Original Article

Effect of Paternal Rat Irradiation Transmitted to the Progeny During Prenatal Development

(ionizing radiation / genome instability / transgenerational transfer / rat progeny)

S. BÁLENTOVÁ[1], L. SLOVINSKÁ[2], E. MIŠÚROVÁ[3], S. RYBÁROVÁ[4], M. ADAMKOV[1]

[1]Institute of Histology and Embryology, Jessenius Faculty of Medicine in Martin, Comenius University, Martin, Slovak Republic
[2]Institute of Neurobiology, Slovak Academy of Sciences, Košice, Slovak Republic
[3]Institute of Biological and Ecological Sciences, Faculty of Science, P. J. Šafárik University, Košice, Slovak Republic
[4]Institute of Anatomy, Faculty of Medicine, P. J. Šafárik University, Košice, Slovak Republic

Abstract. The transgenerational transmission of radiation damage was investigated on the basis of quantitative changes of nucleic acids and histones, the integral index of tissue and organ cellularity. Male Wistar rats, whole-body irradiated with the dose of 3 Gy of gamma rays, were mated with non-irradiated females 25 or 80 days after exposure and their progeny were investigated on the 15th (embryos), 17th (embryos), or 19th (embryonic brain) day of prenatal development (E15, E17, and E19Br, respectively). A significant increase in DNA and RNA concentration and content was found on the 15th day and predominantly on the 17th day of gestation in the progeny of males irradiated 80 days before mating. On the contrary, in the progeny of the same males, concentration of histones was decreased in groups E15 and E19Br. Finally, the radiation alterations in the progeny arisen from irradiated spermatogonia (by paternal exposure 80 days before mating) were more profound in nucleic acids than in histones. Our findings suggest an incidence of radiation-induced genome instability manifested as enhanced proliferating activity of cells in response to DNA damage in the progeny of males, mated at later intervals after exposure.

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Corresponding author: Soňa Bálentová, Institute of Histology and Embryology, Jessenius Faculty of Medicine in Martin, Comenius University, Malá Hora 4, 037 54 Martin, Slovak Republic. Phone: +421 434 131 531; e-mail: sonabalent@yahoo.com.

Abbreviations: BSA – bovine serum albumin, E15, E17 – whole embryos on gestation days 15 and 17, E19Br – whole embryonic brain on gestation day 19, ESTR – expanded simple-tandem repeat, F_0 – parental generation of male rats, F_1 – first generation of progeny of male rats.

Introduction

Ionizing radiation causes chromosomal damage, which results in various types of effects including increased risk of cell death, mutagenesis and carcinogenesis. The initial radiation damage results in elevated mutation rate throughout many cell divisions after exposure, and due to accumulation of multiple changes, a stable normal cell genome becomes unstable. This phenomenon, called genome instability, is characterized by an increased rate of acquisition of alterations in the genome. Multiple endpoints of genome instability include large-scale chromosomal rearrangements and aberrations, aneuploidy, gene mutations and amplification, micronucleus formation, cellular transformation, clonal heterogeneity, delayed reproductive cell death, enhanced frequency of gene mutations *de novo* and tandem repeat instability (Dubrova et al., 1998; Streffer et al., 1998; Little, 2000; Vasilyeva et al., 2001; Smith et al., 2003).

The demonstration of radiation-induced genomic instability in somatic cells initiated an increased interest in the study of potential long-term effects of exposure and possible transmission of delayed adverse effects via the germ-line to next generations, called transgenerational effects.

Radiation-induced genomic instability will contribute to the accumulation of oncogenic mutations and malignant transformation of cells (Little, 2000). Investigation of offspring of irradiated mice after low-dose foetal irradiation (E14 or E17) showed a dose-dependent increase in tumour incidence observed in the liver, spleen and reproductive organs (Uma Devi and Hossain, 2000; Nitta and Hoshi, 2003). In slowly proliferating tissues, such as liver or brain, paternal whole-body irradiation alone or in combination with other genotoxic agents, or partial ablation of organ mass (partial hepatectomy, unilateral bulbectomy of brain) clearly affects cell proliferation in exposed parents, and through the germ-line also influences the non-irradiated subsequent two gen-

erations of rats (Kirschenbaum et al., 1999; Kropáčová et al., 2002; Slovinská et al., 2004; Bálentová et al., 2007a, b; Kožurková et al., 2007). The offspring of normal and chimeric male mice after paternal germ-line irradiation three or six weeks before conception showed altered protein kinase activities and nuclear levels of p53 and p21 proteins even after three generations (Baulch et al., 2001; Vance et al., 2002). Another output of paternal irradiation included teratogenic effects, increased prenatal mortality, incidence of malformed foetuses and decreased fertilization rates for both *in vivo* and *in vitro* fertilization (Burruel et al., 1997; Pils et al., 1999).

The results of numerous studies showed that also an epigenetic phenomenon, connected with long-term changes in the gene expression patterns, contributes to radiation-induced genomic instability. The main epigenetic mechanisms include DNA methylation and histone modifications. Kožurková et al. (2007) demonstrated an increased level of DNA methylation in the liver of non-irradiated first (F_1) generation of progeny of exposed parental (F_0) male rats, probably due to radiation-induced genome damage.

In our previous works, the non-exposed embryos and young of irradiated male rats were characterized by decreased mitotic activity and high incidence of chromosomal aberrations (Slovinská et al., 2004; Šanová et al., 2005). In the non-irradiated F_1 and F_2 generations of adult progeny of irradiated parents, the mitotic activity (represented by mitotic index) in the liver regenerating after partial hepatectomy was lower in comparison with irradiated offspring of the same exposed parents (Kropáčová et al., 2002; Slovinská et al., 2004). Regarding the crucial role of DNA and histones in the processes of cell proliferation, differentiation and ageing, we have focused on delayed effects of germ-line irradiation, transmitted from exposed male rats of parental generation (F_0) to the progeny (F_1). The effects were estimated on the basis of quantitative changes of DNA, RNA, histones and alterations in the relative proportion of individual histone fractions and H1° subfraction of H1 fraction in the whole embryos on the 15th and 17th days, and in embryonic brain on the 19th day of prenatal development (E15, E17, and E19Br, respectively).

Material and Methods

Animals. The experiment was performed with 133 adult Wistar rats of parental (F_0) generation (51 males, 82 females), 3–6 months old at the time of irradiation, and their progeny (F_1 generation). The animals were kept under standard conditions (temperature of 22–24 ºC, natural light rhythm) and provided with food and water *ad libitum*. All animal procedures were performed in accordance with the requirements for ethical standards of welfare and treatment of animals.

The control group, represented by progeny of control males and females, and two experimental groups consisting of the progeny created by irradiated males mated with intact females 25 or 80 days after exposure, were investigated on the 15th, 17th, and 19th days of prenatal development (5–6 animals at each time interval). The intervals before mating correspond to irradiation of the postmeiotic spermatids (25 days before conception) and premeiotic stem spermatogonia (80 days before mating).

Irradiation procedure. Male rats of the parental generation were irradiated with a single whole-body dose of 3 Gy by gamma rays from a ^{60}Co source (apparatus Chisostat, Chirana, Prague, Czech Republic) at a dose rate of 0.187 $Gy.min^{-1}$.

Nucleic acid analysis. Quantitative analysis was performed according to the method of Tsanev and Markov (1960). The whole 15- and 17-day-old embryos (E15, E17), or half of the brain of 19-day-old embryos (E19Br) were homogenized in 5% trichloracetic acid, then deproteinized and purified by washing in methanol, chloroform-methanol, benzene and ether. After DNA and RNA separation by hydrolysis in alkaline (1 N KOH), and acidic media (1 N $HClO_4$), the concentration of nucleic acids was determined by spectrophotometric measurements (Hitachi 1031, Tokyo, Japan) at two wavelengths (DNA at 268 and 284 nm; RNA at 260 and 286 nm) and expressed as mg of DNA or RNA per g of wet tissue. Total content was evaluated by calculation of the nucleic acid concentration compared to the total weight of embryo or embryonic brain and expressed as mg of DNA or RNA per whole embryo or whole brain; therefore, the total content reflects not only the changes in concentration, but also the simultaneous changes in the weight of the whole embryo or organ.

Histone analysis. The whole embryos or half of the brain of 15- 17-, and 19-day-old progeny were homogenized in STKM buffer (0.25 mol sucrose; 0.05 mol Tris; pH 7.9; 0.25 mol KCl; 5 mmol $MgCl_2$,). Cell nuclei were isolated by the method of Grünicke et al. (1989) and histones were extracted with 0.2 mol H_2SO_4 (at 4 °C for 1 hour). After precipitation and repeated washing, the concentrations of samples were determined using the method of Lowry et al. (1951), which is reliable for determination of small amounts of proteins. The method is based on the reduction of Folin-Ciocalteu phenol reagent in alkaline solution. Working standards were prepared from bovine serum albumin (BSA) diluted in 0.5 M NaOH and spectrophotometric measurements of histone concentrations were done at the wavelength of 230 nm (Hitachi). Analyses of individual histone fractions were performed by polyacrylamide gel electrophoresis according to the method of Panyim and Chalkley (1969). After amido black B staining, the relative proportion of individual histone fractions and of H1° subfraction in H1 fraction was detected spectrophotometrically by densitometer Shimadzu CS-930 (Tokyo, Japan).

Statistical analysis. The values analysed by ANOVA and Tukey-Kramer test are given as mean ± SEM. Statistical significance was set at $P \leq 0.05$.

Results

Nucleic acids. In embryos of the control group, DNA concentration moderately decreased between 15th and 17th gestation days (28.40 ± 0.57 *vs.* 22.58 ± 0.88 mg/g; Fig. 1). A comparable trend was also seen within experimental groups; however, relative to control animals, DNA concentration increased on the 15th gestation day in the offspring of males mated on the 25th day after exposure (31.67 ± 0.95 *vs.* 28.40 ± 0.57; $P \leq 0.05$) (the offspring of males irradiated 80 days before conception were not investigated on the 15th day because of small litters). In the offspring of males irradiated 80 days before conception, a significant increase in DNA concentration was found on the 17th day of development (37.45 ± 2.76 *vs.* 22.58 ± 0.88; $P \leq 0.05$). In the brain of 19-day-old offspring of control and irradiated males, the DNA concentration was much lower than in younger (15- and 17-day-old) whole embryos. Due to progressing rise of embryonic weight between the 15th and 17th gestation days, the total DNA content increased in embryos of control and mainly of experimental groups.

The same drift as in DNA was also seen in the concentration and total content of RNA in the offspring of irradiated males of both experimental groups. Comparison of the whole 15- and 17-day-old embryos of control and experimental groups showed an increase in RNA concentration (E15 Ir-25: 60.45 ± 2.68 *vs.* 52.88 ± 2.36 mg/g in control animals; $P \leq 0.05$; E17 Ir-25: 60.34 ± 3.35 *vs.* Ir-80: 71.59 ± 5.23 *vs.* 53.03 ± 2.27 in control animals; $P \leq 0.05$) and total content (E15 Ir-25: 0.10 ± 0.01 *vs.* 0.07 ± 0.01 mg/org. in the control group; $P \leq 0.05$; E17 Ir-25: 0.15 ± 0.02 *vs.* E17 Ir-80: 0.41 ± 0.03 *vs.* 0.14 ± 0.01 in the control group; $P \leq 0.05$) in dependence on the time between paternal irradiation and mating. Similarly to DNA, the preconceptional irradiation had no statistically significant effect on the RNA values in the brain of the 19-day-old embryos.

Histones. Evident changes were also found in histone concentration and content (Fig. 2A, B). In control groups, a marked difference in histone concentration was seen between whole embryos (15-day-old) and brain (of 19-day-old) (11.76 ± 2.04 *vs.* 7.27 ± 0.95 mg/g). Comparison within experimental groups showed that in the progeny of males irradiated 80 days before mating, the concentration of histones was decreased in the 15-day-old whole embryos (4.76 ± 0.70 *vs.* 11.76 ± 2.04 in controls; $P \leq 0.05$) and in the brain of 19-day-old em-

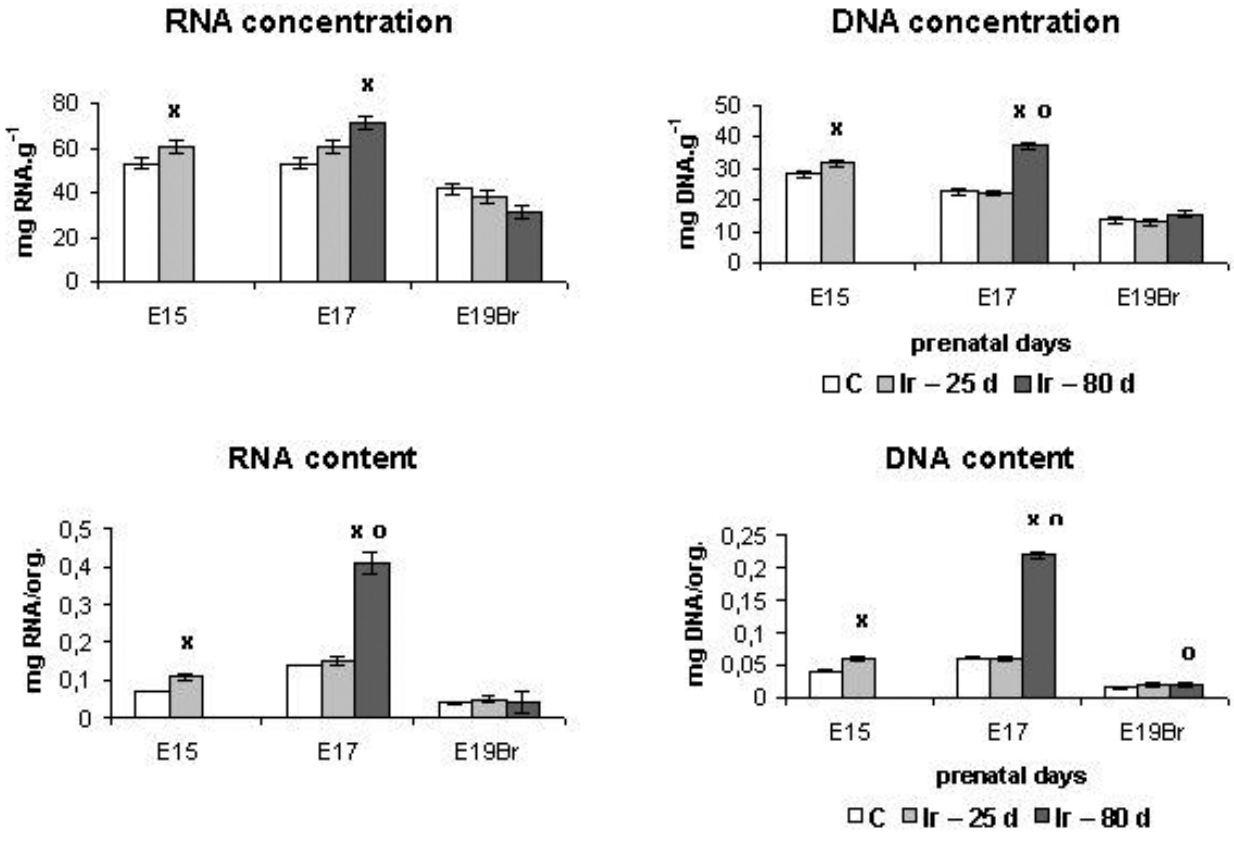

Fig. 1. Concentration and total content of RNA and DNA in the progeny of rat males irradiated with the dose of 3 Gy gamma rays 25 or 80 days before mating (Ir – 25d, Ir – 80d) investigated on the 15th, 17th and 19th days of prenatal development (E15, E17 – whole embryos on gestation days 15 and 17; E19Br – whole embryonic brain on gestation day 19). Data are presented as mean ± SEM. Statistical significance of differences between experimental and control groups: ^{x} $P \leq 0.05$ and within irradiated animals: ^{o} $P \leq 0.05$

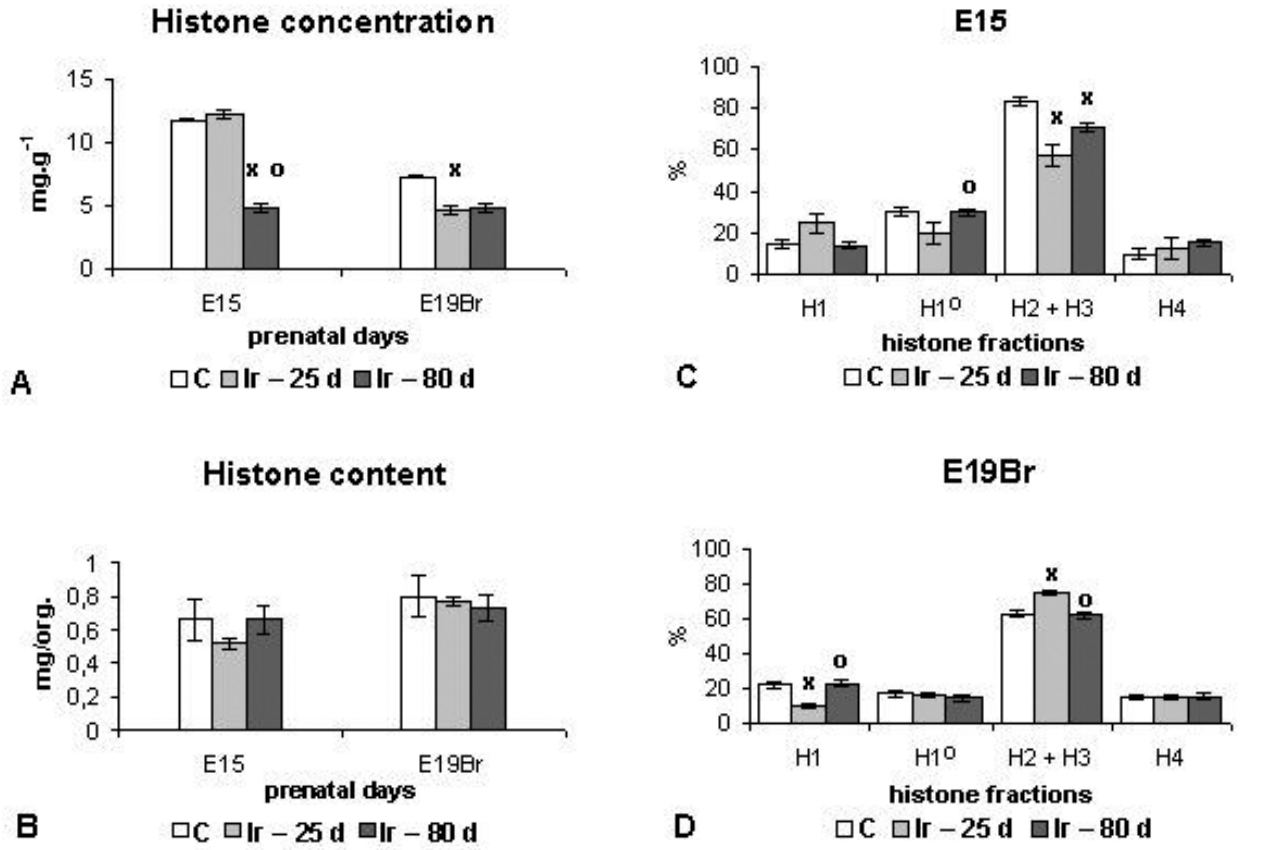

Fig. 2. Concentration, total content (A, B) and relative proportion of H1, H2 + H3 and H4 fractions and subfraction H1° of histones (C, D) in the progeny of rat males irradiated with the dose of 3 Gy gamma rays 25 or 80 days before mating, investigated on the 15th and 19th days of prenatal development (E15, E19Br). For explanation, see Fig. 1.

bryos (4.87 ± 0.47 *vs.* 7.27 ± 0.95). In the brain, the histone concentration was decreased compared to the control values also in offspring of males irradiated 25 days before mating (4.67 ± 0.36 *vs.* 7.27 ± 0.95; $P \leq 0.05$). In experimental groups, the reduction in histone concentration was not accompanied by a decrease in the total histone content due to concomitant increase in the weight of embryos.

Alterations in the relative proportion of individual histone fractions have also been found (Fig. 2C, D). In the offspring of control males, higher relative proportions of H1 and H4 and lower proportions of H2 + H3 histone fractions were found in the brain of 19-day-old embryos than in the whole 15-day-old embryos; moreover, in the brain histone H1 fraction, the proportion of H1° subfraction was significantly lower than in the whole embryos. Irradiation of males of parental generation 25 days before mating resulted in a marked decrease in the proportion of histone H2 + H3 fractions in the whole 15-day-old embryos (57.22 ± 7.95 *vs.* 83.42 ± 3.60 % in control groups; $P \leq 0.05$) and of H1 fraction in the brain of 19-day-old embryos (10.28 ± 1.27 *vs.* 22.03 ± 1.70; $P \leq 0.05$); the decrease in the proportion of H1 fraction in the brain histones was accompanied by an increase in the proportion of H2 + H3 fractions (74.96 ± 1.75 *vs.* 62.97 ± 2.4; $P \leq 0.05$). In the progeny of rats irradiated 80 days before mating, the alterations of histone fractions were milder and they manifested themselves only by a decrease in the proportion of H2 + H3 fractions in the whole 15-day-old embryos (70.86 ± 1.60 *vs.* 83.42 ± 3.60; $P \leq 0.05$).

Discussion

The effects of a sublethal dose of gamma irradiation, transferred to the progeny obtained from irradiated postmeiotic spermatids (on 25th day before mating) or premeiotic stem spermatogonia (on 80th day before mating) manifested themselves by changes of nucleic acids and histones at the prenatal stages of development.

In the progeny of rats, exposed 25 or 80 days before mating, the increase in RNA and DNA concentration and content was observed in the whole embryos on the 15th and 17th gestation day, respectively. The increase in DNA concentration can be related with an increase in the relative number of small cells with a high nucleo/cytoplasmic ratio, i.e. increased cellularity, and decreased ability of cells to recognize the DNA damage (Fiszer-Szafarz and Szafarz, 1984). The increase in the total content of DNA, which was found in the whole control embryos in the course of development (between 15th and 17th day) and mainly in the progeny of males irradiated 80 days before mating, is also due to the concomitant increase in the weight of embryos.

In the course of the experiment, the increase in nucleic acid concentration in the embryonic progeny of irradiated male rats was not accompanied by an increase in the concentration of histones; on the contrary, a decrease in histones occurred in the same groups of embryos. According to our previous finding (Klimová and Mišúrová, 2002), the decrease in histone content without concomitant reduction of DNA content after irradiation was related with the process of acetylation of some histone fractions connected with activation of cell proliferation and transcription. Histone acetylation results in weakening of electrostatic interactions between DNA and histones and leads to destabilization of nucleosomes during the chromatin transition to transcriptionally active stage. Concurrently, it affects the recognition of several regulatory proteins and transcription factors taking place in regulation of gene expression (Grünicke et al., 1989; Lodish et al., 2004).

The presented results showed that paternal irradiation at the sublethal dose of 3 Gy induced alterations in nucleic acids and histones, which were more profound after exposure of fertilizing spermatogonia (80 days before mating) than fertilizing spermatids (25 days before mating). This finding is presumably due to a decrease in genome stability, manifesting itself in the progeny of exposed parents.

These results correlate with those presented in our previous works (Kropáčová et al., 2002; Šanová et al., 2005; Bálentová et al., 2007b) focused on quantification of selected indicators of cellularity and cytogenetic changes in the embryos, suckling and young adult progeny of irradiated male rats. The increase in nucleic acids on the 15th and 17th days in the whole embryos of rats, mated 25 or 80 days after exposure (but not in the brain) was followed by increased cellularity previously observed approximately from the 1st to 14th postnatal day in brain hemispheres and cerebellum. The decrease in mitotic index and increase in the number of postmetaphase cells with severe chromosome damage were not connected with apoptotic fragmentation and loss of DNA (Šanová et al., 2005). Results of another experiment with postmeiotic paternal irradiation by the same dose of gamma radiation also showed striking age-dependent changes in cell proliferation in the forebrain of weanlings (Bálentová et al., 2007b). Dubrova et al. (2000) found an increase in paternal germ-line mutation rate at mouse expanded simple-tandem repeat (ESTR) minisatellite loci rather after irradiation of premeiotic spermatogonia than after irradiation of postmeiotic spermatids. On the other side, some authors refer that the genomic destabilization of the F_1 generation and consequently of the F_2 generation of progeny occurs regardless of the stage of spermatogenesis at the time of irradiation (Vorobtsova, 2000; Barber et al., 2002). In spite of these discrepancies, our results indicated that premeiotic and postmeiotic irradiation did not cause the same cytogenetic alterations (Šanová et al., 2005).

The present knowledge suggests that radiation-induced genome instability is caused by other alterations than the direct DNA damage. The findings of Dubrova et al. (2000) suggested that the radiation-induced changes leading to genomic instability may be inherited by an epigenetic alteration. Epigenetic mechanisms, such as hypomethylation or *de novo* methylation, are proposed as a major contributor to the process of carcinogenesis (Jones and Baylin, 2002). Recent works demonstrated that some kinds of epigenetic changes really can be induced by radiation exposure (Kovalchuk et al., 2004; Kožurková et al., 2007).

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