Abstract. Background: Radioadaptation is a phenomenon whereby cells exposed to a low dose of ionizing radiation are more resistant to a much higher dose delivered some time thereafter. This phenomenon could result from the activation of damage repair and/or antioxidant defense systems by the low dose. Materials and Methods: The existence of a cytogenetic adaptive response in female germ cells was investigated using a recently developed in vitro system. Mouse ovarian follicles were cultured from an early preantral stage up to ovulation. The follicles were X-irradiated with either 2 or 4 Gy (“challenge dose”) preceded or not by 50 mGy (“conditioning dose”, 5 h earlier), on days 0 or 12 of the culture. Ovulated oocytes were collected on day 13, fixed and analyzed for the presence of chromosome aberrations. Results: Irradiation with 2 or 4 Gy on days 0 or 12 did not influence ovulation but had dose-dependent effects on the germinal vesicle breakdown of the oocytes. It also caused dose-dependent chromosome damage, with a greater sensitivity of oocytes to this effect when irradiation occurred on day 12 than on day 0. Prior irradiation of oocytes with the dose of 50 mGy led to a reduction in the yield of chromosome aberrations when irradiation occurred on day 12 but not on day 0. Conclusion: These results suggest that pre-irradiation of mouse pre-ovulatory oocytes with a low conditioning dose could confer on them some protection against radiation-induced chromosomal damage by a subsequent challenge dose of a few Gy.

Radioadaptation is a phenomenon whereby cells exposed to a low dose (conditioning dose) of ionizing radiation are more resistant to a dose in the order of a few Gy (challenge dose) delivered some time thereafter, from a few hours up to 1 month or even more. As a result, cell lethality (1) and the frequencies of chromosomal damage (2-4), gene mutation (5) and neoplastic transformation (6-9) are reduced compared with the effects of the challenge dose alone. This inducible and transient protective effect also seems to occur in humans (10-12) and could result from a stimulation of cell defense and DNA repair systems (13-14).

Compared to somatic cells, germ cells are of greater relevance for the evaluation of genetic hazard because the DNA or chromosome damage induced in germ cells may be transmitted to the next generation where it can cause adverse inheritable effects. Studies performed in male mice showed that either acute or chronic exposure to low doses of ionizing radiation could induce a significant cytogenetic adaptive response in these cells (15-18). To our knowledge, no study has been published with regard to a possible adaptive response in mammalian female germ cells. During recent years, methods have been developed allowing in vitro development of ovarian follicles from the mid-growth stage (1-2 layers of follicle cells) to the pre-ovulatory stage (multilayered Graafian follicle). Upon appropriate hormonal stimulation, Graafian follicles can then be ovulated in vitro, producing a high yield of fertilizable metaphase II oocytes (19-20). We used this system to investigate the possibility of a cytogenetic adaptive response in female germ cells X-irradiated at two well characterized stages of maturation.

Materials and Methods

Animals. F1 hybrid females (♀ C57BL/6J X ♂ CBA/Ca) were used to obtain ovarian follicles. The mice were kept in our animal facilities on a 12-hour light, 12-hour dark cycle, and were used when they were 14 days old, allowing maximization of the number of good-quality preantral follicles suitable for follicle culture.

All animals were housed and bred according to national legislation and with the consent of the local Ethical Committee of the Belgian Nuclear Research Centre (Project number: 02-008).
In vitro culture. Ovaries of the prepubertal mice were dissected and collected at 37°C in L15 Leibovitz medium supplemented with 10% fetal calf serum (FCS; Gibco BRL, Gent, Belgium), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco BRL). The follicles used for the experiments were selected according to their intactness, diameter, presence of 1-2 granulosa cell layers and a clear central oocyte. The diameter of the selected follicles ranged from 100 to 130 μm. Follicles were grown individually in 20 μl of α-minimal essential medium (MEM; Gibco BRL) containing 5% FCS (Gibco BRL), 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (Sigma, Bornem, Belgium), 10 μIU/ml recombinant luteinizing hormone (r-LH) and 100 mIU/ml recombinant follicle stimulating hormone (r-FSH) (both kindly donated by Ares Serono, Genève, Switzerland). Fifteen follicles were cultured per culture dish (60 mm Falcon tissue-culture Petri dishes) covered with 5 ml of embryo-tested paraffin oil (Sigma-Aldrich, Bornem, Belgium). The follicles were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Every 2 days, half of the medium was replaced. On day 12, follicles were inspected for the formation of antral cavities, using a Zeiss axiophot inverted microscope equipped with a 40 X Hoffman Modulation Contrast objective (Carl Zeiss, Zaventem, Belgium). All manipulations and inspections occurred at 37°C.

Hormonal stimulation of in vitro ovulation. After inspection on day 12 of culture, follicles were stimulated to ovulate by addition of 1.5 IU/ml human chorionic gonadotropin (hCG; Serono) and 5 ng/ml epidermal growth factor (EGF; Boehringer, Brussels, Belgium) to the culture medium. In vitro ovulation, as indicated by the mucification of the cumulus oocyte complexes (COC), was assessed on day 13, 16 h after hormonal stimulation.

Irradiation. Follicles were X-irradiated in vitro either on day 0 or on day 12 of culture, based on the results of earlier in vivo studies which reported the highest frequency of mutational events in oocytes irradiated a few hours prior to ovulation (day 12 in the present study) (21-22), its low frequency in oocytes irradiated during the week preceding ovulation, and its two- to three-fold increase in those irradiated 10-14 days before ovulation (day 0 in this study) (23, 24). Irradiation was performed with a Pantak HF420 RX machine (Branford, CT, USA) operating at 250 kV, 15 mA, 1 mm Cu filtration and a dose rate of 0.375 Gy/minute. Four types of treatment were administered: (a) 50 mGy + 2 Gy at a 5-hour interval; (b) 2 Gy; (c) 50 mGy + 4 Gy at a 5-hour interval; (d) 4 Gy. For the various treatments, experiments were repeated 5 times, except for 50 mGy + 2 Gy on day 12 where 3 replicates were performed. In each experiment, 8 culture dishes were used together with 2 culture dishes with unirradiated follicles.

Oocytes irradiated on day 12 were given the small dose 2 hours before hormonal stimulation of ovulation and the high dose 3 hours after it. Microscopic examination of a number of oocytes after fixation and Giemsa staining revealed that at that time all of them were still at the germinal vesicle (GV) stage. Whatever the day of irradiation (day 0 or day 12), the high dose of X-rays (2 or 4 Gy) was always given at roughly the same time (4-5 p.m.).

Cytogenetic analysis of MI oocytes. On day 13, ovulated oocytes were analyzed for the presence of structural chromosome aberrations. The cytogenetic analysis was performed on metaphase I (MI) oocytes. In order to obtain ovulation of oocytes blocked in MI, colchicine was added to the hormonal solution used to induce in vitro ovulation on day 12 (final concentration of colchicine 250 ng/ml). Ovulated oocytes were freed from cumulus cells by repeated pipetting in a narrow glass pipette, then fixed (25) and stained with 2% Giemsa. Fixed oocytes were first examined for germinal vesicle breakdown (GVBD) and those that were in MI were analyzed for structural chromosome aberrations. With two exceptions, at least 100 well-spread MI figures were analyzed in each group. In case of doubt, chromosome preparations were restained with a slightly modified C-banding method (26).

Results

Development of cultured follicles. The in vitro development of follicles in the system used for these experiments has been described earlier (27) and will only be briefly summarized (Figure 1 A-F).

On day 1, theca cells surrounding the granulosa layer(s) of the follicle have attached to the bottom of the culture dish and will start to proliferate. By days 4-6, granulosa cell proliferation is very active, cells have crossed the basal membrane and the follicular diameter has enlarged. During the second half of the culture period, granulosa cells differentiate into two distinct subpopulations: steroid-producing mural granulosa cells separated by an antral-like cavity from the cumulus cells tightly enclosing the oocyte. The administration of the hormonal ovulatory stimulus on day 12 of culture induces detachment of the COC, mucification of the cumulus cells and resumption of meiosis in the oocyte.

In control conditions, approximately 75-85% of the cultured follicles had formed visible antral cavities by day 12 of culture (Figure 1B) and about 85-95% of the COC had undergone mucification (Figure 1C) after hormonal stimulation by day 13. Additionally, almost 90-95% of these “ovulated” oocytes exposed to colchicine had undergone GVBD (Figure 1E) and were in MI (Figure 1F), as expected. The 5-10% remaining oocytes were still at the GV stage (Figure 1D) (Tables I and II).

Figure 1. Summary of the in vitro development of mouse early preantral follicles. (A) On day 1, the oocyte is surrounded by 1-2 layers of granulosa cells, a basal membrane and a single layer of theca cells (arrow) (scale bar = 35 μm). (B) After 12 days of culture, granulosa cells have grown all over the theca cell monolayer, resulting in a considerable enlargement of the follicle, and the cumulus-oocyte complex is completely surrounded by antral cavities (scale bar=250 μm). (C) On day 13, 16 h after hormonal stimulation, in vitro ovulation has occurred, as evidenced by the mucified cumulus-oocyte complex (arrow) (scale bar=250 μm). (D) In vitro ovulated oocyte with an intact germinal vesicle (scale bar = 30 μm). (E) In vitro ovulated oocyte having undergone germinal vesicle breakdown (GVBD) (scale bar=30 μm). (F) First meiotic metaphase of a control oocyte showing 20 bivalents, after Giemsa staining.
Table I. Development of follicle/oocyte complexes after X-irradiation on day 0 of the culture (values in brackets are % relative to observed follicles). For the various parameters, groups irradiated with 2 or 4 Gy are compared to controls, while those irradiated with 50 mGy + 2 or 4 Gy are compared to groups given only 2 or 4 Gy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antral cavities</th>
<th>Mucification</th>
<th>GVBD/MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy</td>
<td>551/643 (85.69)</td>
<td>547/578 (94.64)</td>
<td>337/355 (94.93)</td>
</tr>
<tr>
<td>2 Gy</td>
<td>458/567 (80.78)</td>
<td>501/527 (95.07)</td>
<td>258/306 (84.31)**</td>
</tr>
<tr>
<td>4 Gy</td>
<td>555/676 (82.10)</td>
<td>634/676 (93.79)</td>
<td>366/540 (67.78)**</td>
</tr>
<tr>
<td>50 mGy + 2 Gy</td>
<td>374/485 (77.11)</td>
<td>561/601 (93.34)</td>
<td>346/410 (88.78)</td>
</tr>
<tr>
<td>50 mGy + 4 Gy</td>
<td>532/640 (83.13)</td>
<td>594/625 (95.04)</td>
<td>250/364 (67.78)**</td>
</tr>
</tbody>
</table>

GVBD: germinal vesicle breakdown; MI: metaphase I. Statistically significant at *p≤0.05 and **p≤0.01, respectively, using the Chi-square test with Yates correction.

Table II. Development of follicle/oocyte complexes after X-irradiation on day 12 of the culture (values in brackets are % relative to observed follicles). For the various parameters, groups irradiated with 2 or 4 Gy are compared to controls, while those irradiated with 50 mGy + 2 or 4 Gy are compared to groups given only 2 or 4 Gy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antral cavities</th>
<th>Mucification</th>
<th>GVBD/MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy</td>
<td>461/632 (72.94)</td>
<td>524/601 (87.18)</td>
<td>173/189 (91.53)</td>
</tr>
<tr>
<td>2 Gy</td>
<td>223/305 (73.11)</td>
<td>376/417 (90.17)</td>
<td>246/291 (84.53)*</td>
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<tr>
<td>4 Gy</td>
<td>368/473 (77.8)</td>
<td>419/473 (88.58)</td>
<td>268/367 (73.02)**</td>
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<tr>
<td>50 mGy + 2 Gy</td>
<td>260/318 (81.76)</td>
<td>301/318 (94.65)</td>
<td>181/215 (88.18)</td>
</tr>
<tr>
<td>50 mGy + 4 Gy</td>
<td>242/337 (71.81)</td>
<td>255/293 (87.03)</td>
<td>274/347 (78.96)</td>
</tr>
</tbody>
</table>

GVBD: germinal vesicle breakdown; MI: metaphase I. Statistically significant at *p≤0.05 and **p≤0.01, respectively, using the Chi-square test with Yates correction.

Development of cultured follicle/oocyte complexes after X-irradiation. Up to ovulation, the developmental capacity of follicles irradiated with either 2 or 4 Gy on day 0 of the culture did not much differ from that of the controls, except for a diminution (p≤0.05) of the proportion of follicles able to form antral cavities after a dose of 2 Gy, which was not confirmed after a dose of 4 Gy. However, X-irradiation induced a dose-dependent decrease of the proportion of oocytes undergoing GVBD (Table I). Administration of a conditioning dose of 50 mGy 5 h before the challenge doses of 2 or 4 Gy did not improve the development of the follicles.

Irradiation on day 12 of the culture did not influence ovulation, but again had a detrimental and dose-dependent effect on the GVBD of ovulated oocytes (Table I). Administration of a conditioning dose of 50 mGy 5 h before the challenge dose of 2 Gy seemed to increase the proportion of follicles forming antral cavities and undergoing mucification (p≤0.05). However, such a beneficial effect was not confirmed with the challenge dose of 4 Gy (Table I).

Furthermore, the formation of antral cavities was hardly influenced by the conditioning dose which was administered after the completion of this process. The conditioning dose of 50 mGy neither increased the rate of GVBD in oocytes given either 2 or 4 Gy 5 h later.

Chromosome aberrations in MI oocytes. The numbers of chromosome aberrations found in control and irradiated oocytes are given in Tables III and IV.

No chromosome aberrations were found in the control oocytes, while varying numbers of anomalies were present in irradiated oocytes, depending on the dose and the time at which irradiation had occurred. The types of aberrations that were observed were chromatin interchanges (giving multivalent figures), chromatid breaks, chromatid and isochromatid fragments (Figure 2A-B). An interruption of the continuity of a chromatid was classified as a chromatid break. In such a case, the portions immediately proximal and distal to the site often became out of alignment. Chromatid and chromosome fragments showed complete separation of the chromosomes. Univalent chromosomes (or “achiasmate bivalents”) were observed very rarely and were not classified as abnormal, since they could represent fixation artefacts.

Oocytes irradiated with 2 Gy on day 0 showed some fragments (mostly chromatid fragments) as well as multivalents resulting from chromatid interchanges (Table III). In oocytes given 4 Gy, the frequency of these aberrations was much higher, reaching 46.6% for chromatid/isochromatid fragments and 51.6% for multivalents, respectively. After that dose, a few chromatid breaks were also observed. Administration of a conditioning dose of 50 mGy 5 h before the challenge doses did not result in a decrease in chromosome aberrations (Table III).

A high frequency of chromosome aberrations was found in oocytes irradiated with 2 Gy on day 12 and the proportion of aberrations still strongly increased after 4 Gy (Table IV). In addition to fragments and multivalents, many breaks were observed. Irradiation with 4 Gy induced as many as 58.1% fragments and 61.5% multivalents. When the doses of 2 or 4 Gy were preceded by a conditioning dose of 50 mGy, the yield of abnormal oocytes (i.e. those showing at least 1 chromosome aberration) tended to decrease, as did the frequencies of the various types of chromosome aberration (with the exception of breaks after 50 mGy + 4 Gy). These effects were particularly significant in oocytes given a challenge dose of 4 Gy (Table IV).

Discussion

In vivo studies on X-irradiated mice have shown that structural chromosome aberrations can be induced in female germ cells and that the radiation-induced chromosomal damage strongly depends on the stage of maturation reached by the oocytes at the time of irradiation (21-24). In
Table III. Chromosome aberrations in metaphase I oocytes after X-irradiation on day 0 of the culture. For the various parameters, groups irradiated with 2 or 4 Gy are compared to controls, while those irradiated with 50 mGy + 2 or 4 Gy are compared to groups given only 2 or 4 Gy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oocytes examined</th>
<th>Abnormal oocytes (%)</th>
<th>Multivalents (%)</th>
<th>Breaks (%)</th>
<th>Fragments (%)</th>
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<tbody>
<tr>
<td>0 Gy</td>
<td>119</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2 Gy</td>
<td>136</td>
<td>22 (16.18)**</td>
<td>14 (10.29)**</td>
<td>0 (0)</td>
<td>9 (6.62)*</td>
</tr>
<tr>
<td>4 Gy</td>
<td>120</td>
<td>80 (66.67)**</td>
<td>62 (51.67)**</td>
<td>5 (4.17)</td>
<td>56 (46.67)**</td>
</tr>
<tr>
<td>50 mGy + 2 Gy</td>
<td>130</td>
<td>31 (23.85)</td>
<td>18 (13.85)</td>
<td>1 (0.77)</td>
<td>16 (12.31)</td>
</tr>
<tr>
<td>50 mGy + 4 Gy</td>
<td>114</td>
<td>64 (56.14)</td>
<td>53 (46.49)</td>
<td>7 (6.14)</td>
<td>43 (37.72)</td>
</tr>
</tbody>
</table>

Statistically significant at *p≤0.05 and **p≤0.01, respectively, using the Chi-square test with Yates correction.

Table IV. Chromosome aberrations in metaphase I oocytes after X-irradiation on day 12 of the culture. For the various parameters, groups irradiated with 2 or 4 Gy are compared to controls, while those irradiated with 50 mGy + 2 or 4 Gy are compared to groups given only 2 or 4 Gy.

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<th>Treatment</th>
<th>Oocytes examined</th>
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<tr>
<td>0 Gy</td>
<td>119</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>2 Gy</td>
<td>97</td>
<td>44 (45.36)**</td>
<td>27 (27.84)**</td>
<td>18 (18.56)**</td>
<td>18 (18.56)**</td>
</tr>
<tr>
<td>4 Gy</td>
<td>117</td>
<td>83 (70.94)**</td>
<td>72 (61.54)**</td>
<td>45 (38.46)**</td>
<td>68 (58.12)**</td>
</tr>
<tr>
<td>50 mGy + 2 Gy</td>
<td>97</td>
<td>28 (28.87)*</td>
<td>18 (18.56)</td>
<td>9 (9.28)</td>
<td>12 (12.37)</td>
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<tr>
<td>50 mGy + 4 Gy</td>
<td>100</td>
<td>63 (63.00)</td>
<td>40 (40.00)**</td>
<td>40 (40.00)**</td>
<td>40 (40.00)*</td>
</tr>
</tbody>
</table>

Statistically significant at *p≤0.05 and **p≤0.01, respectively, using the Chi-square test with Yates correction.

Figure 2. First meiotic metaphases of oocytes X-irradiated with either 2 or 4 Gy on day 12 of the culture. Several chromatid interchanges are visible (long arrows), as well as chromosome and chromatid fragments (short arrows) and a chromatid break (arrow head).
agreement with those studies and our recent in vitro study (27), the results of the present experiments confirmed the high radiation sensitivity of oocyte chromosomes 2 weeks prior to ovulation and their even higher sensitivity a few hours before ovulation. Furthermore, they confirmed that irradiation at these two stages is able to inhibit GVBD of oocytes in a dose-dependent manner (27). On the other hand, our results also suggested that prior exposure of pre-ovulatory oocytes to a low conditioning dose of 0.05 Gy could lead to a reduction of the chromosome damage induced in them by a subsequent exposure to a high dose of X-rays.

Somatic cells exposed to a low dose of ionizing radiation prior to a high-dose exposure were frequently reported to show less chromosome damage than expected (28-32), although there are also some publications showing the absence of a cytogenetic adaptive response in various mammalian cells (33-35).

The radioadaptive response appeared to have an optimum dose range below 0.1 Gy (36-37), to occur in metabolically active cells but not in dormant G0 cells (38-39), to represent an immediate early reponse being expressed maximally 4-6 h after irradiation (37-38) and to continue for more than 20 h (37-38).

Data on an adaptive response in embryonic and germ cells are still scarce. A cytogenetic adaptive response was not elicited in the early stages of mouse embryogenesis (40-41), while Wang et al. (42) as well as Boreham et al. (43) presented evidence that priming doses of 0.3 Gy given during late gestation (day 11 or 10, respectively) protected the fetus against the teratogenic effects (limb defects and short tails) of a subsequent exposure to 5 or 4 Gy given 24 h later. The adaptive protection by this unusually large priming dose apparently resulted from a Trp 53-dependent process and could involve a sensitization to apoptosis induced by the subsequent exposure to the challenge dose (43).

With regard to the germ cells, the results of Cai and colleagues (15-17) showed that single (0.05 Gy) or multiple (0.05 Gy x 4) low doses of X-rays induced an adaptive response in mouse male germ cells to chromosomal damage (chromatid breaks and multivalents) and to embryo’s dominant lethality. Furthermore, chronic low-dose exposure to γ-irradiation also induced a marked cytogenetic adaptive response in the male germ cells (18). On the other hand, Boreham et al. (43) reported that a priming dose of 0.1 Gy was able to reduce the induction of germ line mutations at ESTR loci in paternal germ cells exposed to 1 Gy 24 hours later. Studies on female germ cells were not performed in mammals but Fritz-Nigli and Schaeppi-Buechi (44) reported an adaptive response in mature and immature oocytes of Drosophila melanogaster given a low dose of 20 mGy X-rays at various time intervals before a large dose of 2 Gy. The adaptive response was expressed by a reduced dominant mutation lethality.

The results reported here suggest that pre-irradiation of mouse female germ cells with a low conditioning dose of X-rays would not improve their survival and maturation up to ovulation after delivery of a high challenge dose of a few Gy. However, it could possibly confer on them some protection against radiation-induced chromosomal damage by the subsequent high dose. The possibility of a cytogenetic adaptive response was suggested by our results on pre-ovulatory oocytes irradiated just before germinal vesicle breakdown. An adaptive response was definitively not observed in growing oocytes irradiated 2 weeks before ovulation.

Low-dose radiation has been suggested to stimulate damage repair mechanisms (7, 45) and/or the activities of oxidative radical scavengers to minimize the indirect damaging effects of subsequent radiation (8, 46). Protein synthesis was also shown to be required for the induction of a cytogenetic adaptive response by low-dose radiation (47, 48). This suggests that the metabolic state of the cells at the time of the exposure to the conditioning dose is important for the induction of the adaptive response. Obviously, the metabolic activities of pre-ovulatory oocytes enclosed in multilayered antral follicles (day 12 of the culture, in our experimental conditions) markedly differ from those of small oocytes enclosed in secondary follicles (day 0 of the culture). Pre-ovulatory oocytes were given the challenge dose immediately before GVBD which marks the end of the very long prophase and it will be interesting to further investigate the response of oocytes irradiated at the diakinesis stage which follows. During that stage, the sensitivity of oocytes to radiation induced chromosomal damage has been reported to be strikingly high (49-51), a fact which could be related to a decrease of their DNA repair capacity (50, 52-54).

In many studies it has been demonstrated that different sets of genes are activated following high or low doses of radiation, thus suggesting unique biological responses as a function of dose. Identifications of these genes is providing a scientific basis for defining metabolic pathways activated by radiation and determining mechanisms of actions. Cells and tissues that demonstrate an adaptive or protective response following low-dose exposures have up-regulated repair and stress genes (55). Studies of gene expression in pre-ovulatory oocytes exposed to low-dose irradiation during the period from the late diplotene to the metaphase I stage could help to shed some light on a potential adaptive response in female germ cells.

Acknowledgements

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References