effects of melatonin and vitamin E on oxidative status of irradiated rat total bone (bone and bone marrow) has been analysed. The authors concluded that melatonin may protect the total bone from the damaging effects of irradiation exposure, and its actions protect total bone from oxidative stress. Protective effects of Vitamin E were not observed in this study.

As all the substances showed more effective protection against direct radiation damage compared with bystander damage, the nature of bystander factors appears to be complicated and possibly is composed of several factors with different properties. The main possible mechanism of protection against direct and bystander irradiation is the antioxidant properties of all three substances and their ability to neutralise free radicals.

Analysing the present and literature data, the development and manifestation of the bystander effect following ionising radiation influence may be divided into a minimum of two different components. The first of them (small part) is sensitive to the presence of radioprotector in the medium. The effect of the factors of the "second type" (the major part of them) cannot be modified by radioprotectors and can be manifested for a long period since irradiation. It is possible to suggest that these factors are developed as a result of the realisation of different biochemical and cytological cell processes.

The sensitivity of the "first type" factors to radioprotectors indicates their possible free radical nature. The long duration of the effect of the "second type" factors (which cannot be even partially modified by radioprotectors) obviously indicates the formation of substantial genetic instability, which is able to transfer from one cell generation to another.

The possibility to modify RIBE with melanin, melatonin and α-tocopherol – biological substances of human normal metabolism – suggests that the bystander effect is a natural process, which which may have evolved to eliminate potentially damaged cells.

In chapter 3 the effect of serum samples from blood of people affected by the Chernobyl accident on HPV-G cells has been studied using micronuclei and viability assays. There were 3 groups of people affected by the Chernobyl accident: Chernobyl liquidators, PSRER workers and residents of contaminated areas of the Gomel region. Analysis has shown that serum samples from the victims of the Chernobyl accident significantly increased the micronuclei frequency in HPV-G cells compared to cells treated with serum samples from clinically healthy people living in non-contaminated territories. The highest level of micronuclei frequency was observed in liquidators and PSRER workers, while residents from contaminated areas had the lowest increase in micronuclei

frequency, but statistically significantly higher than in control cells. At the same time, serum samples from 4 patients – residents from contaminated territories with acute virus infection induced even higher micronuclei frequency than samples from Chernobyl liquidators. It is possibly the result of extremely high level of oxidants in the active stage of acute virus infection.

Comparative analysis of these bystander experiments using micronuclei assays and cytogenetic analysis of peripheral blood lymphocytes from people affected by the Chernobyl accident showed that there was a statistically significant correlation between micronuclei frequency in HPV-G cells treated with serum samples (i.e. the level of bystander factors) and aberration frequency in human peripheral blood lymphocytes (genomic instability). Thus, even in a remote period after acute and chronic low-dose radiation exposure in human blood *in vivo* there is an accumulation of specific factors, transferring through serum and able to induce increased levels of chromosome aberrations in intact somatic cells.

The effect of serum samples from Chernobyl liquidators and residents from contaminated areas of the Gomel region on the viability of HPV-G cells has shown similar results to the micronuclei assay - the most affected group of populations are liquidators of the consequences of Chernobyl accident 1986-1987 yy., exposed to the highest doses of radiation (the lowest viability, the most expressed genomic instability and sharp increase in micronuclei and aberrations of different types). Less affected were PSRER workers, who continue to stay on contaminated territories and are exposed to chronic radiation doses. The least affected group was the residents of contaminated areas of the Gomel region where the dose load was minimal. The liquidators and PSRER workers groups consist completely of male subjects, while in groups of healthy populations and residents from contaminated

territories, female subjects were also analysed. The dependence of the studied parameters on sex is of great interest, but because of the insufficient number of female subjects for analysis, this can be studied only in future experiments. Also all the persons from the liquidators group and PSRER workers were smokers, which could also significantly influence the level of clastogenic factors in their blood serum. Further experiments should take into account the effect of smoking into the level of clastogenic factors.

Melanin and melatonin were not able to protect cells from the damaging effect of bystander factors from serum samples. This can be explained that bystander factors from serum samples persisted already for 19 years in the blood stream, and perhaps only the ones which could not be neutralised by the donor's own antioxidant system remained and affected HPV-G cells.

The data can be interpreted in the context of genomic instability. Genomic instability mechanisms *in vivo* are not completely understood at present, but it is possible to suggest that they are connected with bystander factors [Lyng *et al.*, 2002]. There are two possible hypothetic mechanisms:

1. Affected stem cells and long-living somatic cells are able to support the elevated levels of bystander factors. As a result, the stimulation of genomic instability can take place (Figure 1).

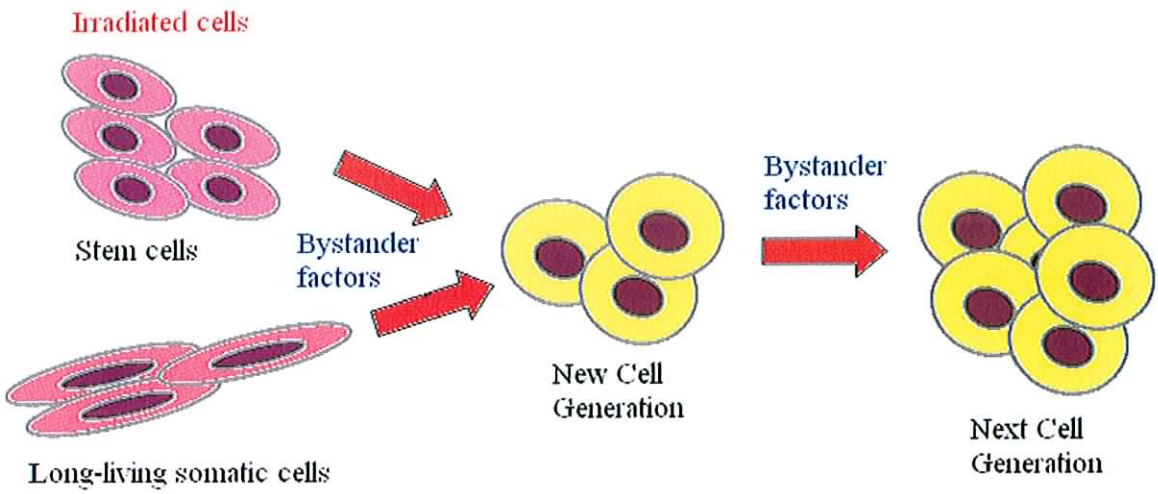

Figure 5.1 First possible mechanism of genomic instability (linear effect – it will decrease with time).

2. Bystander factors could develop their effects based on negative reverse linkage: affected cells are sources of bystander factors, which affect cells of the new generation. These cells of the new generation, in their turn, could directly affect the next cell generations and also to have an effect on precursor cells (Figure 2), thus creating a vicious cycle of damage.

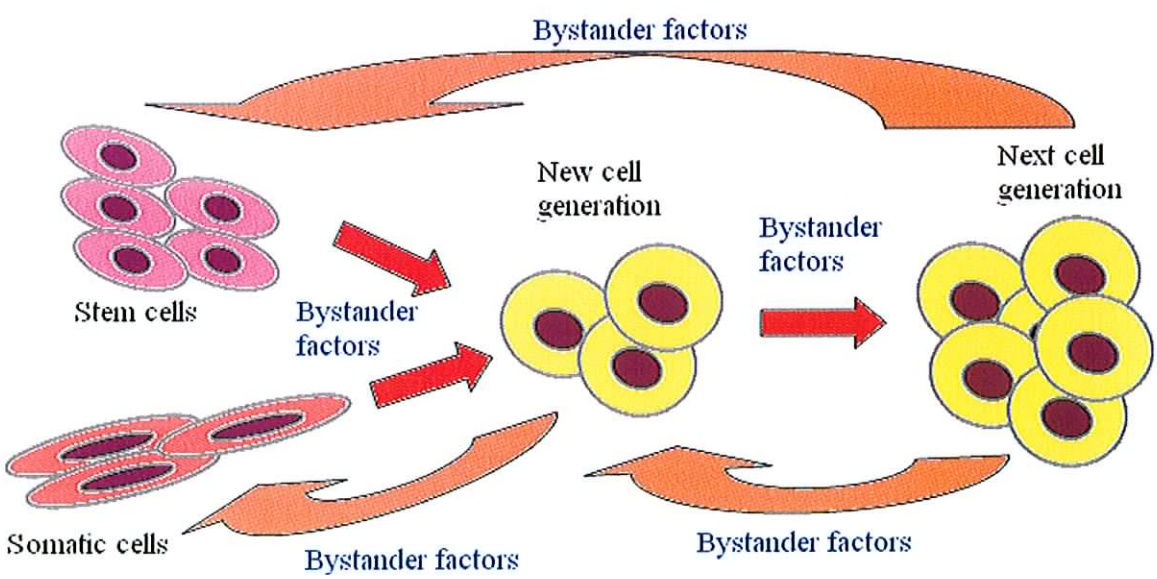

Figure 5.2. Second possible mechanism of genomic instability (vicious cycle).

In such a situation, initial inducers of the bystander factors would be more affected and, possibly, would undergo apoptosis. As a result, a functionally active pool of the cells will be devastated, and tissue or organ activity will be decreased. A similar picture was observed already during ageing processes and recently it was found that rapid ageing syndrome can be also observed for Chernobyl liquidators [Melnov, 2004].

These two possible approaches could be useful in possible interpretation and understanding of the cytogenetic effects observed in analysis of the populations affected by the Chernobyl accident even 20 years after the disaster.

In this case one of the most important problems is the investigation of the RIBE nature and its factors and their effects on germ cell populations. After such investigations we will be able to make prognosis of the possible risk for future generations.

A very interesting question in the field is the pathophysiological relevance of the radioprotectors study. Clastogenic and bystander factors, spreading through the body, may reach the most critical organs and tissues, which are the most vulnerable and on the border of sickness (for example, liver, kidney, heart, endocrine glands, pancreas, etc). As a result, increased level of ROS and other harmful factors in these organs will be observed, and pathologic processes will start. Radioprotectors, used in present research, are able to neutralise some of these factors. This may be essential for avoiding illness.

On the hypothesis that bystander factors from irradiated donor culture medium and from serum samples are the same, it is possible to conclude that only factors of the "second type" persist in the blood serum samples, as they cannot be neutralised by radioprotectors. A possible reason for this may be that all the radioprotectors used in present research are natural and found in human organisms under normal conditions. Possibly, the factors of the

"first type" were already neutralised *in vivo* in the blood stream, and only factors of the "second type" were left. To confirm this hypothesis, it is necessary to perform joint experiments for medium-derived bystander effect and bystander effect induced *in vivo* by serum samples and to see which factors induce stronger cellular damage.

The most sensitive ionising radiation phase of the cell cycle is S-phase [Alberts *et al.*, 1998] – dividing and proliferating cells. Almost all mutations happen in dividing cells [Vogel, 1956]. At the same time, any metabolically active cell may counteract the harmful effects of mutant cells, cells with modified metabolism and surface receptors. It is well known that different cells have very wide spectra of differences in mutation frequency [Neel *et al.*, 1978] and different responses to mutation [Vogel and Motulski, 1986]. That is why the bystander effect may be hypothesised as one of the forms of adaptive reactions of biological systems to radiation influence. The influence of laser, ionising radiation or any other xenobiotics induces in this system damages of any level or character. Small doses of ionising radiation provoke not only gene mutations, but also physiological (metabolic) damage, some of which become self-maintained [Emerit *et al.*, 1996]. In such conditions RIBE may be an adaptive attempt of the transfer of the external effect to other cells, like "request for help" as a response for radiation influence, i.e. an attempt for integration and generalisation of the response, which is not on the cellular, but on tissue, organ or organism level. The mechanism of this response may be something similar to interferon induction as a response to virus infection.

Chapter 4 describes the experiments on a laser-induced bystander effect in HPV-G cells using the clonogenic assay. The results clearly show that laser irradiation is able to

induce bystander factors in cells. The wavelength used in the current study is not used in medicine, so there is a need for a further study of laser effect using therapeutic lasers. Also, it is unknown if laser-induced bystander factors are identical to radiation-induced bystander factors.

Today laser irradiation is becoming more and more popular and is used in different areas of medicine. According to the present study, not all effects of laser irradiation are enough studied and taken into consideration.

Possible coincidence of the bystander data induced by laser irradiation with data induced by ionising radiation and UV light may be possible evidence of the universality of its mechanisms at least in relation to mutagenic factors of physical nature.

The nature of this factor seems to be very similar to the one induced by ionising radiation. This factor is also able to pass through a 0.22 μm filter and does not lose its effect after freezing. But it is necessary to perform special study comparing these two factors.

For the further study of the effects of laser irradiation on the development of the bystander effect, a more profound study of the processes occurring in cells after absorption of the energy is required. It is important to reveal biologically active molecules which are the most sensitive to laser irradiation according to their light absorption spectra. In the present study the laser irradiation with UV A wavelength has been used. The absorption of the light energy of such wavelength may stimulate the formation of a triplet state of the molecules and the vibration of the atomic nuclei and increase of the rotational mobility of the molecules as a whole.

The aim of this study was to study the bystander effects and to find a potential universal radioprotector, able to decrease the damaging effect of these factors. Such a wide-

reaching mission statement is very difficult to be completely accomplished during the course of this work. Bystander effects, which have generated much interest since their discovery, have massive implications for radiation protection. This study has presented evidence that bystander effects are not limited only to ionising radiation and could be partially prevented using radioprotective substances. Further work in the area is warranted to determine whether the nature of such laser-induced effects is similar to those induced by ionising radiation.

In general, the following conclusions can be made:

1. A medium-derived bystander effect was induced by γ-radiation *in vitro* and was partially modified (decreased) by radioprotective substances.

1.1. Melanin was able to neutralise bystander factors induced by γ-radiation *in vitro* when added to culture medium before and after irradiation of bystander donor cells. As melanin is able to adsorb all types of physical energy, it is possible that bystander factors have physical nature or component.

1.2. Melatonin also had the ability to decrease the bystander effect, induced by γ-radiation *in vitro* when added to culture medium after irradiation of HPV-G cells. Melatonin had the best protective effect. This is possibly the result of the ability of melatonin to enter all cells in the organism, to cross all biological membranes and rapid receptor-dependent and independent transport into cells and their subcellular compartments. As melatonin is highly lipid and somewhat aqueous soluble, it may perform the protective effect in both the lipid and aqueous environments of the membrane. Another possible reason is that melatonin may act directly as a free radical scavenger, or it may activate enzymes involved in antioxidant defence.

1.3. α-Tocopherol compared to melanin and melatonin showed the least (slightly significant) protective effect from bystander factors *in vitro*. α-Tocopherol has a completely different chemical structure from melanin and melatonin, and its mechanism is mainly based on the effect on repair processes.

2. Serum samples from the blood of the victims of the Chernobyl accident were shown to contain damaging factors, induced *in vivo* and after filtration to transfer damage to HPV-G cells *in vitro*, similar to bystander factors. These factors persisted in the blood stream for 19 years (in Chernobyl liquidators cohort) and were not neutralised by melanin or melatonin.

3. For the first time it was shown that laser irradiation was able to induce bystander effects *in vitro* into the medium of irradiated cells, which were able to affect non-irradiated cells.

Further research in the field will include possible understanding of mechanisms by which radioprotective substances can neutralise bystander factors and thus, lead to a possible understanding of mechanisms and nature of radiation-induced bystander effects. Also it is necessary to compare factors from serum samples and those induced by ionising and by laser radiation in order to understand if they have a similar or different nature.

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