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PECULIARITIES OF DNA DAMAGE CAUSED BY EXOGENOUS NITRIC OXIDE COMBINED WITH FRACTIONATED LOW DOSE IONIZING RADIATION IN NORMAL AND TUMOR CELLS

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The aim **of this study was to investigate the reaction of normal and tumor cells to genotoxic effect of widespread environmental factors — exogenous nitric oxides and ionizing radiation.** ***Methods:*** **The animals were treated with NO (125 mg/m³) and low dose ionizing radiation (10 acute exposures with 0.1 Gy each). Genotoxicity was estimated *in vivo* in rats peripheral blood lymphocytes, bone marrow cells and tumor cells of Guerin carcinoma. DNA damages were assessed by alkaline single-cell gel electrophoresis.** ***Results:*** **Exogenous nitric oxides as well as irradiation caused significant increase of DNA damage in all types of investigated cells. The genotoxic effect increased in the order: peripheral blood lymphocytes < bone marrow cells < Guerin carcinoma cells. The greatest genotoxic effect was registered in Guerin carcinoma cells on terminal phase of tumor growth in rats exposed to NO and low dose ionizing radiation.** ***Conclusions*****: Long-term exposure to common environmental factors (exogenous nitric oxides and ionizing radiation) capable to induce DNA damage in different cells. Severity of the genotoxic effect depends on cell type and nature of impacting factors. NO caused more significant DNA damage than low dose ionizing radiation but the highest level of DNA damage was observed after their joint action. Obtained results confirm the real threat of cancer risk increase under combined action of common environmental factors of different nature.**

Key Words**: nitric oxide, nitrosative stress, ionizing radiation, tumor cells, DNA damage.**

According to data of the International Agency for Research on Cancer (IARC), a primary cause of human cancers is environmental pollution, especially — air pollution. Emissions from motor vehicles, power plants, domestic combustion of solid fuels are the main sources of air pollution worldwide [1].

Exogenous factors cause 75–80% of all cancer incidents. Moreover, the chemical carcinogens are responsible for the occurrence of 80–90% of all human malignant tumors. Simultaneous action of harmful factors with carcinogenic or co-carcinogenic activity increase the tumor development probability [2].

One of the main environmental air pollutants worldwide are nitrogen oxides (NOx) [3]. Anthropogenic pollution of environment with radionuclides, as well as expanding of X-ray use and radiographic methods in medical research have led to increased external and internal exposure of humans to ionizing radiation [4].

Exposure to low doses of ionizing radiation (LDIR) increases the probability of cancer as well as other types of diseases. Without causing noticeable immediate response in the body, a LDIR lead to numerous delayed negative consequences. Genetic status, overall health and additional effect of environmental factors are modifying the nature and extent of the biomedical consequences of radiation, chemical and combined influences [5].

Final target for the direct or indirect effects of NO and ionizing radiation is genetic material of cells, which leads to implementation of their acute and prolonged biological effects [6]. The reaction of cells to environmental factors of different nature and their combination depend on cells type, epigenetic status, level of energy metabolism. Proliferative activity and phase of the cell cycle determines the state of the genetic material and the activity of the cell repair system as well.

Data of literature on the impact of prolonged (chronic) combined treatment with environmental factors of low intensity, in particular NO and LDIR on the development of genetic instability and as a result increase of cancer risk are mainly absent [7].

We investigated the dynamics of DNA breaks formation in peripheral blood lymphocytes (PBL), bone marrow cells (BMC) and solid tumor Guerin carcinoma (GC) cells.

MATERIALS AND METHODS

Animals and cell lines. Adult random-bred male rats (120–150 g, 48 animals) were obtained from the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine) and kept at steady state conditions with a constant temperature and natural light. The work with animals was performed according to the rules of local Ethic Committee [8–10]. The animals were divided into four groups: 1) intact control (12 animals); 2) animals that inhaled NOx for 1 month (16 h per day, 12 animals); 3) animals were regularly 10 times irradiated at a dose 0.1 Gy over the period of 1 month (total dose was 1 Gy, 12 animals); 4) animals received combined treatment of NO and LDIR (12 animals).

The study was performed on PBL, BMC and GC cells isolated from rats.

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Abbreviations used: BMC – bone marrow cells; GC – Guerin carcinoma; LDIR – low doses of ionizing radiation; NO – nitrogen oxide; NOx – nitrogen oxides; PBL – peripheral blood lymphocytes; $\%DNA_T$ – the DNA percentage in the tail of "DNA-comet".

BMC were isolated according [11]. Fractionation of the obtained cells was not conducted [12]. Modelling of cancer process was performed by inoculation of rats with 0.5 ml of GC suspension (5×10^6 cells/ml) in saline. Viability of obtained cells was estimated using trypan blue according to [13].

Isolation of PBL. Whole blood was diluted in an equal volume of PBS and stratified on Histopaque1077 ("Sigma", St. Louis, MO) for lymphocyte separation according to the manufacturer's instruction. After isolation, lymphocytes were washed in PBS, diluted in 1 ml culture medium. The amount of the isolated cells was counted after trypan blue staining ("Euroclone", Pero, IT) with a Goryaev's chamber. PBL were suspended in PBS and kept at 4–6 °C before use.

NO inhalation. The inhalation treatment of animals with NO was carried out in 0.1 m^3 chamber equipped with device for input of purified gaseous NO mixed inside with air. Air circulation inside the chamber allowing triple total replacement of air per 1 h. NOx concentration at the chamber's output was 150 mg/m^3 of air, 40% corresponds to NO and 60% — NO_2 of their total content. Concentration of NOx was expressed in mg of NO per m^3 of air. Content of NO in the inhalation chamber was controlled as described previously [14].

X-Ray irradiation. X-Ray irradiation was performed using "RUM-17" (RUT — 250–15–2, USSR, the voltage on the tube — 200 kV, stream — 10 mA, filter — 0.5 mm Cu + 1 mm Al, skin-focus length — 50 cm, irradiation dose — 0.89 Gy/min). Measurements of absorbed dose were conducted using an ionization chamber and ferrosulfate dosimeter.

Alkaline comet assay. The single-cell gel electrophoresis was used for visualizing and measuring single-strand breaks and double strand breaks of DNA in individual cells. The method is based on detection of various mobility of damaged DNA [15]. PBL were washed in PBS and suspended in agarose gel at concentration of $0.5–0.7\times10^6$ cells/ml then processed as previously described [16]. Slides were stained with SYBR Green ("Sigma", 15 μg/ml). The images of comets were observed at ×40–100 magnification with a fluorescence microscope equipped with video camera (CCD, Webbers, USA). One hundred images were randomly selected from each sample and analyzed by an image-analysis program "CometScore" (TriTek Corp, Sumerduck, VA, USA).

The degree of DNA damage was estimated by the DNA percentage in the tail (%DNA_T). %DNA_T — the integrated tail intensity ×100 divided by the total integrated cell intensity for a normalized measure of the percent of total cell DNA found in the tail [17].

Statistical analysis. Statistical analysis was performed using Student's *t*-test. Values are reported as mean ± standard error. Significance level was set at $p \leqslant 0.05$ [18].

RESULTS AND DISCUSSION

Results of *in vivo* DNA damage evaluation in PBL of intact animals and in rats with GC, both affected by NO and LDIR, are presented on Fig. 1.

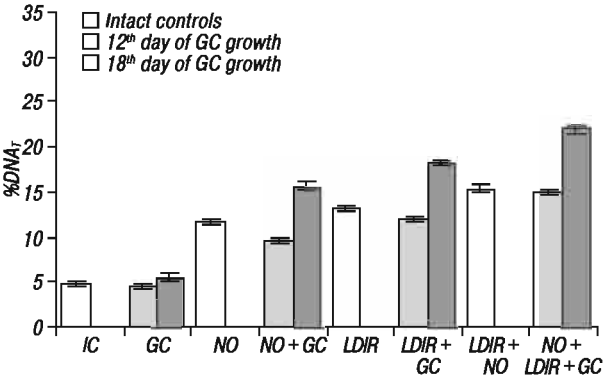

Fig. 1. The level of DNA damage (%) in PBL of rats exposed to NO and/or LDIR

Spontaneous level of DNA damage in animals of the control group was 4.9 ± 0.3%. The level of the studied parameter after GC transplantation varied slightly, rising on 18th day in 1.1-fold.

NO treatment led to increase of DNA damage rate on the 12th day of tumor growth in 2.2-fold compared to intact control demonstrating real genotoxic effect of exogenous NOx due to direct and indirect molecular mechanisms. The difference increased during 18 days, rate of investigated value was in 2.9-fold higher compared to control and in 1.7-fold higher than on the 12th day. Those data indicate the intensification of genetic instability in time after NO inhalation, most likely due to malfunction of cell repair system. LDIR irradiation also caused an increase and persistence of DNA damage in PBL. On the 12th day of GC growth the level of DNA damage exceeded control level in 2.7-fold, and on the 18th day — in 3.3-fold, revealing significant correlation with tumor growth long term after LDIR influence.

Exposure rats to the combined impact of NO and LDIR resulted in significant increase of DNA damage on the 12th day of tumor growth that exceeded control level in 3.4-fold. This value was in 1.6-fold higher than after single treatment with NO and in 1.3-fold when exposed to LDIR. On the 18th day of tumor growth level of DNA damage has exceeded control level in 4-fold. The level of DNA damage in PBL of rats exposed to NO or LDIR alone was exceeded in 1.4- and in 1.2-fold respectively. This indicates the potentiation of genotoxic effect in case of combined impact of both factors, which leads to escalation of genetic instability with term after direct influence.

The results of DNA damage assessment in BMC of intact animals and rats with GC exposed to NO and LDIR are presented in Fig. 2.

Spontaneous level of DNA damage in BMC was 4.7 ± 0.3%. LDIR irradiation caused an increase in DNA damage in 2.6-fold, and inhalation of NO increased genotoxic effects in 3-fold. Combined effect of NO and LDIR resulted in increase of DNA damage in 4-fold compared to controls and, respectively, in 1.5- and

1.3-fold — after individual treatment with both factors. Higher genotoxic effect of NO compared to LDIR demonstrating a real threat of NO to hematopoietic system.

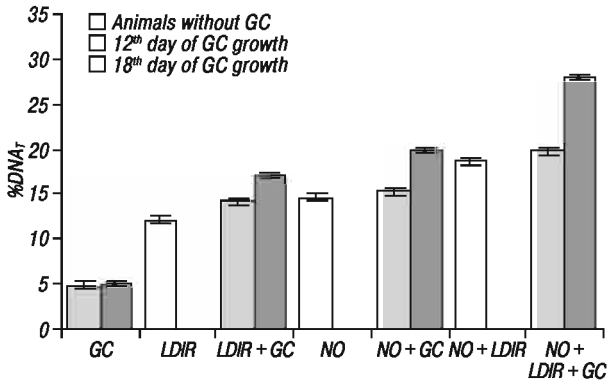

Fig. 2. The level of DNA damage (%) in BMC of rats exposed to NO and/or LDIR

GC transplantation do not resulted in a significant increase of spontaneous DNA damage level in BMC on 12th and 18th days of tumor development. It equals to 5.0% of damaged DNA, which is slightly (in 1.1-fold) higher than its value in intact animals. Thus, GC growth itself does not causes significant changes, leading to damage of the genetic material of hematopoietic cells.

NO inhalation led to an increase of DNA damage in 3.2-fold on the 12th day of GC growth. On the 18th day the level of DNA damage exceeded control value in 3.9-fold, and in 1.3-fold — a value on the 12th day of GC development. It shows increased genotoxic NO impact on more intensively proliferating BMC as compared to PBL on the background of tumor process.

LDIR irradiation caused an increase of DNA damage in BMC in 2.8-fold. On the 18th day after treatment the level of DNA damage was in 3.5-fold higher compared to control and in 1.3-fold higher than on 12th day. Despite the lower overall intensity of LDIR genotoxic effect on BMC it exceeded DNA damage induced by irradiation in PBL.

The biggest increase (4.0- and 5.5-fold) of DNA damage relatively to control group was registered on 12th and 18th days of GC growth in BMC of rats exposed to NO and LDIR. It exceeded an individual effects of NO and LDIR in 1.3- and 1.4-fold, respectively. Joint genotoxic effect of both factors in BMC was in 1.4-fold stronger than relatively less proliferating PBL.

NO caused more significant DNA damage to both types of investigated cells. This can be explained by polymorphism of pathways involved in implementation of genotoxic effect of NO by means of inhibition of repair enzymes, initiation of nitrosative stress, formation of reactive derivatives (such as peroxynitrite) and direct chemical interaction with DNA molecules.

Despite the wide range of identified tumor cells genome anomalities (various types of microsatellite instability and chromosomal aberrations), molecular basis of such violations in the case of each tumor remains uncertain. In this regard, a study of DNA damage in GC cells exposed to separate and combined treatment with NO and LDIR was conducted and results are presented in Fig. 3.

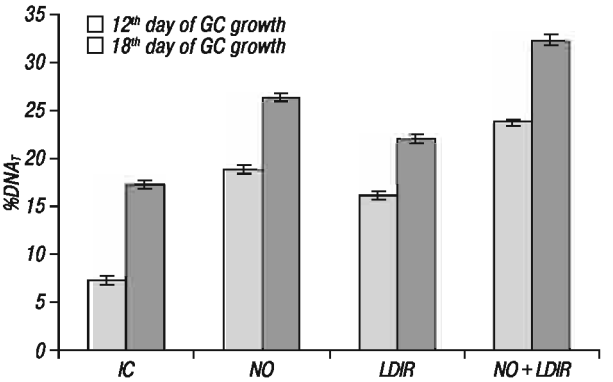

Fig. 3. The level of DNA damage (%) in GC of rats exposed to NO and IR

Spontaneous levels of DNA damage in GC cells on 12th day of tumor growth exceeded values for PBL and BMC in 1.7-fold. On the 18th day of GC growth excess of DNA fragmentation reached 3.5-fold, and was in 1.6-fold higher than on 12th day indicating an intensification of the processes that leads to DNA damage and the development of genetic instability in the GC cells during tumor growth.

LDIR treatment led to an increase in the level of DNA damage after 12 days of GC development — in 2-fold, and on the 18th day — in 1.6-fold compared to controls. Prolonged NO impact caused an increase of DNA damage in 2.3-fold on 12th day and in 1.9-fold on 18th day of GC growth, compared to corresponding control values. Obtained data indicate higher sensitivity of actively proliferating GC cells to both investigated environmental factors, than normally proliferating somatic cells. Combined effect of both factors increased in 2.9-fold the level of DNA damage on 12th days of tumor growth compared to control group thus exceeding in 1.3–1.5-fold separate action of NO and LDIR.

Accordingly to obtained results and literature data [19], the response of cells to separate and combined effects of environmental factors of different nature may depend on the proliferative activity of cells. Proliferative index — dynamic parameter that reflects, as for BMC, existing level of hematopoietic cells generation. Proliferative index also directly related to the changes that occur in the BMC during cells maturation and differentiation of precursor cells to certain subtypes [20].

Genotoxicity of NO and LDIR are more pronounced in somatic cells with relatively higher proliferation rate — BMC [21] *vs* PBL [22]. In 3.5-times intensively proliferating cancer cells (GC cells) [23] exposed to investigated factors revealed even higher level of DNA damage than normal somatic ones, providing the basis for development of genetic instability.

Malignization leads to cell cycle deregulation and modification of proliferative activity. Creating the heterogeneity of cell populations, genetic instability provides the material for the selection of increasingly

autonomous and aggressive cells. Genetic instability provides the preconditions for tumor progression which begins in the precancerous period [24].

Thus, the assessment of DNA damage in cells exposed to NO and LDIR revealed the ability of both factors to induce notable genotoxic effects in the way of formation a single — and double strand DNA breaks. NO caused more significant DNA damage than LDIR but the highest level of DNA damage was observed after the joint action of investigated factors.

The elevated combined genotoxic effect of NO and LDIR (that cannot be reduced to the sum of their individual effects) partially can be explained by existence of common mechanisms for the implementation of genotoxic effects for both factors (formation of reactive oxygen and nitrogen species), as well as individual significant contribution of NO due to chemical DNA modification, peroxynitrite formation and inhibition of repair enzymes. Obtained results about increased level of induced DNA damage confirms the development of genotoxic lesions and real threat of cancer risk increase.

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