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Evaluation of caffeine as a radioprotector in gamma-irradiated C57BL/6N male mice

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Abstract

Caffeine is the main psychoactive ingredient of coffee, tea, even colas with a high frequency of concurrent use in humans. Caffeine has been recently reported as a scavenger of hydroxyl radical in millimolar levels and a potential radioprotector in chronically exposed rodent. This study was performed to investigate the functional radioprotection of caffeine in gamma-irradiated mice. Eight-week-old male C57BL/6N mice were irradiated with 6.5 Gy. A caffeine treated group was administrated 80 mg/ kg body weight by *i.p* injection, a single exposure, at 1 hour before irradiation. The remaining mice were kept as sham controls. At 6 hours after irradiation, we measured the body and organ weight, collected serum, and testes were removed and processed for paraffin sections and isolation of total RNA. Hormonal analysis was performed by means of radioimmunoassay (RIA) in serum. Semiquantitative reverse transcription-reverse chain reaction (RT-PCR) was used to evaluate the expression kinetics of the apoptotic genes after irradiation. The weight of body and organ and H-E stained slide did not show a difference between groups. The circulating testosterone significantly decreased in irradiated group. RT-PCR data represented that the expression of Fas antigen, p21, p53, bax, and bcl2 related radiation-induced apoptosis showed the specific patterns comparable to that of caffeine-untreated group. Specially, bax mRNA dramatically increased in irradiated group, except caffeine-treated irradiated. Taken together, caffeine can protect an early apoptotic initiation against gamma radiation and may act as a radioprotector.

1. Introduction

The radioprotector at present includes phosphorothioates (WR-2721 and WR-3689), bioactive lipids (dimethylprostaglandin E2, platelet activating factor, and leukotriene C4), and immunomodulators (glucan, synthetic trehalose dicorynomycolate, and interleukin-1) [1]. Caffeine, a major component of coffee and tea has significant abilities to scavenge highly reactive free radicals and excited states of oxygen and to protect crucial biological molecules against these species [2]. Recent studies indicated that caffeine had been shown to presumably act as a radioprotector in irradiated mice [3]. Moreover, Lu et al. demonstrated that caffeine had the inhibitory effect on UV-induced carcinogenesis [4]. In cell cycle, caffeine has shown to override the cell cycle checkpoint in particular G_2/M [5]. Knowledge of the clear function and a side effect of caffeine in irradiated individuals are poor.

The testis is known to be sensitive organ to radiation. Ionizing radiation exerted oxidative stress on the testis and induced apoptosis primarily in the germ cells [6]. The p53 and p21 are involved in the control of cell cycle. The G_1/S and G_2/M checkpoints are under the control of p53, which is highly expressed in the normal rat testis during the first round of spermatogenesis [7]. The $p21^{WAF1/CIP1}$ protein is a component of the quaternary cyclin-dependent kinase (cdk) complex, cdk4 or cdk6, and D-cyclin. This protein is a negative regulator of the cell cycle, a role in the control of the G1- to S- phase transition [8]. Its expression, which is largely p53-dependent, may be p53-independent as well.

All apoptotic pathways appear to terminate in the activation of the caspase family. Its activity is regulated by the BCL-2 family of proteins [9]. The BCL-2 family consists of two groups, anti-apoptotic group (BCL-2, BCL-w, BCL-X_L, etc.) and pro-apoptotic group (BAX, BAK, BOK, BID, BCL-X_S, etc.) [10]. From recent studies, the ratio of pro-apoptotic to anti-apoptotic BCL-2 homologues within a cell determines whether the cell will live or die.

The purpose in this study was to investigate the radioprotective effect of caffeine against ionizing radiation. Whole body irradiation of mice leads to change the cell cycle regulator and apoptosis regulators, whereas caffeine may affect the effects of radiation.

2. Materials and Methods

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 animals were maintained under conditions of 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. The caffeine treated group was administrated 80 mg/kg body weight by *i.p* injection, a single exposure, at 1 hour before irradiation. The remaining mice were kept as sham controls. At 6 hours after irradiation, the mice were sacrificed by cervical dislocation.

Histological Observations; Testes were fixed overnight at room temperature with 10% neutralized buffered formalin (NBF), dehydrated, and embedded in paraffin. Sections (6 μm) were performed and mounted on slides. The slides were followed hematoxylin and eosin (H-E) staining for morphological changes.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR); Total RNA was extracted from frozen tissue using a Trizol (Gibco BRL) reagent according to the manufacturer's instructions [11]. The RNA concentration was determined spectrophotometer. For a cDNA synthesis, 10 μg of the total RNA was taken an reverse transcribed to cDNA in a final volume of 40 μl, using AMV transcriptase (Promega) and oligo(d)T15 primers (Promega) following the protocol provided by the enzyme supplier. The PCR was carried out with PCR Thermal Cycler 480 (TaKaRa). Amplication was performed for 35 cycles under the following conditions: denaturation at 95℃ for 1 min, annealing at optimal temperature for each primers (10, 11), elongation at 72℃ for 1min, and after the last cycle an additional elongation step for 5 min at 72℃. The PCR products were loaded in 2% TBE-buffered agarose gel and analyzed after gel electrophoresis by EtBr-staining and UV light illumination. The intensities of the each gene expression signals on film were normalized with the β-actin internal control [10]. The intensities were determined by scanning laser densitometry.

Measurement of Testosterone Levels by Radioimmunoassay (RIA); The testosterone in serum samples was measured by radioimmunoassay kits (Diagnostic Systems Laboratories, USA) with a sensitivity of 0.08 ng/ml. The intra- and interassay coefficients of variations were < 3 % and < 7 %, respectively.

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

3. Results

3.1 Effect of body and organ weights in irradiated mice

As shown in Table 1, the weight of body and testis not showed a significant

differences 6 hrs after gamma-irradiation. Testicular volumes were not different between groups, as well. In case of liver and spleen, irradiated group showed a reduced weight compare to that of the sham control.

Table 1. Body and organ weights and testicular volume of treated and control mice after gamma irradiation

Group	*Body WT (g)*	*Liver WT (g)*	*Spleen WT (mg)*	*Testis*	
				WT (mg)	*Vol (/ml)*
CT	26.4 ± 1.4	1.26 ± 0.1	62.6 ± 8.3	86.6 ± 0.6	93.89 ± 8.7
CF	23.76 ± 0.7	1.22 ± 0.1	54.4 ± 6.1	73.3 ± 0.6	79.85 ± 7.3
RC	26.12 ± 1.4	1.17 ± 0.2	43.7 ± 8.3	87.8 ± 0.6	91.33 ± 6.3
CFR	20.75 ± 1.3	0.96 ± 0.1	36.0 ± 9.6	68.3 ± 1.6	67.55 ± 9.9

Data represent the means ± SD for each group (n = 5).

Abbreviations: WT, weight; Vol, volume; CT, sham-control group; CF, only caffeine treated group; RC, only irradiated group; CFR, irradiated group after caffeine treatment.

3.2 Histological Observation

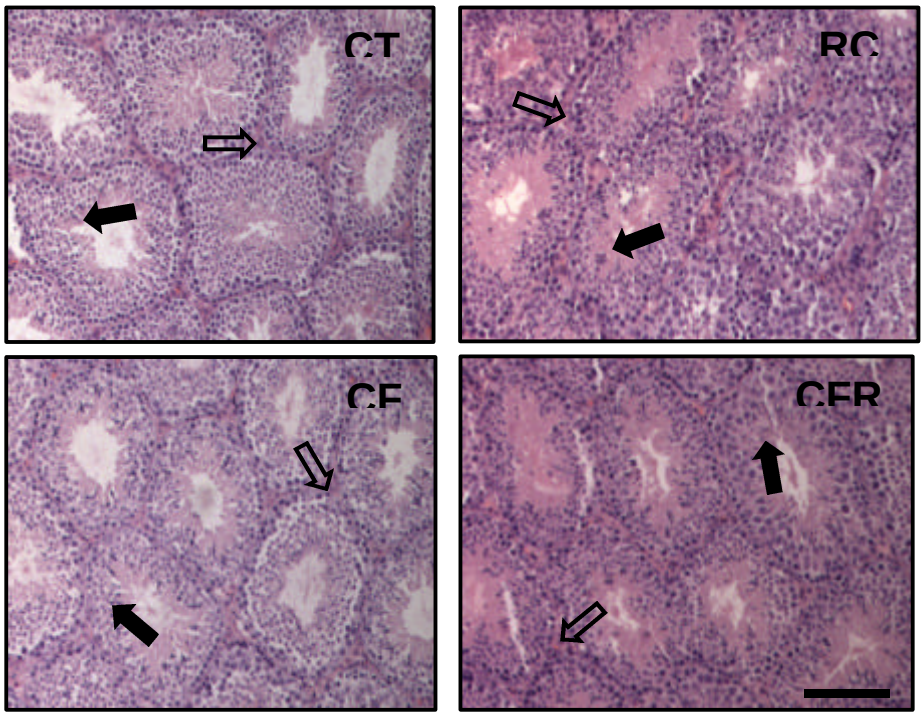

Figure 1. Photomicrographs of section of mouse testes 6 hrs after 6.5 Gy-irradiation. Scale bar is 20 μm. Abbrevations are the same as in Table 1. Arrow, mature spermatids; open arrow, intertitium.

The seminiferous tubules in mice irradiated all groups showed a similar appearance. There had the mature spermatozoa or all stage developing spermatocytes. Diameter of seminiferous tubule was observed a regular size. Interstitial compartment showed normal healthy phase.

3.3 levels of testosterone concentration in serum

The level of testosterone in serum was determined by a radioimmunoassay with sensitivity of 0.08 ng/ml (figure 2). The concentration of circulating testosterone is significantly reduced in irradiated mice (<16%, $p<0.02$). Caffeine treated irradiated group showed similar level of testosterone in serum.

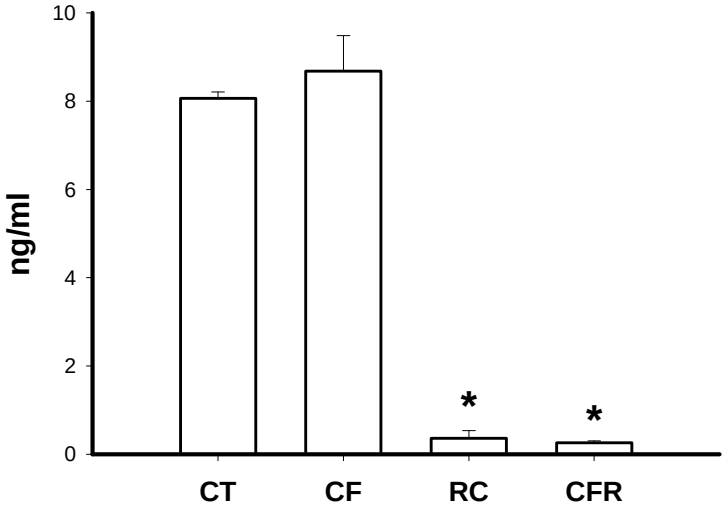

Figure 2. Changes of the testosterone concentration in serum of irradiated C57BL/6N mice. Mice were treated caffeine (80 mg/kg) in saline by *ip* injection and sacrificed at 6 hrs after irradiation. Samples were measured by radioimmunoassay kits with sensitivity of 0.08 ng/ml. The bar represents the mean ± SEM of 5 mice/group. *, $p<0.02$.

3.4 Expression of mRNA related apoptosis

In irradiated testes, the expression of Fas was equal to all groups (figure 3). P21and p53 showed a similar pattern of mRNA expression. Expression of p21 mRNA increased twofold in caffeine-treated and irradiated group than in sham controls. However, p21 expression of irradiated group after caffeine treatment showed a similar to controls. The p53 expression was not statistically changed in all groups 6 hrs after gamma irradiation.

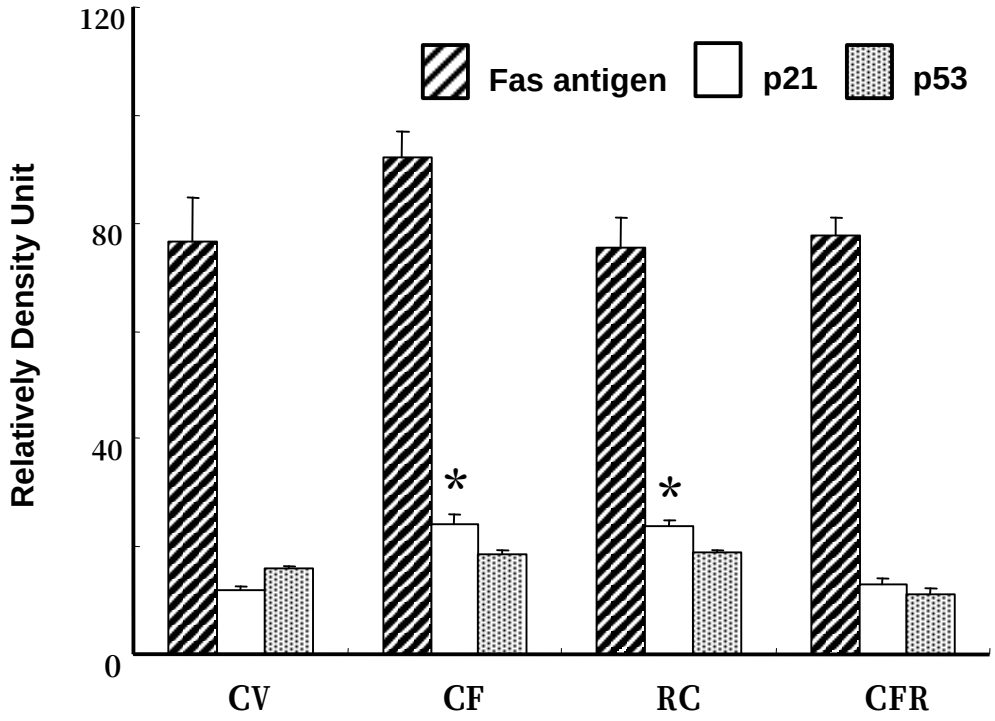

Figure 3. Semiquantitative expression of Fas antigen, p21, and p53 relative to beta-actin mRNA expression, using a reverse transcription-polymerase chain reaction assay. Densitometric analysis was performed by using a scanning laser densitometry

RT-PCT data of BCL-2 family related to mitochondial apoptosis signaling were represented in figure 4. The expression of Bcl-2 mRNA was elevated by threefold in the caffeine and/or gamma irradiation group. The Bcl-2 mRNA in the irradiated group increased slightly higher than that of the sham control. Bax mRNA in the irradiated group showed a marked elevation compared to those of other groups ($p<0.02$). The Bax expressions of the caffeine-treated group and of the sham control group remained at the similar base level.

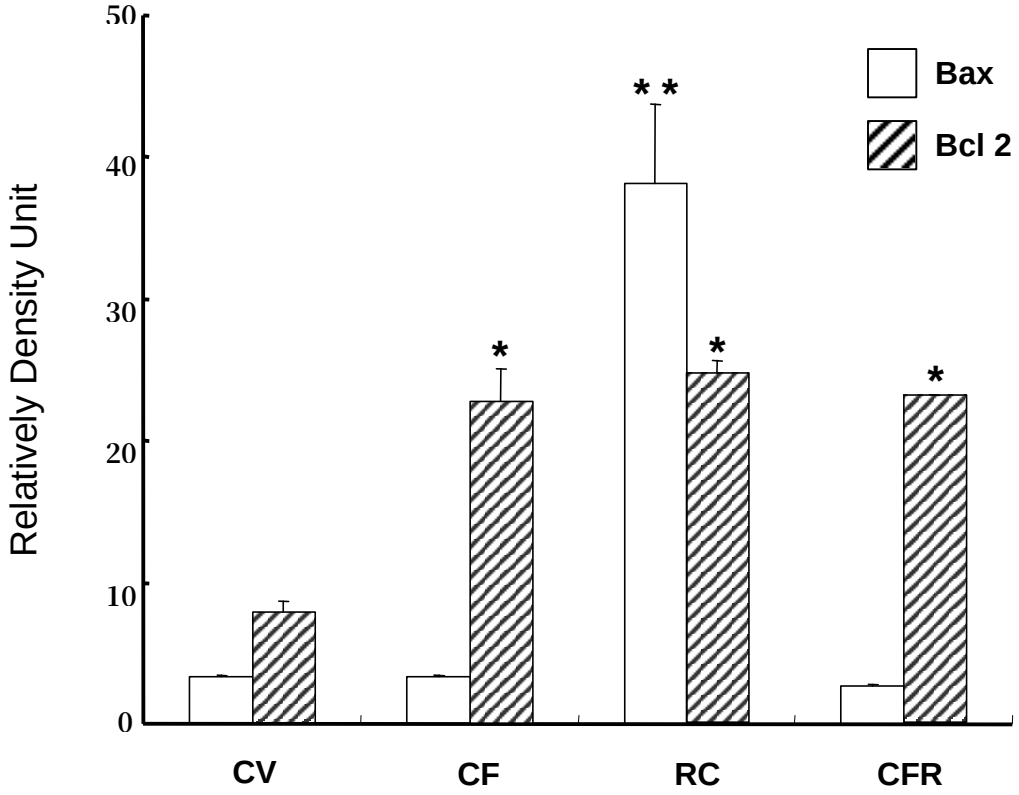

Figure 4. RT-PCR analysis of Bax and Bcl_2 expressions in the testis of C57BL/6N exposed to gamma radiation and/or caffeine. Beta-actin levels represent a loading control. Densitometrical analysis was given as a bar diagram of means ± SD. *, $p < 0.05$, **, <0.02.

4. Discussion

Caffeine has been recently reported as a scavenger of hydroxyl radical in millimolar levels and a potential radioprotector in chronically exposed rodent [2]. The caffeine may be used as a chemical radioprotector for the normal tissues in cancer radiotherapy, chemotherapy, including an everyday occurrence. The testis is known to be sensitive to ionizing radiation, and in particular, during the period when gonocyte proliferation is arrested. The p21 or p53 protein, a regulator of cell cycle, was responded to DNA damage. It has been reported that p53 expression increase in a time- and dose-dependent manner in adult rat germ cells after irradiation [13]. Whereas ionizing radiation increases the amount of p21 in spermatocytes at the pachytene stages and in spermatids, but not in spermatogonia. The increased amount of p21 mRNA in this experiment means the damage of pachytene spermatocytes and spermatids [14]. The lower level of increase in p21 expression means less damage in the cells directly caused by irradiation. Such a fact is also supported by data in the histological

observation in this study. The data of Bax and Bcl-2 analysis indicated the progress in an apoptotic pathway. The expression of Bax mRNA specially increased in irradiated group without pretreatment of caffeine. The group with caffeine and irradiation did not express Bax mRNA, which was same as in the controls. It is indicated that gamma-irradiation cause Bax/Bcl-2 mediated apoptosis and caffeine may inhibit this cascade as a radioprotector. The expression of Bcl-2 mRNA increased in caffeine treated- and/or irradiated group except the sham control. The radio of Bcl-2 and Bax determined the cell fate. However research of other homologous of BCL-2 family should be followed for clear understanding of the mechanism of caffeine protection.

Testosterone in serum was significantly reduced in the irradiated group than in the control (Figure. 2, $p<0.02$). Interestingly, the irradiated group after caffeine treatment showed a decreased testosterone level as well. According to previous reports, endocrine cells in testis were classified into the radio-resistant type compared to spermatogonia or differentiating spermatocytes of the radiosensitive group. But the data in this study indicated that gamma irradiation caused the impairment of steroidogenesis in the irradiated mice and caffeine cannot protect the endocrine defects.

In conclusion, caffeine may act as a radioprotector in BCL2 family-related apoptosis after gamma irradiation. However, it does not have any protective effect on the damage of the steroidogenesis in irradiated testes.

5. Acknowledgement

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6. References

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