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EXOGENOUS NITRIC OXIDE POTENTIATE DNA DAMAGE AND ALTER DNA REPAIR IN CELLS EXPOSED TO IONISING RADIATION

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The aim **of this study was to investigate impact of exogenous nitric oxide (NO) on generation of different types of DNA damages, their transformation, and specificity of DNA repair in cells treated with ionizing radiation (IR).** ***Methods:*** **levels of single-strand and double-strand breaks assessed in peripheral blood lymphocytes (PBL) isolated from healthy humans and treated** ***in vitro*** **with NO donor — S-nitrosoglutathione (GSNO) and IR. The rate of DNA repair estimated after 30 and 60 min of PBL treatment. The visualization and measuring the number of prompt and delayed DNA damages, including strand breaks, apurinic and thermolabile sites performed with single-cell gel electrophoresis.** ***Results:*** **IR caused dose-dependent generation of single strand breaks (SSBs), double strand breaks (DSBs), and heat-labile sites (HLS) in cell DNA. However, particularly destructive was combined treatment IR with GSNO as NO donor that leads to a significant increase of DNA damage and a dose-dependent inhibition of the DNA repair rate. Obtained data proofs the ability of NO to inhibit fast and slow stages of SSBs, DSBs, and HLS repair resulting in significant growth of genotoxic effect. DNA breaks generation from HLS is able to affect DSBs yields especially in cells with altered DNA repair. The process of DNA repair of delayed DSBs formed from HLS was quite different from removal of DNA damages occurring immediately after treatment and was characterized by IR dose dependent inhibition of DNA repair.** ***Conclusion:*** **High level of DNA strand breaks, that are generated after the combined treatment with NO and IR, are accumulated for quite a long time after exposure due to altered DNA repair, indicating the development of genetic instability and increase of carcinogenic risk for organism exposed to combination of harmful environmental factors.**

Key Words: **exogenous nitric oxide, DNA damage, DNA repair, combined environmental factors.**

Influence of environmental factors plays a crucial role in the formation and development of many types of pathology, especially related to the occurrence of about 90% of malignant tumors [1]. Genetic disorders and environmental factors may interact resulting in increase of sensitivity to the action of exogenous toxicants in biological objects [2]. Numerous chemical pollutants of the soil, water, and air are toxic or carcinogenic agents themselves and able to cause pathological changes in the human organism, or give rise to diseases indirectly. The variety of harmful influences endanger the simultaneous action of several factors, and necessitates an experimental study of general regularities of interaction of physical and chemical environmental factors, prediction and optimization of effects induced at the combined influences.

The negative impact of factors can rise under their joint influence and is most dangerous for human organism. In addition to the direct negative effect on living organisms such factors cause long-term effects that can manifest as cancer or diseases manifested in subsequent generations. In this regard, a problem of complex analysis of reactions to common exposure to harmful agents of different nature becomes relevant and essential.

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Abbreviations used: DSB – double strand break; GSNO – S-nitrosoglutathione; HLS – heat-labile sites; IR – ionizing radiation; NO – nitric oxide; RNS – reactive nitrogen species; ROS – reactive oxygen species; SSB – single strand break.

One of the most dangerous to living organism's factor of physical nature is ionizing radiation (IR). In close perspective, nearly two-third of all cancer patients will receive radiation therapy during treatment [3], but diversity of occurring effects and mechanisms of post-irradiation recovery normal and cancer cells is not completely studied.

Certain environmental factors may complicate adverse effects of IR. Exogenous nitric oxide (NO) belongs to the group of common air pollutants with the annual increase in emission to the atmosphere [4]. Acute and long-term effects of the combined action of NO and IR can result in development of genetic instability in the descendants of irradiated cells and can be considered as potentially carcinogenic [5].

Chromosomal DNA is the most sensitive cellular target for IR. The genome is under constant exposure to endogenous and exogenous metabolites and environmental factors that can damage the chemical structure of DNA and alter the expression of certain genes, resulting in the development of genomic instability. Cell death induced by IR partially is the result of altered repair or misrepair of complex lesions in DNA. An increase in chromosomal aberrations, due to inappropriate recombination or repair of DSBs, is a hallmark of chromosomal instability and cancer predisposition disorders.

In vitro studies on cellular models indicate that exogenous NO and NO-containing complexes formed during its metabolism are capable to direct and mediated genotoxic effect [6]. It is shown that gaseous NO are able to cause the formation of oxidized purines, breaks in DNA, deamination, DNA junctions, transitions, transversions [7]. Reactive nitrogen species

(RNS) can damage DNA in a large number of different pathways with formation chemical and structural lesions of all components of the DNA molecule: desoxyribose core, purines and pyrimidines, resulting in the formation of specific sites of damage [8].

Autooxidation of NO with the formation of intermediate deaminating substance — N_2O_3, free diffusion in the form of NO and the formation of peroxynitrite in the reaction with superoxide leads to the formation of a wide range of genotoxic derivatives, carcinogens, DNA strand breaks, deamination and mutagenic effects [9].

NO can inhibit repair enzymes due to nitrosation of cysteine residues in their active site (in the case of ligases) or nitrotyrozyne formation, resulting in partial or complete loss of enzyme activity. NO may affect DNA repair processes not only by nitrosylation reactions but also potentially via kinase signaling cascades. NO activates soluble guanylyl cyclase, generating cGMP, which in turn stimulates protein kinase G activity. Kinase cascades have been shown to modulate DNA repair processes by phosphorylating DNA repair enzymes [10].

Due to single ionizations or ionization clusters formation, IR generates base and sugar damages in the DNA. Many of the lesions induced by IR are chemically similar to those induced as byproducts of oxidative metabolism. However, IR also induces complex damage known as clustered DNA damage sites (CDSs). Clusters of ionization can generate clusters of DNA damage with different sizes and diverse damage composition [11]. CDSs are signatures of DNA modifications induced by IR in mammalian cells.

Both genotoxic factors are able to generate a variety of DNA lesions, such as single-strand breaks (SSBs), double-strand breaks (DSBs), apurinic or apyrimidinic sites (AP sites), a number of base modifications, sugar modifications, DNA-DNA and DNA-protein cross-links [12]. IR is capable to generate about 1000 SSBs and 25–40 DSBs per diploid cell per Gy depending on type of cell. However, the response to these lesions can differ widely for different cell types [13].

Breaks of one or both DNA strands are the most frequently studied lesions. This is because of their important contribution to the toxic, mutagenic, clastogenic and carcinogenic effects of exogenous and endogenous factors. DNA strand breaks arise from attack of the sugar phosphate backbone either by direct DNA-factor interaction or by radicals — attack-induced reactive oxygen and nitrogen species (ROS and RNS) (indirect effect) [14].

There are two types of breaks, "active" and "inactive". An active break is a break that had the possibility to misrejoin with another break, while an inactive break was assumed to never misrejoin. Inactive breaks correspond to 50–70% of all breaks [15].

SSBs in one strand of the DNA and are usually accompanied by loss of a single nucleotide and by damaged 5′- and/or 3′-termini at the site of the break. Without correct repair SSBs pose a serious threat to genetic stability and cell survival. SSBs commonly originate from endogenous ROS DNA oxidation. SSBs can occur directly by disintegration of the oxidized sugar or indirectly during the DNA base-excision repair of oxidized bases, abasic sites, or bases damaged or altered in other ways. Another source of SSBs is malfunction of some cellular enzymes, for example DNA topoisomerase 1. The most likely consequence of unrepaired SSBs in proliferating cells is the blockage or collapse of DNA replication forks during the S phase of the cell cycle, possibly leading to the formation of DSBs [16].

Presence of SSBs in opposite DNA strands in the limits of one or two DNA helical turns can lead to DSBs. Regular repair of the DNA sugar-phosphate backbone within CDS containing base damages or blocked replication forks can be the source of DSBs. Exogenous chemical agents and endogenous ROS also can induce DSB. DSBs are among the most deleterious lesions because they are affect both complementary DNA strands that complicates repair by utilize the complementary sequence on the opposite strand as a template to ensure correct and efficient repair. DSBs result in chromosomal aberrations that may cause apoptosis or tumorigenesis. Several human cancer predisposition syndromes, such as ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS), are characterized by chromosome instability and sensitivity to DSB-causative agents [17].

Additionally, DNA lesions are able to convert to each other. Sugar damage can disrupt the sugar-phosphate backbone to generate SSBs. CDSs containing SSBs or thermal transformations of SSB are able to form DSBs, which can have severe biological consequences [18].

Genotoxic factors, such as NO and IR, can induce heat- or alkaline-labile sites that are repaired by non-DSB pathways in the cells. Such sites are able to convert into DSB during cell lysis and may contribute to approximately 30% of all measured DSBs (delayed breaks). The delayed breaks may not be present as breaks in the cell if they are repaired sufficiently fast (within a few hours), but will only be present as heat-labile sites (HLS) because their hydrolysis is accelerated with increasing temperature. This type of lesion is not observed *in vivo*. The exact nature of HLS is not known, but they are not simply an artifact of lysis protocols. HLS are a spectrum of lesions with different chemical and thermal sensitivities. Their detection and quantification is of great importance considering biological significance and danger of genetic instability [19, 20].

There is evidence for presence of thermally labile DNA lesions in cells maintained under physiological temperatures and their involvement in DSB formation. The proportion of DSBs generated due to HLS during high-temperature lysis is species specific [21].

Because of delayed DBSs formation, the total load of DSBs on cell will be the sum of induced breaks and those generated within a non-DSB-CDS by the conversion of a HLS to a DBSs. It is not known whether prompt DBSs and late DBSs are detected and processed by the cell with the same efficiency. The term "prompt breaks" has been used to describe breaks present immediately after irradiation, and "total

breaks" to describe all those present after the labile sites are converted to breaks [22].

The normal DNA repair is essential to prevent the development of metabolic disorders and cell signaling pathways that may lead to its oncogenic transformation. Repair itself does not always increase survival, and survival is the outcome of several pathways that can be both cell- and tissue-specific. A range of interconnected cellular response mechanisms has evolved to enable efficient repair and thus protect the cell from the harmful consequences of un- or misrepaired breaks that may include early effects such as cell killing and associated acute toxicity and late effects such as cancer [23].

Complex nature of environmental pollution requires the development of an effective system of environmental monitoring. Evaluation of combined effects of adverse factors by methods of molecular biology is complementary to widespread physical and chemical methods and allows assessing the total effect of toxicants directly on the organism.

In this regard, the development of methodological framework and approaches are of great importance to estimate the combined effect of different types of pollutants. The study of total DNA breaks generation and the dynamic of repair processes as biomarkers of genetic instability and carcinogenic risk provides such opportunity [24].

Investigation of association between generation of different types of DNA damages, their transformation, and involved repair mechanisms could provide understanding of molecular mechanisms, involved in carcinogenic effects of multiple environmental factors.

MATERIALS AND METHODS

Genotoxicity was assessed in PBL isolated from healthy humans for an *in vitro* study. An informed consent of donors for taking blood samples and conducting cytogenetic studies was obtained. The total and particular type of DNA damage (SSBs, DSBs, and HLS) estimated in three temporal intervals after PBL treatment: immediately after exposure, 30 and 60 min later to allow processing of DNA lesions by repair enzymes. During this period, cells were kept at 37 °C in humidified atmosphere.

The cells were divided into 4 groups: 1) intact control (IC); 2) cells, treated with nitrosoglutathione (GSNO, 1 mM); 3) cells, irradiated with X-rays at exposure of 2.5; 5; 7.5 and 10 Gy (IR); 4) cells that received combined treatment of GSNO and IR.

Synthesis of GSNO. GSNO was used as a source of NO and synthesized from the reaction between equal molar concentration of reduced glutathione (GSH) and acidified sodium nitrite at 4 °C in the dark for 1 h. GSNO concentration were determined using extinction coefficient $\varepsilon_{334} = 900\ m^{-1}cm^{-1}$ at 334–336 nm [25].

X-ray irradiation. Suspended in gel PBLs were exposed to X-rays with doses range 2.5–10 Gy and the dose rate of 0.89 Gy/min.

PBL isolation. Whole blood was diluted in an equal volume by PBS and loaded on Histopaque-1077 ("Sigma", St Louis, MO) for lymphocyte separation according to the manufacturer's instruction. After isolation, lymphocytes were washed in PBS, counted using trypan blue ("Euroclone", Pero, IT) exclusion staining and kept at 4–6 °C before use.

Alkaline Comet Assay. The single-cell gel electrophoresis was used for visualization and measuring the total number DNA strand breaks including apurinic sites in PBL [26]. Cells were processed as previously described [27].

Neutral Comet Assay. The neutral modification of single-cell gel electrophoresis method was performed for identification and quantification of DNA double-strand breaks. The assay was carried out according to [28].

High-temperature Comet Assay. This modification of neutral comet assay for detection of total DSBs level including prompt and delayed breaks within HLS was conducted as described in [29]. Lysis was carried out at 50 °C for 2 h. Electrophoresis was conducted with 1% agarose gel at 1.0 V/cm for 1 h in TBE.

Slide staining and processing. Stained with SYBR Green I ("Sigma", 15 μg/ml) slides were observed at ×40–100 magnification using a fluorescence microscope equipped with video camera (DCM 520, Science Lab, 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 of comet" (% DNA_T).

Statistical analysis. Statistical analysis was performed using Student's *t*-test. Values are reported as mean ± standard error. Significance level was set at $p \leqslant 0.05$ [30].

RESULTS AND DISCUSSION

The X-ray irradiation of PBL caused elevation of the total level of DNA damage in dose-dependent fashion with maximum genotoxic effect registered after 10 Gy irradiation. An hour after treatment the level of DNA strand breaks decreased 1.5–4-fold due to dose-dependent activity of DNA repair system (Fig. 1).

The treatment of PBL with GSNO alone led to a slight increase in DNA damage (1.25-fold, compared with IC cells). The level of DNA strand breaks did not change during next hour. Combined treatment with GSNO and IR significantly enhanced the level of DNA breaks in comparison with the effect caused by individual agents. DNA damage after joint action of both factors increased with growth of irradiation dose and immediately after exposure exceeded an individual effect of IR 1.5-fold. However, even an hour after treatment the combine genotoxic effect significantly exceeded (2.2–2.3 fold) the level of DNA damages typical for equal dose of IR (see Fig. 1).

This indicates not only persistence of a certain level of DNA damage but also significant inhibitory effect of GSNO on the rate of DNA repair in long periods after irradiation, which may suggest the development

of genetic instability by disrupting of normal functioning of repair system.

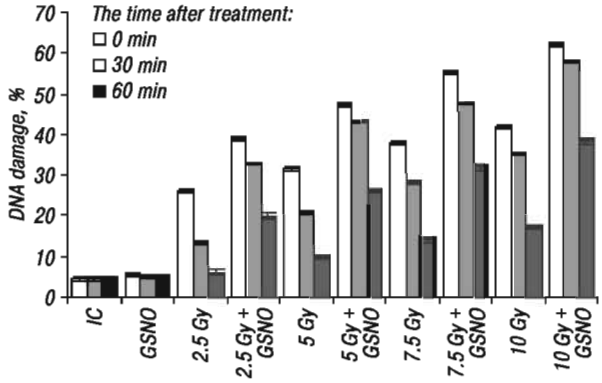

Fig. 1. The total level of DNA damage measured by alkaline comet assay after individual and combined treatment with GSNO and IR

The initial number of DNA damage reduced within an hour but effectiveness of DNA repair was largely dependent on dose and type of damaging factors.

The rate of DNA repair was highest at 2.5 Gy of IR. Increase of irradiation dose reduced rate of repair 1.8-fold, reaching its minimum value at 10 Gy, when only less than a half of the total DNA damages were restored (Fig. 2). This phenomenon suggests inhibitory effect of IR on DNA repair enzymes probably due to generation of ROS and RNS. Additional treatment with GSNO resulted in practically total inhibition of DNA repair system and a dose-independent rate of damage restoration. Thus, combined treatment with GSNO and 2.5 Gy of IR decreased repair efficiency 2.2-fold compared with the effect of IR (see Fig. 2). Similar effect was observed after increased doses of IR (1.5–1.8 fold).

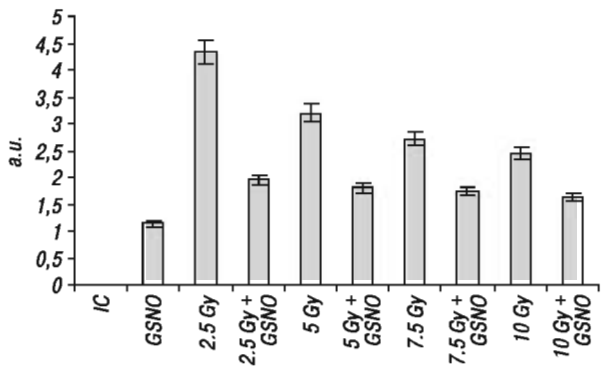

Fig. 2. Dynamics of DNA repair after individual and combined treatment with GSNO and IR as measured by alkaline comet assay

Inhibition of DNA repair processes after GSNO treatment may be caused by release of NO and subsequent RNS generation (for example, peroxynitrite). NO_x are able to inhibit enzymes of repair system due to nitrosylation of cysteine residues in their active site or nitrotyrosine formation resulting in partial or complete loss of its activity [31].

Biological significance and potential danger of DSBs made necessary their distinct detection from the total DNA damage. Formation of DSBs in PBL treated with IR, registered by neutral comet assay, showed dose-dependent increase with irradiation dose. Basic level of DSBs was determined to be 4.0-fold lower compared to SSBs, and amounted to 1.1% of total DNA damage (Fig. 3).

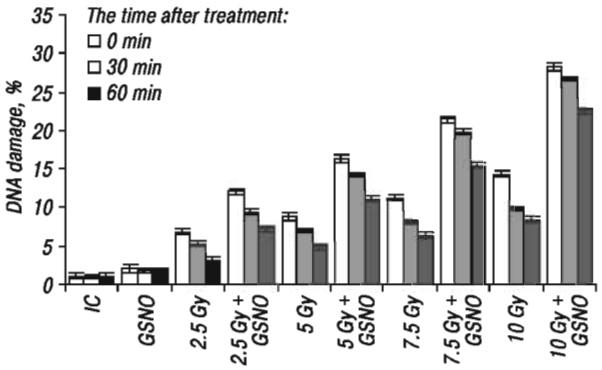

Fig. 3. The level of DSBs measured by neutral comet assay in DNA of PBL treated with GSNO and IR

The level of DSBs increased proportionally to irradiation dose exceeding the control value in 6–13-fold. Treatment with GSNO alone resulted in moderate elevation of DNA damage. However, combined treatment of IR and GSNO caused 1.8–2-fold dose-dependent increase of the DSBs level compared to DNA damage in PBL treated with IR alone. Incubation of PBL during 30 or 60 min after end of the treatment resulted in significant decrease of DSBs level in DNA, but even after this interval relatively high quantity of DNA damage was observed. Additional treatment with GSNO resulted in accumulation of 1.6-2.7 folds more damages compared to observed level in the control groups.

DSBs are thought to be the main lesion involved in cell killing and formation of chromosomal aberrations. DSBs are one of the most toxic and mutagenic DNA lesions detected in human cells: a single DSB can potentially lead to loss of more than 100 mln base pairs of genetic information [32]. Deficiencies in DNA-damage signaling and repair pathways are fundamental to the etiology of most human cancers [33]. The obtained data indicates ability of GSNO to affect and disrupt both mechanisms of SSBs and DSBs repair. High level of DNA DSBs that are generated by the combined action of NO and IR are stored for quite a long time after exposure, indicating the development of genetic instability and rise of carcinogenic risk.

Repair of DNA DSBs is essential to the maintenance of genomic integrity. Genetic polymorphisms in double-strand break repair genes may influence DNA repair capacity and, in turn, confer predisposition to cancer. Repair of DSBs thought to have biexponential kinetics [34].

The effectiveness of DNA DSBs repair in PBL treated with GSNO and IR shown on Fig. 4.

Similarly to dynamics of DNA SSBs repair, the rate of DSBs repair was mainly dependent on the dose of NO and IR. The highest speed of this parameter was observed at 2.5 Gy, and lowest — at 10 Gy dose of IR. Presence of GSNO altered DNA repair. In contrast to the SSBs, differences in the level of inhibition of DSBs repair were almost independent of IR dose, and varied slightly — 1.2–1.4-fold. It should be noted

that the average effectiveness of DSBs repair was 1.4-fold lower compared to SSBs repair.

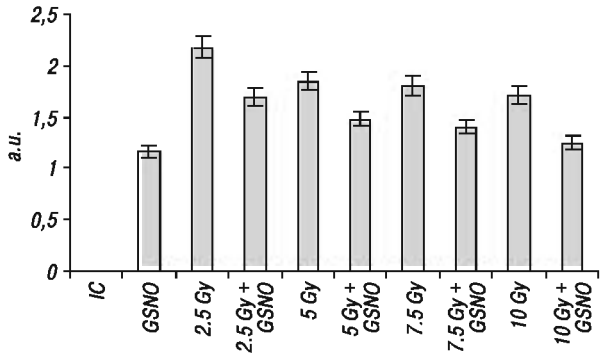

Fig. 4. Dynamics of DNA DSBs repair estimated by neutral comet assay in DNA of PBL treated with GSNO and IR

Accurate repair of DNA DSBs is essential to normal cell. Defective DNA DSB repair can lead to toxicity and large scale sequence rearrangements that eventually may cause cancer. Repair of DSBs is much more complicated and resource-dependent process than recovery of SSBs. There are two distinct and complementary mechanisms for DNA DSB repair — homologous recombination (HR) and non-homologous end-joining (NHEJ), each having several stages and relatively low speed [35]. Many proteins and strictly coordinated activity of numerous genes are required for efficient HR and NHEJ. Substantial amounts of these proteins are cysteine and tyrosine — containing enzymes, potential targets for nitrosylation and inactivation by GSNO. Because of associated lesions, not all DNA ends within DSB or CDSs are readily ligatable. Described above peculiar features of DSBs repair may explain obtained in our experiment inhibition of DNA repair caused by elevated amount of reactive oxygen and nitrogen species due to treatment with NO and IR.

Some kinds of DNA lesions may interfere with the repair of DSBs, affect the measurement of their induction, and repair. To determine the total amount of DSBs including delayed DNA breaks as potential source of DSBs, determination of HLS was performed. Basic level of HLS lesions was 3-fold higher than baseline of DSBs (Fig. 5).

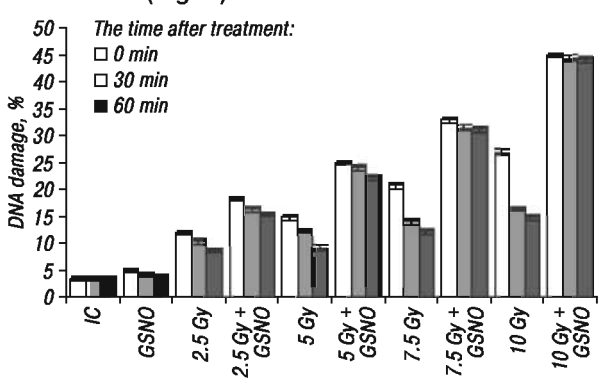

Fig. 5. The level of DNA damage from HLS estimated by high-temperature comet assay in PBL treated with GSNO and IR

Treatment with GSNO resulted in 1,5-fold excess of DNA lesions, which did not change significantly with time, and after an hour their level was 1,3-fold lower than the initial. Irradiation of PBL caused 3.6–8.2-fold dose-dependent increase in the level of DNA damage. Compared dose-by-dose, additional DNA damage originated from HLS in 1.7–1.9-fold exceeded level of DSBs measured by neutral comet assay. Combined treatment with GSNO led to 1.5–1.7-fold increase of DNA damage in comparison with genotoxic effect of corresponding dose of IR alone. During an hour after treatment level of DNA damage decreased 1.4–1.7-fold indicating significant accumulation of DNA breaks.

Interesting to note that cells treated with both factors had relatively low level of DNA repair. Beginning with dose of 5 Gy and higher this type of DNA damage actually was not restored even one hour after exposure. At the same time, the differences between DNA damage in PBL treated with IR alone were well observed and statistically significant.

Increase in the yield of DSBs from the hydrolysis at elevated temperatures caused by transformation of HLS to SSBs and subsequent formation of DSBs. The origins of processes that convert HLS to SSB remain uncharacterized, but may include base-catalyzed hydrolysis or oxidation [36]. Reactive end groups at SSBs can attack the opposite strand at high temperature forming DSB. Double-stranded HLS and/or SSBs opposite to HLS can also be converted into a DSB. Conversion of HLS to DNA breaks and ultimately to DSBs occurs in cells during the first hour of post-irradiation incubation at physiological temperatures [37], thus, HLS-dependent DSBs are not a technique-related artifact.

Regardless of the source of DSBs formation, their additional generation from HLS is able to affect DSBs yields and in cells with altered DNA repair may have important biological consequences and therefore require additional studies [38, 39].

The process of DNA repair of additional DSBs formed from HLS was quite different from removal of the same type DNA damages arisen in PBL immediately after treatment with GSNO and IR as shown on Fig. 6.

Cells treated with IR alone revealed 1.4–1.8-fold dose dependent increase of repair intensity. On the contrary, combined treatment with GSNO and IR caused dependent from IR dose inhibition of DNA repair.

Observed phenomenon can be attributed to the generation of SSBs and DSBs during temperature-dependent HLS transformation with time, which activates the pathways of DNA damage response system, and thus stimulates HR and NHEJ processes by synthesis and activation of signaling or/and ancillary repair proteins [40]. Another explanation is HLS and SSBs causing indirect DSBs may be processed by the cell using repair pathways distinct from the repair of DSBs [41]. Conformingly, repair of damages arisen from HLS was shown to be independent from functional end-joining, XRCC1 or poly(ADP-ribose) polymerase 1 [42].

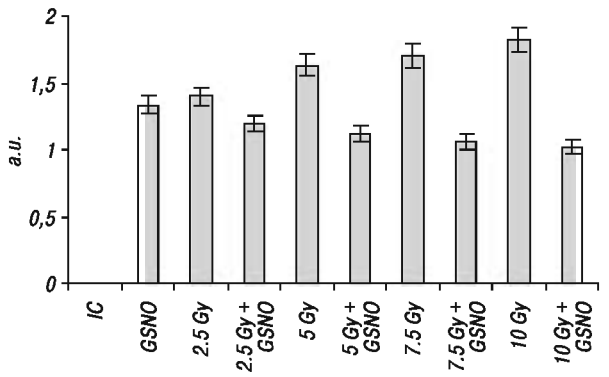

Fig. 6. Dynamics of DNA repair of lesions originated from HLS assessed by high-temperature comet assay in PBL treated with GSNO and IR

Possible interpretation is that fast repair after high-temperature lysis does not reflect repair of DSBs but rather repair of SSBs and HLS within the CDSs that form DSBs only after exposure to the high lysis temperatures. Removal of these lesions by fast-operating non-DSB repair pathways will appear in the high-temperature lysis assay as repair of DSBs. An anticipated consequence of this interpretation is that inhibition of pathways implicated in the repair of SSBs or base damages should inhibit the initial fast rejoining after high-temperature lysis [43].

In conclusion, IR caused dose-dependent generation of SSBs, DSBs and HLS in cell DNA. Additional treatment with GSNO as NO donor leads to a significant increase of DNA damage and inhibition of DNA repair. Attention should be drawn to considerable amount of delayed DNA lesions that are not actually taken into account when using standard techniques. Their ability to convert into DSBs even under physiological conditions may significantly increase total damaging effect on DNA, to be a source of misjudged genotoxic effect, and as a result noticeably increase level genetic instability and rise of carcinogenic risk.

There was also a dose-dependent inhibition of the DNA repair rate, which was most evident after combined treatment with GSNO and IR. Obtained data proofs the ability of NO to inhibit fast and slow stages of SSBs, DSBs, and HLS repair resulting in significant growth of genotoxic effect. A possible mechanism for this phenomenon is the ability of NO to suppress the activity of repair enzymes, resulting in significant disturbances in the normal course of the DNA repair. High levels of DNA strand breaks, that are generated after the combined treatment with NO and IR, are accumulated for quite a long time after exposure, indicating the development of genetic instability and increase of carcinogenic risk for organism exposed to various harmful factors in environment.

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