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Age and sex differences in genome damage between prepubertal and adult mice after exposure to ionising radiation

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The mechanisms that lead to sex and age differences in biological responses to exposure to ionising radiation and related health risks have still not been investigated to a satisfactory extent. The significance of sex hormones in the aetiology of radiogenic cancer types requires a better understanding of the mechanisms involved, especially during organism development. The aim of this study was to show age and sex differences in genome damage between prepubertal and adult mice after single exposure to gamma radiation. Genome damage was measured 24 h, 48 h, and 72 h after exposure of 3-week and 12-week old BALB/CJ mice to 8 Gy of gamma radiation using an *in vivo* micronucleus assay. There was a significantly higher genome damage in prepubertal than in adult animals of both sexes for all sampling times. Irradiation caused a higher frequency of micronuclei in males of both age groups. Our study confirms sex differences in the susceptibility to effects of ionising radiation in mice and is the first to show that such a difference occurs already at prepubertal age.

KEY WORDS: *gamma radiation; micronucleus; oestrogen; radiosensitivity; testosterone*

Radiation protection measures to be applied in cases of nuclear accidents and their link to sex or age differences are very limited (1). A recent report of the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) (2) pointed to the need for further investigations into the health risks to children caused by exposure to ionising radiation - both on human populations and animal models. However, in its evaluation of the current knowledge, UNSCEAR (2013) failed to include the interaction of ionising radiation with sex hormones during development and adulthood, as well as the impact of exposure to xenohormonally active agents on susceptibility to ionising radiation.

The mechanisms by which oestrogen may modify the response of an organism to ionising radiation have been reported only anecdotally; same as for testosterone (3-6). There is growing evidence regarding the impact of oestrogen and its receptors on carcinogenesis (7-10). So far, the biological effects of radiation via interaction with oestrogen receptors have rarely been investigated (4, 11, 12). The significance of interaction between ionising radiation and oestrogen receptors is clearly visible in the differences between mammary carcinogenesis mechanisms in the prepubertal period and in adulthood (13).

The radioprotective effect of phytoestrogens has been described in an animal model showing that the administration of genistein before irradiation significantly improves the survival of progenitor hematopoietic cells (14, 15). The mechanisms involved in modifying the effects of oestrogen on responses to ionising radiation, among others, include an increase in reactive oxygen species, gamma-H2Ax foci levels, and cell-cell signalling (16, 17). Similarly, recent data have shown an interaction between androgen receptors and ionising radiation (18, 19). A decrease in both testosterone and the sex hormone binding globulin has been reported in clinical studies, in men who underwent radiotherapy for rectal cancer (20).

There is no data on possible sex and age differences in genome damage after exposure to ionising radiation between prepuberty and adulthood. Prepuberty is a specific period of development during which complex micronenvironment settings are formed in an organism basically through a strong increase in sex hormones as a preparation phase for the final process of maturation (21). The effects of exposure during postnatal development, due to higher susceptibility of an organism to ionising radiation, may have lifelong consequences (22-24).

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Sex- and age-related differences in the effects of ionising radiation are affected by different levels of oestrogen receptors in tissues during prepuberty in comparison with adults, as reported in human adrenal tissue (25). Increased levels of oestrogen two months after exposure to ionising radiation were described in prepubertal mice (5).

Additionally, the Lifetime Attributable Risk (LAR) of cancer significantly differs during childhood and between sexes. For some radiogenic cancer types there is almost a two times higher risk between prepuberty and adulthood and two times higher risk in males than in females during prepuberty and adulthood (26). Such data show the need for more research into the mechanisms of radiocarcinogenesis in regard to the impact of sex hormones on cancer risks.

The aim of the current study was to investigate sex differences in genome damage in prepubertal and adult mice using an *in vivo* micronucleus assay (27). The main advantage of this method is that it requires a very small sample size, which enables a repeated sampling of the same animal and investigation of small animals during development. It has been shown to be a reliable method for biodosimetry (28). The dose was selected following similar studies in which the biological effects on the cellular level or DNA damage were investigated (14, 29-31).

MATERIALS AND METHODS

Animals

This study included 3-week-old and 12-week-old BALB/CJ mice obtained from a breeding colony of the Rudjer Boskovic Institute (Zagreb, Croatia). During the experiment, four animals were housed per cage. The bottom of the cage was covered with sawdust (Allspan®, Karlsruhe, Germany). Standard food for laboratory mice (4 RF 21 GLP Mucedola srl, Settimo Milanese, Italy) was used. All animals had free access to food and water. Animals were kept under standard conditions with a 12-h light/dark cycle, temperature of 22 °C, and 55 % humidity. All experiments were performed according to the ILAR Guide for the Care and Use of Laboratory Animals, Council Directive (#86/609/EEC) and Croatian Animal Protection Act (OG 135/06) and were approved by the Ethical Committee of the Ministry of Agriculture.

Radiation exposure

Each age and sex group consisted of eight animals. Animals received a single dose of 8 Gy (3,125 cGy s^{-1}) using X6MV photon irradiation (ONCOR linear accelerator, Siemens, Malvern, USA). One half of the dose was applied to the dorsal (PA, SSD=130 cm, bolus RW3 1cm) and the other half to the ventral side (AP, SSD=130 cm, bolus RW3 1 cm) of animals. Animals were sampled before irradiation, 24 h, 48 h, and 72 h after irradiation. During irradiation each animal was placed into a plexiglass cage.

In vivo *micronucleus assay*

The *in vivo* MN assay was performed 24 h, 48 h, and 72 h after exposure in 3-week-old mice, while in adult animals the analysis 72 h after irradiation was not performed due to the reduction of reticulocyte number. Peripheral blood was collected from the tail vein (5 μL per sample) from all animals. Blood smears were prepared on acridine orange (Sigma-Aldrich, St. Louis, USA) coated slides, covered with a coverslip, and analysed according to Hayashi et al. (27). The MN frequency was analysed in 2000 reticulocytes per sample. Analyses were performed by one scorer using a fluorescent microscope under 1000 x magnification (Olympus Provis AX70, Tokyo, Japan).

Statistics

Statistical analysis of data was performed using the Statistica 7.0 software package (StatSoft, Tulsa, USA). An independent sample t-test or one-way analysis of variance (ANOVA) followed by the Tukey *post-hoc* test were used to determine significant differences between the groups. The test value of $P<0.05$ was considered statistically significant.

RESULTS

Changes in the total number of micronucleated cells after irradiation in 3-week- and 12-week-old mice are shown in Figure 1A and 1B, respectively; while the distribution of cells according to the number of MN per cell is shown in Table 1.

Background levels before irradiation were 0.10 % MN in prepubertal males and 0.05 % MN in females. Background values were 0.10 % MN in adult males and 0.08 % MN in females. There was no significant difference between sex and age groups in MN frequencies. Cells with more than one MN were not detected (Table 1).

A significant increase in MN frequencies compared to basal values was observed after 24 h in both 3-week-old and adult mice as well as in both males and females but there were no significant differences between these two sexes (Figure 1).

The most pronounced increase in the incidence of micronucleated cells was observed for both age groups at 48 h after irradiation (Figure 1A and B). At this time, a significantly higher frequency of cells with MN was observed in 3-week-old and adult males than in female animals (indicated by an asterisk in Figure 1A and 1B) and the difference between males and females was more expressed in younger (~33 %) than in adult (~22 %) animals. Further, at 48 h after irradiation, in the same sex group, 3-week-old mice had a significantly higher incidence of cells with MN than 12-week-old mice (indicated by the sign

Table 1 *The incidence of mononuclear (1 MN), binuclear (2 MN), trinuclear (3 MN), and tetranuclear (4 MN) cells in reticulocytes from peripheral blood at 24 h, 48 h, and 72 h after irradiation*

Time	Distribution of cells according to the number of micronuclei (MN) per cell (%) (mean±SD)							
	Prepubertal mice (3-week-old)							
	1 MN		2 MN		3 MN		4 MN	
	Male	Female	Male	Female	Male	Female	Male	Female
0 h (control)	0.10±0.08[a]	0.05±0.06[a]	0	0	0	0	0	0
24 h	0.54±0.09[b]	0.54±0.11[b]	0	0	0	0	0	0
48 h	6.40±0.65[c]*#	4.60±0.57[c]*#	0.68±0.20[a]*#	0.1±0.07*	0	0.04±0.05	0	0
72 h	4.83±0.58[d]	5.30±0.68[c]	0.43±0.06[a]	0.15±0.19	0.07±0.11	0	0	0.03±0.05
	Adult mice (12-week-old)							
Time	1 MN		2 MN		3 MN		4 MN	
	Male	Female	Male	Female	Male	Female	Male	Female
0 h (control)	0.10±0.08[a]	0.08±0.10[a]	0	0	0	0	0	0
24 h	0.58±0.11[b]	0.42±0.13[b]	0	0	0	0	0	0
48 h	3.90±0.69[c]*#	2.92±0.36[c]*#	0.06±0.09*#	0.20±0.07*	0.02±0.04	0	0	0
72 h	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

([a, b, c, d])–significant differences between groups are indicated by different letters (p<0.05, Tukey post hoc *test); (*)–significant differences male* vs. *female mice (p<0.05, t-test); (#)–significant differences prepubertal* vs. *adult mice (p<0.05, t-test); n.a.–not available*

in Figure 1A and 1B). This age difference was more pronounced in male (~45 %) than in female (~35 %) mice.

After 72 h, in 3-week-old mice a significant decrease in cells with MN frequency was observed in males, while in females a slight increase was observed but the difference was not statistically significant (Figure 1A).

Besides determining the total number of micronucleated cells, the analysis of cell distribution according to the number of MN in the cell was performed. From Table 1, it can be seen that the incidence of cells with one MN for both age and sex groups generally follows the trend observed for the total number of micronucleated cells (Figure 1A and B). At 48 h, the incidence of cells with two MN was significantly higher in male than female 3-week-old mice (Table 1). Also, 3-week-old males had a significantly higher frequency of cells with two MN compared to adult male mice (Table 1). In 3-week-old mice, cells with three and three and four MN in one cell were detected at 48 h and 72 h after irradiation but their incidence was very low and was not strictly related with either sex (Table 1).

DISCUSSION

The exposure of general population to ionising radiation due to nuclear accidents such as Chernobyl and Fukushima showed that casualty management is still not satisfactory and that radiation protection plans for specifically susceptible subpopulations such as children according to age groups are still not available. Contrary to the historical approach to cancer risks after overexposure to ionising radiation, which is basically focused on the caused genome damage, current radiation biology is rapidly incorporating complex interactions between ionising radiation and the immune system, epigenetic modifications, sex hormones, and age at exposure. Such approach, however, demands additional investigations into the mechanisms underlying the detected age and sex differences in susceptibility to radiogenic cancers, which should give an insight into the causality of the involved complex pathways. For such studies, animal models as *in vitro* models that simulate developmental process are still not developed.

In order to investigate the differences in the radiosensitivity of prepubertal and adult organisms, in this study prepubertal, 3-week-old and adult mice of both sexes were irradiated with 8 Gy of gamma radiation. Such a high dose of radiation was selected in order to additionally validate the *in vivo* MN assay in regard to difference in haematopoietic potency between young and adult animals.

Ionising radiation caused a significantly higher frequency of MN in prepubertal animals than in adult animals of both sexes. Due to reticulocytopenia, which is already described in applied doses (31) in adult animals, it was not possible to measure the MN frequency 72 h after exposure. As in mice up to four weeks of age reticulocytes make up around 10 % of erythrocytes (32), reticulocytopenia

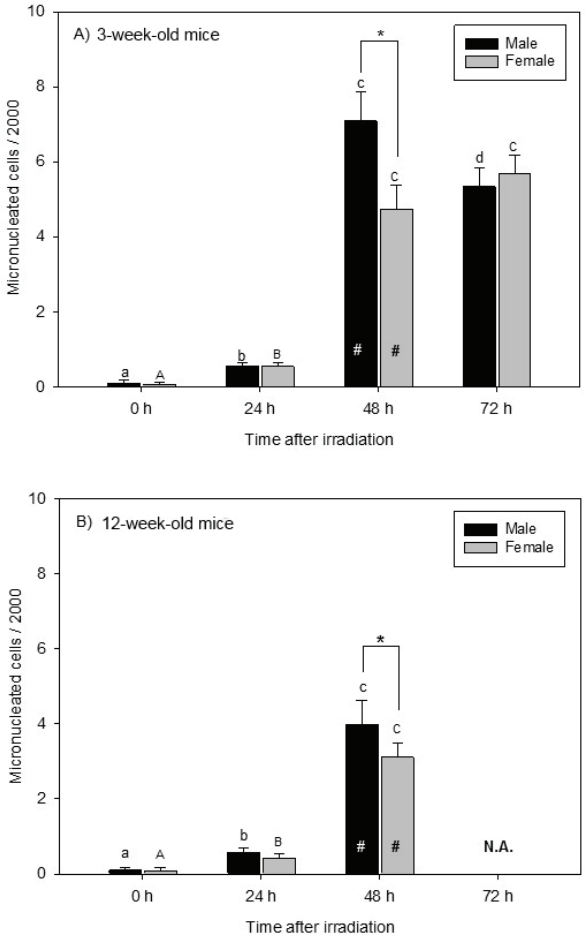

Figure 1 *Frequencies of micronucleated cells in 3-week-old (A) and 12-week-old (B) mice at 24, 48, and 72 h after irradiation. (Mean±SD). Significant differences (p<0.05, Tukey* post-hoc *test) between particular times are indicated by different small and capital letters above the error bars for male and female mice, respectively. Significant differences (p<0.05, t-test) between males and females are marked by an asterisk (*) and significant differences (p<0.05, t-test) between 3-week-old and 12-week-old mice at 48 h are marked by the sign (#) within the bars; N.A.-not available*

was less severe and the scoring in 3-week-old mice was possible. It is suggested that repopulation of reticulocytes may not have an impact on the results of MN frequencies as after irradiation the increase in the reticulocyte number is reported to be only after 72 h (33).

Our results are in concordance with historical data which shows that that the peak in the MN frequency has been observed 48 h after exposure to lower doses of 2 Gy or 3 Gy of gamma radiation (33, 34). This study also confirms the conclusions of previous studies that young animals are more susceptible to the effects of ionising radiation than adults (35-38). The same higher radiosensitivity has also been described in children exposed accidentally, either diagnostically or therapeutically (22, 39).

In our study, a significantly higher MN frequency in males of both age groups was detected. Similarly, although not statistically significant, lower MN frequencies in erythroblasts have been described in female adult mice than in adult male mice exposed to chronic gamma radiation (40). Acute exposure of mice to 5 Gy of X-rays caused different, tissue specific, levels of DNA strand breaks in female and male adult mice (41). In BALB/c mice after irradiation with 1 Gy X-rays, sex-specific changes in methylation were reported. Thus, significantly higher methylation levels were present in males than in females (11). Similarly, the difference in miRNA was also reported to be sex-specific (42). In mice exposed for two weeks to X-rays, adult male genome damage was higher than in females (0.05 and 0.10 Gy) (43).

This study is the first to show that sex differences are present even in prepubertal animals. The higher MN frequency in males than in females in both age groups is suggested to be attributed to the effects of oestrogen.

A possible mechanism by which oestrogen may cause a lower frequency of MN is the quiescence of stem cells (44) or enhanced DNA repair as shown in the case of pretreatment of animals with genistein before irradiation (45). Similarly, dietylstilbestrol, a xenoestrogen, is shown to suppress the proliferation of haematopoietic precursor cells and intensify DNA repair (46). However, in pregnant mice, a significantly increased genome damage in spontaneously dividing bone marrow cells occurred after a high increase in oestrogen and progesterone hormones and this phenomenon disappeared after delivery. It is therefore possible that oestrogen contributed to transient radiosensitivity (47).

Oestrogen as an endocrine, paracrine, and neuromodulator molecule has been shown to interact with the biological effects of ionising radiation. A different age-, sex- and tissue-related distribution of polymorphic oestrogen receptors (alpha, beta, GPR30) enables numerous possible interactions of oestrogen with biological pathways after action of environmental stressors (4). Thus, oestrogen may have radioprotective and radiosensitising effects depending on its level, tissue type, age, type of radiation, and dose.

Contrary to oestrogen, there is no data on the interaction between ionising radiation and pathways regulated by testosterone that may have an impact on radiogenic carcinogenesis. Data on testosterone and oestrogen levels during postnatal development of mice until adulthood are not available. In some organs, such as brain, oestrogen receptor distribution fluctuates during prepubertal period and the impact of the same oestrogen levels differs due to its different half-life (48). Additionally, letrozole, an aromatase inhibitor, is shown to increase radiosensitivity of cancer cells (49). As aromatase inhibitors such as letrozole cause an increase in testosterone, it could be indirectly concluded that testosterone causes radiosensitivity (50).

In both boys and girls, the increase in testosterone and oestrogen levels starts in prepuberty and is simultaneous with a significant reduction in the hormone binding protein, whose level is reduced by half from prepuberty to puberty

(51, 52). In humans, girls have higher levels of 17beta-testosteorne and estradiol during prepuberty, while in boys the estradiol level can be undetectable (53). Thus, changes in the hormonal status are present even before the first clinical signs of puberty (54), which points to the significance of the prepubertal period in the orchestration of physiological conditions for sex differences in response to the environment before puberty, including ionising radiation.

The investigation of the effects of oestrogen and testosterone on the biological effects of radiation is of great importance for (a) the inclusion of prepuberty and puberty group differences in the radiation protection legislation; (b) development of radioprotective xenoestrogen substances whose application would be age- and sex-adjusted; and (c) yielding a contribution to oncology for better effects in cases of combined hormonal and radiotherapy.

In summary, exposure to 8 Gy of gamma-radiation caused significantly increased levels of MN frequency - significantly higher in prepubertal and adult male animals. Further investigations into the mechanisms by which age and sex differences in responses to exposure to ionising radiation set in should include analyses or correlations between oestrogen and testosterone levels, haematopoiesis dynamics, levels of oestrogen, and androgen receptors in bone marrow stem cells and genome damage.

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Dobne i spolne razlike u oštećenju genoma između pretpubertetskih i odraslih miševa nakon izlaganja ionizirajućemu zračenju

Mehanizmi koji uzrokuju spolne i dobne razlike u biološkim odgovorima na izloženost ionizirajućemu zračenju i s tim u vezi zdravstvene rizike još nisu dovoljno ispitani. Kako bi se spoznao značaj spolnih hormona u etiologiji zračenjem izazvanih vrsta tumora, potrebno je bolje poznavanje mehanizama koji su uključeni u taj proces, osobito tijekom razvojne faze organizma. Cilj ovoga istraživanja bio je prikazati dobne i spolne razlike u oštećenju genoma između pretpubertetskih i odraslih miševa nakon jednokratnoga izlaganja gama-zračenju. Primjenom *in vivo* mikronukleus-testa izmjereno je oštećenje genoma nastalo 24 sata, 48 sati i 72 sata nakon izlaganja BALB/CJ miševa, starih tri tjedna i dvanaest tjedana, dozi gama zračenja od 8 Gy. U svim vremenskim točkama mjerenja uočeno je značajnije veće oštećenje genoma u pretpubertetskih u odnosu na odrasle jedinke obaju spolova. Zračenje je uzrokovalo veću učestalost mikronukleusa u muških jedinki u objema dobnim skupinama. Dobiveni rezultati potvrđuju postojanje spolnih razlika u osjetljivosti na učinke ionizirajućega zračenja u miševa, a ovo je prvo istraživanje rezultati kojega pokazuju da do takvih razlika dolazi već u pretpubertetskoj dobi.

KLJUČNE RIJEČI: *estrogen; gama zračenje; mikronukleus; radioosjetljivost; testosteron*