Effect of dose and dose rate of gamma irradiation on the formation of micronuclei in bone marrow cells isolated from whole-body-irradiated mice

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Abstract
It is well-known that the cytotoxicity and mutagenic effects of high dose rate (HDR) ionizing radiation (IR) are increased by increasing the dose but less is known about the effects of chronic low dose rate (LDR). In vitro, we have shown that in addition to the immediate interaction of IR with DNA (the direct and indirect effects), low doses and chronic LDR exposure induce endogenous oxidative stress. During elevated oxidative stress, reactive oxygen species (ROS) react with DNA modifying its structure. Here, BL6 mice were exposed to IR at LDR and HDR and were then sacrificed 3 hours and 3 weeks after exposure to examine early and late effects of IR. The levels of micronuclei, MN, were determined in bone marrow cells. Our data indicate that the effects of 200 mGy on MN-induction are transient, but 500 and 1000 mGy (both HDR and LDR) lead to increased levels of MN up to 3 weeks after the exposure.

KEYWORDS
DNA damage, dose rate, ionizing radiation, micronuclei, systemic effects

INTRODUCTION

Ionizing radiation (IR) targets primarily DNA molecules and gives rise to a range of lesions, including base damages, single-strand breaks (SSBs), and double-strand breaks (DSBs) (Belli et al., 2002; Li et al., 2001). The IR dose associated with a nuclear accident, occupational exposure or a diagnostic radiation is usually in the low or medium dose range (below 200 mGy) and sometimes at a low dose rate. Currently, cancer risk estimates for low doses of IR are based on extrapolation of results from epidemiological studies of populations exposed to relatively high doses at high dose rates. This causes uncertainty in the estimation of risks associated with low doses and low dose rates.

Exposure of cells to an acute dose of IR leads to dose-dependent production of different types of DNA damage, for example, 1 Gy gamma radiation produces ~40 DSBs, ~1000 SSBs, and ~1000 DNA base damages. Depending on its linear energy transfer (LET), IR can form different levels of complex DNA damage which contains one or several DSBs in close proximity to base damages and other types of DNA damage (Hada & Georgakilas, 2008). A link between the induction of complex DNA damage, incomplete DNA repair and continuous activation of the immune system has already been suggested by Mavragani et al. (2017). A complex and persistent DNA damage can also activate pro-inflammatory macrophages (Eken et al., 2019; Nastasi et al., 2021; Teresa Pinto et al., 2016) which then resulted in increased production of myeloperoxidase, MPO (Mishra et al., 2020).
MPO is involved in the production hypochlorous acid, a precursor of free radicals (Ndrepepa, 2019) which leads to elevated oxidative stress. Chronic inflammation and elevated oxidative stress have been suggested to be involved in the adverse health effects of exposure to IR (Eken et al., 2019; Halle et al., 2010; Rodrigues-Moreira et al., 2017).

Our previous study pinpointed that the level of the oxidative stress marker, extracellular 8-hydroxy-2’deoxyguanosine (8-oxo-dG), from cells exposed to a low dose of gamma radiation was several fold higher than expected to be formed by the immediate action of radiolysis of cellular water (Haghdooost et al., 2005). Our studies indicate that low doses and low dose rates of gamma radiation are potent inducers of reactive oxygen species (ROS) (Godoy et al., 2020; Haghdooost et al. 2005, 2006; Sangsuwan & Haghdooost, 2008) probably through mitochondrial dysfunction (Yoshida et al., 2012). ROS at high concentration react with intracellular biomolecules, such as lipids, proteins, and DNA, and modify their structures. At the cellular level, ROS can induce mutations through direct induction of DNA damage or through oxidation of deoxyribonucleotide triphosphates which are then incorporated into DNA during replication (Michaels & Miller, 1992).

Our hypothesis was that exposure of an organism to equal doses of IR at low and high dose rates, respectively, may induce different levels of genotoxicity shortly after and long after irradiation. For this reason, we examined the influence of dose, dose rate and time after exposure on micronucleus (MN) levels as a marker for DNA damage in cells isolated from bone marrow of whole body irradiated mice. The mice were exposed to doses relevant for radiation protection; 100, 200, 500, and 1000 mGy administered at low dose rate (LDR 1.4 mGy/h) or high dose rate (HDR, 6 mGy/s) and the animals were sacrificed 3 h and 3 weeks after exposure.

2 | MATERIALS AND METHODS

2.1 | Animals and irradiation

4–6 weeks old female wild type mice C57BL/6N Crl (Scanbur, Karlskunde, Denmark) were housed 1 week in the animal facility prior to exposure. The mice were exposed to total doses of 100, 200, 500, or 1000 mGy administered at 1.4 mGy/h for LDR or 6 mGy/s for HDR at the Experimental Core Facility-Stockholm University for animal studies (ECF). The LDR animal exposure was performed in a cabin that fits four cages on top of each other containing a low-active $^{137}$Cs source at the bottom. The dose rate was adjusted by using shielding and modifying the distance to the source. The dose rate at the highest cage was 1.4 mGy/h, measured by a PTW UNIDOS E Universal Dosimeter equipped with a Farmer Ionization Chamber Type 30010. For HDR irradiation, we have used the machine Scanditronix, ($^{137}$Cs) with a dose rate of 6 mGy/s.

A corresponding number of control animals were housed in a sham setting to have identical conditions where temperature and humidity were controlled daily. The age of the animals and the time points for the start of the irradiation were kept similar for different exposures. A total number of 96 mice were used for the present investigation, three animals per dose, dose rate and time after exposure, and three animals sham irradiated at the same conditions. The irradiated and corresponding sham-irradiated animals were sacrificed by cervical dislocation 3 h and 3 weeks after exposure, as illustrated in Table 1. The bone marrow cells were isolated and processed for MN assay. All experiments were conducted at the ECF in accordance with the Swedish Ethical Committee (SBA) (Dnr N 112/15).

2.2 | Bone marrow extraction

Dissection was performed immediately after cervical dislocation, working in teams of two persons. After isolating the bone and removing the muscle tissue around, the epiphyses of the femurs were removed and the bone marrow was flushed using the RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) containing 20% bovine calf serum (Thermo Fisher Scientific, Waltham, MA). The media containing bone marrow were kept in a freezer at −150 °C after adding 10% DMSO (Sigma-Aldrich).

2.3 | Cytokinesis-block micronucleus assay

The bone marrow cells were cultured in RPMI 1640 medium (Sigma-Aldrich) containing 20% fetal bovine serum, FBS (HyClone, Thermo Fisher Scientific), 1% penicillin/streptomycin (Sigma-Aldrich), and 1% l-glutamine (Sigma-Aldrich) in a 24-well flat bottom plate (Corning, NY). Lipopolysaccharides from Escherichia coli 0111 (Sigma-Aldrich) at a final concentration of 10 μg/mL, concanavalin A from Canavalia ensiformis (Sigma-Aldrich) at a final concentration of 3 μg/mL, phytohaemagglutinin M-form (Thermo Fisher Scientific) at a final concentration of 25 μg/mL and 2-mercaptoethanol (Sigma-Aldrich) at a final concentration of 10−5 M were added to the medium (Bartenbach & Wittmann, 1976; Doenhoff et al., 1976). The concentration of the bone marrow cells was 105 cells/mL in each well. The cells were incubated in a humidified incubator at 37 °C with 5% CO2 for 20 h. Following the incubation, the cells were treated with cytochalasin-B (Sigma-Aldrich) at a final concentration of 6 μg/mL and then incubated for further 28 h in the cell culture incubator. Following the incubation, the cells were washed and suspended in RPMI 1640 medium containing 10% FBS. Then, the cells were fixed with methanol and acetic acid based on a protocol used in our previous publication (Fotouhi et al., 2015). The fixed cells were then dropped on a slide and stained with Giemsa staining solution (Sigma-Aldrich). The slides were scored at 400× magnification using a bright-field microscope (BX61: Olympus, Tokyo, Japan). At least 500 binucleated cells (BNCs) were scored per slide and the results were normalized and expressed as number of micronuclei per 1000 BNCs.

2.4 | Statistical analysis

The results from 3 animals for each group of treated and sham-irradiated control animals were used for statistical analysis. To
investigate if a particular dose has an effect on the levels of MN induction, the Student’s t-test was used to compare the irradiated with the corresponding sham-irradiated control group. To investigate whether HDR resulted in higher levels of MN than LDR, the increases of MN relative to the corresponding control for each condition were calculated and then the levels of MN for LDR and HDR for each particular dose and time point were compared by t-test. A p-value < 0.05 was considered significant.

3 | RESULTS

The mice were irradiated and euthanized 3 h or 3 weeks after IR. The bone marrow was collected and the cells were used for MN assay. For each comparison, we had a group of sham-irradiated controls. The results at 3 h post exposure are summarized in Figure 1 and indicate that, as compared to the corresponding controls, 100 mGy did not increase MN in the bone marrow cells, while significant increases were induced by 200 mGy HDR (from 21 ± 1.5 up to 32 ± 6 MN/1000 BNC, p = 0.037) and LDR (from 15 ± 4 up to 26 ± 4 MN/1000 BNC, p = 0.027) as well as 1000 mGy HDR (from 24 ± 6 up to 80 ± 10 MN/1000 BNC, p = 0.002) and LDR (from 26 ± 7 up to 56 ± 1 MN/1000 BNC, p = 0.003). Although there appeared to be an increase at 500 mGy for both HDR (from 29 ± 13 up to 43 ± 18 MN/1000 BNC) and HDR (from 28 ± 1 up to 48 ± 17 MN/1000 BNC), large differences between individual animals were responsible for higher standard deviations and nonsignificant results (Figure 1). We also found that the relative level of MN was higher for HDR compared to LDR at 1000 mGy (p = 0.012).

The results at 3 weeks after exposure are summarized in Figure 2 and indicate that, as compared to the corresponding controls, the levels of MN were not significantly increased by 100 mGy or 200 mGy, while, significant increases were induced by 500 mGy LDR (from 23 ± 4 up to 44 ± 9 MN/1000 BNC, p = 0.01) and 1000 mGy HDR (from 26 ± 5 up to 48 ± 12 MN/1000 BNC, p = 0.027). The data also indicate that the doses of 200 mGy HDR (from 13 ± 1 up to 23 ± 1 MN/1000 BNC, p = 0.058) and 500 mGy HDR, (from 31 ± 6 up to 41 ± 4 MN/1000 BNC, p = 0.086) also induced greater amounts of MN/1000 BNC, although nonsignificant (Figure 2). After 3 weeks, we

| Dose (mGy) | Post exposure euthanasia | HDR (6 mGy/s) Exposure time (s) | LDR (1.4 mGy/h) Exposure time |
|------------|--------------------------|---------------------------------|-----------------------------|
| 100        | 3 h                      | 17 sec                          | 3 days                      |
| 200        | 3 h                      | 34 sec                          | 6 days                      |
| 500        | 3 h                      | 84 sec                          | 14 days 21 h                |
| 1000       | 3 h                      | 167 sec                         | 29 days 18 h                |

**FIGURE 1** The levels of micronuclei (MN) per 1000 binucleated cells in bone marrow cells isolated from whole-body-irradiated mice exposed to doses ranging from 100 to 1000 mGy and sham-exposed control animals (C). The doses were administered at high dose rate (HDR) or low dose rate (LDR) and the animals were sacrificed 3 h post-irradiation. Three animals per control or irradiated group were used. *p-values ≤ 0.05; **p-values ≤ 0.005. Error bars represent standard deviation

**FIGURE 2** The levels of micronuclei (MN) per 1000 binucleated cells in bone marrow cells isolated from whole-body-irradiated mice exposed to doses in range of 100 to 1000 mGy and sham-exposed control animals (C). The doses were administered at high dose rate (HDR) or low dose rate (LDR) and the animals were sacrificed 3 weeks post-irradiation. Three animals per control or irradiated group were used. *p-values ≤ 0.05. Error bars represent standard deviation
found that the relative level of MN was higher for LDR compared to HDR (p = 0.032) for 500 mGy. However, this might be due to the high level of MN found in the sham irradiated group for 500 mGy at HDR.

4 | DISCUSSION

Historically, the carcinogenic properties of IR have been attributed to targeted effects arising from the direct induction of DNA damage in the cell nucleus and mutations through direct interaction of energy with the DNA molecule or by formation of ROS through radiolysis. At the cellular level, we and others have shown that radiation increases the formation of ROS not only by radiolysis of water molecules but also by induction of endogenous oxidative stress (Haghdoost et al., 2005, 2006) and mitochondrial dysfunction (Lafargue et al., 2017; Yoshida et al., 2012). However, most of the studies have been performed in simple cell culture models. Therefore, the effects of IR on the immune system and systemic oxidative stress are less well known.

It was shown that the monocyte chemoattractant protein-1, MCP-1 (CCL2) and macrophage activation play important roles in the induction of distant complex DNA damage in organisms that carry a tumor (Redon et al., 2010). Induction of CCL2 and macrophage activation resulted in chronic tissue stress and inflammation (Georgakilas et al., 2014; Mavragani et al., 2017). Upregulation of CCL2 as well as macrophage activation have also been reported to be involved in response to IR (Mavragani et al., 2016, 2017; Teresa Pinto et al., 2016) and as a possible cause of low-grade chronic inflammation and systemic DNA damage (Chen et al., 2012; Siva et al., 2016) after exposure. In the present investigation, micronuclei were used as a marker for DNA damage (Helleday et al., 2014). MN are chromosome/chromatid fragments or whole chromosomes that are not included in the daughter nuclei during mitosis. The fragments are then enclosed by a nuclear membrane and are visible as small nuclei in the cytoplasm (Fenech et al., 2011). The lesions that lead to MN are DSBs (Fenech et al., 2011; Pfeiffer et al., 2000) and perhaps complex DNA damage.

Only a few reports have documented the effects of dose rate on mutation levels in mice exposed to ionizing radiation (Lyon et al., 1972; Russell, 1965; Russell et al., 1958; Tucker et al., 1998). (Lyon et al., 1972; Russell, 1965; Russell et al., 1958; Tucker et al., 1998). Notably, dose rate effects were reported for chromosome translocations in mouse spermatogonia in the range between 36 Gy/h and 0.37 mGy/h (Bayakova et al., 1987). No differences were reported in frequencies of chromosome translocation in blood lymphocytes of mice exposed to gamma-radiation at dose rates of 2 mGy/h up to 16.5 mGy/h for 90 days (Sorensen et al., 2000), and of HPRT gene mutations in mice exposed at 6 mGy/h and 41.5 mGy/h (Lorenz et al., 1994).

Our previous in vitro results indicated that at the same accumulated dose, HDR exposure resulted in less survival than LDR, while the levels of point mutations were similar for HDR and LDR in the range of 1 mGy/h up to 15 mGy/h (Shakeri Manesh et al., 2014, 2015, 2017), indicating a dose rate effect at the level of survival but lack of dose rate effect at the level of mutations.

The results presented in the Figure 1 indicate increases of MN (approximately 10–50%), induced by 200 mGy up to 1000 mGy comparing exposed and sham-exposed animals. Slightly higher levels of MN were observed in animals exposed to 200, 500 and 1000 mGy HDR in comparison with the corresponding LDR exposed animals, but significant only for the 1000 mGy group, indicating that declining dose rate leads to lower levels of MN. When the dose rate is decreased, the accumulation of DNA damage is also decreased, and the efficiency or fidelity of DNA repair is expected to be high (Dikomey & Brammer, 2000). For the purpose of radiation protection, a dose and dose-rate effectiveness factor (DDREF) for reducing cancer risk of low linear energy transfer (low LET) ionizing radiation (e.g., gamma and X-rays) is defined. Today, a DDREF factor of 2 is recommended for radiation protection purposes, which means that the cancer risk observed at high acute doses of IR should be divided by a factor of 2 in the risk assessment for the same doses when delivered at a low dose rate. Our data from cell experiments (Shakeri Manesh et al., 2014, 2015, 2017) indicate slight differences in mutation levels induced by LDR compared to HDR which argues against a DDREF factor of 2. However, the direct application of experimental results from cell cultures and mice to humans may not be reasonable but the results may show us the way forward.

No increases of MN were found in mice exposed to 100 mGy. These results suggest that mouse bone marrow cells repair the DNA damage induced by 100 mGy efficiently. The results are in accord with our previously published article in which we reported no increase of MN or translocation after in vitro irradiation of human blood with 100 mGy (Nakamura et al., 2017).

The results presented in Figure 2 indicate that the levels of MN in mice exposed to 200 mGy return to the levels of the shame-exposed control animals within 3 weeks while for the 500 mGy and 1000 mGy groups, increases of MN are observable 3 weeks after exposure. This can be explained by the fact that some of the cells exposed to 500 and 1000 mGy may carry DNA damage without repairing them for a long time (3 weeks in our study) after exposure.

It is known that persistent MN can lead to prolong activation of DNA damage signaling as well as activation of immune signaling (Gekara, 2017; Nakad & Schumacher, 2016; Xu, 2006). The activated immune cells may induce chronic inflammation (Halle et al., 2010) and DNA damage through release of cytokines such as the monocyte chemoattractant protein-1, MCP-1 (CCL2) (Georgakilas et al., 2014; Mavragani et al., 2017) and oxidative stress (Rodrigues-Moreira et al., 2017). Notably, the levels of MN in the LDR group 3 weeks after exposure appear to be higher in animals exposed to 500 mGy than in those exposed to 1000 mGy. This might be due to; (a) DNA repair and DNA damage response are more efficient at 1000 mGy LDR than 500 mGy LDR (low dose hyper sensitivity) (Nagle et al., 2018); or (b) the inflammatory response induced by 500 mGy is greater than of that induced by 1000 mGy LDR which leads to elevated MN in bone marrow cells. This is an interesting observation to
be explored mechanistically but it is outside the scope of the present investigation.

Our conclusions from the present investigation are that the doses of 200 mGy and above, may lead to an increase of the MN levels in the bone marrow cells 3 h after exposure as compared to the corresponding nonirradiated controls. The results at 3 weeks indicate that the mice repair DNA damage or eliminate cells that carry DNA damage induced by 200 mGy. However, 500 and 1000 mGy whole body irradiation may induce MN which are difficult to be repaired and probably activate immune response (Gekara, 2017). The data also indicate slight differences between MN levels induced by LDR and HDR, arguing against a DDREF factor of 2 for MN induction as marker for genotoxicity.

Limitations of the study need to be acknowledged. Although we used 2 time points, 3 h and 3 weeks, the generalizability of the response needs to be confirmed by including time points later than 3 weeks. The number of animals in the study might be increased to improve statistical analysis.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Conceptualization; SH, PG, APK, methodology; APK, TS, APK, formal analysis; AN, PG; writing original draft preparation; AN, SH, PG; writing—review: APK, AN, TS, SH, PG and editing; SH; project administration, SH and PG; funding acquisition SH. All authors have read and agreed to the published version of the manuscript.

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