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ORIGINAL PAPER

Reduction of transgenerational radiation induced genetic damages observed as numerical chromosomal abnormalities in preimplantation embryos by vitamin E

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Abstract. To study the effects of parental γ-irradiation (4 Gy) of NMRI (Naval Medical Research Institute) mice on the numerical chromosome abnormalities in subsequent preimplantation embryos in the presence of vitamin E (200 IU/kg), super-ovulated irradiated females were mated with irradiated males at weekly intervals in successive 6 weekly periods. About 68 h post coitus, 8-cell embryos were fixed on slides using standard methods in order to screen for abnormalities in chromosome number. In embryos generated by irradiated mice, the frequency of aneuploids dramatically increased compared to control unirradiated groups ($p < 0.001$), while no significant difference were observed within irradiated groups mated at weekly interval. Administration of vitamin E significantly decreased chromosomal aberrations in all groups ($p < 0.05$). Data indicate that γ-irradiation affects spermatogenesis and oogenesis and causes DNA alterations that may lead to chromosome abnormalities in subsequent embryos. Vitamin E effectively reduced the frequency of abnormalities. The way vitamin E reduces genotoxic effects of radiation might be via radical scavenging or antioxidative mechanism.

Key words: paternal irradiation • γ-rays • preimplantation embryo • chromosomal abnormalities • vitamin E • radioprotection • NMRI mouse

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Introduction

Germ cell mutagens produce heritable gene mutations, and heritable structural and numerical chromosome aberrations in germ cells. The consequences of germ cell mutations in subsequent generations include genetically determined phenotypic alterations without signs of illness, or reduction in fertility, or embryonic or prenatal death, more or less severe congenital malformations, or genetic diseases with various degrees of health impairment [1].

Male and female germ cells vary in their sensitivity to the mutagenic effects of chemotherapy and radiotherapy, depending on their stage of maturation and the agent used [2, 40].

In irradiated male mice, post irradiation spermatozoa exhibit increase in the incidence of abnormal shaped spermatozoa, preimplantation loss [7, 46] and transmission of tumors to the first (F1) progeny [38]. It has been shown that DNA-damaged sperms have the ability to fertilize the oocyte, but that embryonic development is very much related to the degree of DNA damage. The majority of *de novo* structural chromosome aberrations in fetuses and newborns are considered as being of paternal origin that is of sperm origin [39].

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, 33]. However, the result of numerous publications suggests that radiation may also have an indirect effect on genome stability which is transmitted through the germ line of irradiated parents to their offspring [11, 13].

Ionizing radiation triggers the formation of free radicals, which interact among themselves to form newer reactive oxygen species (ROS). Some of these stable free radicals may be responsible for the genomic instability mediated by the microenvironment [6]. Normally, cells contain enzymatic antioxidant defense mechanisms, which serve to scavenge free radicals. However, ionizing radiation is such a potent free radical former that intracellular enzyme systems fall short of ridding the cell of excessive amounts of free radicals [3, 53]. Numerous studies have examined the radioprotective effects of antioxidant substances, known as free radical scavengers, which protect the cell and its organic constituent molecules from free radical damage.

Antioxidants are one such a class of agents, which are non-toxic and moderately radioprotective. These antioxidants include tocols (tocopherols and tocotrienols), soy-isoflavones vitamin A, β-carotene, selenium (organic and inorganic), zinc, copper, and the enzyme supper oxide dismutase and its mimetics [27–30, 47, 53, 54]. Among these compounds, vitamin E has attracted considerable attention. Previously the radioprotective effects of vitamin E on cytotoxicity, DNA single strand breaks, chromosomal aberrations and mutation in Chinese hamster V-79 cells exposed to ultraviolet-b light [31, 51], human HCC cell line [17], the intestinal lumenal route [18], radiation-induced cataract [25] and on mice spermatogenesis [37, 49] were investigated.

Despite of numerous published studies on radiation, relatively little information is available on parental irradiation and the incidence of chromosomal abnormalities in their preimplantation embryos. In our previous study we have shown a high frequency of chromosomal aneuploids in embryos following paternal irradiation during various spermatogenic cycle and vitamin E could effectively reduced the frequency of abnormalities [37]. Study of parental irradiation and its genetic consequences is a very important issue in case of radiological and nuclear accidents, where both males and females are affected. To our knowledge, there is no such report describing the extent of chromosomal damage transmitted through parental irradiation to their preimplantation embryos. Therefore, the aim of this investigation was to study the effect of pre-mating parental (both mother and father) whole body gamma irradiation of mice alone or in combination with vitamin E as a relatively known antioxidant on the frequency of numerical chromosome aberrations in subsequent preimplantation embryos.

Materials and methods

Animals

Adult 8–11 weeks male and female albino NMRI mice with a mean weight of 30 ± 5 g (Razi Institute, Karaj, Iran) were used in this study. The animals were housed in a room kept in mesh cages at 22°C with a cycle of 10 h darkness and 14 h light and fed with standard mouse peletes and water *ad libitum*. This study was approved by the Ethical Committee of the Tarbiat Modares Universiy and animals were treated according to the university regulations.

Vitamin E treatment

All reference to vitamin E in this paper will be to α-tocopherol, and these two terms (α-tocopherol and vitamin E) may be used interchangeably. Vitamin E (Darupakhsh, Iran) was administered intraperitoneally (i.p.) one hour before irradiation, at a dose of 200 IU/kg. Vitamin E was dissolved in a sufficient amount of olive oil before injection. The rationale for using this dose of vitamin E was based on our previous experience in which this dose could effectively reduce teratogenic effects of radiation exposure during organogenesis in mice [42] and also low dose reduction factor yield reported with a lower dose of vitamin E (100 IU/kg) against killing effects of gamma rays [50]. There was a control group receiving only vitamin E to study possible genotoxic effect of the dose used in this investigation.

Gamma irradiation and coupling

Mice were whole body irradiated alone or in the presence of vitamin E with 4 Gy gamma rays generated from a cobalt-60 source (Theratron II, 780 C, Canada) at a dose rate of 132 cGy/min, with a source to sample distance (SSD) = 82 cm, field size: 20 × 20 cm at room temperature (23 ± 2°C).

For maternal (female) irradiation study, female mice were whole body irradiated alone or in the presence of vitamin E after induction of super ovulation in females using intraperitoneal (i.p.) injection of 10 international units (IU) of pregnant mare's serum gonadotrophin folligon (PMSG; Intervet, Holland) followed by injection of 10 IU of human chorionic gonadotrophin (HCG; Organon, Holland). Female mice were irradiated approximately 18–20 h after PMSG injection and 24 h prior to HCG injection. Two irradiated female mice were transferred with a male (unirradiated) in a cage for an overnight to mate. The next morning female mice were checked for vaginal plug (VP). A VP positive female was considered as a pregnant mouse.

For parental (male and female) irradiation study, male mice were whole body irradiated alone or in the presence of vitamin E with an irradiation condition described above. Four days after gamma irradiation, irradiated male mice were mated with super-ovulated irradiated females in successive 6 weeks at weekly intervals. Three to five irradiated mice were assigned for coupling in each experimental group. Two irradiated female mice were transferred with an irradiated male in a cage for an overnight to mate. The next morning female mice were checked for VP. A control unirradiated and also only vitamin E treated animals were assigned for each experimental group. All experiments were repeated for three times.

It is worthy to mention that several authors used the dose of 4 Gy for studying radiation induced genomic instability [26], *in vitro* cytogenetic studies on human and mouse germ cells [21, 52] and prenatal effects of gamma irradiation [5]. This dose of radiation was chosen for induction of enough abnormalities to observe the effect of vitamin E.

Embryo recovery

About 68 h post coitus (p.c.), the pregnant females were sacrificed by the cervical dislocation method and their oviducts were flushed using a special flush syringe (Supa, Iran) filled with a 37°C incubated T6 medium [ingredients for pH of 7.2–7.4; NaCl (4.73 mg/ml), KCl (110 μg/ml), NaH_2PO_4 (50 μg/ml), $MgCl_2{\cdot}6H_2O$ (100 μg/ml), $CaCl_2{\cdot}2H_2O$ (260 μg/ml), $NaHCO_3$ (2.10 mg/ml), Phenol Red (10 μg/ml), ethylenediaminetetraacetic acid (EDTA, 6 μg/ml), glucose (1 mg/ml) and Na-pyruvate (30 μg/ml) purchased from Sigma, St. Louis, MO, USA; Penicillin G (60 μg/ml) and Streptomycin (50 μg/ml) from Seromed, Berlin, Germany and Na-lactate (1.98 μg/ml) from Merck, Darmstadt, Germany]. The flushing was done under a stereomicroscope (Hund-Wetzlar, Wetzlar, Germany) to obtain 4–8 cell embryos. The collected morphologically normal embryos were transferred to a fresh T6 medium supplemented with 15 mg/ml Bovin Serum Albumin, (BSA, Sigma, St. Louis, MO, USA) containing 0.2 μg/ml colcemid (Gibco BRL, Lifetech, Paisley, UK) incubated in a humidified CO_2 incubator (Lifetech, Paisley, UK) at 37°C for 16–20 h.

Cytogenetic analysis

For cytogenetic analysis, the Dyban method, which is a suitable method for analysing chromosomes of embryo cells, was used with some modifications [14]. Briefly, the zona Pellucida was removed by the use of Tyrode's acid (ingredients for pH = 2.5; NaCl (8 mg/ml), KCl (2 mg/ml), $MgCl_2{\cdot}6H_2O$ (0.1 mg/ml), $CaCl_2{\cdot}2H_2O$ (0.25 mg/ml), glucose (1 mg/ml) and polyvinylpyrrolidone (4 mg/ml) all from Sigma, St. Louis, MO, USA). This process was followed under a stereomicroscope to avoid damage to the blastomers. Then, the embryos were transferred to a watch glass containing 1% sodium citrate (Sigma, St. Louis, MO, USA) as a hypotonic solution for 30 min. The embryos were placed on a precleaned slide and fixed with a drop of fixative consisting of methanol and acetic acid (3:1) (Merck, Darmstadt, Germany). After leaving overnight at room temperature, the slides were stained in 3% Giemsa (Merck, Darmstadt, Germany) for 3 min and cells were analyzed under a light microscope (Nikon, Kawasaki, Japan) at ×1000 magnification to screen numerical chromosome abnormalities. The numerical chromosome abnormalities were divided into hyperdiploids, aneuploids, hypodiploids and polyploids.

Statistical analysis

Data were statistically analyzed and the significance of any inter-group differences was evaluated with χ^2 and the Mann-Whitney U-test to compare two groups using SPSS (version 12) software. *P*-value of less than 0.05 was considered as significant.

Results

Results are summarized in Table 1 and shown in Fig. 1. About 8% of metaphase plates in the control group showed aneuploids. As seen, radiation dramatically increased the frequency of chromosomal aberrations in preimplantational embryos compared to the embryos generated from non-irradiated female mice.

The total number of embryos generated from the preovulatory stage irradiated female group was 203 embryos from which 171 embryos were 4–8 cells and morphologically normal. About 50% of the observed metaphase plates in the irradiated female group showed numerical chromosome abnormalities which is significantly higher than the control ($p < 0.001$).

The total number of embryos generated from the irradiated parents was 652 embryos from which 288 embryos were 4–8 cells and morphologically normal and were divided into six groups according to the time of mating post irradiation. In the embryos generated from irradiated parents from the 1st till 6th weeks post irradiation, 87.5, 82.5, 71, 81, 90 and 93.5% of metaphase plates, respectively showed numerical chromosome abnormalities which are significantly higher than the control ($p < 0.001$). As these data showed, the frequency of numerical chromosome abnormalities in all the six test groups was significantly higher than those in the control group. This increase is also significantly higher than the observed chromosome abnormalities in the test groups where only female mice (maternal) were irradiated (Fig. 1).

Administration of 200 IU/kg vitamin E resulted in a lower frequency of chromosomal abnormalities in all radiation test groups. The frequency of numerical chromosome abnormalities in embryos generated from

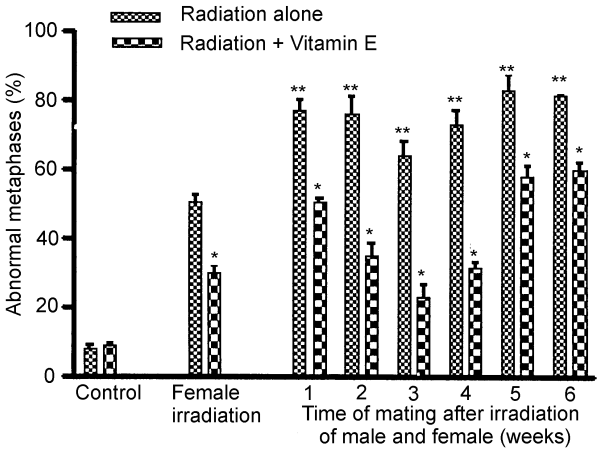

Fig. 1. Frequency of abnormal metaphases observed following mating of irradiated male with an irradiated female mouse in the presence and absence of 200 IU/kg vitamin E at a weekly interval. Error bars show the standard error of mean values obtained from three independent experiments. * indicates statistically significant difference with irradiated groups ($p < 0.05$). ** indicates statistically in significant difference between irradiated male groups ($p > 0.05$).

Table 1. Comparison of the frequency of numerical chromosomal abnormalities in the embryos generated from gamma irradiated (4 Gy) male and female NMRI mice in the presence or absence of vitamin E (200 IU/kg) after mating at weekly intervals 1 to 6 weeks post irradiation

Treatment	No. pregnant mice[@]	Total[#] embryo	4–8 cells normal embryos	Observed metaphase plates	Normal metaphase	Abnormal metaphase	Aneuploids	Hyper-diloids	Hypo-diploids
Control	**9**	120	117	95	87	8	8	0	0
Vitamin E alone	**8**	100	93	83	75	8	8	0	0
				Female*					
Irradiated	**13**	**203**	**171**	**121**	**60**	**61**	**34**	**14**	**13**
+ vitamin	**10**	159	94	50	35	15	8	3	4
				Week 1 (W1)**					
Irradiated	**9**	**137**	**67**	**40**	**5**	**35**	**31**	**2**	**2**
+ vitamin	**15**	250	189	97	38	59	45	6	8
				Week 2 (W2)					
Irradiated	**9**	**150**	**112**	**40**	**7**	**33**	**29**	**2**	**2**
+ vitamin	**10**	160	115	38	20	18	16	0	2
				Week 3 (W3)					
Irradiated	**10**	**155**	**69**	**45**	**13**	**32**	**29**	**2**	**1**
+ vitamin	**12**	162	92	35	23	12	10	1	1
				Week 4 (W4)					
Irradiated	**6**	**100**	**20**	**32**	**6**	**26**	**22**	**2**	**2**
+ vitamin	**7**	100	52	28	16	12	10	1	1
				Week 5 (W5)					
Irradiated	**4**	**50**	**10**	**35**	**3**	**32**	**24**	**5**	**3**
+ vitamin	**7**	100	12	43	13	30	19	6	5
				Week 6 (W6)					
Irradiated	**5**	**60**	**10**	**31**	**3**	**28**	**21**	**3**	**4**
+ vitamin	**7**	98	17	38	9	29	23	3	3

[@] Number of pregnant mice (sacrificed VP+) used to retrieve the total number of embryos.
[#] Total embryos indicated in this table represent all retrieved embryos including, degenerated, blocked and morphologically normal embryos.
* Irradiated female mated with unirradiated male mouse.
** W1–W6: One–six week post gamma irradiation, mating time of irradiated male with irradiated female mouse.

γ-irradiated parents in the presence of vitamin E from 1st to 6th weeks post irradiation decreased to 60, 47.3, 34.2, 42.8, 69.7, 76.3%, respectively which are statistically significant for the data obtained with radiation alone ($p < 0.05$). Dose reduction factor (DRF), calculated as the ratio of abnormal metaphase induced by radiation alone to the number of abnormal metaphase in the presence of vitamin E, for all male and female irradiated groups show a range of 1.18–2.07 with a mean value of 1.61, which is comparable with the DRF calculated for irradiated female mice at 1.7 (Table 2).

Table 2. Dose reduction factor (DRF) calculated for treatment with gamma rays in the presence of vitamin E. DRF was calculated as the ratio of abnormal metaphase induced by radiation alone to the number of abnormal metaphase in the presence of vitamin E

Treatment	DRF
Irradiated female only	1.7
Male and female irradiated	
Week 1*	1.44
Week 2	1.74
Week 3	2.07
Week 4	1.89
Week 5	1.31
Week 6	1.18
Mean	1.61

* Mating time post irradiation.

Discussion

The need for good quality metaphases for cytogenetic analysis of preimplantation embryos is a major limitation of such studies because only one-third of the mitoses are analysable (Table 1) [41, 44]; the obtaining morphologically normal embryos from irradiated parents is very difficult as well. As shown in Fig. 1, the frequency of numerical chromosome abnormalities in subsequent embryos, generated from pre-ovulatory stage gamma irradiated female mice, is significantly higher than the control group ($p < 0.001$). These abnormalities may be due to translocations and other chromosomal abnormalities induced in oocytes which can lead to generation of abnormal embryos. The induction of structural chromosome changes by ionizing radiation in female germ cells has been reported previously [21]. These anomalies are often lethal for cells or embryos, and only a small proportion is transmitted to the F1 progeny [43]. Oocytes at different stages of maturation

vary in their radiosensitivities from those a few hours from ovulation, being considerably more sensitive than maturing dictyate stages [2, 4, 21, 23].

It was shown that oocytes irradiated at the beginning of the oestrous cycle had a low frequency of chromosome aberrations, which those irradiated at the middle of the oestrous cycle (when growing Graafian follicles are clearly visible at the surface of the ovaries), exhibited heavy chromosome damage [22]. Our observation shows that pre-ovulatory maternal irradiation has an important role in the chromosomal abnormalities of generated embryos.

In an investigation on *in vitro* fertilization rate of mouse eggs with sperm after X-irradiation at various spermatogenesis stages, [34] have shown that the number of fertilized eggs seemed to remain constant almost at control level until the 4th week after X-irradiation reaching to a minimum level in the 6th week. We have previously shown that the frequency of numerical chromosome abnormalites in embryos generated from paternal (male only irradiated) gamma irradiation for all 6 weeks mating post irradiation was significantly higher than the control group, moreover, the frequency of abnormalities sharply increased from the 4th through the 6th weeks post irradiation [37]. Other researchers have also shown that the frequency of morphologically abnormal spermatozoa from irradiated male mice significantly increased from the 4th till 6th weeks post irradiation [7, 20, 46].

In spite of the high radiosensitivity of spermatozoa, it is shown that spermatozoa can retain a high fertilizing ability even after a high dose of irradiation. This suggests that radiation-induced DNA damages in spermatozoa may be transmited to the next generation without being selected out at fertilization [24]. Required time for spermatogenesis in mice for spermatozoa development from the stem cells is more or less constant (about 6 weeks). Therefore, the fertilizing spermatozoa in the first week post gamma irradiation has been in its spermatid stage at the time of irradiation, also gamma-irradiated early spermatid, secondary spermatocyte, early spermatocyte and spermatogonia stages act as a fertilizing spermatozoa in the 2nd, 3rd, 4th, 5th and 6th weeks post irradiation, respectively [36].

Data shown in Table 1 and Fig. 1, clearly indicate that the frequency of abnormal metaphases following paternal and maternal irradiation is significantly higher than those obtained following only maternal irradiation. This increase might be attributed to the effects of gamma irradiation on all stages of spermatogenesis cycle in the male mice. These abnormalities may be due to translocations induced in chromosomes and meiotic recombination involving a chromosome inversion that affects chromosome pairing and meiotic segregation in male mice as well as centromere spliting causing aneuploids. Results in the present study suggest that when both parents are gamma irradiated at different stages of spermatogenesis and pre-ovulatory stage of oocyte in male and female respectively, the frequency of numerical chromosome abnormalities increased significantly compared with those when only a female was irradiated (Fig. 1).

It has been established that cell damage induced by radiation is mediated by free radicals produced by radiation [10, 18]. In cells, free radicals are the cause of formation of other free radicals, lipid peroxidation [3, 48, 53], DNA damage mainly double strand breaks (DSB), mutagenesis and cancer [9, 19, 35]. Free radicals are removed from cells by the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase [3, 12, 53]. It has been proposed that apart from these antioxidant enzymes, other substances which are not enzymes, when taken at supra-physiological doses, might also serve to protect cells against radiation damage mediated by free radicals, notable among these substances are some vitamins including vitamin E (alpha-tocopherol). Administration of vitamin E, 2 h prior to gamma irradiation led to the reduction of chromosomal damages in mouse bone marrow cells [45]. Recently, the radioprotective effect of vitamin E was shown on the human HCC cell line [17], on radiation-induced cataract [25], on mice spermatogenesis [49] and on the frequency of chromosomal abnormalities in preimplantation embryos generated from male mice [37]. In the present study administration of 200 IU/kg vitamin E using i.p. injection, an hour prior to acute gamma irradiation of mice significantly decreased the frequency of numerical chromosome abnormalities in subsequent embryos in all the test groups ($p < 0.05$) (Fig. 1).

Various protection mechanisms against these effects have been proposed for vitamin E. Vitamin E either donates a hydrogen atom or possibly interacts with arachidonic acid to protect the membrane lipid bilayer. In addition, it may exert its protective effect by stabilizing the polypeptide chains of proteins [15, 32]. Vitamin E can scavenge molecular oxygen, proxide and hydroxyl radicals and atomic oxygen radicals [8, 16].

Decrease of chromosome aberrations in the presence of vitamin E is probably due to antioxidant effects of this vitamin and scavenging free radicals induced by gamma rays in mice gametes environment. Achievement of a mean DRF of about 1.61 by vitamin E when both parents are irradiated and DRF of 1.7 when only female mouse is irradiated is a notion for protection of individuals at risk of exposure to ionizing radiation.

In conclusion, the presented results indicate that irradiation of mice during various stages of gametogenesis could lead to morphologically normal preimplantation embryos with high frequency of chromosomal aneuploids, and administration of vitamin E before irradiation could effectively reduce this effect.

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