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Differential Functions of Caffeine and Ascorbic Acid in γ-Irradiated Male Mice

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Abstract - Radioprotection is of practical importance for the normal tissues of tumor patients subject to radiotherapy, people with planned or accidental exposure to radiation, and the public and radiation workers. Since oxygen enhances radiation-induced biological damage, antioxidants should be related with the function as a radioprotectors. Ascorbic acid or caffeine is an essential component and antioxidant in the diet of humans and a small range of other mammals. The present study investigates functional radioprotection of caffeine and ascorbic acid against gamma radiation in irradiated C57BL/6N mice. Eight-week-old male C57BL/6N mice were irradiated with 6.5 Gy. A caffeine treated group was administered with 80 mg kg^{-1} body weight by intraperitoneal injection, a single treatment 1 hr before irradiation. Ascorbic acid was administered 330 mg L^{-1} in drinking water through all the experimental period. According to time schedules, animals were sacrificed by cervical dislocation. And the samples were collected 2 weeks after whole-body gamma irradiation. The caffeine treated group showed lower decrement of body and organ weights than the other experimental groups. The qualitative analysis of circulating testosterone in serum was performed by means of radioimmunoassay (RIA). The normal level of circulating testosterone was maintained by the treatment of caffeine and ascorbic acid. The change of weight of body and organ and the appearance of seminiferous tubules were improved by an effect of caffeine or ascorbic acid against irradiation. Taken together, caffeine and ascorbic acid protects impairment of spermatogenesis against gamma radiation and may act as a radioprotector.

Key words : caffeine, ascorbic acid, gamma irradiation, male, mice

INTRODUCTION

A requirement of radioprotectors has been rising because of the scattered possibility of exposure to radiation; accidental external or internal exposure, chronic exposure of the lungs to environmental alpha-particles, cosmic radiation exposure during a manned space flight and working over the long haul in an airplane, and therapeutic exposures to radiation. The oxygen effect in radiation biology is well known. Radiation injury occurs through the formation of reactive oxygen species. Since oxygen also enhances radiation-induced biological damage, antioxidants should be radioprotectors. Numerous antioxidants have proven beneficial to radioprotection, among which well known are cysteine, cysteamine, glutathione, WR-compounds, tempace, vitamine A, E, and C, bioactive lipids (dimethylprostaglandin E2, platelet activating factor, and leukotriene C4), and immunomodulators (glucan, synthetic trehalose dicorynomycolate, and interleukin-1) (Goa and Spencer 1995; Nair *et al.* 2001). Ascorbic acid is a well-known antioxidant and an essential component in the diet of humans and a

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small range of other mammals. Ascorbic acid is known to react with free radicals (Singh and Gaby 1991). Because ionizing radiation leads to the production of the activated oxygen species, increased glutathione levels in tissues by the vitamins may contribute to scavenging free radicals generated from irradiation and *in vivo* protection against radiation injury (Tubiana *et al.* 1990). Radioprotective effects of ascorbic acid have been demonstrated in certain cells and animals, which would result from scavenging free radicals (O'Connor *et al.* 1977; Stratford *et al.* 1988). Because the vitamins do not modify tumor growth delay induced by irradiation and concomitantly exhibit radioprotective effects in normal tissues (Okunieff and Suit 1987), these agents have great therapeutic potential as radioprotectants.

Caffeine (1, 3, 7-trimethylxanthine) is one of the most extensively studied ingredients in the food supply. Caffeine exists in diet from a variety of sources-primarily coffee, tea, chocolate, drinks, and both prescription and nonprescription drugs. Recently, caffeine in a cell cycle has shown to override the cell cycle checkpoint in particular a G_2 delay (Powell *et al.* 1995). Caffeine inhibits ATM and ATR in a dose-dependent fashion. The concentration that inhibits ATM by 50% *in vitro* is 1 mM and the 50% inhibitory concentration for ATR is about 3 mM (Sarkaria *et al.* 1999). Studies by George *et al.* (1999) showed that a dose of 80 mg kg^{-1} administered 60 min before a whole-body exposure to 7.5 Gy was optimal for a maximal radioprotection (George *et al.* 1999). Caffeine and its xanthine metabolites had an oxygen radical absorbing capacity comparable with that of ascorbic acid or uric acid (Chul 2000; Azam *et al.* 2003). Moreover, Lu *et al.* (2000) demonstrated that caffeine had an inhibitory effect on UV-induced carcinogenesis. Knowledge of the clear function and side effects of caffeine in the irradiated individuals has been poor. Thus the present studies are to examine the effects of caffeine treatment before whole-body irradiation in male mice. According to the previous investigations, the testis is now recognized as one of the most radiosensitive organs of the body. Interestingly, differential sensitivity of irradiation depends on cell types in the testes (Inano *et al.* 1989). The male mouse gonad contains both radiosensitive (premeiotic germ cells) and radioresistant (somatic) cells. Indeed, irradiation ($\geq$5 Gy) of the male gonad selectively destroys premeiotic (differentiating spermatogonia) germ cells, whereas other cells, i.e., somatic (Leydig and Sertoli cell) cells and postmeiotic (spermatids) germ cells are unaffected (Rodriguez *et al.* 1997). However, it has been reported that fetal irradiation is associated with an increase of hCG binding and testosterone production per Leydig cell, and a decrease in the number of these Leydig cells per testis (Jansz and Pomerantz 1984; Pinon-Lateillade *et al.* 1991). The previous data suggest that somatic cells may be biochemically and physiologically radiosensitive. Therefore, the present study is designed to investigate and compare the radioprotective effects of caffeine and ascorbic acid on spermatogenesis of mice after whole-body gamma irradiation.

MATERIALS AND METHODS

1. Animals and irradiation

The animal was obtained from Daehan Biolink (Chungbuk, Korea). C57BL/6N mice (male, 8-week-old) were used throughout the investigations. All the mice were maintained under the following conditions; temperature (23°C) and lighting (14 hr light : 10 hr dark) and allowed free access to food and water. Irradiated groups were exposed to γ-irradiation using a ^{60}Co source with a total dose of 6.5 Gy, and a dose rate of 12.8 Gy hr^{-1} (Kim *et al.* 1999).

2. Treatments of caffeine and ascorbic acid

The caffeine treated group was administrated with 80 mg kg^{-1} body weight by *i.p* injection, a single treatment, one hour before irradiation (George *et al.* 1999). The ascorbic acidtreated group was administered with 330 mg L^{-1} in drinking water during experiment (Maeda *et al.* 2000). The remaining mice were kept as sham controls. According to the experiment schedules, mice were sacrificed by cervical dislocation 2 weeks after whole-body irradiation. The body weight (BW) and organ weight (OW) were recorded and the indices were calculated with the equation; OW × 100/BW.

3. Immunohistochemical analysis for Bcl2 and Bax in testis

Testes were fixed overnight at room temperature with

10% neutralized buffered formalin (NBF), dehydrated, and embedded in paraffin. Sections (6 μm) were prepared and mounted on slides. The slides were then hematoxylin and eosin (H-E) stained for morphological observations.

For the immunohistochemical analysis, a rabbit polyclonal IgG antibody against Bcl-2 (N-10) and a mouse monoclonal IgG26 antibody (B-9) from Santa Cruz Biotechnology (Santa Cruz, CA) were used. Sections (5 μm) were mounted onto poly-lysine-coated slides. After rehydration, the slides were washed with distilled water (DW) each followed by the antigen retrieval at 121°C for 15 min in 10 mM citrate solution (pH 6.0). After the washes with DW, enough drops of 0.05 mL of peroxide blocking solution (0.3% H_2O_2 in methanol) were applied to each section and incubated for 10 min. After washing the slides, enough drops of block solution (1% BSA in phosphate buffer) to minimize the non-specific binding of the antibodies were placed at each section and incubated for 5 min. After blocking, a primary antibody (1 : 400 diluted in phosphate buffered saline) was applied to each section and incubated at 4°C overnight. Incubation with a secondary antibody and visualization was performed using InnoGenexTM IHC kit (InnoGenex, CA) according to the mansufacturer's instructions. The slides were subsequently counterstained with Mayer's hematoxylin (Sigma).

4. Measurement of testosterone levels by radioimmunoassay (RIA)

The concentration (ng mL^{-1}) of serum testosteone was measured by radioimmunoassay kits (Diagnostic Systems Laboratories, USA) with a sensitivity of 0.08 ng mL^{-1}. The intra- and interassay coefficients of variations were $<3\%$ and $<7\%$, respectively. All experimental groups were assayed in duplicate. The 50 μL of serum or standards was mixed with Testosterone [I-125] reagents. After gentle mixing of the test tube rack by hand, all tubes were incubated in a water bath at 37±2°C for 60 to 70 min. Then, all tubes except the total count tubes were decanted. Radioactivity was measured by a liquid scintillation counter (LS 6500S, Beckman Instruments Int., Fullerton, CA).

5. Statistical analyze

Statistical analysis was performed by Student's *t* test for a simple comparison of the two groups using Sigma Plot® software (Jandel Scientific, Germany). They are expressed as mean±SEM and $P<0.05$ was considered significant.

RESULTS AND DISCUSSIONS

1. Effect of body and organ weights in irradiated mice

The present studies were designed to investigate the effect of caffeine and ascorbic acid before whole-body irradiation. The dosage and treatment route of caffeine and ascorbic acid used in this experiment referred to the previous reports (George *et al.* 1999; Maeda *et al.* 2000). The rate of increase in the body weight during the experimental period was presented in Figure 1. Two week after whole-body irradiation, the mean body weight of the irradiated control (RC) group showed a significant decrease (−4.08 g, $P<0.02$) compared with those of the control group. The decrease in the body weights of the caffeine-treated irradiated group (AR) was not marked. In the ascorbic acid-treated irradiated (AR) group, the decrease was higher than the RC group. While the unirradiated groups (CT, A and B) showed increases in the body weights, the irradiated groups

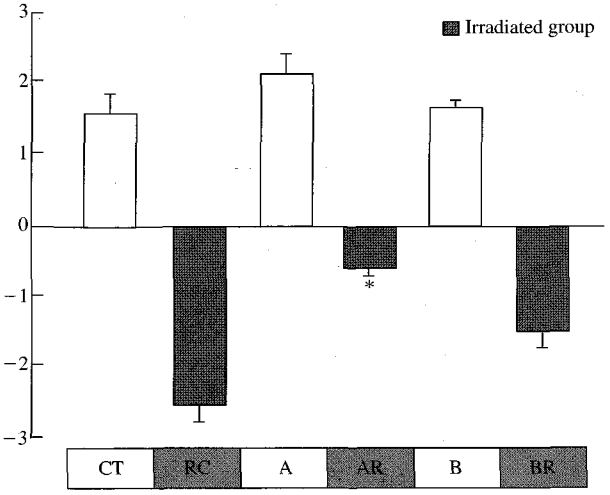

Fig. 1. Increased ratio of the body weights (g) during the experiments. Zero value indicated the mean of the body weights of the experimental groups at the irradiation time. Each bar showed an increase or decrease in body weights 2 weeks after whole-body irradiation. Abbreviations: CT, the control group; RC, the irradiated control group; A, the caffeine-treated group; AR, the caffeine treated before irradiation; B, the ascorbic acid-treated group; and BR, the ascorbic acid-treated before irradiation. *, significantly different from the irradiated control group ($P<0.05$).

(RC, AR and BR) resulted in decreases from the mean body weights at the irradiation point on the whole. It is noteworthy that the treatment of caffeine before whole-body irradiation improved the radiation-induced body weight decreases and that the effect of caffeine was superior to that of ascorbic acid.

To investigate the organ-sensitive defects corresponding to the reduction of the body weights of each group, we were calculated the organ indices of liver, kidney, spleen, and testis. The indices for the experimental groups were shown in Table 1. The index (%) was calculated from the organ weights divided by the body weights as mentioned before. Liver indices in Table 1 did not show difference among the experimental groups. Because kidney indices were affected by gamma irradiation, the value of the RC group was reduced to 87% of those in the control group.

The values of spleen and testis in the RC group decreased significantly by 50% compared with those of the control group ($P<0.05$). However, the values of the AR group reflected a marked recovery, especially in spleen indices, compared with those of the RC group. While spleen indices of the AR group showed a similar level to the control group, the values of the BR group was not improved into the level of the RC group. Because spleen and testis were the most sensitive to radiation, pretreatments of caffeine and ascorbic acid played important roles in the organ-specific influence on the irradiated groups. In case of testis indices (*ie*, gonad indices), both of the AR and BR group did not differ from the values of the RC group. Though testis indices of the AR and BR group were not better than the RC group, the spleen indices of the AR group indicated that caffeine protected the organ against whole-body irradiation.

Table 1. Calculated organ indices of the experimental groups 2 weeks after whole-body irradiation

	Organ indices after whole-body irradiation			
	Liver	Kidney	Spleen	Testis
CT	3.45±0.04 (1.00)	0.62±0.01 (1.00)	0.27±0.002 (1.00)	0.38±0.04 (1.00)
RC	3.83±0.01 (1.11)	0.54±0.02 (0.87)	0.11±0.001* (0.41)	0.20±0.02 (0.53)
A	4.22±0.01 (1.23)	0.61±0.01 (0.98)	0.24±0.001 (0.89)	0.34±0.02 (0.89)
AR	4.09±0.01 (1.18)	0.50±0.01* (0.81)	0.28±0.001* (1.03)	0.20±0.01 (0.53)
B	3.82±0.02 (1.10)	0.62±0.08 (1.00)	0.20±0.002* (0.74)	0.36±0.03 (0.95)
BR	3.60±0.02 (1.04)	0.58±0.02* (0.93)	0.10±0.001 (0.37)	0.23±0.02* (0.61)

*, significantly different from the value of the irradiated control group. Each value was calculated as described in 'Materials and methods' and represented the mean ±SEM of data from 5 mice/group. A parenthesized value showed a relative potency compared with those of the control group. Abbreviations in this table are the same as in Figure 1.

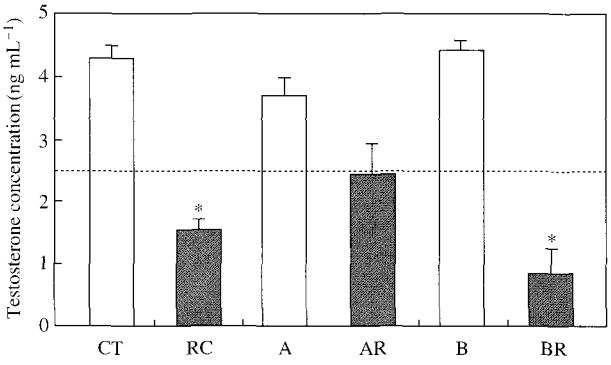

Fig. 2. Changes of the testosterone concentration (ng mL^{-1}) in serum of C57BL/6N mice after whole-body irradiation. Mice were treated with caffeine (80 mg kg^{-1}) in saline by *i.p* injection or ascorbic acid (330 mg kg^{-1}) in drinking water and sacrificed 2 weeks after irradiation. Samples were measured by radioimmunoassay kits with a sensitivity of 0.08 ng mL^{-1}. Striped bars correspond to the irradiated groups. Abbreviations are the same in Figure 1. *, $P<0.05$.

2. Concentration of the circulating testosterone on irradiated mice

As a functional parameter of radiation-induced testicular defects, we investigated concentration of the circulating testosterone. The level (ng mL^{-1}) of testosterone in serum was determined by a radioimmunoassay with a sensitivity of 0.08 ng mL^{-1} (Fig. 2). In the RC group, the concentration of testosterone was significantly reduced, compared with the value of the CT group (<36.2%, $P<0.02$). The caffeine pretreated group showed a similar level to the caffeine only group. However, the level of the AR group did not show a difference from the value of the RC group. We have previously shown that the concentration of circulating testosterone in all the irradiated groups was significantly reduced irrespective of caffeine pretreatment six hours after irradiation (Kim *et al.* 2003). The results obtained in the present study lead to supposition that caffeine pretreatment was associated with protection or recovery of the androgen productive system. As a result of the function of caffeine against γ-ray, the concentration of

testosterone two weeks after irradiation showed a similar level compared with those of the A group. Moreover, ascorbic acids did not play a role like the action of caffeine in the steroidogenic system in the present study.

3. Histological observation

Many studies have been performed by qualitative or quantitative examination to detect the testicular impairments via histological investigations (Russell *et al.* 1990). Qualitative evaluation involves the Leydig cells and their subcellular features, blood vessels, intertubular fluid build-up, cell infiltration, basement membranes, multinucleated giant cells, Sertoli cell morphology, delayed spermiation, missing germ cells, abnormalities of germ cells and germ cell development, absence of the tubular lumen, and disruption of the Sertoli cell barrier. Quantitative evaluation includes testis, fertility, tubular diameter, determination of sperm production, cell ratios, and cell numbers by morphometry.

Irradiation of male reproductive system caused a loss of spermatocytes, missing germ cells, and absence tubular lumen in seminiferous tubules (Muller *et al.* 1999). Results of the morphological assessment using H-E staining 2 weeks after whole-body irradiation showed that, compared with the control mice, the seminiferous tubules of the irradiated group significantly decreased in their diameters and increased a complete atrophy and vacuolation inside the tubules (Fig. 3). While vacuoles of the AR group also remained in quantities, they were not observed inside the tubules of the BR group. However, the BR group showed a reduction in the size of seminiferous tubules and disturbance of the spermatogenesis process. The earlier studies demonstrated that pretreatment with ascorbic acid resulted in a significant reduction of radiation-induced delay in wound healing such as increased collagen contents and fibroblast and vascular densities (Jagetia *et al.* 2003). In the present investigations, ascorbic acid may provide an assistance to regeneration of defects by reducing the vacuole and upkeep the spermatogenesis.

Immunohistochemistry using an antibody raised against Bcl-2 and Bax showed their presence in the seminiferous tubules although the staining did not gave rise to a particularly intense spot. The Bcl-2 family of proteins is a widely recognized group of regulation of apoptosis. This family consists both of pro-(Bax, Bad, Bak, Bik, Bok, Diva, Hrk, and Bid) and anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, Blc-w, and Bfl-1/A1) proteins that modulate the execution phase of the cell death (Richberg, 2000). Numerous studies have indicated an important role of Bcl-2 family for functional spermatogenesis. Bax-deficient male mice are infertile due to an inappropriate accumulation of premeiotic germ cells (Knudson *et al.* 1995). Transgenic

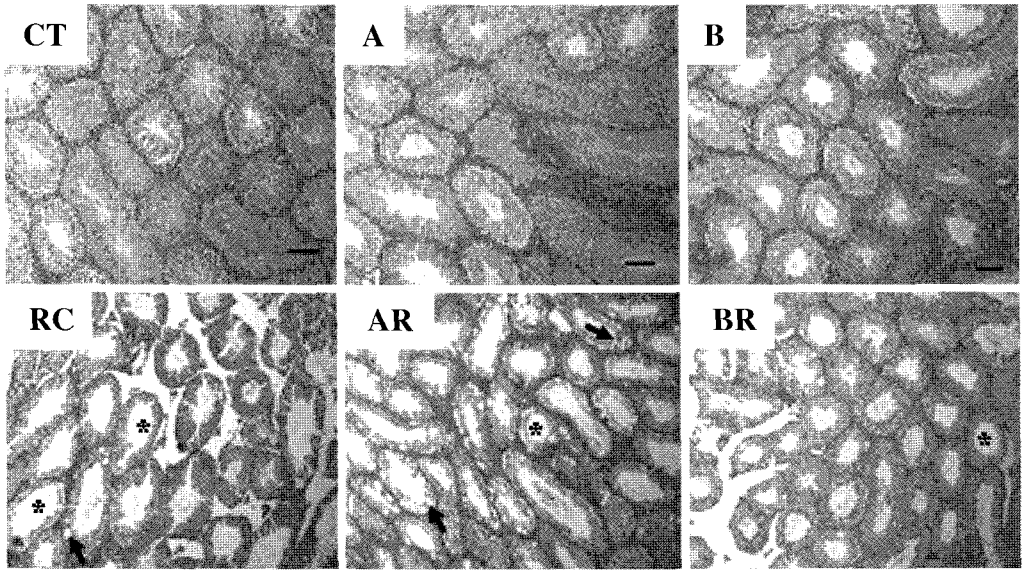

Fig. 3. Photomicrographs of the hematoxylin-eosin staining of the seminiferous tubules from the experimental group two weeks after whole-body irradiation. Abbreviations are the same in Figure 1. An asterisk or arrow indicates a complete atrophy and vacuolation in seminiferous tubules. A scale bar indicates 100 μm.

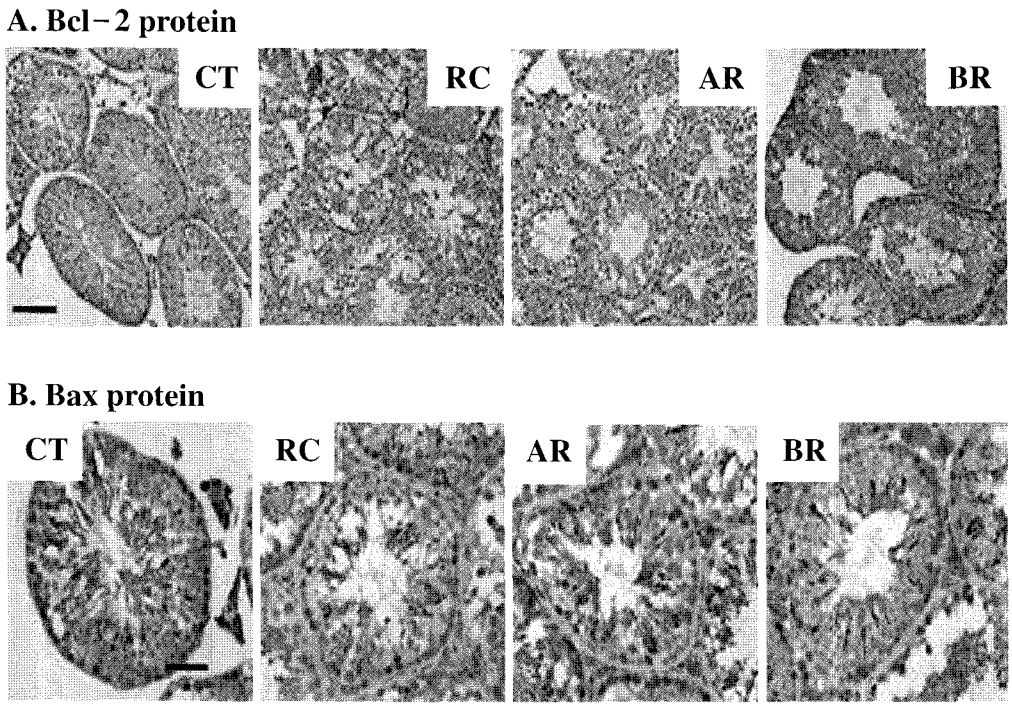

Fig. 4. Bcl-2 (upper panel) and Bax (lower panel) immunohistochemistry in the testis of C57Bl/6N mice two weeks after whole-body gamma irradiation. Abbreviations are the same in Figure 1. A scale bar indicates 50 μm.

mice that overexpress Bcl-2 are infertile and show a disorganization of the cells of the seminiferous tubules (Rodriguez *et al.* 1997).

Two weeks after irradiation, Bax in all the irradiated groups was strongly expressed in the nuclei of the round spermatids and a few primary spermatocytes, but it was also stained in the cytoplasm of the seminiferous tubules and the interstitium (Fig. 4-B). Expression of Bcl-2 was weak in the tubules of all the experimental groups. In case of the CT and BR group, Bcl-2 expression was localized in the cytoplasm of the differentiating spermatids close to the luminal surface (Fig. 4-A). These data were not sufficient to identify the differential expression of regulators among the irradiated groups (RC, AR, and BR group), but further researches for qualitative analysis and isolation upon the cell type in the seminiferous tubules will give the answer to the question whether the treatment of caffeine or ascorbic acid before γ-irradiation affects regulatory proteins which act executively in radiation-induced alteration.

CONCLUSIONS

As radiation-induced defects were concomitant with the oxidative stress, antioxidants should be radioprotectors. Caffeine and ascorbic acid in the present study were essential components in the diets in human and a small range of other mammals. The antioxidative ability of caffeine and ascorbic acid was evaluated in numerous reports, but there was no quantitative approach to their radioprotection role *in vivo*. Therefore, the present study investigated the functional radioprotection of caffeine and ascorbic acid against gamma radiation in irradiated C57BL/6N mice. The caffeine treated mice before irradiation showed higher body weights, spleen indices, and concentrations of the circulating testosterone than those of the other irradiated groups. Ascorbic acid treatment before irradiation exhibited high gonad indices, diameters of the seminiferous tubules, and decrease of vacuoles in cytoplasm of the Sertoli cells. In conclusion, the several defects related with male reproduction caused by γ ray in mice were improved by pretreatment of caffeine or ascorbic acid. Especially, the ascorbic acid treated group was distinguished in structural improvement compared with those of the other irradiated groups and the caffeine treated group showed the excellent steroidogenesis in testis.

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